

Trophic ecology, habitat use and ecophysiology of Audouin's Gull (*Larus audouinii*) in the Ebro Delta

Manuel García-Tarrasón

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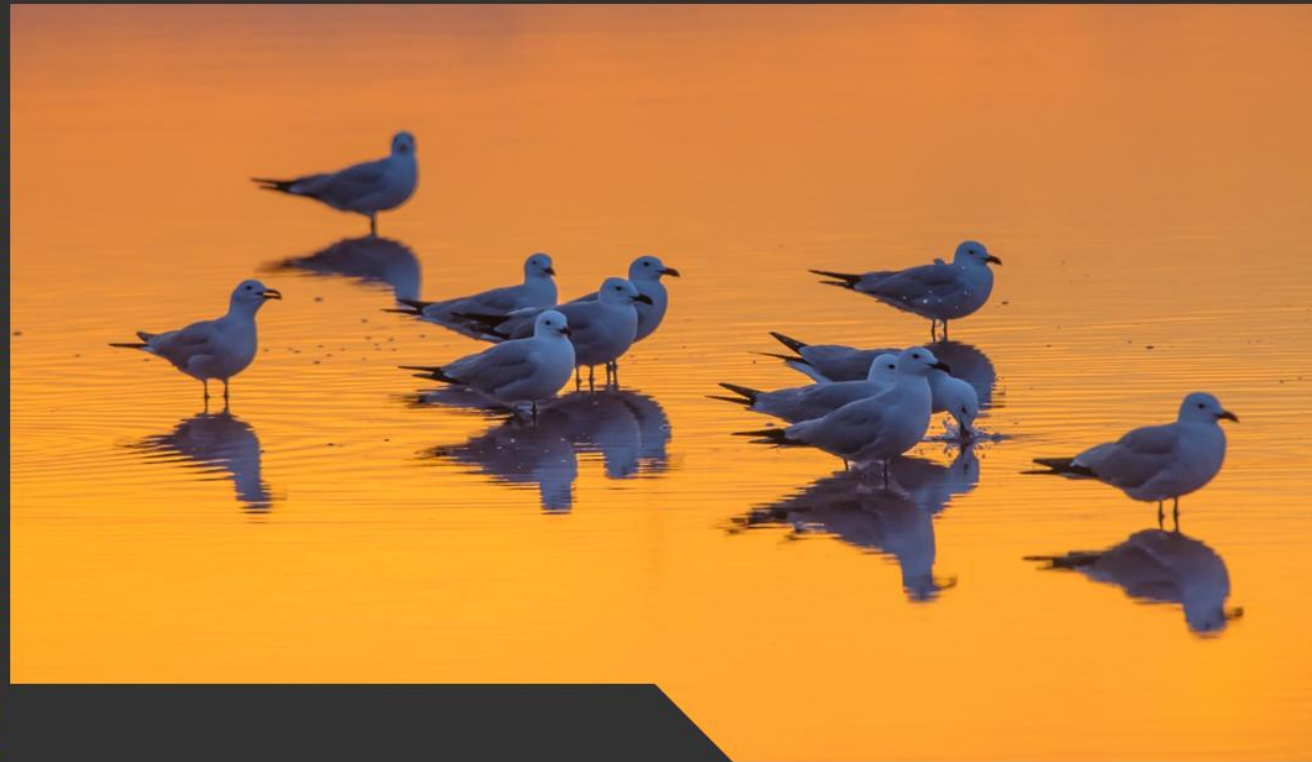
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MANUEL GARCÍA TARRASÓN
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Memoria presentada por **Manuel García Tarrasón** para optar al grado de
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“Le vent se lève!... il faut tenter de vivre!
L'air immense ouvre et referme mon livre,
La vague en poudre ose jaillir des rocs!
Envolez-vous, pages tout éblouies!
Rompez, vagues! Rompez d'eaux réjouies
Ce toit tranquille où picoraient des focs!”
— Paul Valéry, *Le cimetière marin*

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Abstract: The results presented in this thesis have shown the strong dependence of Audouin's gull from the Ebro Delta on fisheries. Foraging movements of breeding birds showed a great association with fishing activities (specially trawling), determining the most of its at-sea distribution on work days. An increase in the use of rice field habitats was also detected in absence of fishing activities (during weekends). However, a key aspect presented in this thesis was the significant sex-differences in their foraging behaviour in relation to fisheries. Female Audouin's gull tended to perform longer foraging trips on weekends compared to males. This great dependence on fishing activities was also reflected in the resource allocation into the clutch. An isotopic $\delta^{15}\text{N}$ enrichment in the albumen of eggs synthesized during the weekends was observed, which we attributed to an increase in the consumption of rice field resources as well as to the mobilization of female reserves. However, rice field diets were associated to lower antioxidant capacity in the eggs. Also, rice field diets were related to smaller egg size. Finally, the limiting nature of calcium (in constraining egg synthesis) and antioxidant molecules were also revealed, decreasing their concentration especially in the latter eggs of the clutch.

Resumen: Los resultados presentados en esta tesis han mostrado la gran dependencia de las gaviotas de Audouin del Delta del Ebro sobre las pesquerías. Los movimientos de búsqueda de alimento de las aves durante la reproducción mostraron una gran asociación con las actividades pesqueras, determinando la mayor parte de su distribución en el mar los días laborables. Un incremento en el uso de arrozales fue detectado en ausencia de actividades pesqueras (durante los fines de semana). Sin embargo, un aspecto clave presentado en esta tesis fue las diferencias significativas entre sexos para el comportamiento de alimentación en relación a las actividades pesqueras. Las hembras de gaviota de Audouin tendieron a realizar viajes de alimentación más largos que los machos durante los fines de semana. Esta gran dependencia sobre las actividades pesqueras fue también reflejada en la asignación de recursos sobre la puesta. Un enriquecimiento del $\delta^{15}\text{N}$ fue detectado en el albumen de los huevos sintetizados durante los fines de semana, lo cual puede ser atribuido a un incremento en el uso de recursos de arrozal así como a la movilización de reservas de la hembra. Sin embargo, un mayor uso de dietas de arrozal se asoció con una menor capacidad antioxidante de los huevos. También, dietas de arrozal estaban relacionadas con tamaños del huevo más pequeños. Finalmente, se puso de manifiesto la naturaleza limitante del calcio (restringiendo la síntesis del huevo) y las moléculas antioxidantes del huevo, decreciendo su concentración especialmente en los últimos huevos de la puesta.



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Audouin's gulls at "Punta de la Banya" colony (Ebro Delta, Spain).
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GENERAL INTRODUCTION

1. ECOLOGICAL STUDIES IN SEABIRDS: INTRODUCTION AND BACKGROUND

“Seabird” is a rather loose term traditionally used to cover those species which obtain at least a part of their food from the sea by travelling some distance over its surface. The most commonly used seabird classifications include about 471 species in four orders: **Sphenisciformes** (penguins), **Procellariiformes** (tubenoses), **Pelecaniformes** (pelicans, gannets and boobies, cormorants, frigatebirds and tropicbirds) and five families of **Charadriiformes** (skuas, gulls, terns, skimmers and auks). Seabirds are widespread and exploit a broad spectrum of marine habitats, from littoral to pelagic and from tropical to polar, breeding at higher latitudes and in colder environments than any other vertebrate on Earth. However, despite the enormous amount of food potentially available in surface waters, less than 5% of the approximately 10,000 described bird species exploit these two-thirds of the Earth's surface represented by oceans and seas. Moreover, the most generalist groups (as gulls or some Pelecaniformes) can also feed on terrestrial and freshwater resources. The 98% of them breed colonially, mainly on offshore islands or coastal areas (Furness & Monaghan 1987, Hamer *et al.* 2002, Schreiber & Burger 2002, Gaston 2004).



Figure 1. Examples of seabird diversity, from left to right and top to bottom:
Salvin's Albatross *Thalassarche salvini* (Order Procellariiformes)
Red-billed Tropicbird *Phaethon aethereus* (Order Pelecaniformes)
King penguin *Aptenodytes patagonicus* (Order Sphenisciformes)
Audouin's gull *Larus audouinii* (Order Charadriiformes)

Although seabirds vary greatly in lifestyle, behaviour and physiology, in general they are long-lived species, with a delayed sexual maturation and breeding, and a low annual reproductive rate with extended chick-rearing periods (Hamer *et al.* 2002). According to life-history theory, species are subjected to a trade-off so that any investment in current reproductive attempt may reduce an individual's future fitness, for example, by reducing fecundity or decreasing survival of parents until future breeding seasons (Williams 1966, Stearns 1992). In long-lived species, such as seabirds, allocation of resources between self-maintenance and reproduction is of particular interest since a small reduction in adult survival may have a large negative effect on their lifetime reproductive success. Parents are therefore limited in how they can increase effort in current reproductive investment in order to moderate the impact on future reproduction attempts (Croxall 1982, Furness & Monaghan 1987, Russell 1999, Croxall *et al.* 1992, Gaston 2004). Therefore, tracing resource allocation to their clutch during reproduction becomes clue to understand evolved seabird reproductive strategies.

Another important point determining the population dynamics and breeding success is food availability (Oro *et al.* 2004, 2014). In this regard, there is clear evidence that human activities in this globalized world have a strong impact on marine ecosystems where seabirds feed (Halpern *et al.* 2008). The anthropogenic factors that traditionally have had a greater effect on the foraging habitats of seabirds are fishing activities and pollution (Boersma *et al.* 2002). Regarding pollution, seabirds are exposed to a wide range of contaminants because they spend most of their time in aquatic environments, where contaminants move faster than in terrestrial environments (Burger and Gochfeld 2002). Pollutants may have a diffuse effect (for example heavy metal and organochlorine accumulation incorporated through diet and ingestion of plastics; Morera *et al.* 1997, Cotín *et al.* 2012) or concentrated effect with devastating consequences for seabirds (for example oil spills; Peterson *et al.* 2003, Moreno *et al.* 2013) (see a review in Nisbet 1994). The magnitude of a given impact will depend largely on the degree of association with the habitat and also on the level of aggregation of the species, being especially vulnerable when they are concentrated in high densities (Nel *et al.* 2000).

On the other hand, the industrialization and drastic development of commercial fisheries over the last century has exerted an important pressure in our seas and oceans. This has resulted in a significant impact in marine environments by depleting the stocks of some fishes (Casey and Myers 1998, Coll *et al.* 2013), transforming the structure of the food webs (Camphuysen *et al.* 1995, Pauly *et al.* 1998) or providing

huge quantities of extra food in the form of trawling discards (Arcos 2001, Furness 2003). Therefore, the establishment of trophic preferences and habitat use of seabirds seems essential in several key areas of applied ecology, such as the conservation of endangered species, management of problematic species or interaction with human activities (Tasker *et al.* 2000, Louzao *et al.* 2006, Votier *et al.* 2010, Ramos *et al.* 2011).

This thesis was developed somewhere between these two aspects discussed above, using the Audouin's gull as a model species: habitat use and trophic ecology on one hand, and resource allocation to the clutch during the breeding event on the other. Moreover, both issues are interrelated since a precise knowledge of the trophic ecology is necessary to understand changes in resource allocation.

2. INSIGHTS INTO TROPHIC ECOLOGY AND HABITAT USE IN SEABIRDS

i. Trophic ecology: Isotopic approaches to dietary analysis

The use of conventional methods to characterize the diet of a given population, such as stomach contents analysis (pellets and regurgitates) or direct observation, have some limitations related to biases in differential persistence and conspicuity of the resources consumed by individuals. In particular, while studies based on direct observation tend to overestimate conspicuous prey, their differential digestibility skews results of the stomach content into an overestimate of prey with hard or little digestible parts (González- Solís *et al.* 1997a; Votier *et al.* 2003, Ramos *et al.* 2009a). On the other hand, these methodologies provide punctual information about individual diets (Votier *et al.* 2001). Therefore, obtaining information at a population level involves an exhaustive monitoring of individuals over time which increases the difficulty and costs associated to these studies (Jordan 2005). In this regard, the use of intrinsic tracers such as the **stable isotopes** for trophic ecology studies of wild populations strongly emerges as a feasible alternative (see BOX 1 for further information about what a stable isotope is). The main advantage of this approach relies in the fact that this technique provides integrated temporal information of the assimilated diet by means of a punctual sampling (Hobson *et al.* 1994, Kelly 2000). Thus, despite losing some detail in the taxonomic study of the consumed prey, the approaches based on the use of stable isotopes allow for a broad scope in which the use of different resources may be identified and quantified at an individual or population level (Ramos *et al.* 2009a, Moreno *et al.* 2010). That's the reason why the use of the Stable Isotopes Analysis (SIA) for feeding ecology studies has undergone an exponential increase in recent decades (Boecklen *et al.* 2011).

At a local scale, the most commonly used stable isotopes to infer the origin of the food resources and trophic relations in dietary studies are the stable isotopes of carbon ($\delta^{13}\text{C}$) and nitrogen ($\delta^{15}\text{N}$) (Peterson & Fry 1987). The applicability of $\delta^{13}\text{C}$ in ecological studies lies in tracing the carbon source in the process of primary production and, therefore, the matter flow through the ecosystems. This allows us to establish the origin of the resources used by the final consumer (Chisholm *et al.* 1982, Hobson *et al.* 1994). Typically, values of $\delta^{13}\text{C}$ tend to differ in relation to the photosynthetic machinery between the different primary producers. For example, $\delta^{13}\text{C}$ values of marine producers are typically higher than those of terrestrial producers as a result of the different carbon sources used in the photosynthetic processes (Marshall *et al.*

2007). The $\delta^{13}\text{C}$ is represented in the three trophic macromolecules (proteins, lipids and carbohydrates). On the other side, $\delta^{15}\text{N}$ is generally used as an indicator of the trophic position. During protein catabolism (nitrogen is generally absent from lipids and carbohydrates), amino acid retention is biased toward those that contain amino-groups

BOX 1 | Stable Isotopes as an ecological tool

Elements in nature occur in more than one stable form due to varying numbers of neutrons in the nucleus. These various forms are known as stable isotopes (as they do not decay over time) of an element and have identical chemical but varying kinetic properties due to their mass differences.

Mass spectrometers are instruments that provide an extremely accurate estimate of the ratio of the heavier to lighter isotope in an unknown sample relative to an international standard. Isotope ratios are expressed conventionally as δ values in parts per thousand (‰) according to the following delta notation:

$$\delta X = [(R_{\text{sample}}/R_{\text{standard}}) - 1] \times 1000$$

Where X (‰) is ^{13}C and ^{15}N and R are the corresponding ratio $^{13}\text{C}/^{12}\text{C}$ or $^{15}\text{N}/^{14}\text{N}$. R standard for C is Pee Dee Belemnite (PDB) and for N is atmospheric nitrogen (AIR).

Although the properties of isotopic forms do not change dramatically amongst themselves, they behave differently in various biogeochemical processes that ultimately results in changes in the relative abundance of the heavier to the lighter isotope (isotopic fractionation). Enrichment happens when the heavier stable isotope is accumulated in the product (relative to the substrate), while the lighter isotope is preferably eliminated. On the other hand, depletion occurs when the lighter isotope is favored. Changes in the isotopic signal between diet and consumer tissues are as a consequence of three main aspects: fractionation, discrimination and routing. The metabolic processes of enrichment and depletion are known in general as **isotopic fractionation**. **Discrimination** refers to the difference in isotopic composition between a tissue and diet while the differential allocation of isotopically distinct dietary components to different tissues is called '**isotopic routing**'. Hobson & Clark (1992) described the following function:

$$D_t = D_d + \Delta d_t$$

Where D_t is the isotopic signature of consumer tissue, D_d is the isotopic signature of the diet, and Δd_t is the discrimination factor between diet and consumer tissue. From the ecological point of view, as energy transfer happens throughout ecosystems isotopic fractionation takes place, resulting in alterations of the consumer's stable isotope signatures relative to its diet. That is, this isotopic fractionation occurs in each trophic level and it sums throughout trophic chains. Therefore, once the appropriate assumptions are considered, stable isotope analysis is a powerful tool for reconstructing quantitatively elementary flows throughout food webs, which can help answer relevant questions in trophic ecology (Gannes *et al.* 1997, Bearhop *et al.* 2004).

with the heavy nitrogen isotope ($^{15}\text{NH}_2$ respect $^{14}\text{NH}_2$), with therefore, a greater nitrogen excretion of the light isotope (Peterson & Fry 1987). As a result, the tissues of organisms are generally enriched in ^{15}N respect of the consumed food resources. This enrichment with each trophic position is variable, but we usually assume values of approximately 3‰ (DeNiro & Epstein 1981, Post 2002). However, the use of $\delta^{15}\text{N}$ as an indicator of trophic position must be taken carefully, and reliable estimates of isotopic values at the base of food chains are needed. For example, it is known that nutrient enrichment as a result of anthropogenic activity increases eutrophication of the

ecosystems, leading also to a $\delta^{15}\text{N}$ enrichment not related to trophic position (McClelland *et al.* 1997, Abdennadher *et al.* 2011, Cotín *et al.* 2012).

Finally, the selection of the suitable tissue is a very important issue in the design of any isotopic study. The time-space frame integrated by any biogeochemical marker as stable isotopes depends on the growth and renewal rate of the analyzed tissue (Hobson & Clark 1992, Rubenstein & Hobson 2004). Tissues with a high renewal rate typically integrate isotopic signatures incorporated in recent times, while tissues with a slow renewal rate integrate isotopic forms from a longer period of time (Hobson 1993). Keratinous tissues from a variety of organisms (not only seabirds), such as hair, nails, feathers or scales are widely used in ecological research (Hobson & Clark 1992, Hobson *et al.* 1996, Bearhop *et al.* 2003, McCue *et al.* 2008) because allow a nonlethal sampling and once formed these structures remain inert integrating the isotopic information from the local food-web were synthesized. On the other side, other tissues as whole blood integrates the diet of a period of up to a few weeks in a small passerine and months in larger-bodied animals, whereas blood plasma typically represents information over a period of 3 days or so (Hobson 2005).

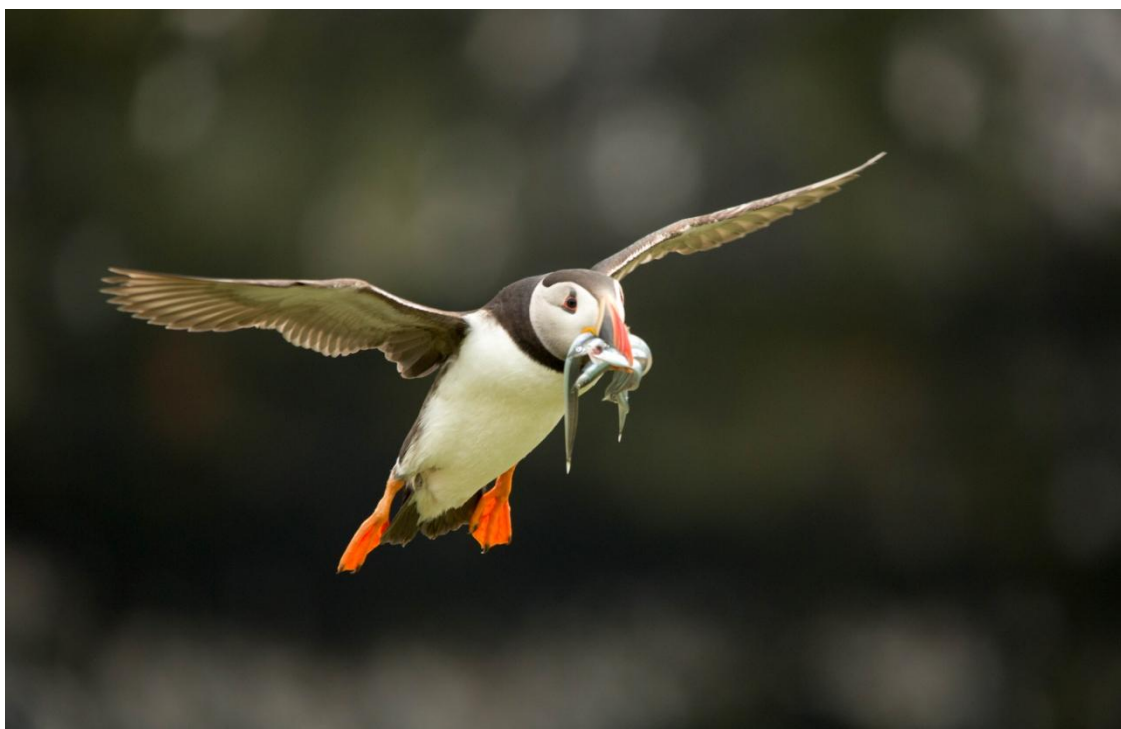


Figure 2. An **Atlantic puffin** *Fratercula arctica* flying on Skomer Island (Wales, United Kingdom) and carrying sand eels back to its chick in the nest.
© Tony Wood/Wikipedia Commons.

ii. Foraging movements and interactions with fisheries

As important as identifying the main food resources for seabirds, is to locate their main feeding areas and understand their movements. Studies of the foraging patterns of seabirds have been conducted primarily during the breeding season, when seabirds must return periodically to the nest site to assume incubation duties or to feed nestlings. Because of these obligations, it is generally assumed that foraging ranges of seabirds are constrained during the breeding season. Nevertheless, some species continue to range widely in search of prey (Shealer 2002).

The distribution of seabirds over the oceans and seas is not random, mainly as a result of localized concentrations of prey (Shealer 2002). They concentrate in areas where suitable preys are abundant, such as regions of high productivity where interaction between water currents and bathymetry force nutrient-rich water toward the surface (upwelling) or coastal areas where nutrient-rich freshwater inputs from rivers contribute to increase the productivity in a significant way (Furness & Monaghan 1987, Shealer 2002). Often this at-sea distribution of seabirds interact with human activities, mainly because the most productive waters also hold important fishing fleets operating in the area (Abelló *et al.* 2003). In this regard, due to both management and conservation reasons, it is important to study the interactions of seabirds with fisheries.

From a marine bird point of view, seabird-fisheries interactions may affect in different directions (reviewed in Tasker *et al.* 2000, Montevecchi 2002, Furness 2003). Fisheries can cause direct mortality of seabirds due to the entanglement of the birds in lost gear or due to by-catch in gillnets and longlines. The last point is of special concern, since longlines are considered one of the major threats to pelagic seabirds nowadays. Given life-history traits of seabirds (see above), this source of adult mortality is depleting some seabirds populations at a too fast rate to be compensated through reproduction (Belda & Sanchez 2001, Gilman *et al.* 2005). Paradoxically, some seabird populations can take advantage of fishing activities. Demersal trawlers and other fisheries return large amounts of fishery waste to the sea in the form of offal (intestines and other remains of gutted fish) and discards (fish either under minimum legal landing size, of little commercial value, exceeding the quota established for individual vessels, or damaged during the fishing process) (Arcos 2001). These practices allow surface-feeding seabirds have easy access to demersal and bigger prey otherwise inaccessible, and this extra food is provided in abundance and predictable in the space and time (Camphuysen *et al.* 1995, Garthe *et al.* 1996, Cama *et al.* 2012). Consequently, by provisioning huge quantities of this novel and abundant

food has led to changes in seabird movement patterns (Bartumeus *et al.* 2010, Votier *et al.* 2010), breeding success (Oro 1996), wintering strategies (Hüppop & Wurm 2000), population dynamics (Oro *et al.* 2004) and community composition (Votier *et al.* 2004a). This extra food resource is considered to be a key factor in the growth that some generalist and opportunist seabird populations (as skuas or some gull species) experimented during the last century due to its efficient exploitation (Furness 2003, Oro *et al.* 2013). However, given global demand for protein and severe threats to some fish stocks, these trawling activities are not sustainable for our seas (Furness 2003, European Commission 2007). For example, during the period 1992-2001, the Food and Agriculture Organization of the United Nations (FAO) globally calculated an average of 7.3 million tonnes of discarded fish each year (Kelleher 2005). Eliminating discards is necessary to ensure that fisheries are economically and environmentally sustainable (Bicknell *et al.* 2013). For that reason in 2011 the European Commission proposed major changes to the Common Fisheries Policy. This reform proposed a complete ban on discarding in all European fisheries not later than 2019 (Symes 2009, Bicknell *et al.* 2013). Although necessary, a significant reduction in or complete removal of discards is likely to have direct and indirect negative consequences for these scavenger communities. Understanding the impact of these reforms on seabird ecology is therefore important for management and conservation purposes, but is constrained by our limited knowledge of scavenging ecology (Votier *et al.* 2013).

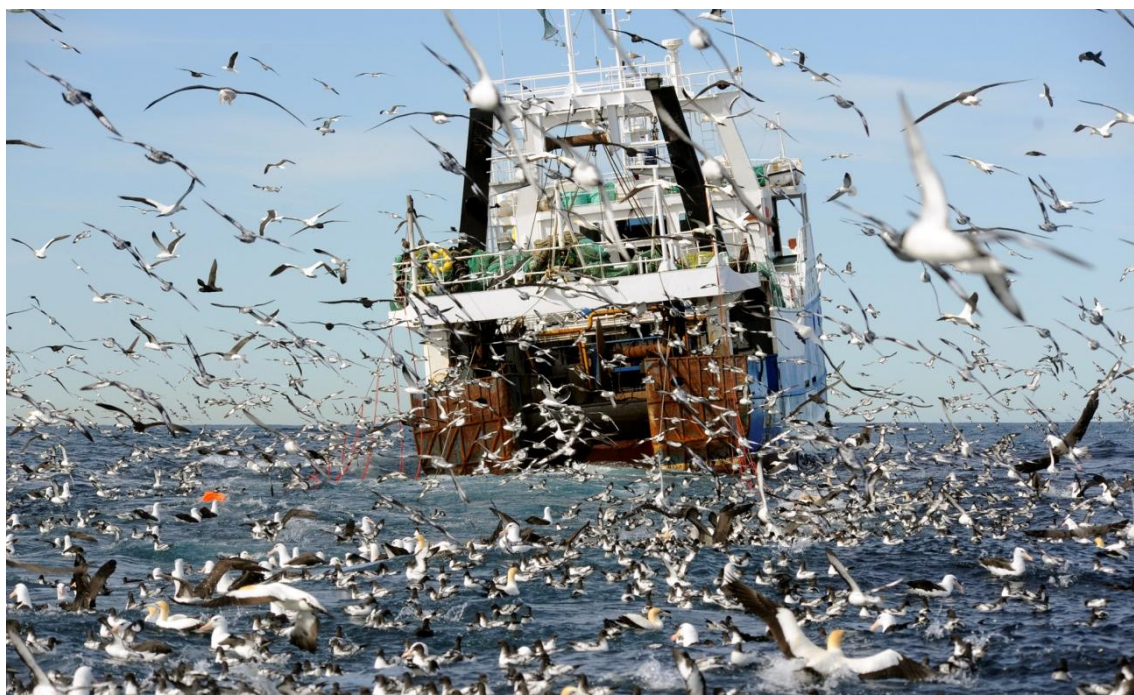


Figure 3. Hundreds of **Black-browed albatrosses** *Thalassarche melanophrys*, **Shy albatrosses** *Thalassarche cauta*, **White-chinned petrels** *Procellaria aequinoctialis*, **Cape gannets** *Morus capensis*, **Brown skuas** *Stercorarius antarcticus* and **Cape gulls** *Larus dominicanus vetula* following a trawler at Cape Town (South Africa). © Daniel López-Velasco

Traditionally, the associated problem when studying seabird movements and their interaction with fisheries has been the inability to study at-sea foraging behaviour in detail, while simultaneously recording feeding behaviour and fishing vessel activity (Votier *et al.* 2010). Until recently, these studies were based mainly on systematic observations from ships, which started in the 1970s (Wakefield *et al.* 2009). However, in the past two decades, advances in electronic technology have greatly changed the way we study seabirds, providing unprecedented insights into their biology, demography and exposure to anthropogenic risks at sea (see BOX 2 for more details in tracking devices) (Burger & Shafer 2008). For example with GPSs, locations can be recorded every second at accuracies within meters of true location, and GPS tags are now relatively inexpensive and small enough (10-20 g) to be used on many seabirds

BOX 2 | Characteristics of the most commonly used tracking devices

Device type	Precision	Signal Frequency	Weight (g)	Battery life	Equipment cost
Radio Transmitters	Medium to low at sea (1-10 km)	Variable. It depends on the effort	Low (<1)	Medium (weeks-months)	Low-medium
Geolocators (GLS)	Very low (> 100 km)	Low (1-2/day)	Low (<1)	High (years)	Low-medium
Satellite transmitters (PTT)	Medium to high (<1-10 km)	Medium-low (<10/day)	Medium (> 5)	Medium to high (up to >> 1 year in solar PTT)	Medium-high
Compass Register	Medium to high (<1 to 10 km, cumulative)	High (seconds-minutes)	Medium-High (≥ 17)	Low (days-weeks)	Medium
GPS Loggers	Very good (<10 m)	High (seconds-minutes)	Medium-High (> 10)	Low (days-weeks)	Low
GPS Satellite Transmitters	Very good (<10 m)	Medium-low (<10/day)	High (≥ 22)	Medium to high (up to > 1 year in solar PTT)	Very high
Mobile phone tracker GSM- GPS	Very good (<10 m)	Medium-high (min-hours)	Very high (> 100-150)	Medium (weeks-months)	High

Adapted from: Bécares *et al.* 2010.

(Grémillet *et al.* 2004, Votier *et al.* 2011, Harris *et al.* 2012). Such advances have enabled studies at costs compatible with obtaining appropriate sample sizes for rigorous statistical analysis. The high temporal and spatial resolution reveals unparalleled details of ground speed, micro-movements, and area restricted searching behaviour (Ropert-Coudert & Wilson 2005). Furthermore, Vessel Monitoring Systems (VMS) have been adopted by many fishing nations and provide high resolution data on the spatial and temporal distribution of fishing effort (Mills *et al.* 2007; Witt & Godley 2007). In Europe, the European Commission has introduced legislation to monitor fishing activity so that all vessels >15 m long are required to transmit their locations, estimated by GPS, at intervals of 2 h or less, so that data are comparable with data provided by remote animal sensing (Votier *et al.* 2010).

Used in combination, these developments, as well as access to remotely sensed environmental data, form a robust suite of techniques to study in detail individual foraging behaviours of large marine predators in relation to fisheries.



Figure 4. Audouin's gull tagged with a GPS-logger (CatTraq™) fixed in the back with a Teflon adjustable harness at "Punta de la Banya" colony (Ebro Delta, Spain). © Juan Bécares

iii. Biomonitoring pollutant levels

Anthropogenic activities have long been exerting an enormous pressure on marine ecosystems either through the exploitation of their resources as well as pollution (Figure 5) (Furness 1993, Furness & Camphuysen 1997). Processes as mining, fossil fuel burning, production of chemicals and agricultural activities have become an important source of heavy metals to marine environments (Furness & Rainbow 1990, Monteiro & Furness 1997, Dietz *et al.* 2009). In addition, the oceans are the ultimate sink in the biogeochemical cycles of many pollutants (Clark 1992). Therefore, pollution of the marine food webs today is of special concern for the society.

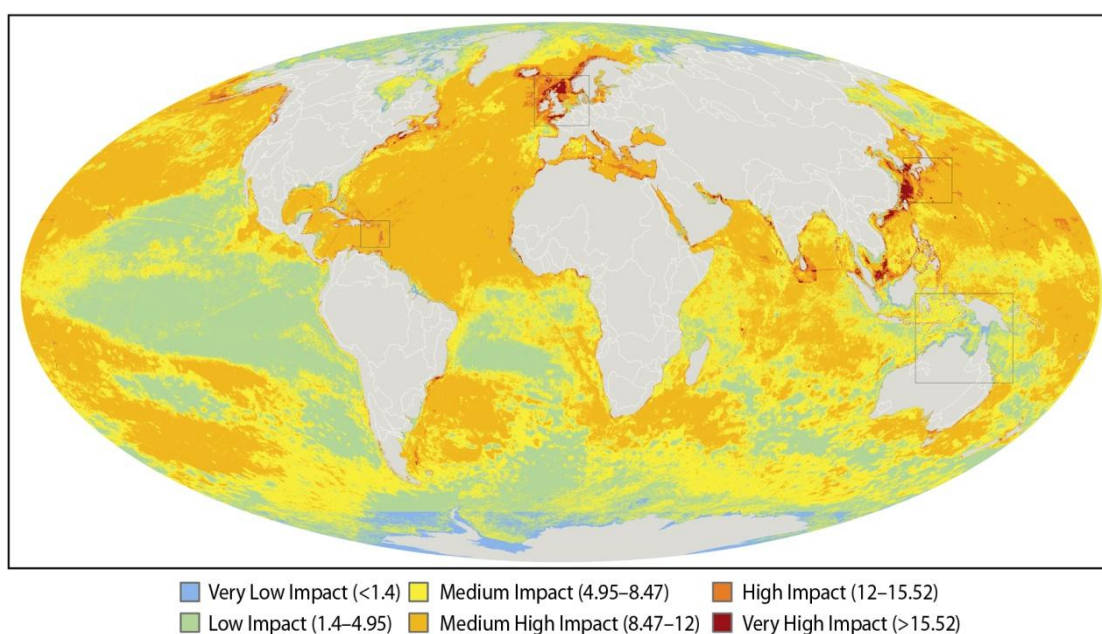


Figure 5. Global map of cumulative human impact across 20 ocean ecosystem types. Adapted from Halpern *et al.* 2008.

Some marine animal groups have been widely used as bioindicators of pollutant levels such as heavy metals. This is the case of seabirds, particularly adequate due to the detailed knowledge about their ecological habits and their relative high trophic position in the marine food webs. Their colonial nature also allows collecting large sample sizes from a particular site in a relatively short period of time (Furness & Camphuysen 1997). Seabird feathers provide non-destructive samples for monitoring heavy metal pollution in marine food webs (Monteiro & Furness 1995). In particular, chick feathers reflect the availability of some metals of a local area during the chick rearing phase (Furness 1993).

Among heavy metals, mercury (Hg) and lead (Pb) have prompted many investigations mainly because they are extremely toxic (Bryan 1979). Mercury is the heavy metal which is more bioavailable in aquatic ecosystems, where there is the major transformation to methylmercury (Wright & Welbourn 2002). Inorganic Hg is biotransformed into methylmercury and this form is accumulated in the biota, increasing its concentration as it travels up aquatic food chains (bioaccumulation) (Nriagu 1989). In marine systems, methylation is especially important in mesopelagic environments (Monteiro *et al.* 1998, Arcos *et al.* 2002). Levels of mercury in feathers have been shown to be significantly related with its diet intake (Furness *et al.* 1986), for that reason a trophic assessment of the bird ecology is necessary in order to carry out better interpretations of the mercury levels. Fortunately this is now feasible thanks to the stable isotopes (see above). On the other hand, other heavy metals as lead appears to originate mainly from atmospheric deposition onto feather surfaces, whereas ingested Pb become firmly bound in bones and only enter in feathers in trace amounts (Furness 1993, Furness & Camphuysen 1997). Lead levels can help us to elucidate the characteristics of a foraging area since the main part of atmospheric lead is from anthropogenic origin (Wright & Welbourn 2002). Thus, the levels of some heavy metals are complementary indicators for determining diet and foraging habitat in seabirds.

Finally, seabirds may have an important role as bioindicators or “sentinel organisms” (Furness & Camphuysen 1997, Burger & Gochfeld 2004). Generalist species as gulls benefit from the same resources as humans do, as for example fishing discards or refuse dumps (Ramos *et al.* 2009b). In that way, the study of the pollutant levels in these birds may also be important in fields related to public health, helping us to identify potential sources of environmental pollution and later recovery of the ecosystem as has been shown in many studies (Koeman 1969, Swennen 1972, Hyrenbach *et al.* 2009, Moreno *et al.* 2011).

3. INSIGHTS INTO LIFE-HISTORY TRAITS IN SEABIRDS

i. Resource allocation to egg production

As stated in the introduction, parental investment in a particular reproductive event is limited by the negative effect on future reproductive success (Williams 1966). One of the main issues of reproduction is to understand how individuals allocate their resources within a given reproductive event in order to maximize their fitness. In this context, intra-clutch patterns in egg size, represented in many species by relatively small size of the last laid egg (Slagsvold *et al.* 1984, Leblanc 1987), have been related to an adaptive character addressed to maximize the efficiency of maternal investment during reproduction. This hypothesis was first postulated by British ornithologist David Lack and is based on the existence of selective pressures favouring both the hatching asynchrony and the smaller size of the last egg (Lack 1947, 1954, 1968). He summarized the adaptive value as follows: 'Asynchronous hatching ...means that in a food shortage only some, instead of all, the young die.' (Lack 1947, p.332). In fact, intra-clutch pattern in egg size results in differences in siblings' size and reserves due to the positive effect of egg size on the chick mass (Wilson 1991, Grant 1991). If we combine intra-clutch size differences with an asynchronous hatching, the result is the establishment of a hierarchy among siblings that favours the elimination of the weakest individual (the chick from the last egg) under conditions of low food availability (the **brood reduction hypothesis**). However, other adaptive hypotheses state the possible ability of females to invest resources differentially among the laying sequence to ensure the survival of those chicks that are most likely to hatch and survive. For example, in the Lesser Snow geese (*Chen caerulescens caerulescens*) the larger size of the eggs placed in the middle of the laying sequence has been interpreted to be the ones more likely to hatch: while the firsts eggs are more exposed to predation (incubation starts later), the last ones are more likely to be abandoned (Williams *et al.* 1993).

Intra-clutch egg-mass variation may be also determined by non adaptive processes such as nutritional constraints or physiological conditions during the laying period. Nutritional constraints could generate intra-clutch egg-size variation by depletion of stored reserves of laying females (Houston *et al.* 1983, Pierotti & Bellrose 1986), restrictions in females' foraging options associated to the early onset of the incubating behaviour (Bollinger *et al.* 1990, Bollinger 1994), daily changes in food resources exploited by laying females (Perrins 1970) or habitat quality (Viñuela 1997). On the other hand, these intra-clutch variation patterns have been also related to

physiological factors acting both during the egg-formation and the onset of the incubation period (Mead & Morton 1985, Sockman *et al.* 2000). The incubation period occurs as a result of hormonal changes, mainly as a consequence of an increase in prolactin levels (Sockman *et al.* 2000). This hormonal control is also related to the regression of the female reproductive system, leading to the relatively small size of the last egg of the sequence (Leblanc 1987). Hatching asynchrony and the smaller size of the last egg of the sequence would be, therefore, a by-product of this hormonal control (Bollinger *et al.* 1990, Bollinger 1994). In this way, some authors have pointed the possible allocation of the absorbed material from the regression of the reproductive system in the synthesis of the last egg to optimize the investment of females in reproduction (Ramírez *et al.* 2011). All these hypotheses (adaptive or not adaptive) are not mutually exclusive, and may act simultaneously on the same species.

Tracing nutrient acquisition and allocation to eggs is now feasible through the application of isotopic approaches, mainly focused in determining the contribution of endogenous/exogenous nutrients to the eggs (reviewed in Hobson 2006). Stable isotopes analysis can help us to quantitatively track the contribution of reserves in cases where local (breeding season) diets differ isotopically from endogenous reserves typically acquired on the wintering or stop-over grounds prior to arrival to breed (e.g. Hobson *et al.* 2005, Oppel *et al.* 2010) or in opportunist species subject to dietary shifts (e.g. Ramírez *et al.* 2011). In this context, albumen may be the most suitable egg compartment for an intra-clutch insight since it is synthesized during non-overlapping short time periods (Ruiz *et al.* 2000). However, all these processes of nutrient-allocation are by far not fully understood. The gap of knowledge is especially obvious in determining the differential allocation of resources throughout the laying sequence. Further research in this field is needed to achieve a better comprehension of nutrient-allocation to reproduction, an issue of particular importance in long-lived species such as seabirds.

ii. Limiting nutrients in egg production: Calcium and Antioxidants

The egg is a complex structure comprised of three main components (yolk, albumen and shell) which develop separately. Several of the resources invested into the egg are essential, thus, influencing the offspring's growth and survival through allocation of resources such as proteins, lipids, carotenoids and antioxidants, calcium, antibodies, hormones and water (reviewed in Mousseau & Fox 1998, Christians 2002, Nager 2006). Moreover, many of these resources invested in the egg have limited availability in the environment, trading against female self-maintenance. However, the role of specific nutrients (particularly micronutrients) in shaping the costs of egg production in an intra-clutch frame remains largely unclear (Bernardo 1996, Christians 2002). We will focus on two of these nutrients: calcium and antioxidants.

There are many studies highlighting the limited nature of calcium for breeding females, as it is required in unusually large quantities for eggshell formation (Robbins 1993). Although some species store calcium in bone before laying (Reynolds 1997), calcium mobilization from bone appears to contribute relatively little to total calcium requirements during laying, especially in small birds (whose clutches often contain more calcium than is present in the entire skeleton of the breeding female) (Williams 2005). Furthermore, effects of limited calcium availability have been also reported in large birds such as the European Herring Gull (*Larus argentatus*), laying a reduced number of relatively small eggs (Pierotti and Annett 1991). For that reason calcium intake increases markedly in laying females, and birds apparently target on calcareous substances during egg laying (Creutz 1953, Graveland *et al.* 1994). However, field studies commonly suffer from methodological limitations associated with difficulty in measuring Ca availability in the wild (i.e., exogenous Ca: Wilkin *et al.* 2009; Reynolds and Perrins 2010). Alternatively, temporal trends in plasma Ca levels for breeding females could be considered a likely reflection of females' physiological response to increased Ca demand associated with clutch production. Plasma Ca levels, therefore, could be used as an indicator of overall Ca (both exogenous and endogenous) available to females during the clutch-production period, thus facilitating investigations into the role of this micronutrient as a proximal factor constraining avian reproduction.

Antioxidant compounds also play an important role influencing many life-history traits of animals in general and birds in particular (see Box 3 for an introduction in these concepts). Going back in time, around 2.45 billion years ago it took place the *Great Oxidation Event*, an increase of oxygen levels in the atmosphere as a consequence of the emergence and proliferation of photosynthetic organisms that fix carbon from light and release oxygen to the air (Sessions *et al.* 2009). As a consequence, living organisms not only developed physiological mechanisms to produce energy in more efficiently way using resultant oxygen but also had to develop a range of mechanisms to protect themselves against the toxicity of oxygen (a highly reactive molecule that produces reactive species during oxidative metabolism) and to mitigate the negative effects of oxidative processes (Halliwell & Gutteridge 2007). For that reason, oxygen free radicals exerted an important selective pressure during the incipient stages of oxidative metabolism (Costantini 2008). However, this pressure still shapes the life-history of actual living organisms.

BOX 3 | Some concepts in oxidative stress studies

Reactive Species (RS): the most part of these molecules are Reactive Oxygen Species (ROS), although we can also find Reactive Nitrogen Species (RNS). The ROS include oxygen ions, free radicals and peroxides. They generally are highly-reactive small molecules due to the presence of a layer of unpaired valence electrons. These species are naturally formed as a byproduct of the normal metabolism of oxygen. When their levels are increased can result in significant damage to cell structures. This leads to a situation known as **oxidative stress**. We have to avoid the idea that all RS are “bad” because some of them are also essential components of cell signaling and regulation (e.g. redox messengers).

Hormesis: there is also evidence that exposure of an organism to a low dose of a chemical agent or environmental stressor (that can cause damage in high doses) induces an adaptive advantage to the organism. Thus, the hormesis is the no linearity in the response to an environmental stressor.

Oxidative Stress (OS): The OS is caused by an imbalance between the production of RS and the ability of a biological system to rapidly detoxify the intermediate reactive or to repair the resulting oxidative damage. This biochemical stress cause loss of cell functions and cell death. The damage can be to the DNA, the polyunsaturated fatty acids or to amino acids. In humans, the OS is involved in many diseases, such as atherosclerosis, Parkinson's disease, Alzheimer's disease and may also be important in aging processes.

Antioxidants (AO): they are the other side of the coin. An antioxidant is a molecule capable of slowing or preventing the oxidation of other molecules. Generally, water soluble AO react with oxidants in the cell cytoplasm and plasma, whereas soluble AO protect cell membranes from lipid peroxidation.

In the last 20 years, ecophysiological studies on birds have mainly focussed on the antioxidant pigments underlying sexually selected body colourations (McGraw 2005). Among them, carotenoids have been the main target in these antioxidant works (e.g. Blount *et al.* 2002, 2004, Hipfner *et al.* 2010a, 2010b). Carotenoids are lipid-soluble hydrocarbons that are synthesized only by photosynthetic plants and bacteria. Animals are incapable of synthesizing carotenoids *de novo* and must obtain them through their diet (Goodwin 1984). However, recent studies have shown that carotenoids offer limited protection against oxidative stress (Costantini & Møller 2008, Isaksson and Andersson 2008, Simons *et al.*, 2012). For instance, a recent meta-analysis showed that carotenoids explain < 0.002% of the antioxidant capacity of birds (Costantini & Møller 2008). Therefore strongly emerges the importance of looking at other dietary antioxidants as well, not only carotenoids. Indeed, diet quality and dietary antioxidants are interrelated factors that influence many traits of animals (not only oxidative stress, but also sexual ornamentation, fertility and fecundity or brood growth) (reviewed in Catoni *et al.* 2008). The use of biogeochemical markers as the stable isotopes stand, again, as a useful tool to trace the diet of wild birds in order to relate it to their total antioxidant capacity (not only carotenoids). Only a few experimental studies have looked at the link between diet and antioxidant content in wild birds (e.g. Beaulieu *et al.* 2010, Hipfner *et al.* 2010a, 2010b). Consequently we still know relatively little about how the intra-clutch allocation of antioxidants throughout the laying sequence is produced, a very important issue for a better interpretation of how females modulate resource allocation to the eggs.



Figure 6. Chicks and egg of **Audouin's gull** at Illa de l'Aire (Balearic Islands, Spain).
© Manuel García-Tarrasón

4. CASE OF STUDY: AUDOUIN'S GULL AT THE EBRO DELTA

i. Study area: the Ebro Delta (Western Mediterranean)

The “*Mare Nostrum*” (‘Our Sea’ for ancient Romans) or “*Medi Terraneum*” (‘in between lands’) is the Latin form in which the Mediterranean Sea has been defined since ancient times. Many important civilizations have flourished on its basin due to its privileged situation in between the continents of Africa, Asia and Europe. The Mediterranean basin is the largest (2,969,000 km²) and deepest (average 1,460 m; maximum 5,267 m) confined sea on Earth (Coll *et al.* 2010). The Mediterranean is a semi-closed, elongated and rather low productive sea, divided in two basins that are connected by the Strait of Sicily (Estrada 1996). The basin is generally oligotrophic (Figure 7), though with heterogeneous features which enrich coastal areas such as climatic conditions, thermoclines and discharges of nutrient-rich waters from currents, rivers and municipal sewage (Coll *et al.* 2002). In summer, the regime is oligotrophic and characterized by low primary productivity due to a strong stratification phenomenon. In winter, however, the primary productivity increases to a mesotrophic regime due to oceanographic processes such as deep mixing, upwelling and oceanographic fronts (Allen *et al.* 2002).

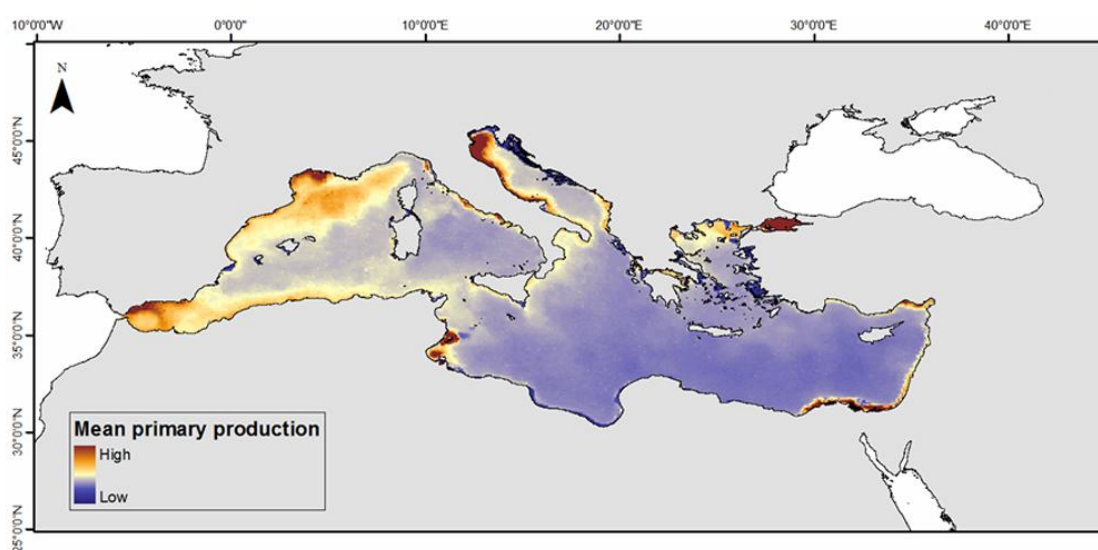


Figure 7. Annual mean relative primary production in the Mediterranean Sea. Adapted from Coll *et al.* 2010. doi:10.1371/journal.pone.0011842.g001.

Despite being an area with relatively low productivity, the Mediterranean Sea holds a considerable diversity of species with a high degree of endemism (Zotier *et al.* 1999, Bianchi & Morri 2000, Coll *et al.* 2010). New estimates on the total diversity of species inhabiting the Mediterranean show revenues of approximately 17,000 species,

with an average of 20% of endemic species depending on the group (Coll *et al.* 2010). This is why the Mediterranean Sea is often considered as a worldwide hotspot of diversity. Seabirds are represented only by 15 species, but four of them are endemic: the Mediterranean shearwater (*Puffinus yelkouan*), the Balearic shearwater (*Puffinus mauretanicus*), the Scopoli's shearwater (*Calonectris diomedea*) and the Audouin's gull (*Larus audouinii*). The high degree of endemic species is mainly the result of a tormented geological history, including isolation from the world ocean, that led to its near drying out during the Messinian crisis (5.96 million years ago) and to drastic changes in climate, sea level, and salinity (Bianchi & Morri 2000, Coll *et al.* 2010). However, the distribution of this diversity is not random. It is primarily associated with a productivity gradient from west to east (Coll *et al.* 2010). The most productive areas occur mainly in the Western Mediterranean, and this area displays the highest values of species richness and holds most of the endangered species (Figure 8).

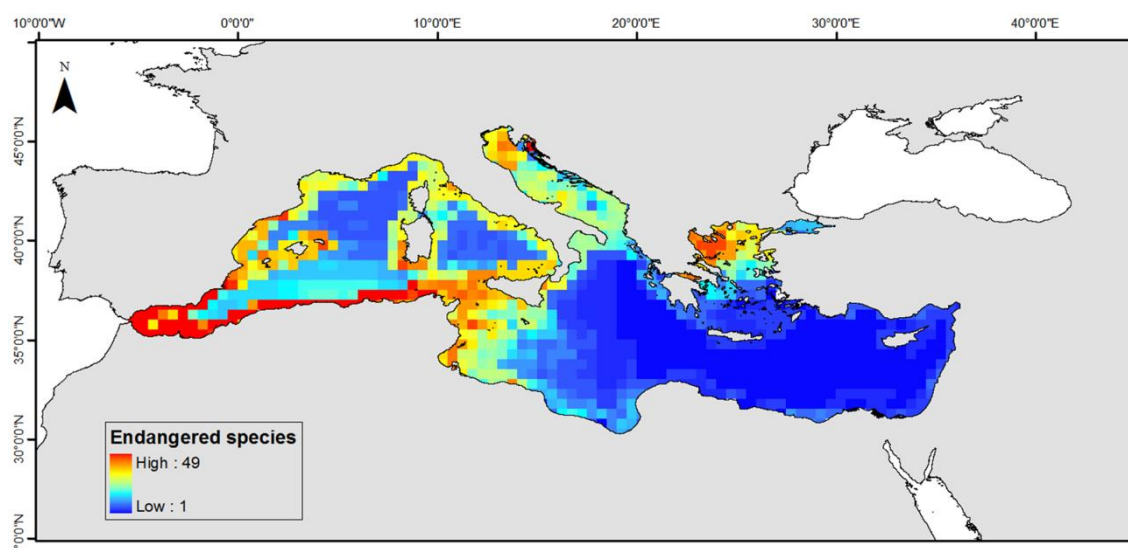


Figure 8. Biodiversity hot spots for Mediterranean vertebrate species of special conservation concern. This figure includes 110 critically endangered, endangered, vulnerable, or near threatened species. Colours express species occurrence from blue (little occurrence) to red (highest occurrence). Adapted from Coll *et al.* 2010. doi:10.1371/journal.pone.0011842.g012

The Western Mediterranean is currently a highly exploited area: most fish stocks are fully exploited or overexploited, inducing major changes in the ecosystem (Bianchi & Morri 2000, Tudela 2004, Coll *et al.* 2006, 2010). Some ecological models have shown that demersal trawling is the fishing gear with more impact and less effectiveness, in both target and non-target species (Coll *et al.* 2006). Trawl fisheries in the Mediterranean discard between 20% and 70% of total captures, with an average of 17,954 tonnes of discards every year (Kelleher 2005). In developed Mediterranean countries, the rate of discards from trawl fisheries can be up to 400% of commercially

valuable catches (Coll *et al.* 2010). In this way, one of the most important trawl fleets in the Mediterranean is located in the Western basin: the Ebro delta shelf (Figure 9).

The Ebro Delta (NE Iberian Peninsula) occupies an area of 320 km² and is one of the largest wetlands in the western Mediterranean region, home to extensive aquatic bird colonies. This is a predominantly agricultural area, where 75% of this surface corresponds to rice fields (ca. 20.000 ha). The surrounding marine area is a region with a particularly high productivity in a Mediterranean context. This is due to the nutrient-rich freshwater input from the Ebro River, together with the prevailing and strong northwest winds and a relatively wide continental shelf (Salat *et al.* 2002). These productivity conditions make this area particularly favourable for the marine biological community, for example for spawning of many fish species or as a feeding area for seabirds (Palomera 1992, Estrada 1996, Arcos 2009, Coll *et al.* 2010). Indeed, the Ebro delta area is one of the most important foraging areas for seabirds in the whole Mediterranean, especially during the breeding season. Remarkable are the numbers of three endemic species that feed in these waters: Balearic shearwaters, Scopoli's shearwaters and Audouin's gulls (Arcos 2009). In the case of the Audouin's gull, about 60% of the world population nests in the Ebro Delta area. For all this, the Ebro Delta shelf has recently been designated as important area for the conservation of seabirds (marine IBA ES409) (Arcos 2009).

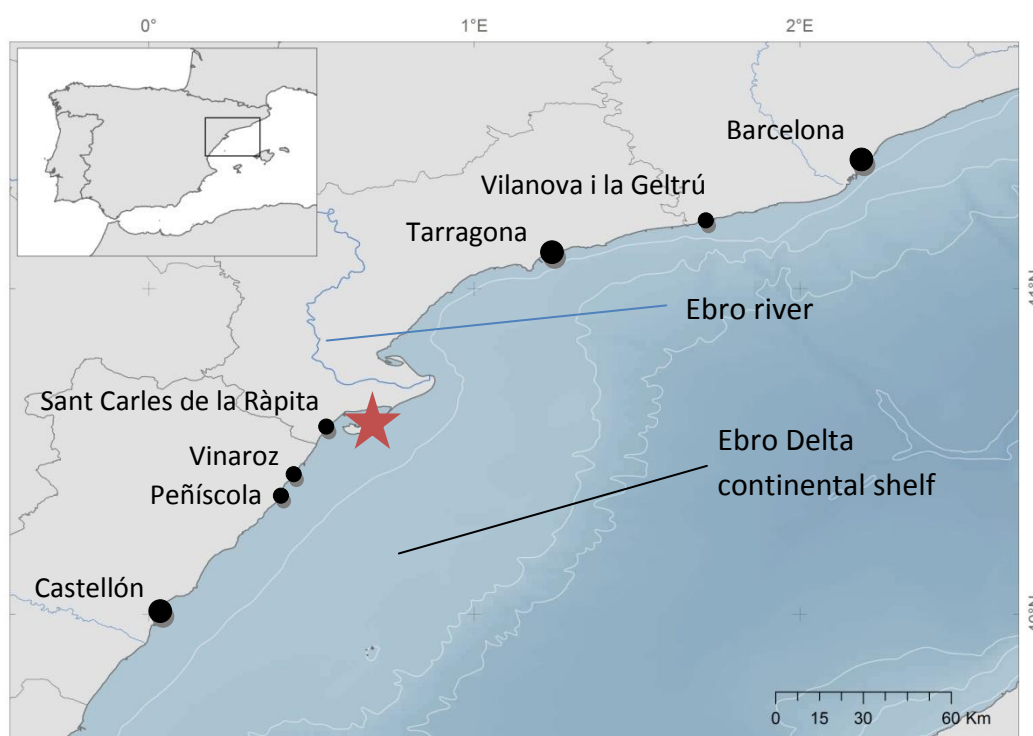


Figure 9. Situation of the Ebro Delta continental shelf with the "Punta de la Banya" colony (red star) and the main fishing harbours (black dots).

ii. The species: the Audouin's gull (*Larus audouinii*)

The Audouin's gull (*Larus audouinii* Payraudeau 1826) is a medium sized gull endemic from the Mediterranean as breeder. It measures 48-52 cm height, with a weight of 500-750 g, being males usually bigger than females (Ruiz *et al.* 1998, Genovart *et al.* 2003). It's considered a migratory species. After the breeding period (which finalizes in June - July) Audouin's gull start their way south from their breeding grounds, leaving the Mediterranean through the Strait of Gibraltar and reaching their winter quarters in West Africa (mainly situated in southern Morocco, Mauritania, the Gambia and Senegal). In late winter they undertake the journey back, arriving to the nesting places mainly during march (Cramp & Simmons 1983, Oro & Martínez-Vilalta 1994a, Pedrocchi & Oro 2005). However, some individuals spend the whole winter in the Mediterranean, mainly associated with the fishing harbours (Bécares *et al.* 2012). The Audouin's gull is categorized as "near threatened" at global level by the International Union for Conservation of Nature (IUCN) (BirdLife International 2012). In Spain, the species is listed as "vulnerable" (Martínez-Vilalta & Oro 2004).



Figure 10. Adult Audouin's gull at "Punta de la Banya" colony (Ebro Delta, Spain).
© Juan Bécares

Formerly, it was considered one of the most endangered seabirds in the world (Burger & Gochfeld 1996). In the late 1960s the world population was estimated in only 800-1,000 breeding pairs (De Juana 1984). The situation reverted in 1981, when 36 pairs of Audouin's gulls established at "La Punta de la Banya" (40° 35'N, 0° 42'E; a sandy peninsula hosting a salt pan industry) in the Ebro Delta. Since then, this new colony experienced a significant growth, reaching a more or less stable figure of 11,000 pairs from 1997 (Figure 11, Pedrocchi & Oro 2005).

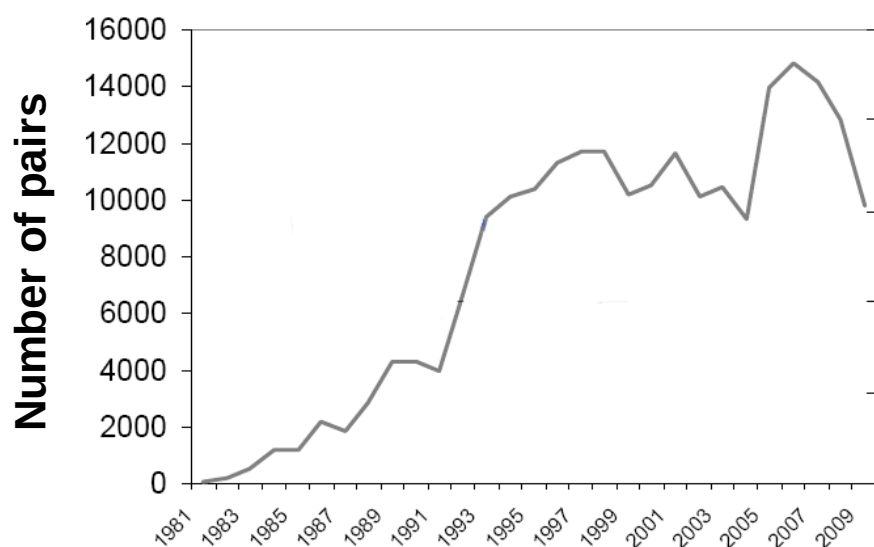


Figure 11. Population trend of **Audouin's gull** at "Punta de la Banya" colony (Ebro Delta, Spain) since the establishment of the colony in 1981 to 2009. Adapted from Oro *et al.* 2010.

The current world population is estimated to be about 21,000 pairs (BirdLife International 2012), so nowadays, the Ebro Delta holds more than a half of the global population. There, the Audouin's gull nests in both coastal dunes with vegetation and in the salt pans. The spectacular population increase in the Ebro Delta has been considered the result of an opportunistic and efficient utilization of trawling discards, together with effective protection measures of the breeding area (Ruiz *et al.* 1996, Arcos *et al.* 2001, Oro & Ruxton 2001). In the past this species was considered a piscivorous gull, specialized and adapted for the capture of Clupeiform fish during the night (Wallace 1969, Cramp & Simmons 1983). However, since its new establishment, the Audouin's gull has learned to take advantage of the abundant fishery discards from trawlers that operate in the area. As a result it has been suggested that, at present, this new food resource accounts for more than 75% of the energy required by this species and represent the bulk of its marine diet (Arcos 2001, Oro *et al.* 2013). In addition to marine fish, the other resource with an important contribution for Audouin's gull's diet is

rice field prey. In the rice fields, the Audouin's gull takes advantage of the abundant American crayfish (*Procambarus clarkii*) (Ruiz *et al.* 1996; Oro *et al.* 1997). This invasive and easily available species is present in the Ebro Delta since the 1980s (Gutierrez-Yurrita *et al.* 1999, Suárez-Serrano *et al.* 2010). In this way, it has been suggested that this species increases its importance in the Audouin's gull diet during periods without trawling activity (Oro *et al.* 1996a, Ruiz *et al.* 1996, Oro *et al.* 1997).

Many past studies have emphasized the relevance of these new food resources (especially discard availability) for this species, shaping its reproductive parameters (Pedrocchi 1997, Oro *et al.* 1996b, Oro *et al.* 1999, Oro *et al.* 2004), trophic ecology (Oro *et al.* 1996a, Ruiz *et al.* 1996, Pedrocchi *et al.* 2002) and behaviour (Oro 1995, Arcos & Oro 1996, Mañosa *et al.* 2004). In the case of diet studies, the most part of the available work is based mainly in observational studies, with the according biases associated to these methodologies (González-Solís *et al.* 1997a). Few studies have used isotopic approaches to achieve a better understanding of Audouin's gull trophic ecology, and usually they lack a correct assessment of the isotopic prey scenario (e. g. Sanpera *et al.* 2007a, 2007b, Navarro *et al.* 2010). Also, previous works on foraging movements in this species (Mañosa *et al.* 2004, Christel *et al.* 2012) deal with positioning techniques (radio tracking and PTTs loggers, respectively) with some kind of positioning restrictions and errors, and these studies largely neglected to compare periods with different fishing activities (for example work days vs. weekends). The information on the trophic ecology provided by the stable isotopes together with the information on the habitat use and foraging movements provided by new accurate positioning devices (e.g. GPS loggers) will allow us a very powerful approach on the feeding habits of the Audouin's gull in the Ebro Delta. Finally, a better comprehension of the trophic ecology (using stable isotopes) will be helpful when studying the resource allocation into the clutch. In this regard, available studies on clutch formation and oologic parameters (e. g. Pedrocchi 1997, Ruiz *et al.* 2000) lack the incorporation of a biogeochemical marker of the diet in order to relate the foraging ecology with the allocation of different nutrients into the clutch.

OBJECTIVES

Current thesis was designed to deepen and update the knowledge about the trophic and reproductive ecology of Audouin's gull. To do this, we used new methodologies and techniques currently available that allow a more precise study of the species. The results obtained in the present work, together with the previously generated information on this widely studied species will allow us a better interpretation of the species' ecology.

The study area was mainly the Ebro Delta colony (40° 35'N, 0° 42'E), although the newly formed colony of the Llobregat Delta (41° 18' 18"N, 2° 07' 50"E) was also used to compare heavy metal levels between the two areas. The fieldwork was conducted during the 2009-2011 breeding seasons (from April to June).

This thesis is divided into two sections. The **first section** is focused in the trophic ecology and habitat use of Audouin's gull, especially potential differences related to fishing activities. Also geographical differences in relation to pollutant levels were assessed. The **second section** revolves around ecophysiological processes and resource allocation into the clutch, with special emphasis in how diet can influence these processes.

1. TROPHIC ECOLOGY AND HABITAT USE

- To study the time-space distribution of the species in the Ebro Delta area according to fishing activities (work days vs. weekends) and time intervals, modelling GPS data from the individuals with information from VMS and environmental variables (**Chapter 1.1**).
- Deepen the knowledge on the foraging behaviour, trophic ecology and habitat use in the same area combining the information provided by the GPS loggers with the stable isotopes analysis. Possible differences between sex and fishing activities (work days vs. weekends) were assessed (**Chapter 1.2**).
- To evaluate geographical differences in the heavy metal levels (mercury and lead) between two Audouin's gull colonies subject to differential anthropogenic inputs of pollutants (**Chapter 1.3**).

2. RESOURCE ALLOCATION AND ECOPHYSIOLOGY

- To explore the intra-clutch pattern of $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ in the albumen of three-egg clutches during two consecutive breeding seasons, with special emphasis on the potential effects of fishing activities (work days vs. weekends and moratorium periods) on these allocation patterns. Also the possible relation of the diet with the egg size was tested (**Chapter 2.1**).
- To examine the relationship between diet (traced through stable isotopes analysis) and the total antioxidant capacity (not only carotenoids) of plasma females and their clutches (**Chapter 2.2**).
- To investigate the potential role of the calcium in constraining the egg synthesis, evaluating the relationship between plasma calcium levels in incubating females with several traits of the clutch (egg size, egg shape and eggshell thickness) (**Chapter 2.3**).

Supervisors' report

Informe de los Directores

Dr. Carolina Sanpera and **Dr. Lluís Jover**, co-supervisors of the PhD thesis entitled “**Trophic Ecology, Habitat Use and Ecophysiology of Audouin’s Gull (*Larus audouinii*) in the Ebro Delta**” certify that the dissertation presented here has been carried out by Manuel García-Tarrasón in its totality and grants him the right to defend his thesis in front of a scientific committee.

As supervisors, we have participated in designing, guiding and correcting earlier drafts of the chapters and manuscripts written by the PhD candidate. The contribution of the PhD candidate to each manuscript is detailed below:

Modelling terrestrial and marine foraging habitats in Audouin's gulls: timing matters

J Bécares, **M García-Tarrasón**, D Villero, S Bateman, L Jover, V García-Matarranz, C Sanpera, JM Arcos

PLOS ONE (In prep.)

Impact Factor (2013): 3.534

The PhD candidate M.G.T. has contributed in the study design, collecting data, as well as in scientific writing.

Sex-specific foraging behaviour in response to fishing activities in a threatened seabird

M García-Tarrasón, J Bécares, S Bateman, JM Arcos, L Jover, C Sanpera

Ecology and Evolution (Under review)

Impact Factor (2013): 1.658

The PhD candidate M.G.T. has contributed in the study design, collecting and analyzing data, as well as in scientific writing.

Anthropogenic input of heavy metals in two Audouin's gull breeding colonies

M García-Tarrasón, S Pacho, L Jover, C Sanpera

Marine Pollution Bulletin, 74(1), 285-290

Impact Factor (2013): 2.793

The PhD candidate M.G.T. has contributed in the study design, collecting and analyzing data, as well as in scientific writing.

The Weekend's Hangover: intra-clutch patterns of $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ reveal the ecological footprint of fisheries in Audouin's gull eggs

M García-Tarrasón, C Sanpera, JL Carrasco, L Jover

Proceedings of the Royal Society B: Biological Sciences (Under review)

Impact Factor (2013): 5.292

The PhD candidate M.G.T. has contributed in the study design, collecting and analyzing data, as well as in scientific writing.

Levels of antioxidants in breeding female Audouin's gulls and their deposition in eggs across different environments

M García-Tarrasón, C Sanpera, L Jover, D Costantini

Journal of Experimental Marine Biology and Ecology, 453(4), 116-122

Impact Factor (2013): 2.475

The PhD candidate M.G.T. has contributed in the study design, collecting and analyzing data, as well as in scientific writing.

The role of calcium in constraining egg synthesis in the Audouin's Gull (Ichthyaeetus audouinii)

F Ramirez, **M García-Tarrasón**, L Rami, M Genovart, L Jover, C Sanpera

Canadian Journal of Zoology, 91(9), 640-645

Impact Factor (2013): 1.346

The PhD candidate M.G.T. has contributed in the study design, collecting and analyzing data, as well as in scientific writing.

We also certify that none of the manuscripts included in this PhD thesis has been used as a part of another PhD thesis.

Barcelona, 25th September 2014

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SECTION 1:
TROPHIC ECOLOGY, HABITAT USE AND
POLLUTANT LEVELS

Audouin's gull flying over the Mediterranean Sea
© Juan Bécares

CHAPTER 1.1. Modelling terrestrial and marine foraging habitats in Audouin's gulls: timing matters

Título: Modelizando los hábitats de alimentación terrestres y marinos en la gaviota de Audouin: la importancia del momento temporal

Resumen:

Aunque la ecología trófica y reproductiva de la gaviota de Audouin ha sido ampliamente estudiada, su distribución espacial ha recibido mucha menos atención. Además, esta especie puede usar recursos de hábitats tanto marinos (pescado) como terrestres (principalmente el cangrejo americano en los arrozales), lo que dificulta el análisis de los patrones de distribución. Para ello, durante el período de incubación de 2011, se obtuvieron los viajes de alimentación (usando registradores GPS) de 36 gaviotas de Audouin adultas que criaban en el Delta del Ebro. Modelamos su distribución mediante la combinación de estos datos GPS con variables ambientales (incluyendo las actividades pesqueras utilizando el Sistema de Seguimiento de Buques, VMS en inglés). Este análisis para la modelización del hábitat fue llevado a cabo con el programa Maxent para intervalos horarios de 2h y para dos períodos de actividades pesca diferente (días laborables vs. fines de semana), permitiendo una visión sin precedentes sobre los patrones de distribución espacial de la gaviota de Audouin, así como su grado de asociación con las actividades pesqueras. Todos los modelos generados presentaron un gran poder predictivo. Durante los días laborables, la actividad en el mar de la gaviota de Audouin encajó perfectamente con las actividades pesqueras, tanto de arrastre (durante las horas de luz) como de cerco (durante la noche). Las gaviotas tendían a evitar el área sujeta a una moratoria en la pesca de arrastre, confirmando la dependencia de esta especie sobre los descartes de este tipo de pesca. Por otro lado, durante los fines de semana, las gaviotas presentaron una tendencia a incrementar el uso de los arrozales después del mediodía. Durante la noche de los días festivos la actividad en el mar estaba más dispersa que en días laborables, debido a la ausencia de barcas de cerco, y centrándose en áreas que presentaban una alta concentración de clorofila donde las gaviotas de Audouin presumiblemente se alimentan de forma natural. Estos resultados proporcionan importantes datos para la gestión de la especie, así como su interacción con las actividades pesqueras. Es de especial interés la relación con la prohibición de los descartes pesqueros propuesta en 2013 por la Comisión Europea como parte de la última reforma de la Política Pesquera Común. Este nuevo escenario no parece el más óptimo para esta especie dada su gran dependencia sobre los descartes pesqueros.

Title: Modelling terrestrial and marine foraging habitats in Audouin's gulls: timing matters

Authors: Bécares J., García-Tarrasón M., Villero D., Bateman S., Jover L., García-Matarranz V., Sanpera C., Arcos JM.

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Modelling terrestrial and marine foraging habitats in Audouin's gulls: timing matters

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Abstract:

Although the trophic and reproductive ecology of Audouin's gull has been widely studied, the spatial distribution of this species has received much little attention. Moreover, this species uses resources from both marine (fish) and terrestrial (specially the American crayfish in rice fields) environments, which difficulties the analysis of the foraging patterns. In this regard, during the 2011 incubation period, we obtained the foraging movements (using GPS loggers) of 36 adult Audouin's gull breeding at the Ebro Delta. We modelled the distribution by combination of these GPS data with environmental variables (including fishing activities from Vessel Monitoring System, VMS). This habitat modelling analysis was conducted with Maxent software for 2h time intervals and for 2 fishing activity periods (workdays vs. weekends), allowing an outstanding insight into the spatial distribution patterns of Audouin's gull and the degree of association with fisheries. All models generated showed a high predictive power. During work days, at sea activity of Audouin's gull fully matched with fishing activities, both trawling (during daylight) and purse-seine (at night time). The gulls tended to avoid the area under trawling moratorium, confirming the dependence of this species on trawling discards. On the other hand, on weekends, gulls tended to

increase the use of rice field habitats after noon. During weekend's night time, at sea activity was more disperse than on work days (due to the absence of purse-seine vessels) and focusing in areas of high chlorophyll concentration where Audouin's Gulls are feeding in a natural way. These results provide important data for the management of the species in relation to their spatio-temporal distribution and its interaction with fishing activities. It is of special interest the relationship with the discard ban proposed in 2013 by the European Commission as part of the last reform of the Common Fisheries Policy. This new scenario does not seem the most optimal for Audouin's gull given the great dependence of this species on trawling discards.

Key words: *Larus audouinii*, GPS-Tracking, purse-seine, trawling, fisheries, Habitat modelling, Maxent, Seabirds, rice fields, Western Mediterranean.

1. Introduction

Human activities have traditionally strongly impacted on both marine and terrestrial ecosystems (Halpern *et al.*, 2008). At sea, a particularly impacting activity along the last century has been the industrialization of commercial fisheries (Pauly *et al.*, 1998; Votier *et al.*, 2010). Among many other elements of the ecosystem, seabirds have been significantly influenced by such activity (Furness & Monaghan, 1987; Montevecchi, 2002). Indeed, fisheries have produced changes in the abundance of natural prey for seabirds, often reducing their availability markedly. At the same time, commercial fisheries have made available to seabirds large amounts of easily accessible and predictable food in the form of discards (Wagner and Boersma, 2011). Although this anthropogenic/artificial resource can benefit seabird populations in the short term, it also carries counterparts such as lower quality food intake (Grémillet *et al.*, 2008), higher levels of heavy metals associated to bottom-dwelling prey (Arcos *et al.*, 2002), changes in the seabird community in favour of the most opportunistic species (Votier *et al.*, 2004) or, ultimately, a high fishing pressure that leads to overexploitation of natural prey (Montevecchi 2002, Arcos *et al.*, 2008). On the other hand, in the terrestrial environment, the proliferation of some invasive species may also constitute a new food resource for predators, especially those with greater adaptability (Tablado *et al.*, 2010).

The Western Mediterranean has been for centuries a highly humanised region and, therefore, the local seabird community has been highly influenced by anthropogenic activities. This is the case of the waters off the Eastern Iberian Peninsula, which hold one of the most important Mediterranean fishing fleets (especially trawlers and purse-seine vessels) (Abelló *et al.*, 2003; Arcos *et al.*, 2009; Cama *et al.*, 2013). Moreover, since the 80s, the introduced American crayfish *Procambarus clarkii* has colonized the adjacent coastal freshwater bodies in the area (mainly the rice fields) (Gutierrez-Yurrita *et al.*, 1999; Suárez-Serrano *et al.*, 2010). This new resource has become very abundant in the rice fields of the Ebro Delta and the Albufera of Valencia, where it represents an important proportion of the diet for some species of the local bird community (Longoni 2010). This great availability of abundant and predictable food resources, together with the protection of some potential breeding sites, allowed an increase of gulls and terns in the region. Of particular relevance is the case of Audouin's Gull *Larus audouinii*, an endemic species to the Mediterranean. This gull was once considered one of the most scarce and endangered seabirds in the world in the 1970's (Burger and Gochfeld, 1996). However, it has notably increased its

population afterwards, becoming a common species in the Iberian Mediterranean coast, especially in the Ebro Delta (Oro and Ruxton 2001; Bertolero *et al.*, 2009).

The feeding ecology of Audouin's Gull has been widely studied in NE Iberian waters. Fisheries and the resulting discards are one of the most important factors influencing Audouin's gull ecology. Trawlers, which operate during the daylight hours, appear to be the most influencing fleet for these gulls, due to the high amounts of discards produced (Arcos, 2001). However, Audouin's gulls also associate with purse seine vessels, which operate at night targeting shoals of small pelagic fish. In this case, gulls take advantage mainly from discards, but also feed on small epipelagic fish attracted to the surface by the lamps of purse-seine vessels (González-Solís *et al.*, 1998; Arcos & Oro 2002a; González-Solís, 2003). According to other isotopic studies (Navarro *et al.*, 2010), 39-48 % of the diet of adult breeding Audouin's gulls comes from demersal fish (and, therefore, discards), 29-30 % from small pelagic fish (which come from either discards, feeding associated to purse-seines or natural feeding) and 20-31 % from the American crayfish.

Although there are several studies linking trophic and reproductive ecology of Audouin's gull with fisheries, the study of the use of space has received little attention. Mañosa *et al.* (2004) addressed this issue through radio tracking, providing interesting information about the association with fisheries and the nocturnal activity of breeding birds, but the results present some limitations inherent to the radio tracking system (low frequency and precision of signals). Other studies performed with PTT-loggers (Christel *et al.*, 2012), show patterns of activity in some time intervals, but the small sample size and the low spatial accuracy of PTT provide little detailed information on the use of space (Wilson, 2002; Bécares *et al.*, 2010). Changes in distribution range and activity patterns influenced by the fishing activity were also assessed through direct observations, but results are again limited (Oro, 1995; Arcos & Oro, 1996, Cama *et al.*, 2013),

In this paper we aim to overcome the methodological limitations of previous studies and address in detail the spatio-temporal distribution of Audouin's Gull during the breeding season and their interaction with human activities, particularly fisheries, taking advantage of the developments on GPS tracking devices (Bécares *et al.* 2010). The study was conducted at the Ebro Delta breeding colony, and encompasses for the first time the analysis of the terrestrial and marine environments altogether, through generating habitat suitability models from GPS bird tracking data. These models pretend to show the habitat use by the species, in presence or absence of fishing

activities, and in different time-intervals. Results will contribute to the understanding of the ecology of this near threatened seabird, as well as its threats, thus giving solid and effective arguments for the conservation policies in this species (Madroño *et al.*, 2004).

2. Material and methods

Study Area

The study area was defined from the movements of GPS-tracked Audouin's gulls breeding at the *Punta de la Banya* colony (Ebro Delta, NW Spain, 40°35'N, 0°40'E), where the world largest colony of this near threatened species is located (Oro *et al.*, 2000, Bertolero *et al.*, 2008), holding about the 60% of the world population (Gold *et al.*, 2009), 11,967 pairs in 2011 (DAAM / Generalitat de Catalunya). The area comprised the Levantine coast of Spain, from *Cape de la Nao* (Alicante) to the *Tordera* river mouth (Barcelona), and extended from the coast over the continental shelf off to the upper slope (Fig. 1). To encompass all GPS locations, the 1000 m isobath was used to define the offshore limit, whereas a 2 km band from the coast inland defined the terrestrial limit. This coastal band was extended further inland wherever rice fields were present (up to 11 km from the coast), since Audouin's gulls also exploits this habitat (Ruiz *et al.*, 1996; Navarro *et al.*, 2010; García-Tarrasón *et al.*, 2013). In this way the study area comprises three main habitats: the sea, rice fields and fishing ports.

Marine waters in this area have a high productivity, influenced by strong prevalent winds from the NW, the Liguro-Provençal-Catalan shelf-slope current and the Ebro river inputs, as well as by the wide continental shelf that extends up to 70 km offshore (Sabates *et al.*, 2007; Estrada, 1996; Salat, 1996). The concentration of Chl-a in this area is high in a Mediterranean context, which is often reflected in the high numbers of planktonic predators such as sardines (*Sardina pichardus*) or anchovies (*Engraulis encrasicolus*), which exploit these areas to spawn (Sabates *et al.*, 2007). These species are the natural prey of Audouin's gull and other seabirds. This richness also supports an important fishing fleet, mostly trawlers (with 321 vessels within the study area) and purse-seiners (90 vessels) that operate locally across the whole continental shelf and slope, on a workday basis (from Monday to Friday) (Bécares & Cama 2013). Trawling is restricted to daylight hours (7:00-17:00h local time), while purse-seiners operate at night and dawn. The study period coincided with a trawling moratoria established from the Ebro river mouth northwards up to south of *Vilanova i la Geltrú* harbour (Fig.1).

1.1. Spatio-temporal distribution

The coastline is highly urbanized in the study area, with few suitable foraging sites for gulls. Exceptions are some important wetlands, mainly the Ebro Delta and the Albufera de Valencia, and to a lesser extent the Llobregat Delta (Fig. 1). The former two areas include large extensions of rice fields, with high densities of American crayfish (Gutierrez-Yurrita *et al.*, 1999). Other sites include the numerous fishing ports scattered along the coast (Fig. 1).

During the study period the weather remained calm and roughly uniform, with sunny sky and without strong winds.

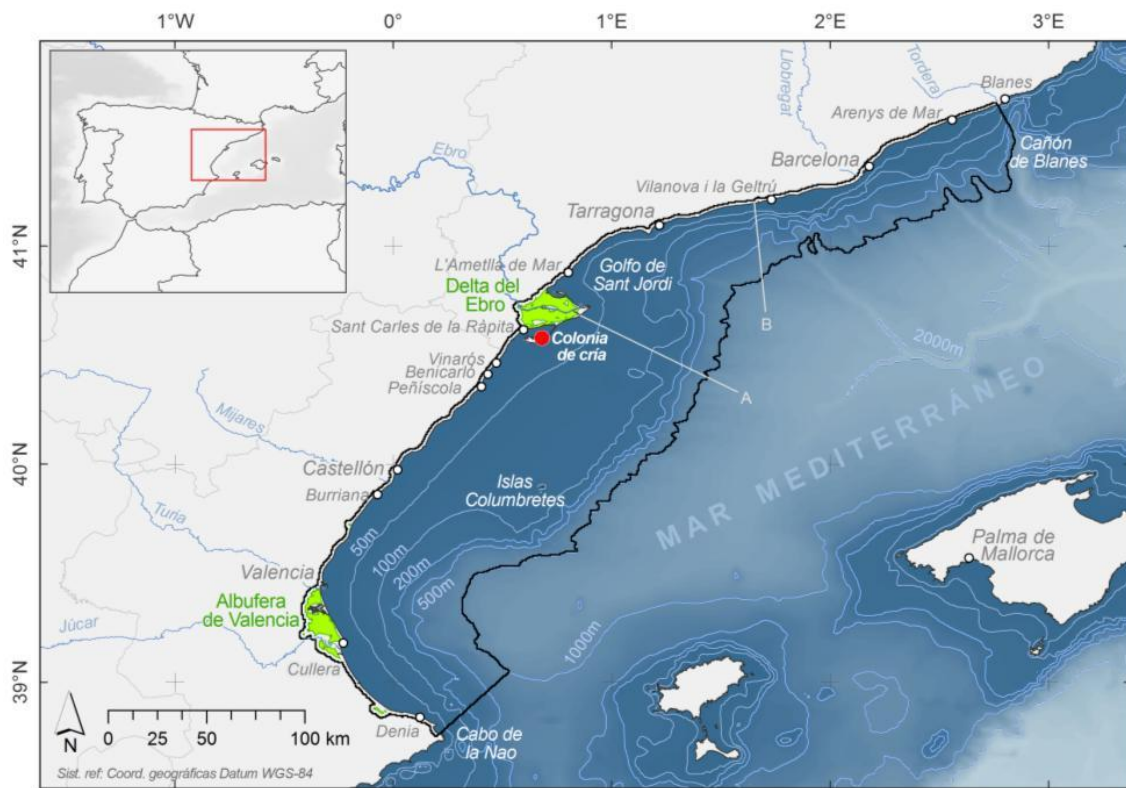


Figure 1. Study area. The red circle shows the Audouin's gull breeding colony (Punta de la Banya, Ebro Delta) where the birds were trapped and GPS-tagged. The modelling area was defined by the movements of these gulls, and is shown bounded by a solid black line, covering both marine and terrestrial areas. Green areas indicate rice fields. The most important fishing ports are also shown (white circles). The area between lines A and B defines the trawler moratorium area (see text for details).

Audouin's gull remote tracking

The study was conducted between 8th and 26th of May 2011 (data registered until 25th), coinciding with the incubation period of Audouin's gulls. Breeding gulls were captured in the nest with either box or tent-labelled traps (Bub *et al.*, 1991), and were then ringed, measured and equipped with CatTrack GPS loggers (Perthold, 2011). These loggers were programmed to record locations every 5 minutes, which allowed for a battery life of 10-15 days. Accuracy is in most cases within the 10 m range (Perthold, 2011). The devices were sealed using a rubber shrink tube to ensure waterproof.

The GPS loggers were attached to the back of the gulls using a Teflon adjustable harness (Bécares *et al.*, 2010), to ensure that the birds could not tear off the devices. The harness had a single stitch to minimize the time of attachment in case that the birds could not be recaptured. The weight of the sealed devices plus the harness was 25g, roughly representing 3-5% of the bird's body mass, just on the limit recommended by most authors (Wilson *et al.*, 2002, Phillips *et al.*, 2003). Tagged birds were recaptured between one and two weeks after being tagged, using the same fieldwork procedure.

Spatial patterns analyses

GPS locations were assigned to either foraging trips or colony locations. The foraging trip was defined as the locations since a bird leaves the colony until it returns (BirdLife International, 2004). For each GPS-location during the foraging trip, the speed was calculated and we estimated if the bird was resting (vel. < 1 km/h) or moving (vel. $\geq$ 1 km/h). The main habitats (sea, rice-field, and fishing port) were also assigned to each GPS-location. Spatial distribution models (SDM) were built by combining the foraging trip locations and habitat information (see details below). To account for differences in daily patterns and fishing activity, separate models were produced for different periods of the day, namely 12 time intervals (TI) of two hours each. Different models were also produced for work days and weekends, to account for differences in fishing activity. Thus, 24 SDMs were finally conducted, accounting for 12 TIs in workdays and 12 TIs in weekends. TI followed the standard local time (summer time, GMT + 2).

Data filtering

For each TI, a single location per bird and day was selected at random from all the available data, both to ensure equal contribution by all individuals to the models

1.1. Spatio-temporal distribution

and to reduce spatio-temporal autocorrelation (Dale and Fortin 2002; Dormann, 2007). Moreover, all individuals used to build the models contributed with the same selected number of days (sND) to each SDM, although it could differ between models depending on data availability (which differed according to the type of day and the TI). Indeed, for each SDM we tried to find a compromise between sND used and the number of individuals reaching such sND. Thus, birds with $ND \geq sND$ were used to build the model (calibrate), whereas birds that did not reach sND (i.e. $ND < sND$) did not contribute to build the models, although they contributed to the model validation process (see below). For workdays, sND used to calibrate the models varied from 1 to 4 days depending on TI (Table 1), while in weekends the information was more limited and sND was set at 1 in all cases.

For the validation, only one location per bird and day was selected at random for each TI, both for workdays and weekends. Birds not used for the calibration ($ND < sND$) were used for validation. Furthermore, birds with $ND > sND$ were also used to validate the model, excluding the days used previously to build the model (Table 1).

	Workdays					Weekend				
TI	NB	sNBc	sND	sLc	sNBv	NB	sNBc	sND	sLc	sNBv
00-02	25	25	1	25	16	17	17	1	17	6
02-04	25	25	1	25	15	17	17	1	17	5
04-06	33	20	3	60	26	20	20	1	20	7
06-08	34	24	3	72	28	22	22	1	22	11
08-10	36	27	3	81	29	25	25	1	25	10
10-12	32	22	4	88	26	26	26	1	26	11
12-14	32	21	4	84	27	26	26	1	26	14
14-16	34	31	3	93	26	30	30	1	30	15
16-18	33	24	4	96	27	29	29	1	29	15
18-20	34	22	4	88	27	28	28	1	28	14
20-22	33	30	2	60	21	28	28	1	28	14
22-24	27	19	2	38	21	26	26	1	26	8

Table 1. Data selected for calibration, from the number of birds with available data by time interval for workdays and weekends. TI: time interval, NB: number of birds. sNBc: selected number of birds to calibrate. sND: selected number of days. sLc: selected locations to calibration. sNBv: selected number of birds to validate, one location for bird.

Environmental variables

To build the SDMs, we preliminarily selected those explanatory variables that could likely influence the distribution patterns of the Audouin's gulls, based both on published information on the species and on modelling studies with other seabirds in the Mediterranean region (Arcos *et al.*, 2009, 2012; Louzao *et al.*, 2006, 2009, 2012; Cama *et al.*, 2013). Differing to previous studies on spatial modelling of seabirds, which focused on the marine environment, here we combined information from both marine and terrestrial environments altogether, since Audouin's gulls forage in both of them regularly (Ruiz *et al.*, 1996; Mañosa *et al.*, 2004; Navarro *et al.*, 2010). The combination of these two environments with noticeable differences in available spatial resolution (SR) forced to find a compromise to define the working SR. This resolution was selected at 0.31 minutes of arc ('), roughly equivalent to 500 m, using a geographic coordinate system in WGS-1984 datum. This resolution can differentiate well the land-sea border and terrestrial habitats. Working with this resolution forced to increase the pixel size for the terrestrial variables, as well as to refine the resolution for the marine variables (Table 2). Since Maxent cannot model in areas where one of the variables is *nodata* (see *Modelling approach*), and each environment had its own specific variables, we assigned arbitrary and meaningless values to marine variables in terrestrial areas, and the opposite was true for terrestrial variables in marine areas.

The marine variables selected were bathymetry (sea depth), slope, chlorophyll concentration (Chl-a), sea surface temperature (SST), and densities of both trawlers and purse-seiners for each time interval. The *bathymetry* and *slope* are related to the marine topography and subsequently with oceanographic circulation, productivity and prey distribution (Salat, 1996). Chl-a was taken as a proxy of productivity, and hence of food availability, while SST can also influence primary production. We used the average of daily data of both variables, and were called *chl2011may* and *sst2011may* (for more details see Supplementary Information). Information on the distribution of fishing vessels was available from the Vessel Monitoring System (VMS), which was provided by the Spanish Ministry of Environment (MAGRAMA). This system collects a vessel GPS position every two hours, though it only works for fishing vessels larger than 15m long. All VMS data within the study area coinciding with the study period were incorporated to the models considering separately trawlers and purse-seiners. These data correspond to the 92.2% (n=83) purse-seine vessels present in the ports of the study area and the 90.1% (n=221) of active trawlers (in ports without moratorium). Other fishing practices in the area are mostly conducted by smaller vessels of artisanal type that do not provide VMS information, and thus were not considered here.

Table 2. Variables considered for the Audouin's gull habitat modelling process, indicating their original spatial resolution (oSR), as well as their temporal resolution (TR). Those variables with an asterisk (*) were eventually not used to build the models (see text for details).

Variable	Units	oSR	TR	Source
<i>Bathymetry</i>	meters	1'	-	ETOPO1 Global Relief Model (NGDC and NOAA, 2009) ¹
Seafloor slope; <i>Slope*</i>	percentage	1'	-	Derived from ETOPO1 Global Relief Model (NGDC and NOAA, 2009) ¹
Distance to the shoreline; <i>CoastDist*</i>	degrees	0.31'	-	Derived from GSHHS shoreline ² (Wessel and Smith, 1996). Calculated as negative (land) and positive (sea)
Distance to the continent; <i>ContinentDist*</i>	degrees	0.31'	-	
Altitude above sea level; <i>MASL</i>	meters	30m	-	Derived from ASTER GDEM (METI & NASA, 2009) ³
Rice fields cover; <i>RiceFieldsCov*</i>	percentage	100m	-	From Corine Land Cover 2006 (level 3) (Bossard <i>et al.</i> , 2000). Downloaded from Centro Nacional de Información Geográfica (CNIG) ⁴ .
Rice fields altitude; <i>RiceFieldsMASL</i>	meters	30m	-	Derived from ASTER GDEM (METI y NASA) ³
Distance to the breeding colony; <i>ColonyDist</i>	degrees	0.31'	2011	Calculated as cost distance (<i>Cost distance tool</i> , ArcGis)
Fishing ports cover; <i>PortsCov</i>	percentage	200m	-	From BCN200 (Spanish National Base Cartographic) of CNIG ⁴
Distance to the active trawling ports <i>TrawPortDist*</i>	degrees	0.31'	-	Calculated as cost distance (<i>Cost distance tool</i> , ArcGis). Information about fishing ports from MAGRAMA (MAGRAMA, 2012) <i>Trawling ports in trawling moratorium areas are not considered.</i>
Distance to the purse seine ports <i>PurSePortDist*</i>	degrees	0.31'	-	
Distance to both trawling & purse seine ports; <i>PortsDist</i>	degrees	0.31'	-	
Chlorophyll concentration; <i>chl2011may</i>	mg/m ³	1km	Daily	From Aqua-Modis (level 2) ⁵ (Feldman, 2012). Averaged for the days 8 to 25 May 2011
Sea surface temperature (SST); <i>sst2011may</i>	brightness temperature	1km	Daily	From Aqua-Modis (level 2) ⁵ (Feldman, 2012). Averaged for the days 15 to 17 May 2011
Purse seine density (estimated separately for each TI)	VMS locations /km ²	0.31'	≤ 2h (8-25 may)	Purse seine VMS data supplied by <i>Centro de Seguimiento de Pesca (MAGRAMA)</i> . Calculated by <i>Point Density tool</i> (ArcGis)
Trawlers density (estimated separately for each TI)	VMS locations/km ²	0.31'	≤ 2h (8-25 may)	Trawlers VMS data supplied by <i>Centro de Seguimiento de Pesca (MAGRAMA)</i> . Calculated by <i>Point Density tool</i> (ArcGis)

¹ <http://www.ngdc.noaa.gov/mgg/global/relief/> ² <http://www.ngdc.noaa.gov/mgg/shorelines/shorelines.html>

³ http://ide.unex.es/wiki/geocatalogo/index.php?title=MDE#SRTM_DEM ⁴ <http://centrodedescargas.cnig.es/CentroDescargas/buscadorCatalogo.do>

⁵ <http://oceancolor.gsfc.nasa.gov/>

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Nevertheless, trawlers and purse-seiners are the vessels that most frequently attract seabirds in the area (Arcos & Oro 2002). Densities of both trawlers and purse-seiners were calculated for the different TIs when these vessels operate: 6 TI from 22:00 to 10:00h in the case of purse-seiners (*PurSeDe*), and 6 TI from 06:00h to 18:00h in the case of trawlers (*TrawDe*). To calculate these densities, only one location per vessel, day and TI was selected, at random.

The terrestrial variables selected were altitude above sea level (*MASL*), fishing ports cover, rice fields cover and rice fields altitude. *MASL* was taken as the topographic variable on land. Fishing ports often congregate Audouin's gulls, so fishing ports cover (*PortsCov*) was created (this variable includes inner port waters). To convert this categorical variable into a continuous variable, we calculated the coating of port per pixel (0 to 1). This was done because the original SR is greater than the working one (Table 2 and for more details see Supp. Inf.). Another inland habitat used by the Audouin's gulls is rice fields (Ruiz *et al.*, 1996; Navarro *et al.*, 2010), and rice fields cover (*RiceFieldsCov*) was thus added to the list of variables. It was calculated using the same procedure as with *PortsCov*. *MASL* of rice fields (*RiceFieldsMASL*) was considered as a proxy of soil salinity, and hence of crayfish presence or abundance probability since crayfish do not tolerate saline soils, which occur in lowlands, even if rice is cultivated there by flooding the fields with freshwater (Canicio *et al.*, 2008). To create this variable we cut *MASL* using a mask of rice use; an arbitrary value of -1 was assigned to the rest of the study area (both land and sea).

Distance variables were created for both the marine and terrestrial environment, and were calculated as Cost Distance in ArcGis, restricting the passage only to the study area. Distance to the coast (*CoastDist*; either island or continental coastline) and only to continent (*ContinentDist*) were considered and calculated as positive values for locations at sea and negative for locations on land. We also calculated the distance to the breeding colony where birds were marked (*ColonyDist*). Distance to fishing ports was considered because of Audouin's gulls often follow fishing vessels to take profit of their discards when they come back to port (Arcos, 2001; Cama *et al.*, 2013). Hence we computed the distance to fishing ports, differentiating between trawling ports (*TrawPortDist*), purse-seining ports (*PurSePortDist*) and both trawling and purse-seining ports (*PortsDist*).

Finally, to avoid the excessive collinearity between variables included in each model, we conducted a Spearman correlation matrix in R software, and for each TI we

excluded highly correlated variables; $|r_s| \geq 0.70$ (Table S5 in Supp. Inf.). This reduced relevant variables from 16 to 10 to feed the modelling process.

Modelling approach

We used a modelling method based on maximum entropy implemented in the software MAXENT (Phillips *et al.*, 2006), because of its flexibility when handling different types of data and responses. This revealed as one of the most practical methods for modelling species distributions, which allows working only with presence data (Elith *et al.*, 2006). Hence it is particularly useful to deal with tracking data, which only provides positive information (i.e. presence-only data) (Edrén *et al.*, 2010). The models were calibrated constraining the response of environmental variables to linear and quadratic functions, due to the difficulty of interpreting more complex relationships, and because these are the functions that better reflect the response of the species to the environment (Louzao *et al.*, 2012). A setting *hinge feature* in Maxent was also added, allowing to identify turning points in the linear or quadratic response settings (Elith *et al.*, 2010). This adjustment was made due to the complexity of using at the same time variables at both sea and land, with an abrupt change in the biological significance between two environments (see *Environmental variables*).

We performed SDMs for the 12 daily TIs, separately for work days and weekends, thus producing 24 final SDMs. Data selected for calibration and validation were far lower than the total data available (we recorded up to 24 locations for each TI, day and bird), and hence potentially relevant information could have been missed. To minimize related biases, all the filtering and model building procedure was repeated 10 times per TI and fishing situation (work day and weekend), independently (Edrén *et al.*, 2010). Thus we obtained 10 replicates for each TI and fishing situation, which were averaged to provide a single output map. Mapping units obtained correspond to a habitat quality index comprising values between 0 (minimum) and 1 (maximum).

Model evaluation

We tested the predictive ability of the SDMs using cross-validation with data that were not used in model calibration to avoid autocorrelation (see details above; Guisan and Zimmermann, 2000). Validation samples used represented an average of 31.4% data (SD=5.8, range 21.9-45.7). The predictive reliability of the models was evaluated with the AUC statistic (Area Under the Curve). This statistic measures the area under the ROC (Receiver Operating Characteristics) curve, which provides a measure of the models predictive capability ranging from 0.5 (no predictive power) to 1

(a perfect model) (Boyce *et al.*, 2002; Pearce and Ferrier, 2000; Swets, 1988). To test the differences between the models generated on weekends and on workdays, an AUC cross validation (cvAUC) of samples between workdays and weekends was conducted.

Ethic statement

All work was carried out with the necessary permits, awarded by both the Wildlife Service of the Catalan Government and the Ebro Delta Natural Park. Handling time was minimized to reduce any inconvenience to the birds, not exceeding 10 minutes in any case. Birds were captured in daylight hours, avoiding periods when either low (dawn) or high temperatures (noon) could pose a problem for the eggs. Whenever a bird was reluctant to get captured, we left it. The tags represented 3-5% of the weight of the birds, about the recommended limit (Cochran, 1980; Wilson *et al.*, 2002; Phillips *et al.*, 2003).

3. RESULTS

A total of 60 adult breeding Audouin's gulls were tagged with GPS loggers in three days of fieldwork (8-10 May 2011). From these, GPS data from 36 Audouin's gulls were obtained, providing 9.6 ± 5.5 days of data (median $\pm$ IQR) (range 0.8-15.6 days) (more details in Table S1 in Supp. Inf.). The remaining birds either could not be recaptured (n=20) or presented GPS sealing failure and did not provide any data (n=4). In total, 89,800 locations were obtained, of which 38,090 corresponded to foraging trips. Of the latter, 28,844 locations corresponded to workdays, and 9,246 to weekends.

Movements and activity patterns

Foraging trips lasted 8.9 ± 6.14 hours (median $\pm$ IQR) (range 0.5-77.3 hours) (Fig. S3 in Supp. Inf.). Audouin's gulls were mostly diurnal, although birds were also active at night (Fig. 2). Birds spent almost half of the daylight hours outside the colony (49.8%, SD=11.1). At night the activity out of the colony was reduced to about a half (25.8%, SD=20.5) and in some birds was negligible (14.3 % of individuals spent less than 5% of night time hours outside the colony). Most of the time spent outside the colony corresponded to resting behaviour (50.5%, SD=14.01). Bird's activity was markedly different across environments: they were mostly resting in ports (84.0% of the time, SD=11.5), about half of the time in rice fields (53.0%, SD=17.5) and over a quarter of the time at sea (25.16%, SD=12.6). At sea, the gulls mainly used the

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continental shelf waters south of the Ebro Delta, largely avoiding areas subject to trawl fishing moratorium (Fig. 3). Considering data for working days between 10 and 16h (peak trawling activity), the Audouin's gull density was more than 6 times higher in trawling areas than in areas under moratorium. All birds went directly out to the sea, while most often followed the coastline in their return trips. Many rested in the main fishing ports or in other breeding colonies (Castellón and Llobregat Delta) before returning to the colony. Although birds mainly visited neighboring rice fields at the Ebro Delta, two of the individuals were recorded to feed and to rest in the rice fields of the Albufera de Valencia, about 150 km southward from the study colony (Fig. 1).

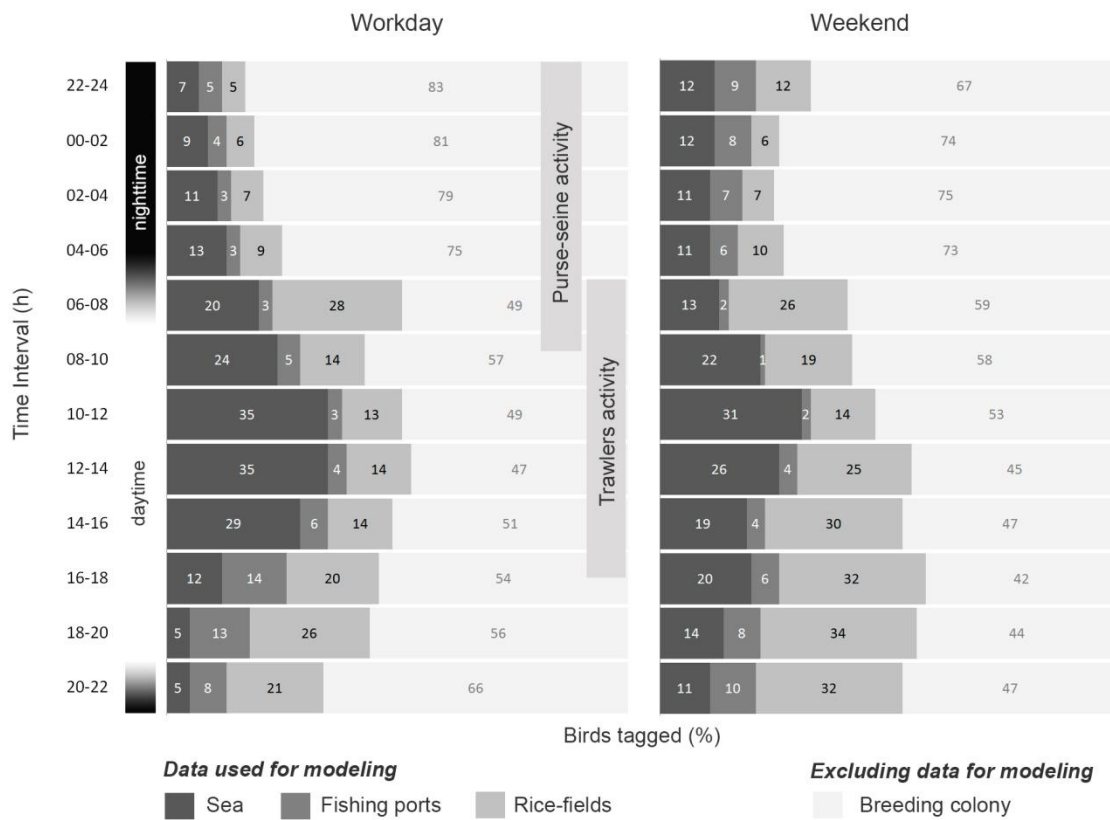


Figure 2. Distribution of tagged Audouin's gulls in the different time intervals for workdays and weekends

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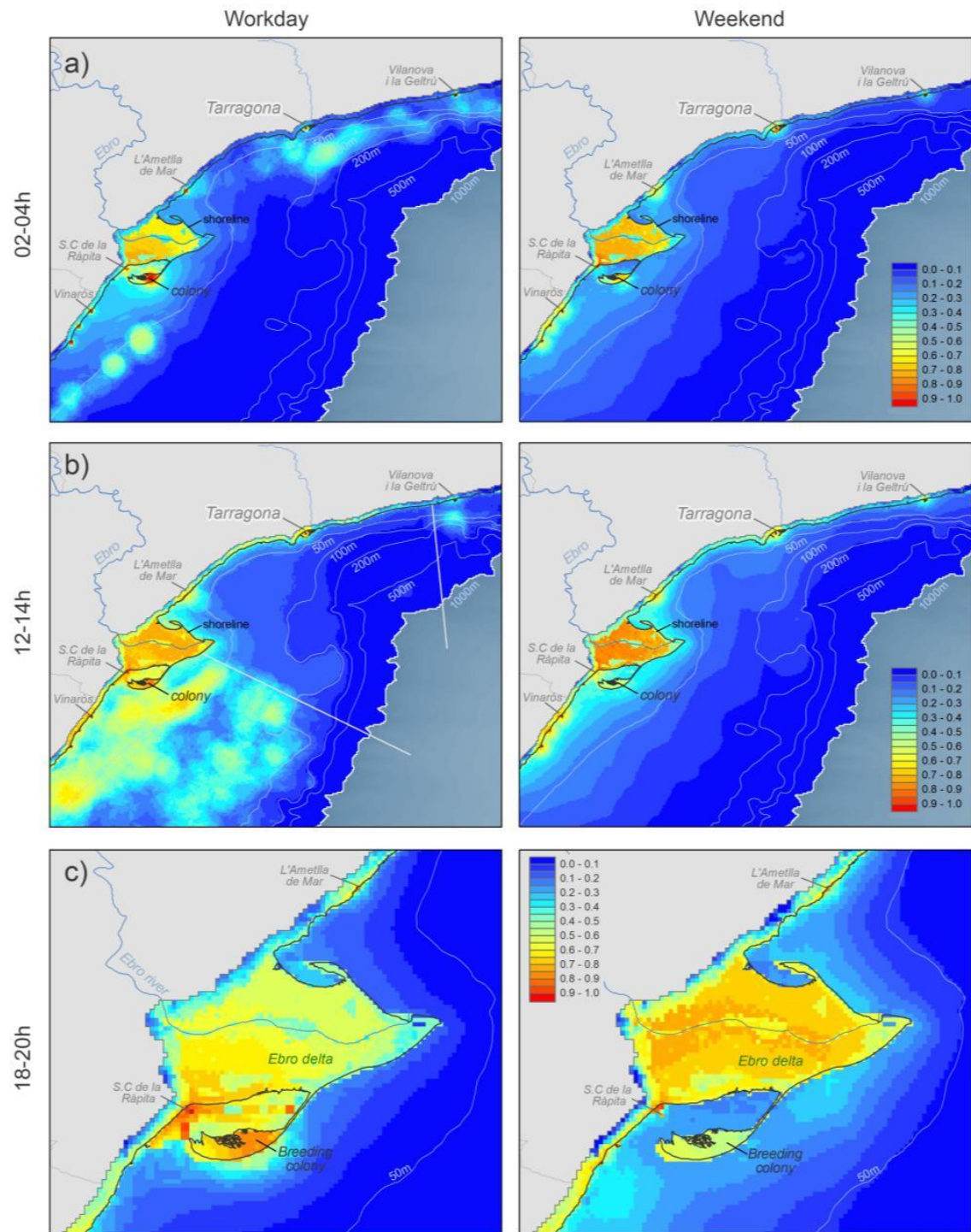


Figure 3. Some examples of Audouin's gull distribution models for both workdays and weekends: a) purse-seine activity (TI: 02-04h), b) trawling activity (TI: 12-14h) and c) no fishing activity (TI: 18-20h)

Species Distribution Models (SDM)

All models generated (n=240) showed a high predictive power (workdays AUC: mean=0.93, SD=0.04, range 0.76-0.99; weekends AUC: mean=0.90, SD=0.06, range 0.67-0.97). The best results (AUC > 0.90) and, therefore, with an excellent level of prediction, were obtained in TI with a larger validation sample (mainly during daylight hours and in workdays). For TI in workdays (tested with weekends) between 14:00 and 24:00, the cvAUC showed significant statistical differences (one factor Anova test $p < 0.05$).

Three representative time intervals were selected to illustrate the Audouin's gull SDMs (Fig. 3). Between 02-04h (night time) in relation to purse-seine activity (Fig. 3a), time interval 12-14h (daylight) associated to trawling activity (Fig. 3b), and finally 18-20h (daylight), a period without fishing activity, in this case focusing in the Ebro Delta rice fields since suitability indexes at sea are very low (Fig. 3c). In all cases the activity is compared between work days (fishing activity) and weekends (without fishing activity). Figures corresponding to 24 TIs are provided in the Supp. Inf. (Figs. S30 - S54).

Activity of individuals at night time (25.8%) took place mainly in the rice fields or at sea (Fig. 2 and Fig. 3a). On work days birds concentrated their activity at sea during the study period in areas around 50m depth, coinciding with areas of high intensity of purse-seine activity. It is remarkable that on weekends night activity at sea was similar to work days, although there is not purse-seine activity. In these cases birds distributed at sea between the coast and 50-75m depth or concentrated near the main fishing ports (Fig. 2 & Fig 3a). Activity observed on rice fields at night was similar between workdays and weekends.

During the morning and the central hours of the work days (10-16h), the SMDs showed the better suitability indexes at sea, and the Audouin's gull activity at sea was maximum, while on weekend's suitability indexes and activity was lower (Fig. 2 & Fig. 3b). By contrast, the activity in the rice fields increased during the weekend. At sea the gulls showed a widespread distribution over most of the continental shelf off the Ebro Delta, but they avoid the marine area under trawling moratorium (Fig. 3b). In subsequent hours (16-18h), when trawlers return to the port, gull abundance at sea was reduced (Fig. 2), and better suitability indexes at sea were close to the coast, especially near main fishing ports (Supp. Inf.). After 18h, Audouin's gulls were not at sea on work days. Normally, at this time we observed birds returning to the colony or concentrating in fishing ports and rice fields (Fig. 2 and Fig. 3c).

The daily pattern at this time differs on weekends, when birds made a far lower use of the open sea, but tended to show some activity both during the central hours of the day and also in the late afternoon. In this case the cells with the best suitability indexes at sea were relatively near the coast, in depths around 50m and both north and south of the breeding colony, independently of the moratorium area, and also in areas with high *Chl-a* concentration (Fig. 3b). Rice fields were more important during weekends, especially from noon until night (12-24h, Fig. 2), and rice fields situated above 4 m.a.s.l. showed a better suitability index than those located at lower altitudes (Fig. 5j).

The variables that most contributed to the SDMs were *ColonyDist*, *PortsDist* (for both further distance implies lower probability of presence; Fig. 5f,g), *Chl-a* and *SST* (Fig. 4). *ColonyDist* is for most time intervals the variable with a higher contribution. This variable has a similar response in work days and weekends during night time and until noon. However, after noon *ColonyDist* has lesser contribution in weekends than in work days (Fig. 4e). *PortsDist* is important, especially at night time (specifically between 22-04h) in both weekends and workdays. Variables related to productivity, especially *Chl-a*, showed an important contribution to the model, especially on the afternoon and the night time during weekends (Fig. 4b). *Bathymetry* did not show an outstanding contribution, probably because other variables related to productivity (*Chl-a* and *SST*) or *PortsDist* (closely related to the distance to shoreline), masks its effect. In TIs with fishing activity, both *PurSeDe* and *TrawDe* take important values of contribution (Fig. 4a), higher vessel density implies a greater probability of presence (Fig. 5a,b). Those variables exclusively terrestrial have a lower contribution to the overall model, probably because these habitats occupy a small percentage of the total study area. In that case the variable with more influence on Audouin's gull distribution is *RiceFieldsMASL*, especially on weekends and highlighting from noon and during all afternoon on weekends (Fig. 3c and Fig. 4i), followed by *PortsCov* that is relevant (despite its small size) during weekend night hours (Fig. 4g).

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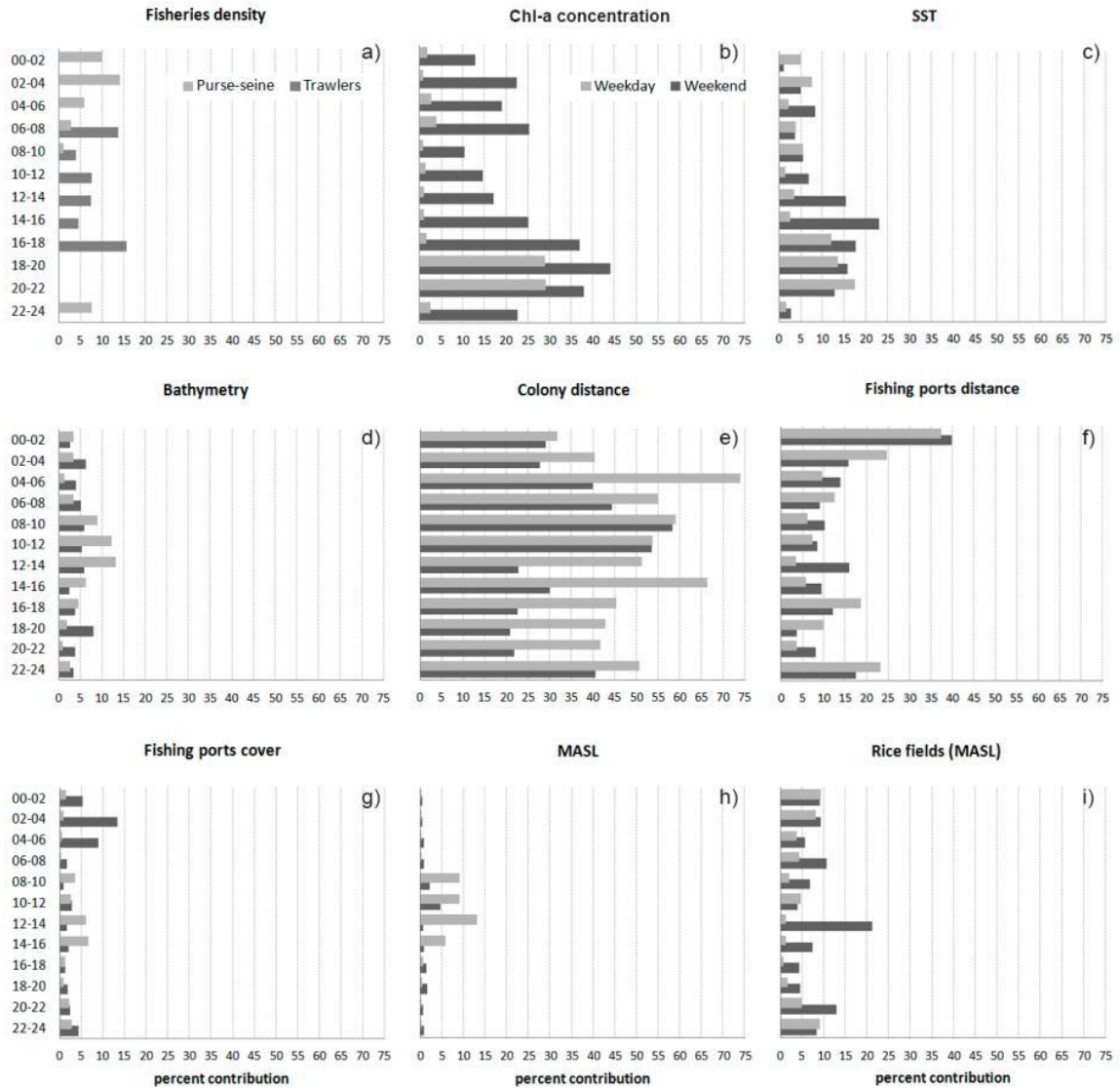


Figure 4. Variables percent contribution by time interval. Contribution of both purse-seine and trawlers (a) are showed in the same picture (only for workdays). For all other variables (b-i) the percent contribution for workdays and weekends are showed jointly.

- a- Fisheries density
- b- Chlorophyll a concentration (Chl-a)
- c- Sea Surface Temperature (SST)
- d- Bathymetry
- e- Colony distance
- f- Fishing ports distance
- g- Fishing ports cover
- h- Altitude above sea level (MASL)
- i- Rice fields altitude (Rice fields MASL)

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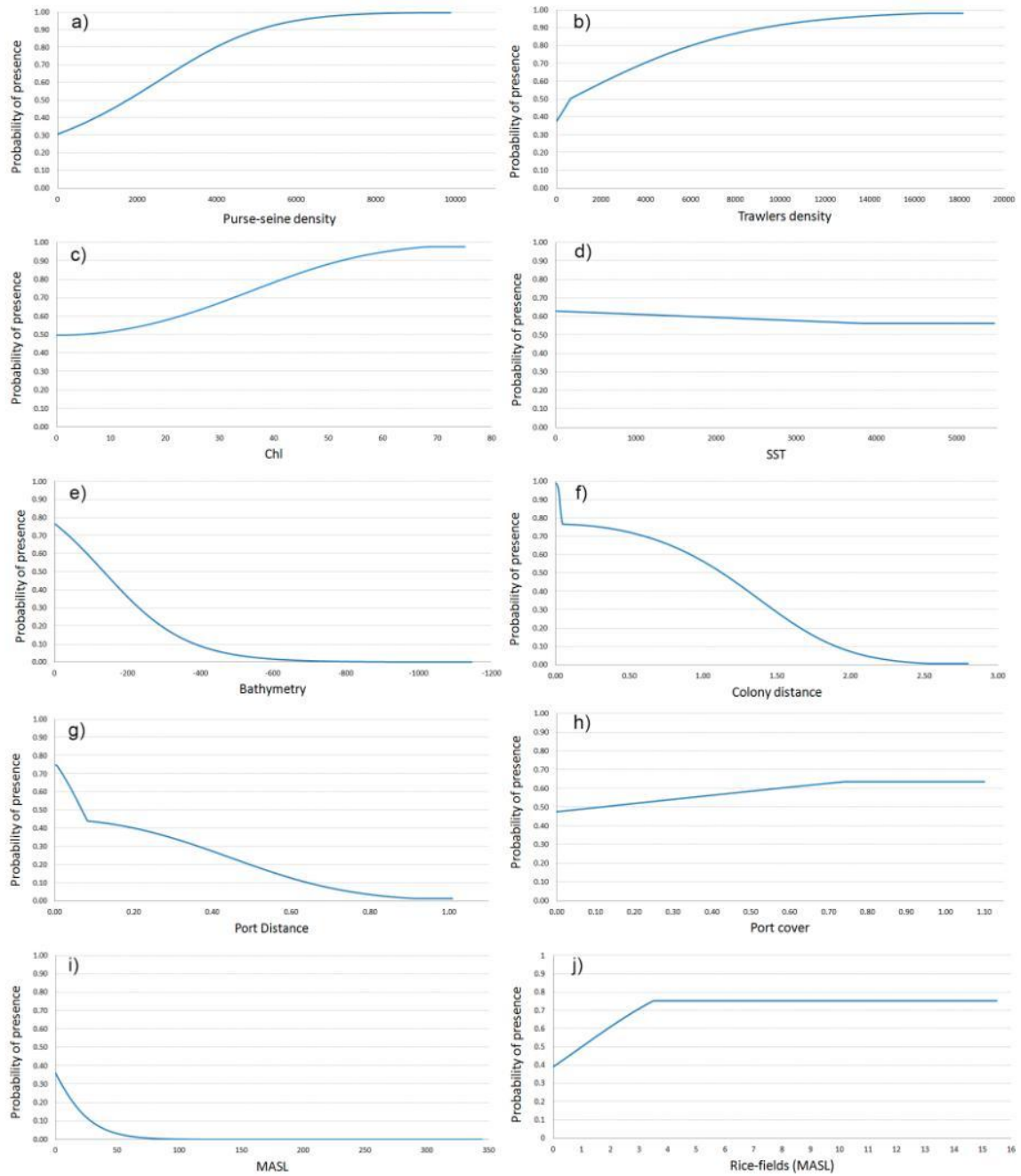


Figure 5. Response curves relating the probability of presence of Audouin's gull. Higher values correspond to higher probability of presence. The curves show how the logistic prediction changes as each environmental variable is varied, keeping all other environmental variables at their average sample value. The figure shows only a representative response curve for all time intervals, both for workdays and for weekends.

4. Discussion

The present work is the most accurate spatio-temporal study carried out in Audouin's gull so far, thanks to: (1) the high precision and frequency of GPS data; (2) the use of detailed information on fishing activity (VMS system); (3) the joint modelling of both marine and terrestrial environments; (4) the consideration of different time intervals and different fishing situations (workdays vs. weekends); and (5) the implementation of a trawling moratoria in half of the study area. Indeed, all these factors together allowed an accurate assessing of distribution and activity patterns in breeding adult Audouin's gulls under different situations, and provide an outstanding insight on how different factors influence the ecology of this near threatened seabird. As far as we know, the combination of marine and terrestrial environmental variables in the same modelling area had not been addressed before. This is important to get a complete view of the ecology of many gulls and terns, since they commonly use both environments extensively, although studies are usually focused on only one of them. On the other hand, this could also reduce the accuracy of the model distribution results in both environments, masking information that might be relevant, but providing an overview of high interest. In the case of Audouin's gull, only two works have considered before the marine and freshwater distribution simultaneously (Mañosa *et al.* 2004, Christel *et al.*, 2012). However, the methodologies used in these studies present some spatial and temporal limitations (see Introduction) and they did not apply any modelling methodology.

The study also confirms the wide foraging range and foraging flexibility of Audouin's gulls, with trips lasting up to 3 days (77.3 hours), alternating different daily periods and habitats, and extending up to 190 km from the colony (Figure S2 in Supp. Inf.). This is quite an extreme strategy for a gull, since in most species trip duration does not exceed a few hours and is restricted to a few 10s of km (Mañosa *et al.*, 2004).

Fisheries define spatio-temporal Audouin's gull distribution at sea and on land

During trawling activity (8:00h to 18:00h), at sea activity of Audouin's gulls was higher on work days than on weekends, fully matching with the distribution of trawlers. Moreover, the gulls tended to avoid the area under trawling moratorium. This confirms the great influence of trawling discards, an easily accessible and predictable food resource, on the ecology of this species. In fact, some authors have estimated that trawling discards account for more than 75% of the energy required by breeding Audouin's gulls and represent the bulk of their marine diet (Arcos 2001, Oro *et al.*, 2013). When trawling activity finishes (18:00h), gulls head towards the fishing ports and

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towards the colony after having met their energy requirements. Afterwards, the probability of finding gulls at sea is very low.

Regarding nights on work days, purse-seine activity appeared to define the spatio-temporal distribution of the gulls at sea. Both gulls and purse-seiners concentrated during the study around the 50 m depth isobath, where these vessels most often target sardines. During this association, gulls could be fishing by themselves (taking advantage of the vessel light) or exploiting the purse-seine discards (Arcos & Oro, 2002a). However, during the weekend's night time (when purse-seiners do not operate) the gull activity at sea was similar -or even higher- than on workdays, although birds were more disperse (up to 75-100 m depth). These best suitability areas during this night periods on weekends match with high productive areas, as shows the contribution of the variable *Chl-a*. Probably, almost full moon and clear sky conditions during the study period would facilitated the natural fishing in this species, adapted to the capture of Clupeiform fish at night (Wallace 1969, Witt *et al.*, 1981; Cramp & Simmons, 1983). This is also supported by the greater activity observed both, at sea and in rice fields during the moonlight period, especially obvious for the first weekend of data which comprise the 84.4% of total weekend data (moonlight from dusk until 04:40 (TPE, 2013); Table S4 in Supp. Inf.).

Fishing activity also appeared to influence the distribution of Audouin's gulls in neighbouring rice fields. It is especially remarkable that on weekends the rice field's suitability index and the activity patterns were significantly higher than on workdays, especially from noon until midnight (12-24h), probably due to the food shortage caused by the absence of fishing activities. This suggests that this habitat is of secondary importance for the gulls, although the American crayfish represent an abundant and accessible food resource. The fact that rice fields showed higher suitability indexes that the sea is probably due to the far smaller area occupied by this resource compared to the marine environment, thus concentrating this habitat all the gulls in a very limited extension. Moreover, Audouin's gulls spend more time resting at rice fields than at sea. It is noteworthy that rice fields below 4 meters above sea level showed lower suitability index than rice fields located at higher altitudes. This is probably related to the higher salinity of these soils which prevent crayfish development (Canicio *et al.*, 2008).

Synthesis and applications

In spite of the great influence that fishing activities appear to exert on the distribution and activity patterns of Audouin's gulls, data presented here can not properly assess the relative importance of natural foraging relative to scavenging about

fishing vessels. Moreover, the overexploitation of small pelagic fish stocks, as well as their pelagic predators such as tunas (that during their foraging maneuvers force small pelagic fish to the surface), reduce the availability of natural marine prey to non-diving seabirds, such as Audouin's Gull (Arcos *et al.*, 2008). This situation implies that natural fishing would imply, nowadays, even more increased energy expenditure than the consumption of discards or crayfish in the rice fields. This can be a problem, because several studies suggest that diet from discards is energetically worse than coming from pelagic fish (Wanless *et al.*, 2005; Grémillet *et al.*, 2008). Nevertheless, we observed that when there are not availability of discards Audouin's gull also feeds at sea to some extent, both during the day and at night, most probably feeding small pelagic fish.

Fishing pressure has led to widespread overexploitation of fish stocks worldwide, with major impact in long exploited areas such as Europe (Watson *et al.*, 2004). Added to the genuine removal of commercial fish, several fleets (particularly trawlers) also produce large amounts of discards, adding unnecessary pressure to the marine environment (Kelleher 2005; Coll *et al.*, 2006). Reducing discards has become a priority to help reducing fishing pressure over the oceans, and a first step towards a model of economically and environmentally sustainable fisheries (Penas, 2007; Symes, 2009;). Within this context, the European Commission took in 2013 the resolution of banning discards in European waters, as part of the last reform of the Common Fisheries Policy. Nevertheless this new scenario does not seem the most optimal for Audouin's gull given the great dependence of this species on trawling discards. Several studies have shown how the lack of discards produced by trawling moratoria have a negative effect on the breeding performance of this species (Oro, 1999; Oro *et al.*, 2004, Bicknell *et al.*, 2013). The new European scenario could have severe effects on the breeding population of Audouin's gull, as the availability of food at sea might not be enough to sustain the current population, and the American crayfish seems a lower quality prey compared to marine fish (García-Tarrasón *et al.*, 2014). On the other hand, the ban or the reduction of discards would also affect other seabird species in the region, and this might increase competition and predation, posing an added potential threat for species of conservation concern (Arcos *et al.*, 2001, Votier *et al.*, 2004; Martínez-Abraín *et al.*, 2003). Therefore, the ban on discards should be closely monitored, and efforts should be directed at finding mitigation measures to minimize the impact on the seabird community. Ideally, discard reduction should be based on increased selectivity, and accompanied of improved management of the local fisheries to ensure the recovery of the exploited fish stocks.

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SUPPLEMENTARY INFORMATION***Tagged bird's information (data collected)*****Table S1.** Tagged Audouin's gulls information. FT: foraging trip; WD: workday; WE: weekend.

Metallic ring	Track time (days)	N° locs (Total)	N° locs (FT)	N° FT	Average FT time (hours) $\pm$ SD	N° locs (WD)	N° locs (WE)	Available days	
								WD	WE
5107912	11.23	3175	915	17	4.49 $\pm$ 3.68	704	211	9	3
5107913	4.79	1380	669	5	11.15 $\pm$ 10.21	514	155	4	1
5107916	9.91	2834	1110	8	11.56 $\pm$ 9.02	687	423	7	2
5107917	12.20	3508	1981	18	9.17 $\pm$ 8.23	1483	498	10	3
5107918	9.89	2794	747	12	5.19 $\pm$ 2.92	577	170	7	3
5107919	3.06	875	215	4	4.48 $\pm$ 1.92	191	24	3	1
5107922	9.55	2741	1092	15	6.07 $\pm$ 4.04	792	300	8	3
5107923	0.82	237	119	2	4.96 $\pm$ 6.31	113	6	1	1
5107925	11.55	3300	1245	10	10.38 $\pm$ 8.62	1144	101	10	2
5107927	9.79	2796	897	16	4.67 $\pm$ 2.71	706	191	8	2
5107928	6.44	1826	745	7	8.87 $\pm$ 5.25	629	116	5	1
5107929	11.48	3280	803	12	5.58 $\pm$ 3.22	690	113	9	2
5107930	3.17	911	431	4	8.98 $\pm$ 8.5	431	0	4	0
5107932	1.69	509	187	3	5.19 $\pm$ 3.05	187	0	3	0
5107935	14.75	4222	2386	15	13.26 $\pm$ 10.53	1880	506	12	4
5107936	11.75	3395	1807	10	15.06 $\pm$ 16.55	1330	477	10	3
5107938	4.49	1283	410	9	3.8 $\pm$ 3.16	410	0	5	0
5107940	11.96	3428	2189	7	26.06 $\pm$ 24.37	1465	724	9	3
5107941	8.92	2561	1353	8	14.09 $\pm$ 16.05	966	387	7	2
5107942	15.50	4418	1861	23	6.74 $\pm$ 4.2	1378	483	12	4
5107943	15.60	4467	2401	8	25.01 $\pm$ 22.55	1392	1009	9	4
5107944	9.53	2693	879	11	6.66 $\pm$ 4.44	757	122	8	2
5107948	9.77	2795	1283	12	8.91 $\pm$ 8.16	991	292	9	2
5107950	12.80	3652	950	13	6.09 $\pm$ 4.22	598	352	8	4
5107953	8.15	2324	1021	7	12.15 $\pm$ 10.7	798	223	7	2
5107955	8.59	2461	1051	12	7.3 $\pm$ 6.44	721	330	7	2
5107956	11.01	3140	1427	12	9.91 $\pm$ 4.05	1118	309	9	2
5107957	8.72	2495	829	13	5.31 $\pm$ 3.24	605	224	7	2
5107959	6.94	1986	542	6	7.53 $\pm$ 2.06	423	119	5	2
5107961	3.63	1046	421	6	5.85 $\pm$ 2.58	376	45	4	1
5107962	9.64	2691	1215	11	9.2 $\pm$ 10.05	993	222	9	2
6030764	9.85	2795	1580	8	16.46 $\pm$ 15.06	1235	345	7	2
6068786	2.08	600	244	2	10.17 $\pm$ 0.12	244	0	2	0
6072630	11.59	3339	1079	24	3.75 $\pm$ 3.22	839	240	9	3
6132642	5.55	1615	1031	6	14.32 $\pm$ 10.44	736	295	5	2
6135339	7.76	2228	975	7	11.61 $\pm$ 9.05	741	234	6	2

1.1. Spatio-temporal distribution (Supplementary Information)

Figure S1. Locations of tagged Audouin's gulls (yellow). The red star shows the breeding colony. The study area is delimited by a white line on sea and by black line on land. A and B lines delimited the area under trawler moratorium.

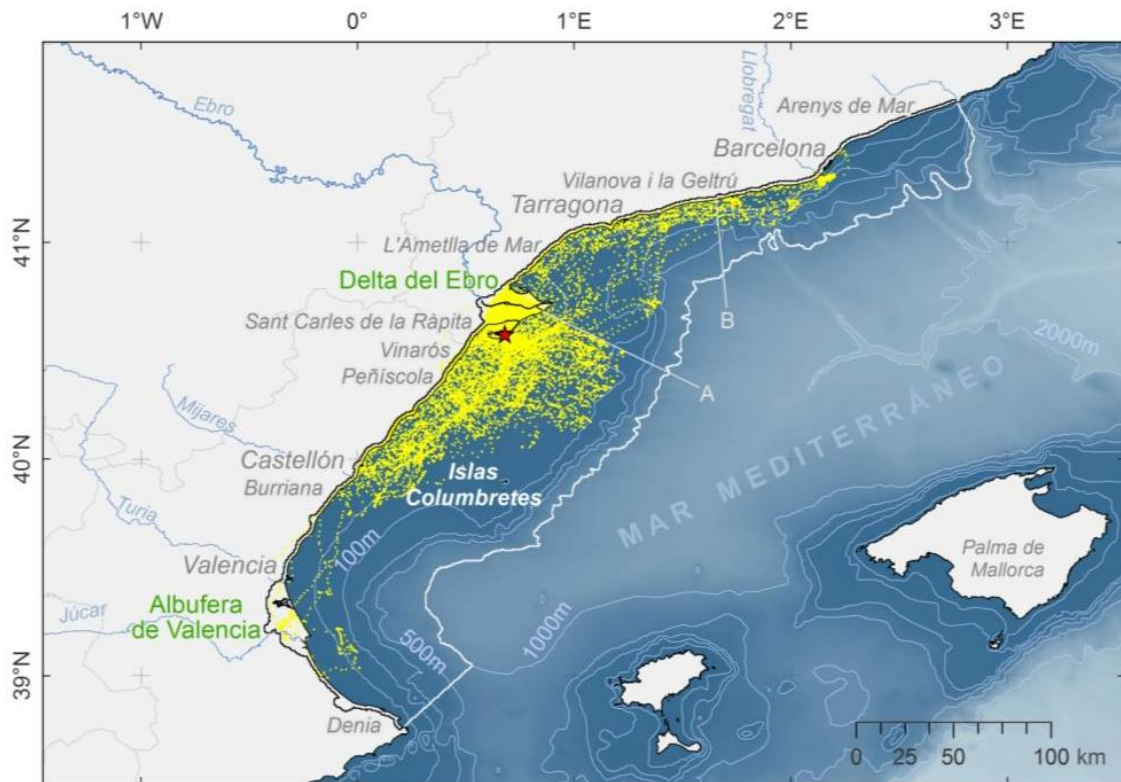
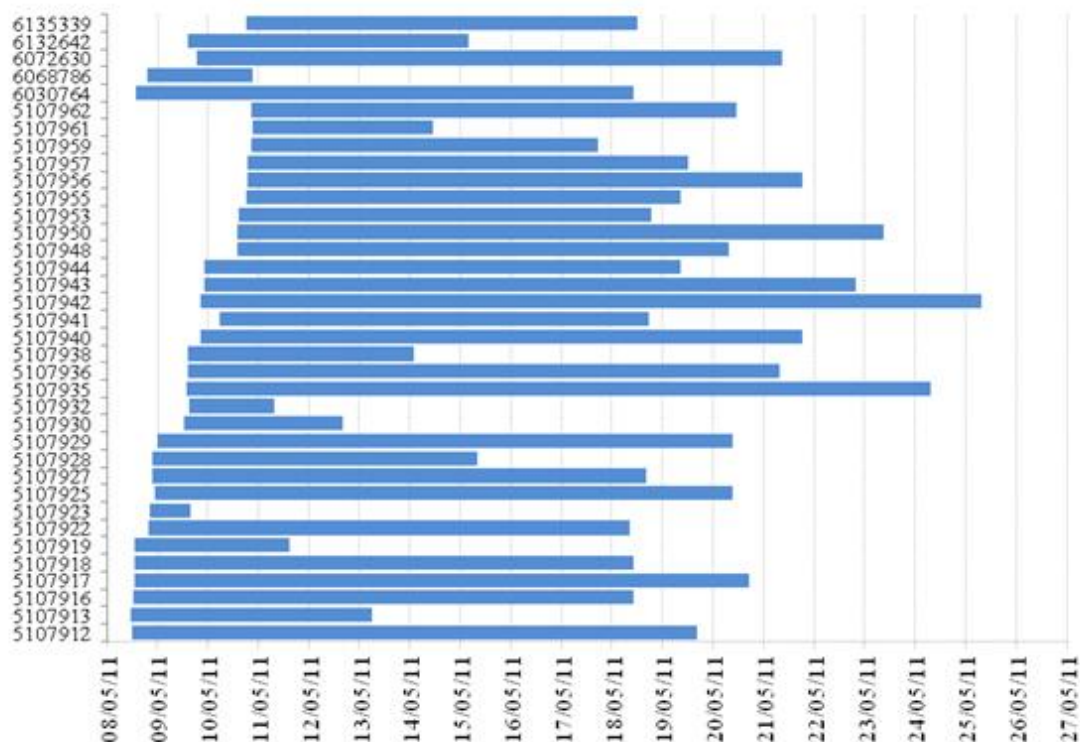


Figure S2. Available data for each Audouin's gull tagged. Weekends are showed in light gray.



1.1. Spatio-temporal distribution (Supplementary Information)

Figure S3. Duration of foraging trips (hours) Audouin gulls. The median (line), interquartile ranges (box) and minimum and maximum (dashed lines) are shown. Data that fall outside the interquartile range $\times 1.5$ are drawn as circles.

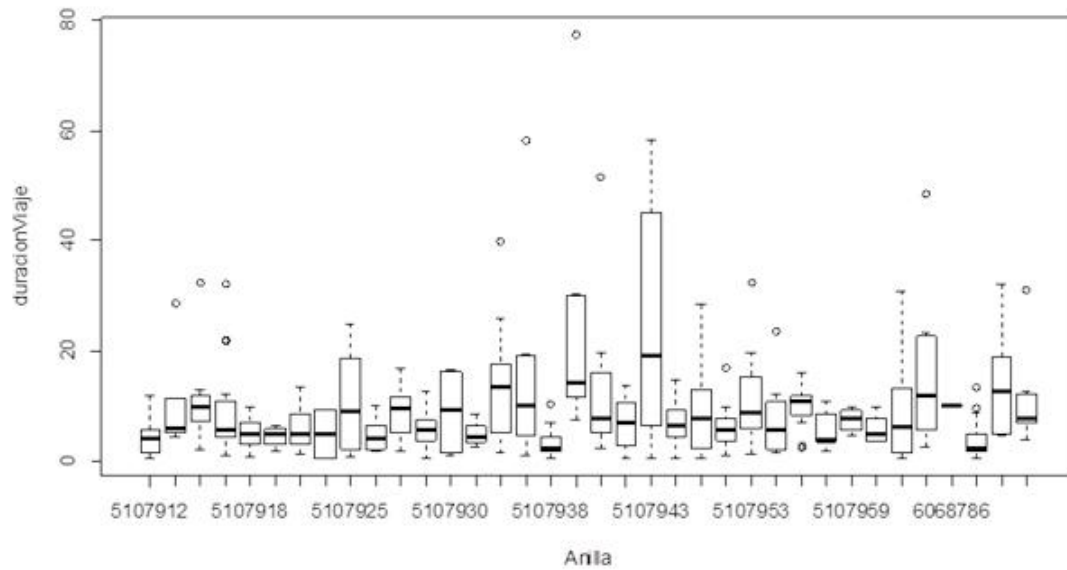


Table S2. Number of individuals with at least one data per day and per time interval for weekends. Gray shadow shows the days selected to calibrate the models for each time interval.

Available days of data	00-02	02-04	04-06	06-08	08-10	10-12	12-14	14-16	16-18	18-20	20-22	22-24
1 day	15	15	18	20	23	24	24	28	27	26	26	24
2 days	6	5	7	11	10	11	14	15	15	14	14	8
3 days	2	2	4	5	3	3	2	1	3	5	4	2
4 days	0	0	0	1	2	1	1	1	1	1	0	0
5 days	0	0	0	0	0	0	0	0	0	0	0	0

Table S3. Number of individuals with at least one data per day and per time interval for workdays. Gray shadow shows the days selected to calibrate the models for each time interval.

Available days of data	00-02	02-04	04-06	06-08	08-10	10-12	12-14	14-16	16-18	18-20	20-22	22-24
1 day	25	25	33	34	36	32	32	34	33	34	33	27
2 days	16	15	25	29	30	30	29	32	30	32	30	19
3 days	8	7	20	24	27	25	25	31	27	25	18	13
4 days	5	6	13	18	20	22	21	23	24	22	13	10
5 days	3	4	10	16	16	16	16	22	18	15	8	4
6 days	2	2	8	11	10	11	12	13	16	14	6	3
7 days	1	1	5	9	6	6	9	10	10	6	5	2
8 days	0	0	3	6	2	2	4	3	2	3	1	1
9 days	0	0	0	1	1	1	0	1	1	1	1	1
10 days	0	0	0	0	0	0	0	1	1	1	1	1
11 days	0	0	0	0	0	0	0	0	0	0	0	0
12 days	0	0	0	0	0	0	0	0	0	0	0	0
13 days	0	0	0	0	0	0	0	0	0	0	0	0

Environmental information

Table S1. Moonlight information during the study period. Weekends are showed in light gray. Data from TPE. The Photographer's Ephemeris. 2013. Software available <http://photoephemeris.com/>

Date	Day	illuminated lunar disk	moonrise	moonset	sunrise	sunset
09/05/11	Monday	30.30%	11:56	1:41	6:47	21:00
10/05/11	Tuesday	41.05%	13:04	2:17	6:46	21:01
11/05/11	Wednesday	51.80%	14:14	2:49	6:45	21:02
12/05/11	Thursday	63.10%	15:25	3:20	6:44	21:03
13/05/11	Friday	73.90%	16:38	3:50	6:43	21:04
14/05/11	Saturday	83.60%	17:52	4:20	6:42	21:05
15/05/11	Sunday	91.40%	19:07	4:54	6:41	21:06
16/05/11	Monday	96.90%	20:23	5:32	6:40	21:07
17/05/11	Tuesday	100.00%	21:35	6:17	6:39	21:08
18/05/11	Wednesday	99.60%	22:40	7:09	6:38	21:09
19/05/11	Thursday	96.80%	23:36	8:07	6:37	21:10
20/05/11	Friday	91.70%	0:00	9:11	6:36	21:11
21/05/11	Saturday	84.70%	0:23	10:16	6:35	21:12
22/05/11	Sunday	76.40%	1:02	11:21	6:34	21:13
23/05/11	Monday	67.20%	1:34	12:24	6:33	21:14
24/05/11	Tuesday	57.60%	2:03	13:24	6:33	21:15

Environmental variables

Habitat use cover calculation

To convert this categorical variable to a continuous variable, we calculated the coating of habitat per pixel (0 to 1). This has been done because of the BCN200 spatial resolution is greater than working spatial resolution. The procedure is as follows:

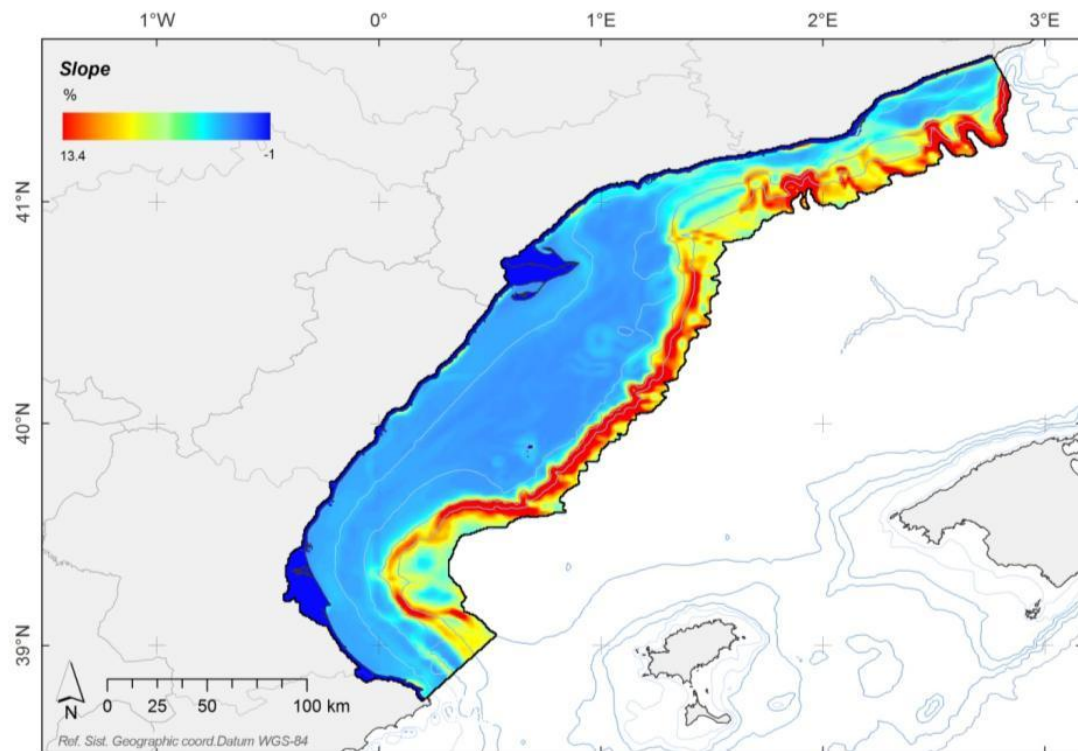
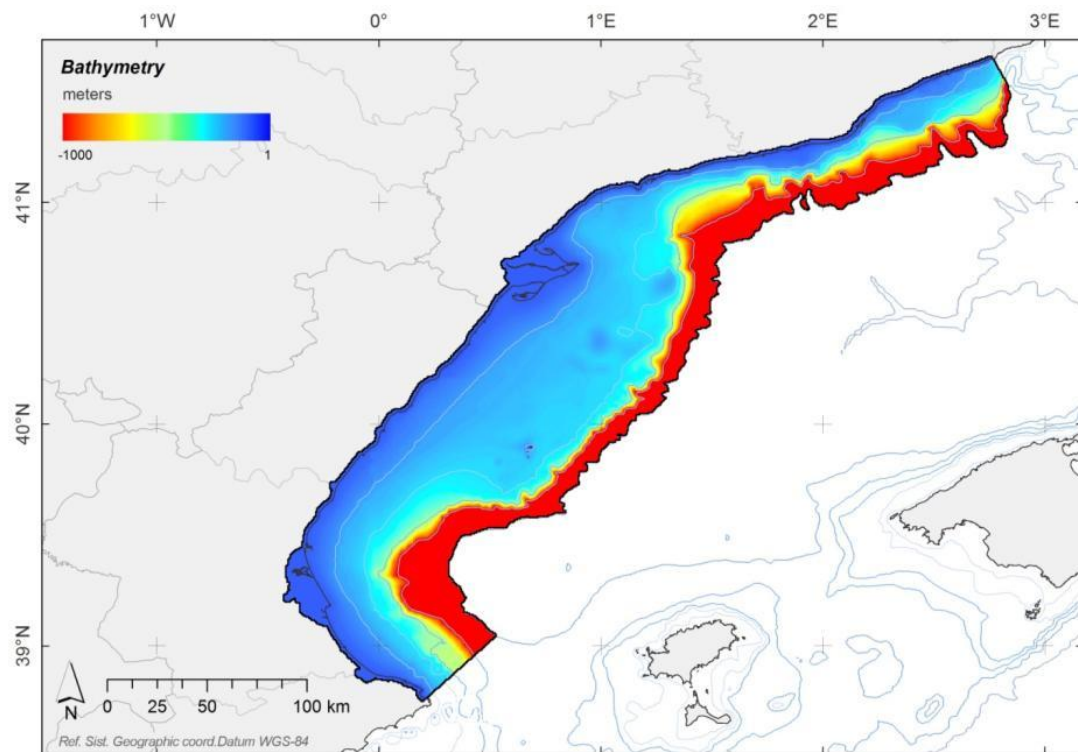
- a) Polygons (rice fields) are rasterized to a size less than 5 times the work spatial resolution (approximately coinciding with the Corine SR 100m). We used the same spatial origin in x, in this way the 25 pixels resulting are overlapping with the work grid.
- b) Using the ArcGis tool *focal statistics* and a window of 5x5 pixels, we calculated the average rice field cover of 24 pixels adjacent to each of the pixels of the study area
- c) Finally the resultant raster has densified to the spatial resolution work by bilinear interpolation. In this way is obtained for each pixel the percentage of rice field cover.

Chl-a and SST calculation

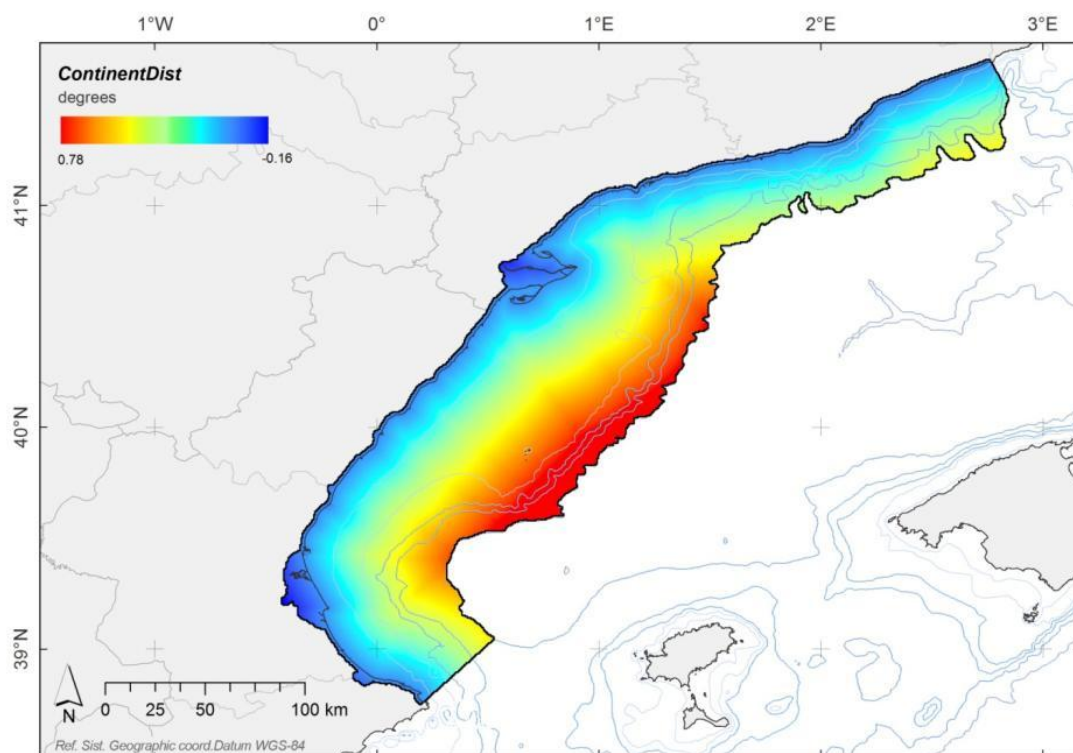
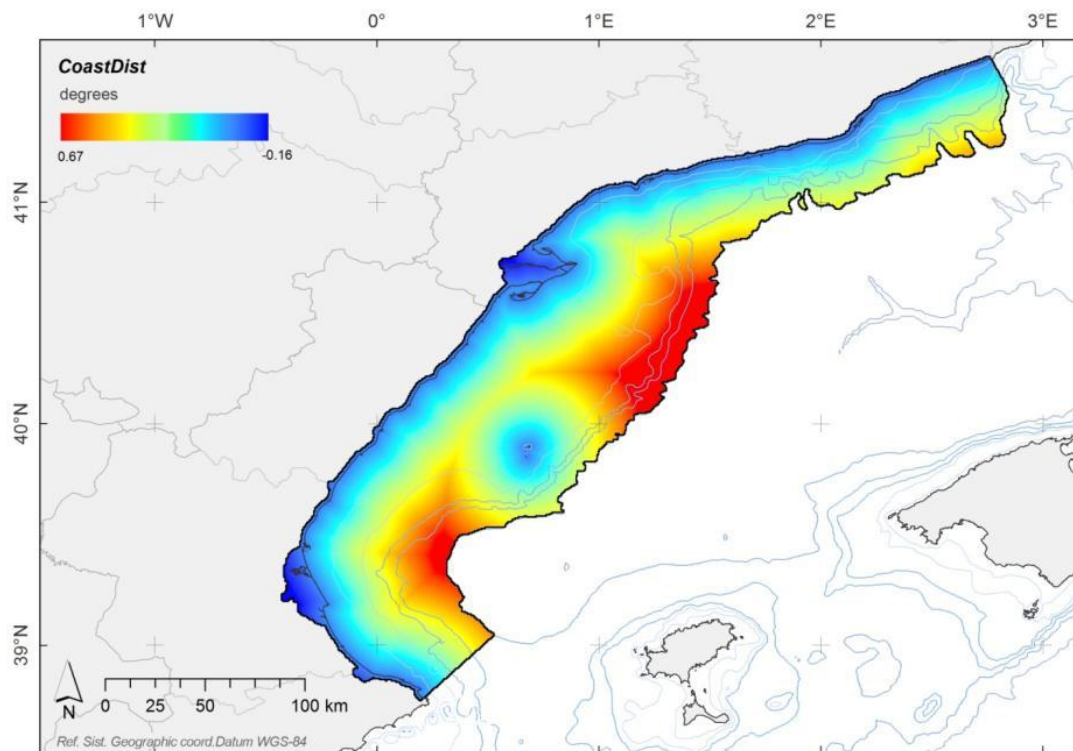
Both variables have been downloaded from the [Oceancolor web http://oceancolor.gsfc.nasa.gov/](http://oceancolor.gsfc.nasa.gov/) (Feldman, 2012) in HDF format and level 2 processing (daily images). Level 2 was selected because of provides a spatial resolution of 1 km, unlike other downstream MODIS products, whose spatial resolution is 4 km (level 3). Thus we have obtained the maximum resolution according to the working spatial resolution.

- a) All available images between 8 and 24 May 2011, both Chl-a (n = 14) and SST (n = 20), have been downloaded (both Aqua and Terra) at Level 2.
- b) The georeferencing of these images has been made from the grid points of latitude and longitude provided in the HDF itself. For this we used ENVI software tool *Georeference MODIS*. We used for reprojection the nearest neighbor to not alter the radiometric image values.
- c) Once georeferenced, have been exported to MiraMon software. In the case of the Chl-a, NODATA values correspond to areas with clouds or land, so the selection of the information has been very simple (in the absence of cloudless images for the study period, it was decided to make the average of available images).
- d) For each image (day), a mask with data areas (without clouds) has been done with MiraMon and assigned a value = 1. For each image, Nodata values have been reclassified to 0, and the sum of all this images, have been divided by the sum of the masks, to obtain the average.
- e) For SST images, clouds do not have Nodata value, but take the value of its temperature. Most of the clouds have been removed automatically since they have values outside the range of the sea temperature. In some cases had to be removed by hand digitized masks. To average SST images obtained have proceeded in the same manner as for Chl-a.

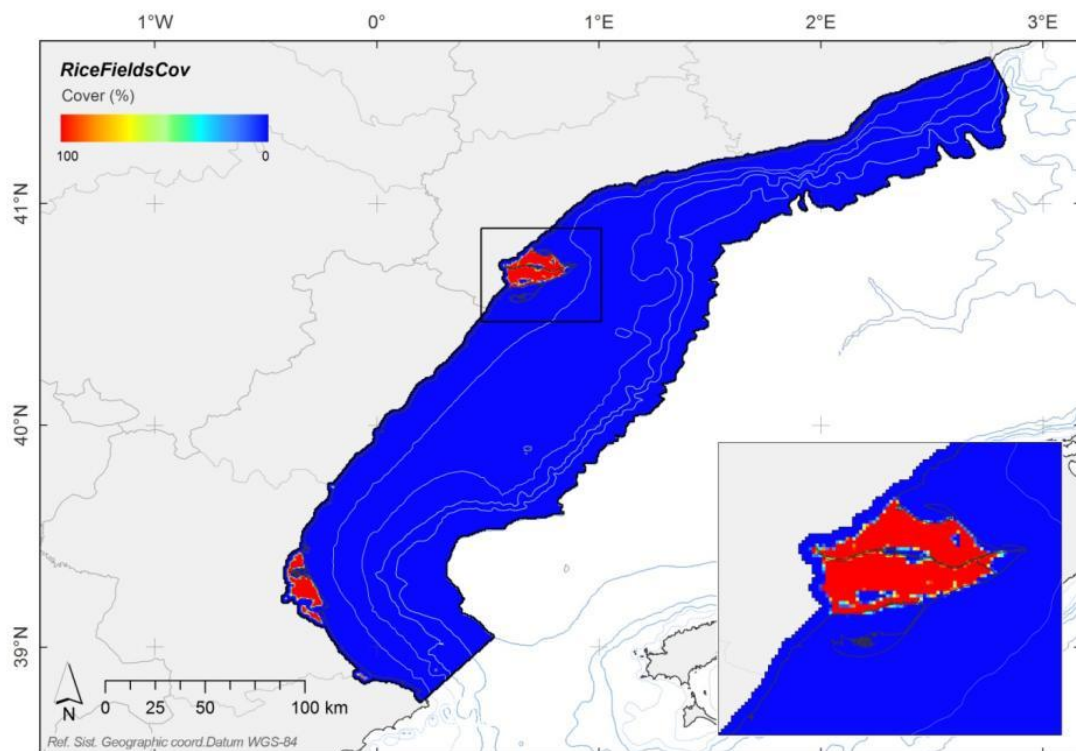
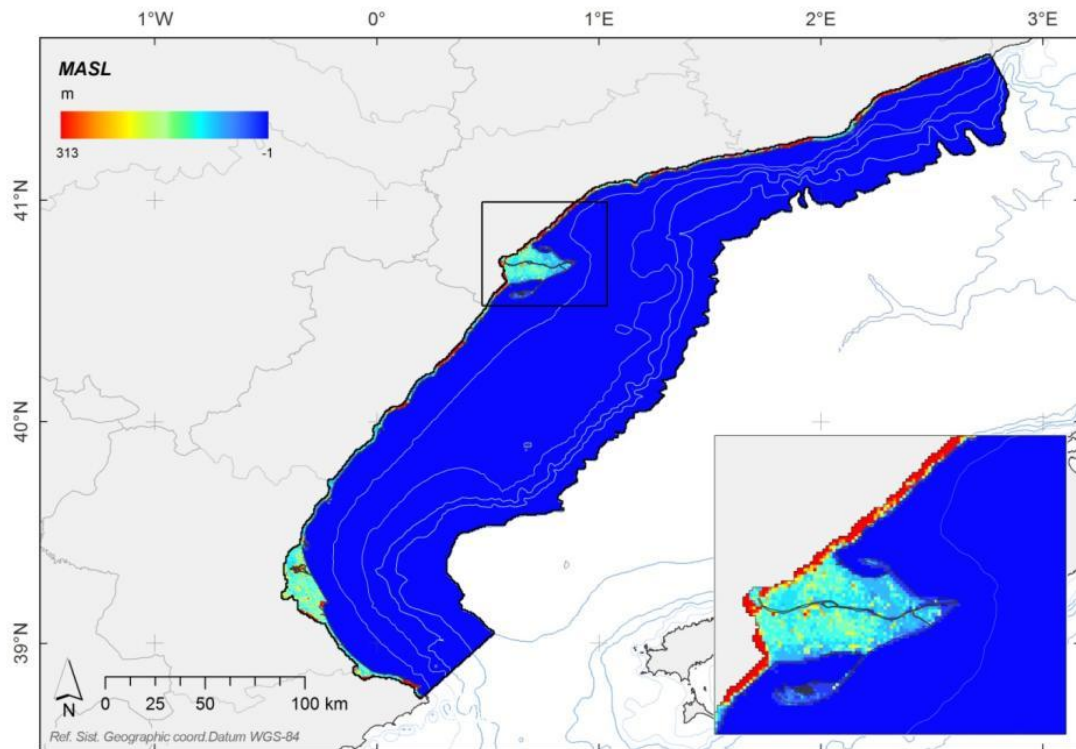
Figures S4 – S29. Environmental variables (shown in the same order as in Table 2, see article).



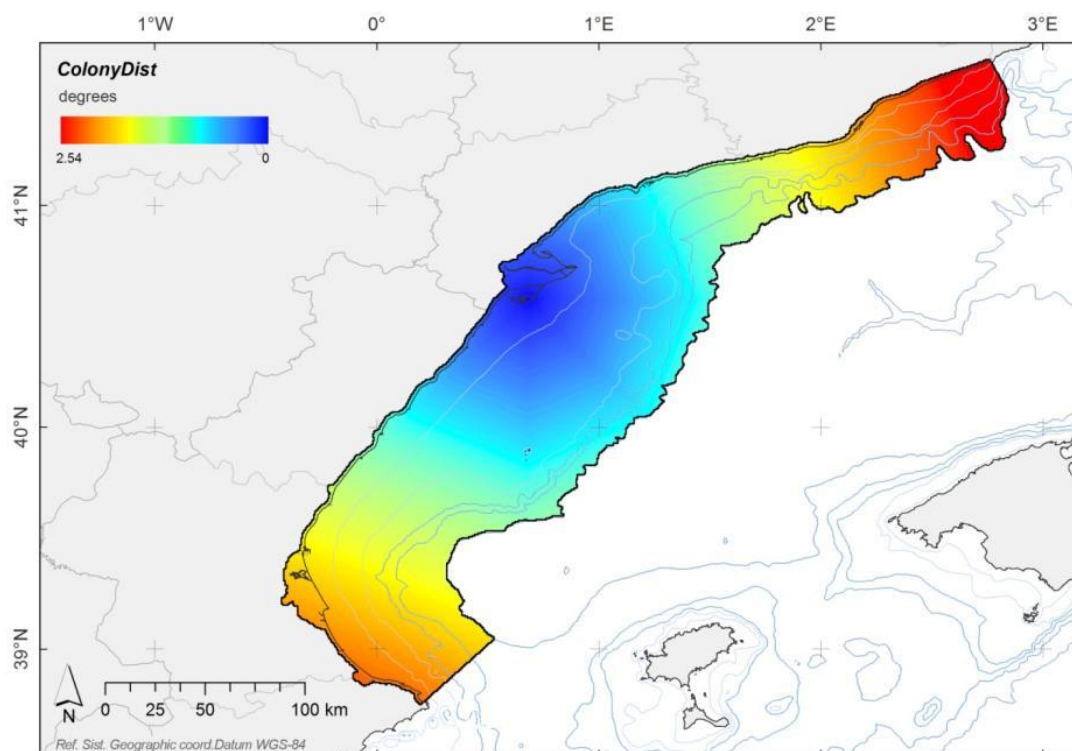
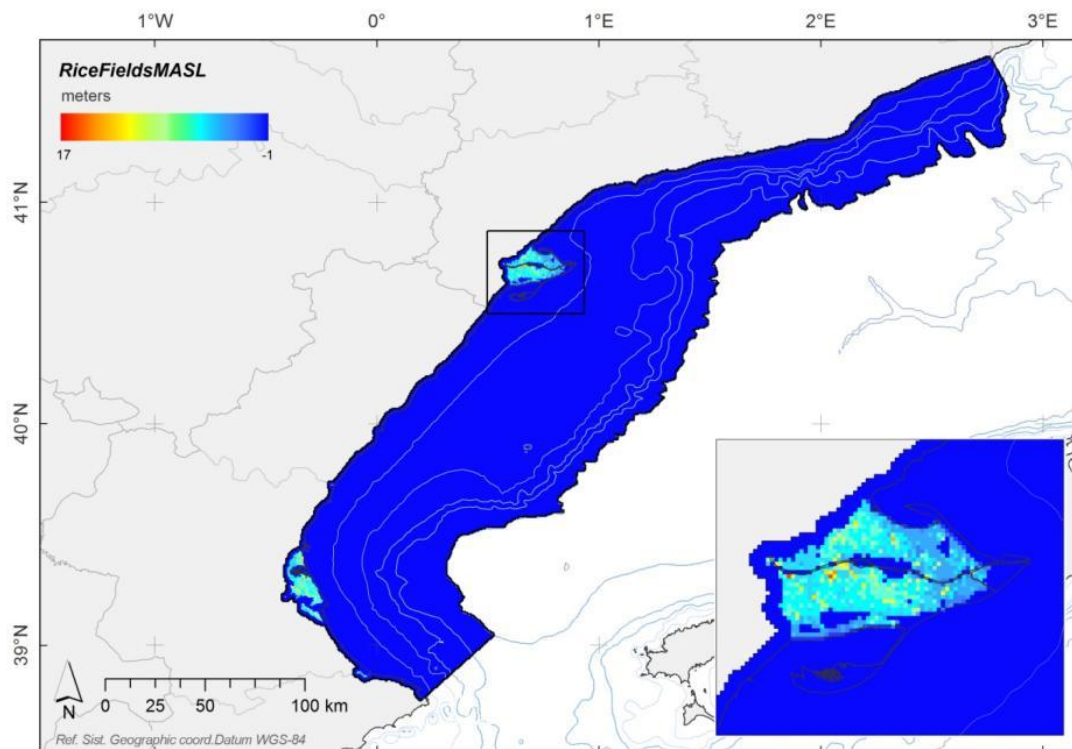
1.1. Spatio-temporal distribution (Supplementary Information)



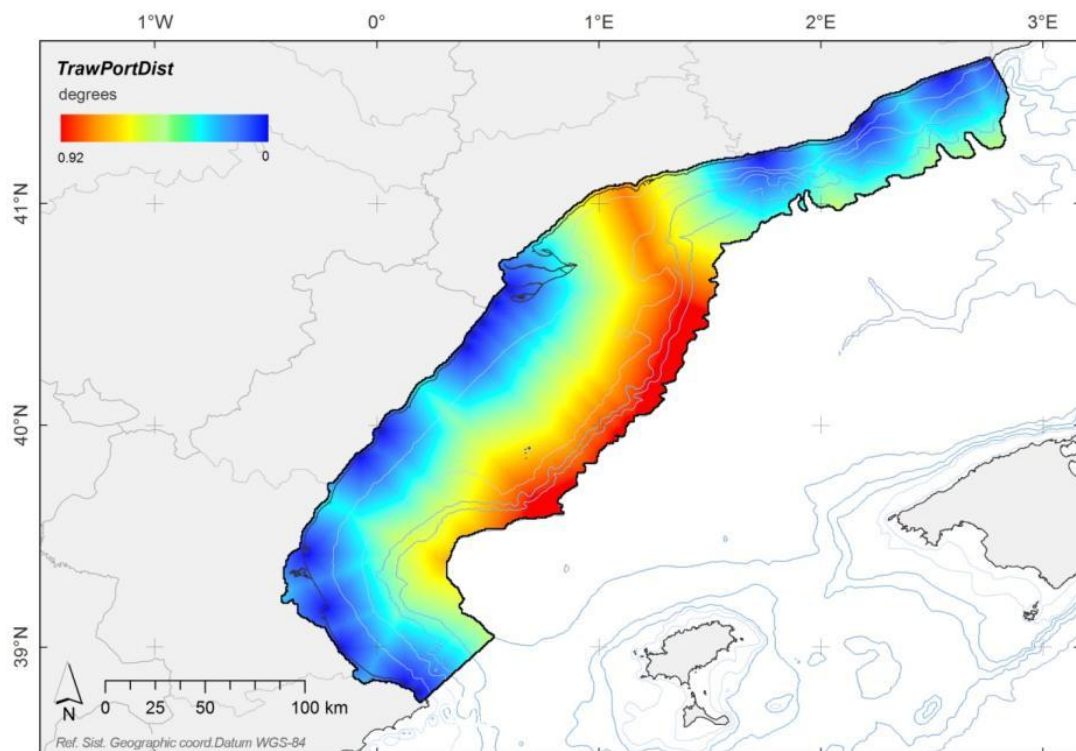
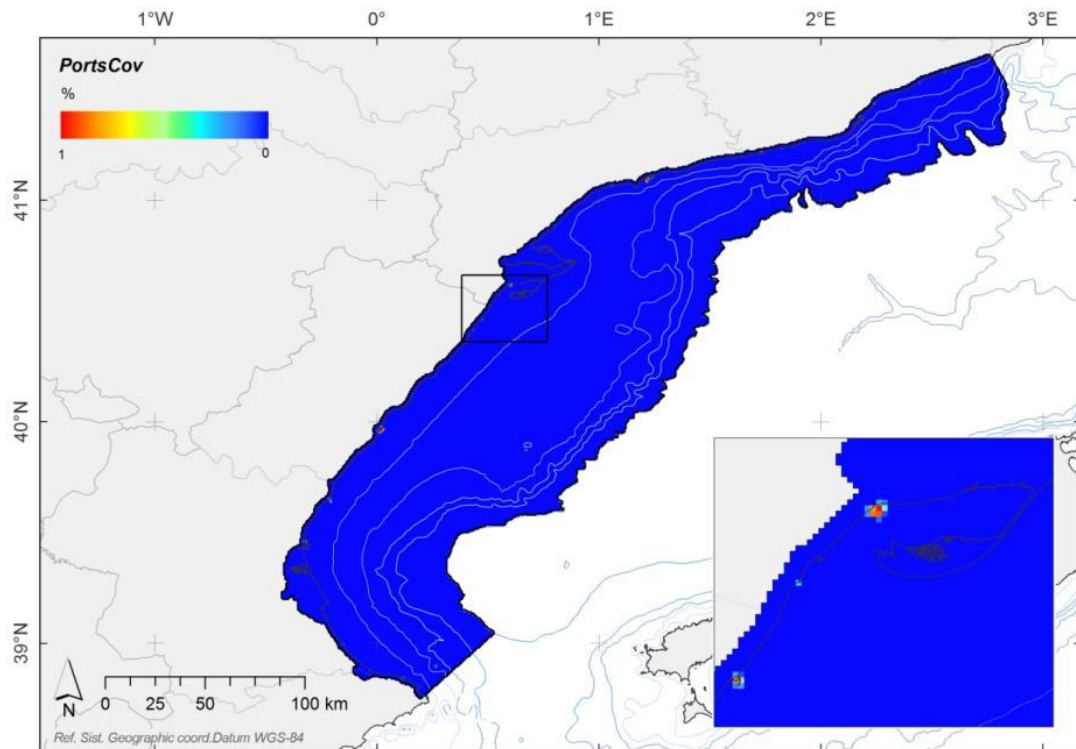
1.1. Spatio-temporal distribution (Supplementary Information)



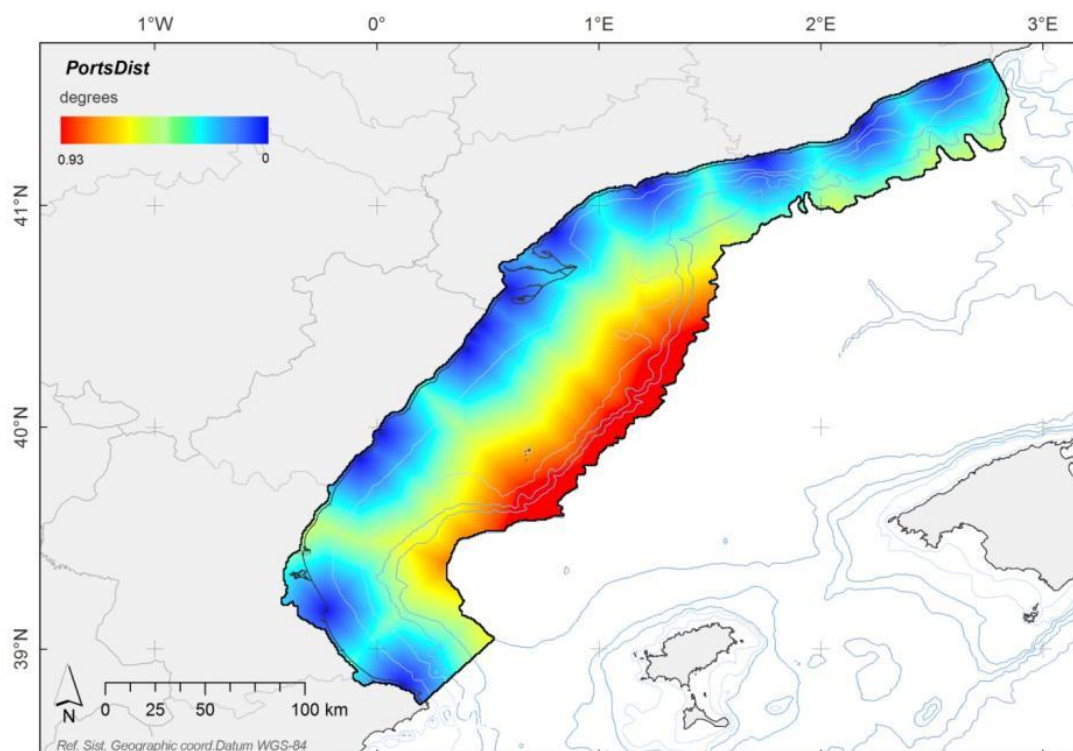
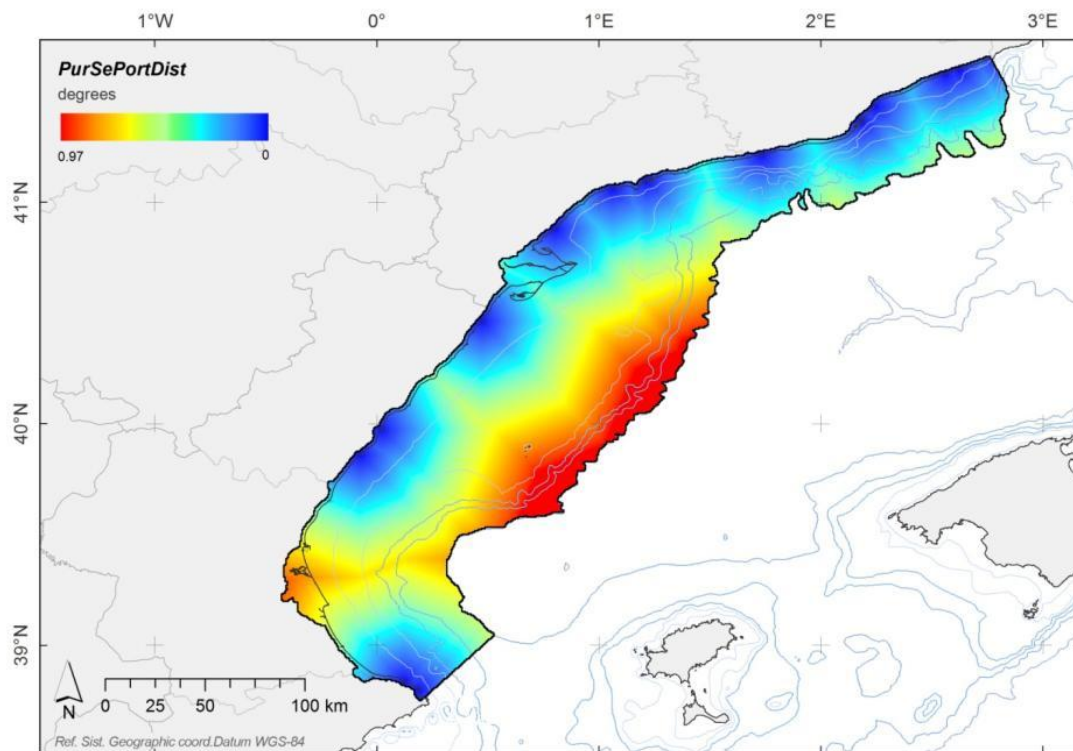
1.1. Spatio-temporal distribution (Supplementary Information)



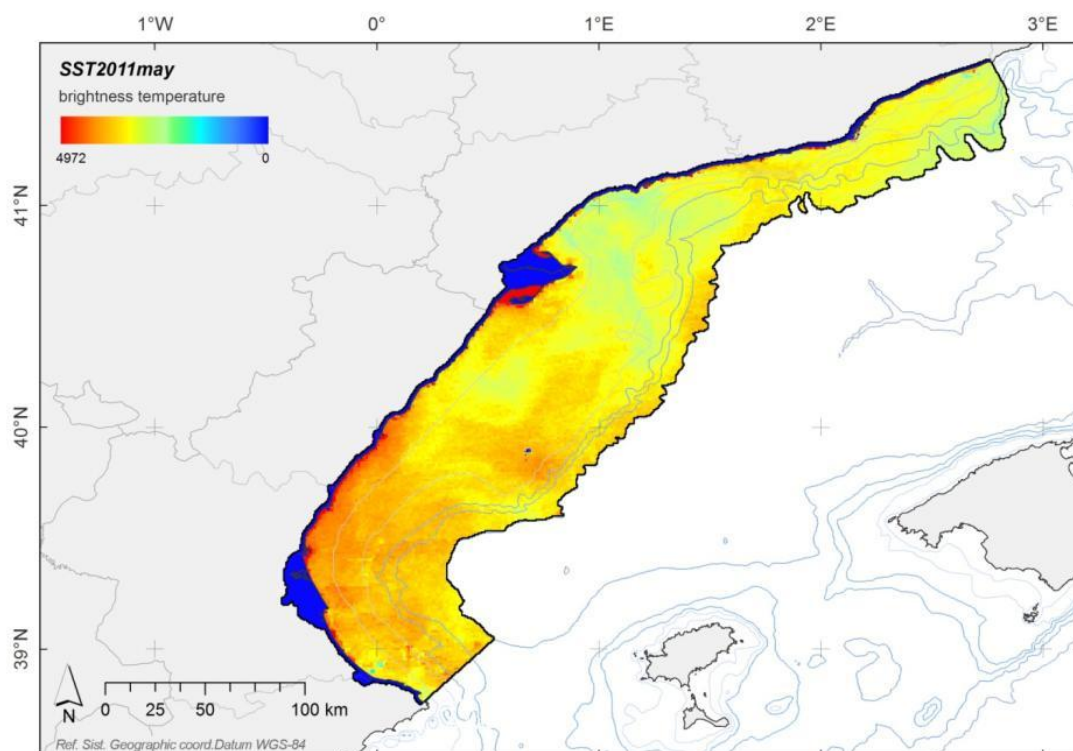
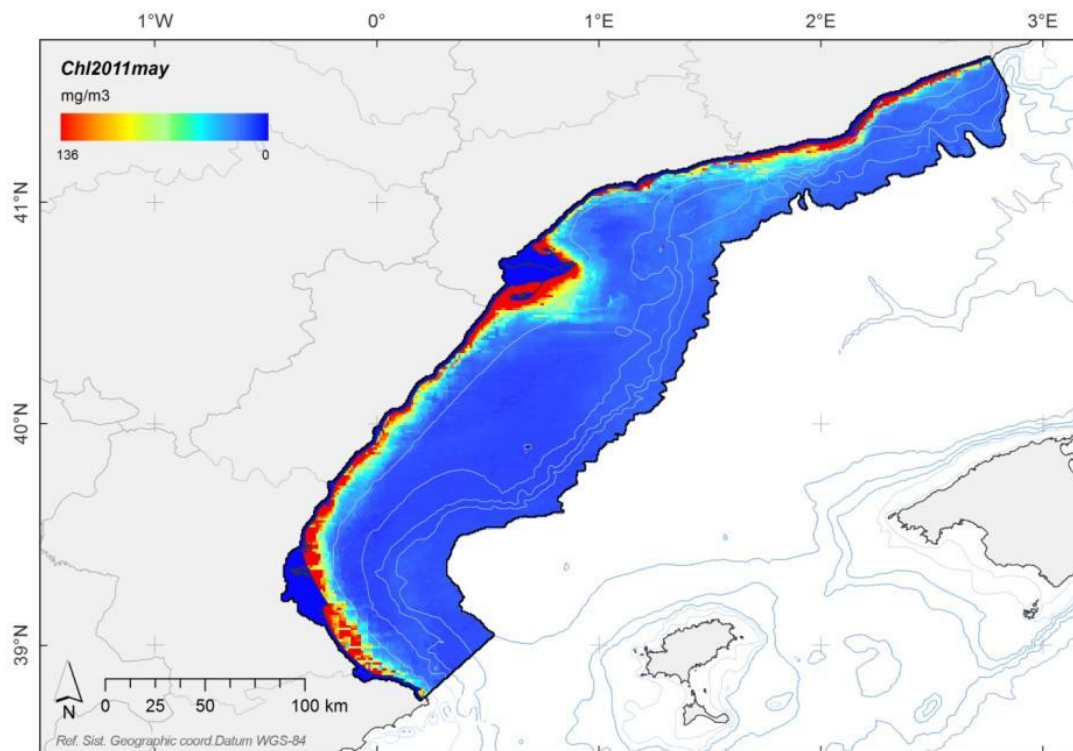
1.1. Spatio-temporal distribution (Supplementary Information)



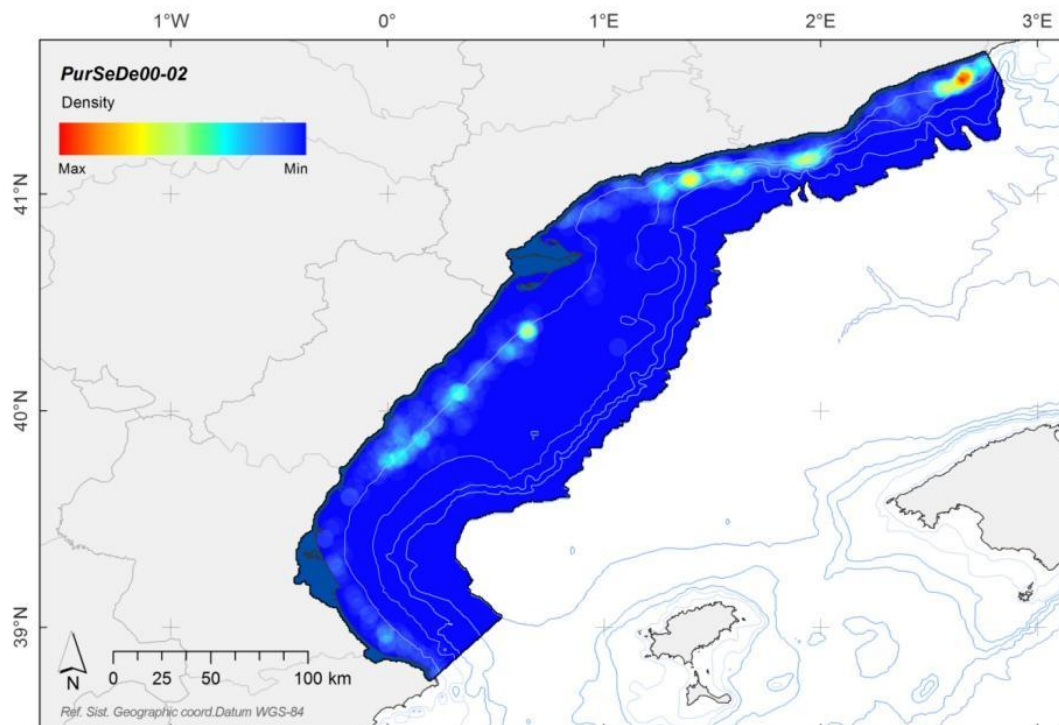
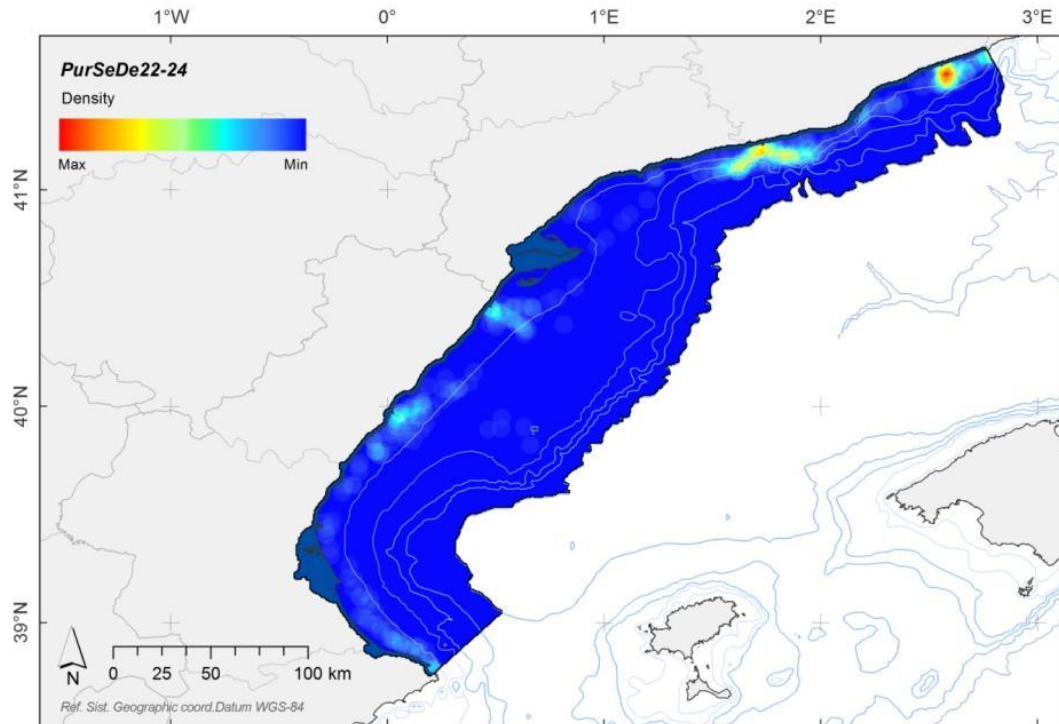
1.1. Spatio-temporal distribution (Supplementary Information)



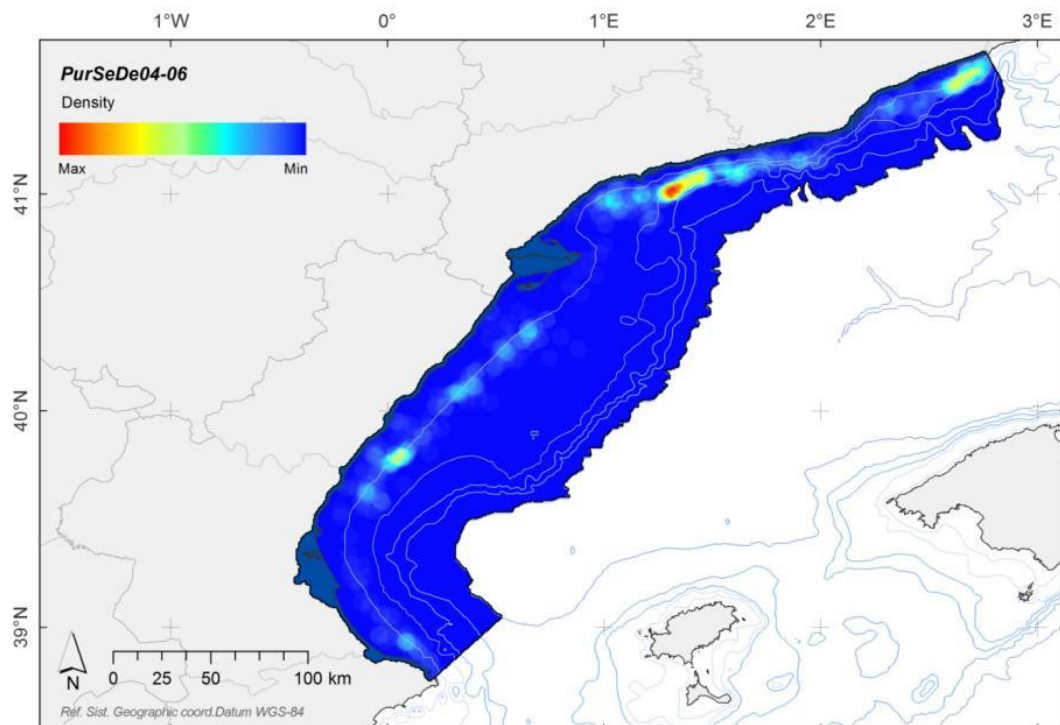
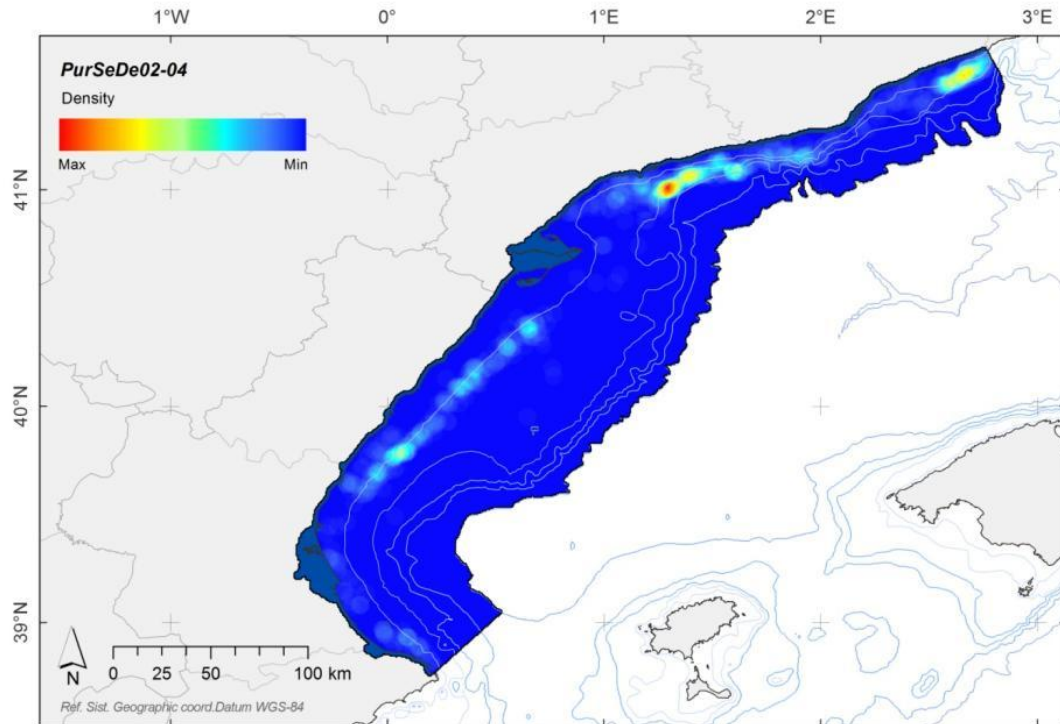
1.1. Spatio-temporal distribution (Supplementary Information)



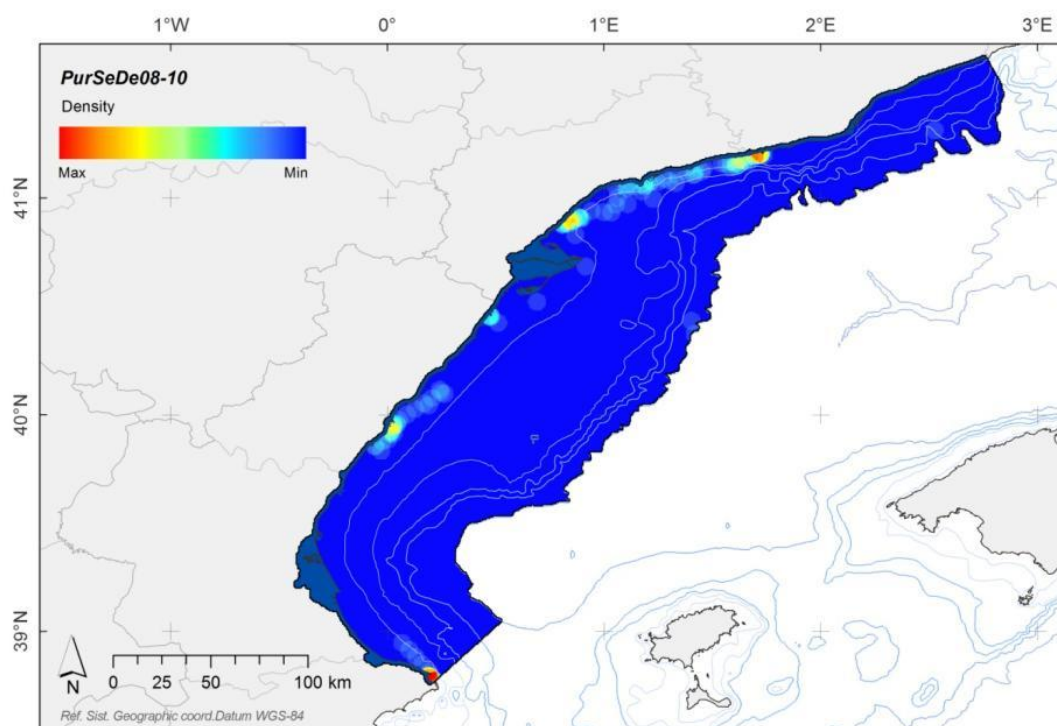
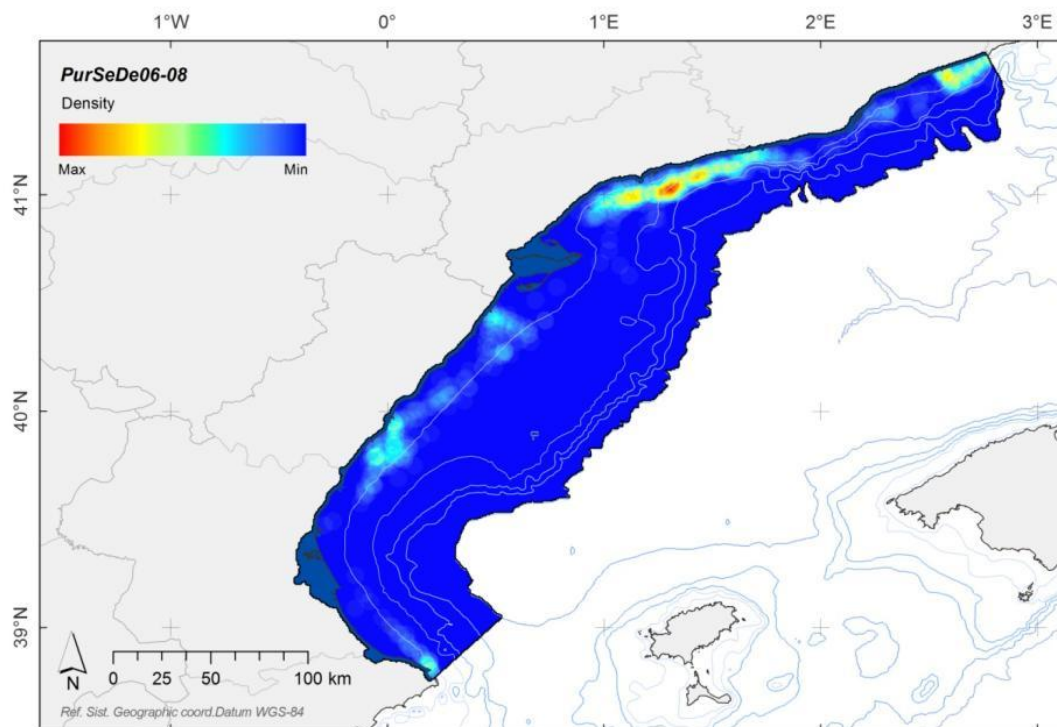
1.1. Spatio-temporal distribution (Supplementary Information)



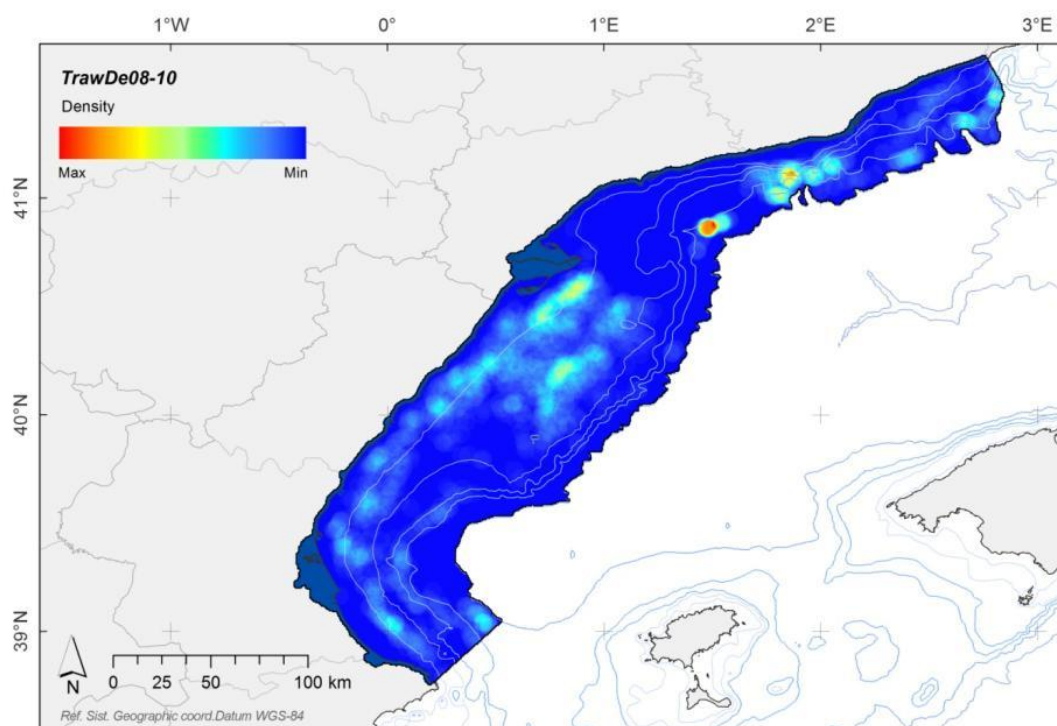
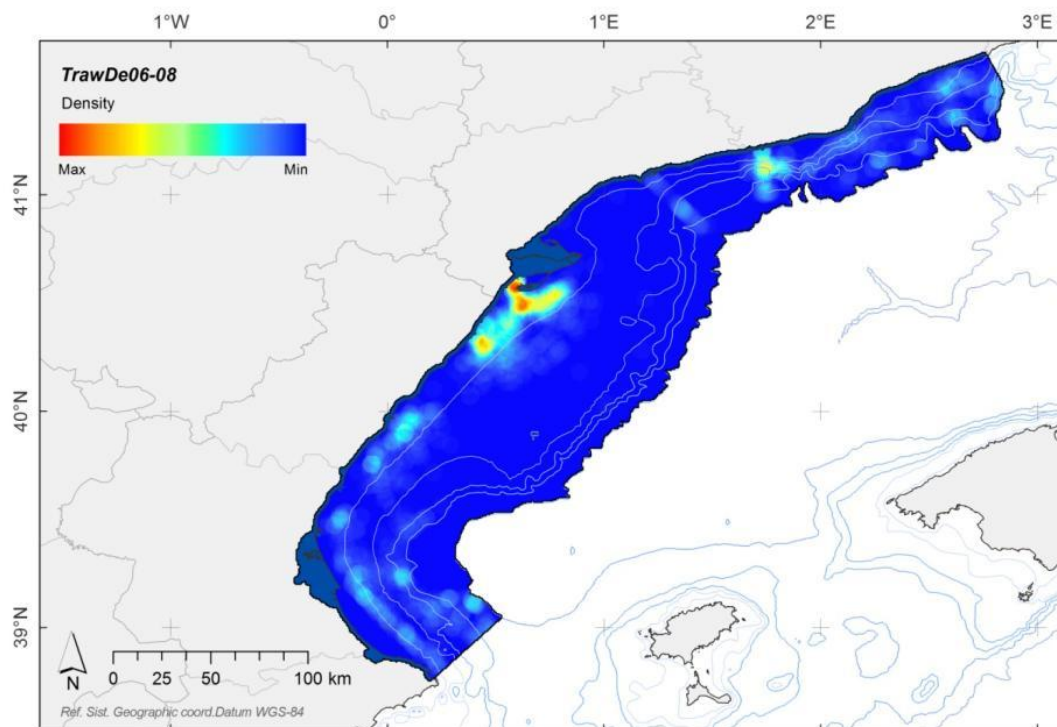
1.1. Spatio-temporal distribution (Supplementary Information)



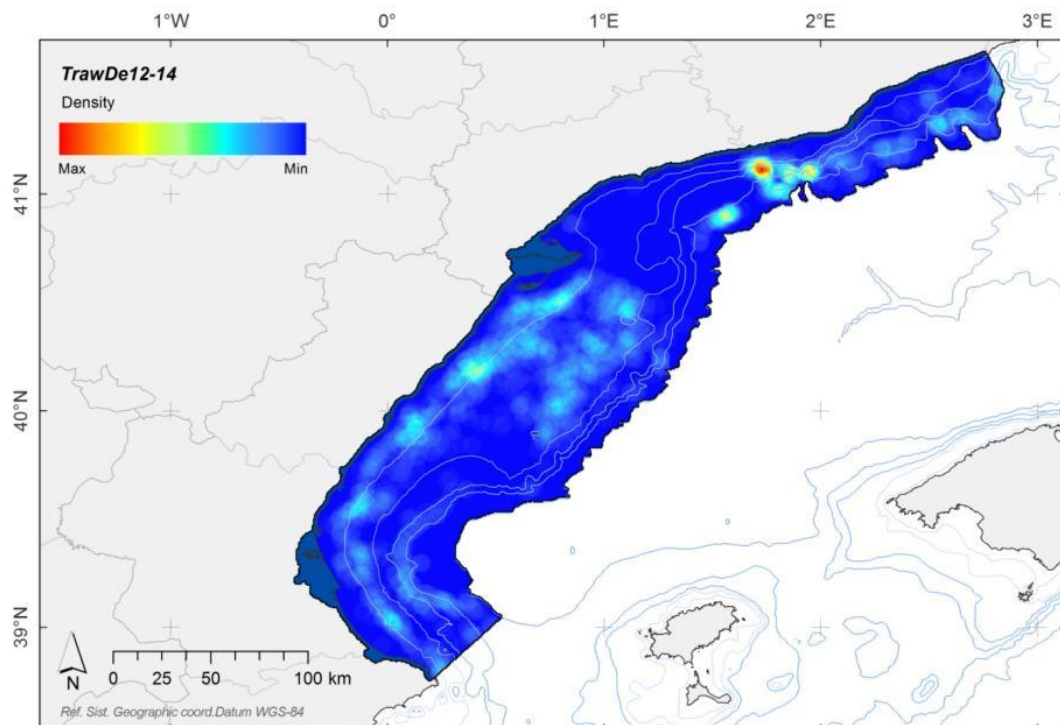
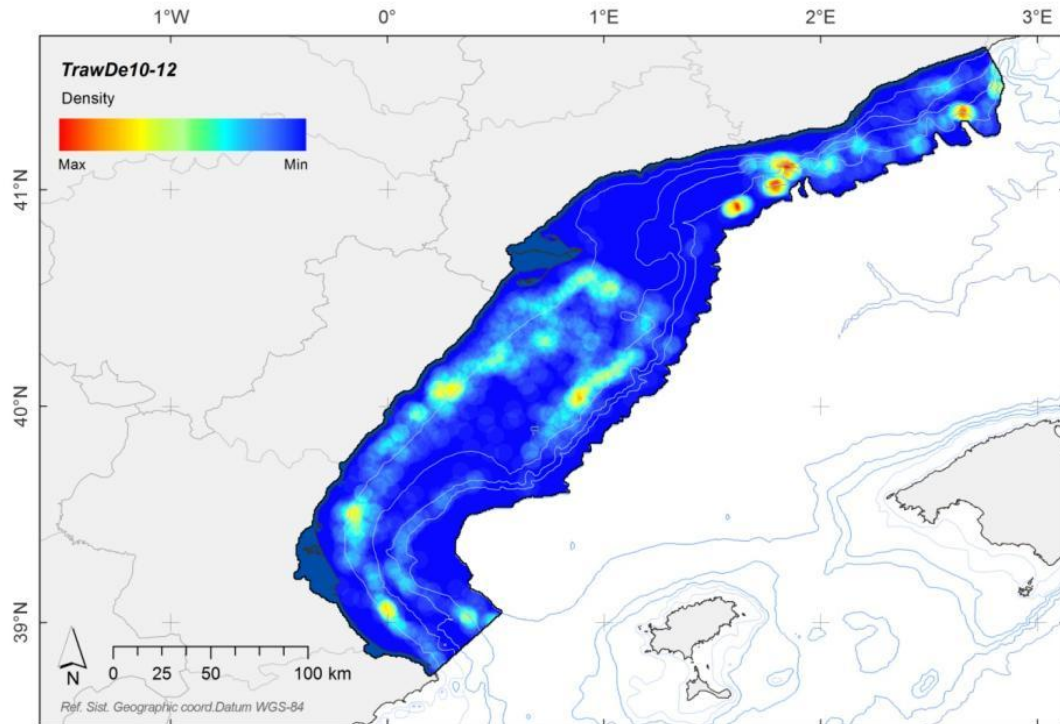
1.1. Spatio-temporal distribution (Supplementary Information)



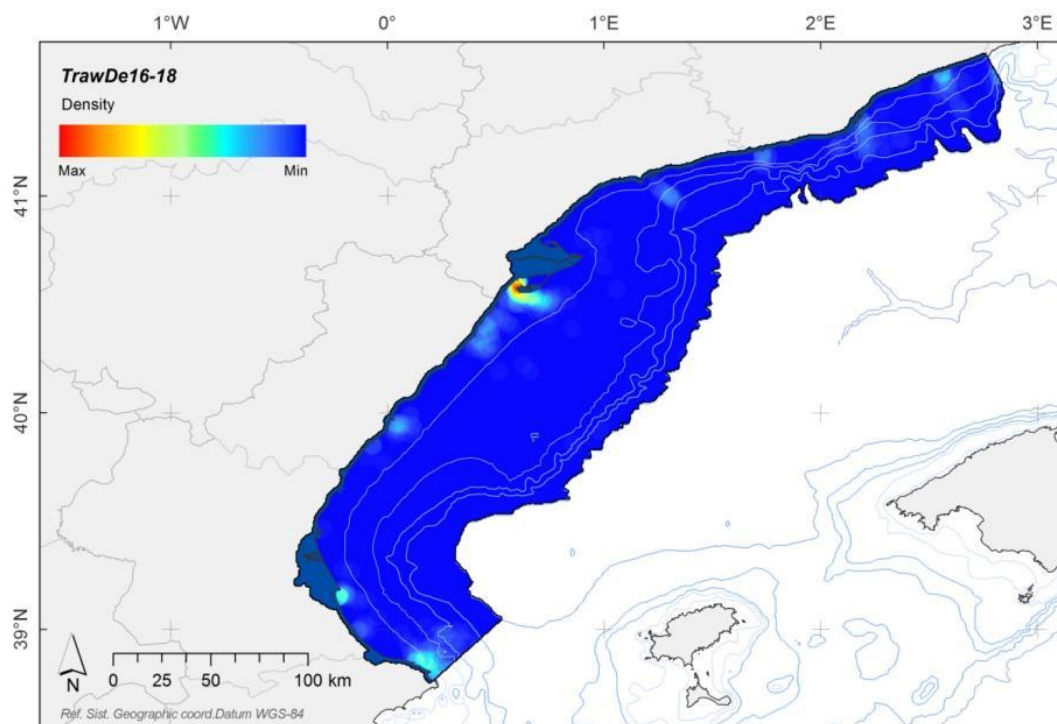
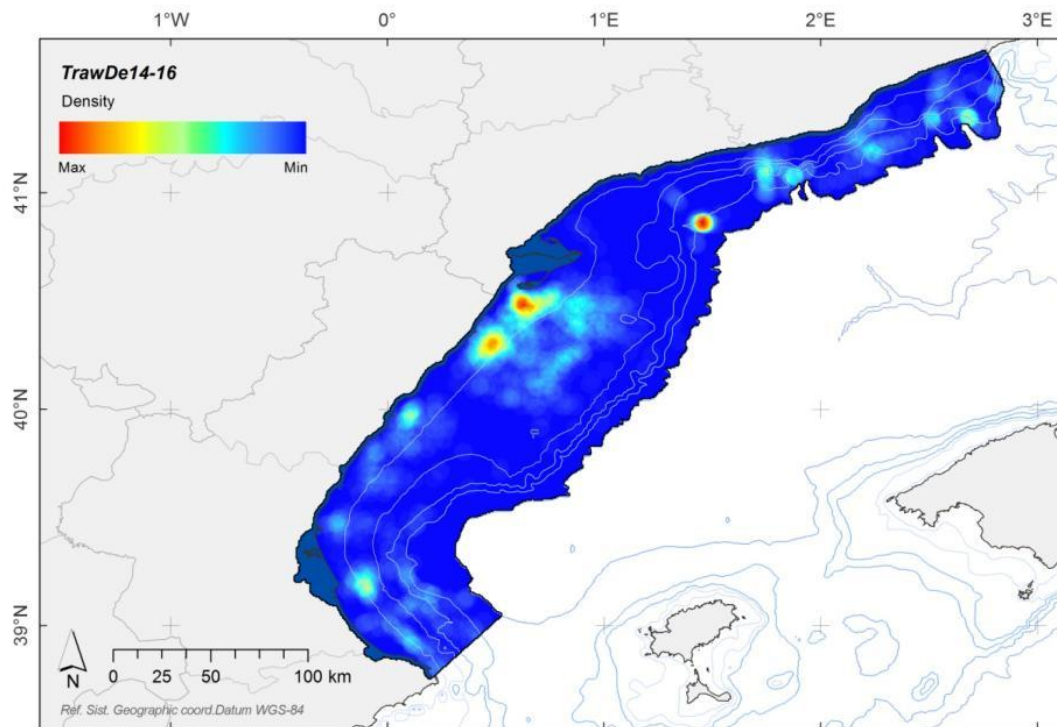
1.1. Spatio-temporal distribution (Supplementary Information)



1.1. Spatio-temporal distribution (Supplementary Information)



1.1. Spatio-temporal distribution (Supplementary Information)



Environmental variables selection

Once created environmental variables, we calculated the correlation between these. For this we have created a grid of points every 5 pixels and extracted the value of each variable using the ArcGIS tool *Extract Multi Values to points*. With data obtained has been made a Spearman correlation matrix in R software and the highly correlated variables $r_s \geq 0.70$ were discarded. Thus six variables were eliminated. Fishing variables for different time intervals may be highly correlated between them, but have not been eliminated because of will not be used simultaneously.

Table S5. Spearman's correlation matrix. In red are shown correlation coefficient values $|r_s| \geq 0.70$. *Variables eliminated to the modelling process.

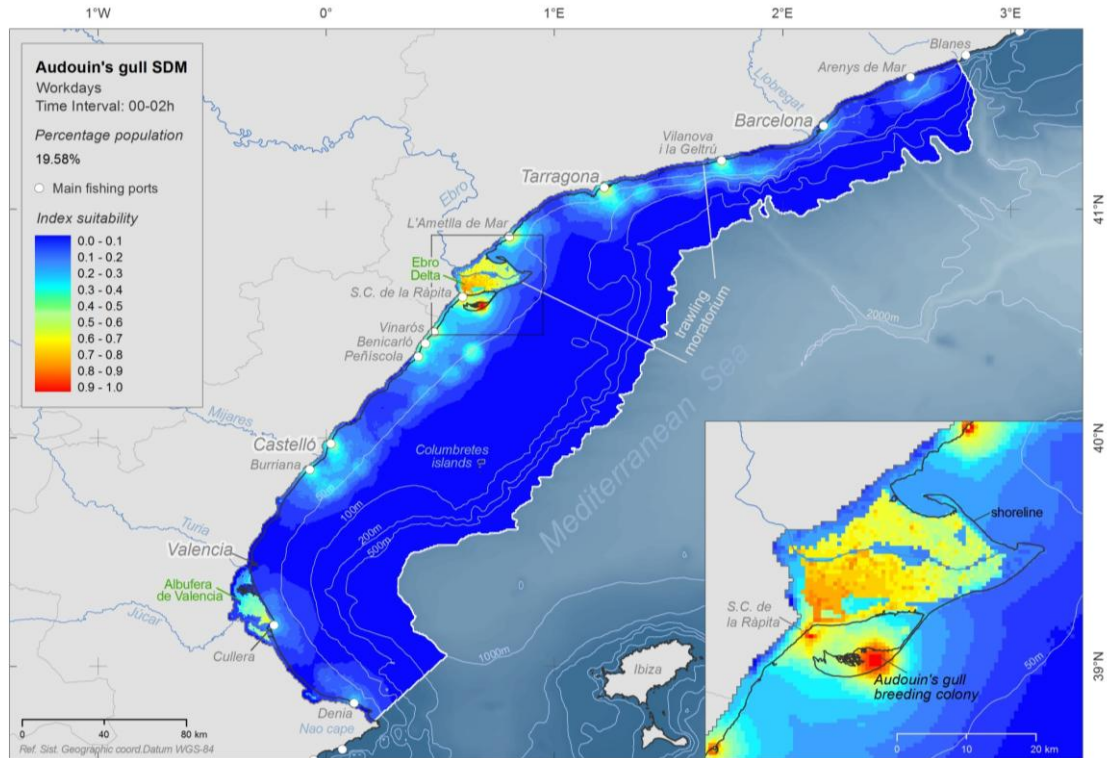
	TrawDe06-08	TrawDe08-10	TrawDe10-12	TrawDe12-14	TrawDe14-16	TrawDe16-18	RiceFieldsCov*	MASL	RiceFieldsMASL	Bathymetry	PurSeDe00-02	PurSeDe02-04	PurSeDe04-06	PurSeDe06-08	PurSeDe08-10	PurSeDe22-24	Chl2011may	ColonyDist	ContinentDist*	CoastDist*	TrawPortDist*	PurSePortDist*	PortsDist	Slope*	PortsCov	SST2011may
TrawDe06-08	1	0.59	0.57	0.60	0.75	0.59	-0.25	-0.47	-0.22	0.00	0.45	0.43	0.50	0.49	0.36	0.48	0.38	0.19	-0.17	-0.05	-0.42	-0.35	-0.34	0.22	-0.03	0.30
TrawDe08-10	0.59	1	0.78	0.83	0.70	0.30	-0.24	-0.45	-0.22	-0.04	0.30	0.30	0.36	0.29	0.26	0.30	0.16	-0.05	0.10	0.15	-0.15	-0.02	0.02	0.04	-0.05	0.26
TrawDe10-12	0.57	0.78	1	0.81	0.62	0.32	-0.24	-0.45	-0.21	-0.09	0.30	0.30	0.35	0.29	0.26	0.30	0.16	-0.01	0.15	0.17	-0.10	0.03	0.07	0.10	-0.05	0.24
TrawDe12-14	0.60	0.83	0.81	1	0.73	0.33	-0.24	-0.45	-0.21	-0.06	0.32	0.31	0.35	0.30	0.28	0.31	0.19	-0.03	0.11	0.15	-0.15	-0.02	0.01	0.07	-0.05	0.26
TrawDe14-16	0.75	0.70	0.62	0.73	1	0.55	-0.24	-0.45	-0.22	0.02	0.33	0.34	0.38	0.36	0.30	0.37	0.30	0.01	-0.05	0.04	-0.33	-0.21	-0.20	0.08	-0.03	0.26
TrawDe16-18	0.59	0.30	0.32	0.33	0.55	1	-0.31	-0.58	-0.28	-0.03	0.44	0.42	0.45	0.53	0.50	0.56	0.51	0.09	-0.07	-0.05	-0.22	-0.26	-0.22	0.24	0.01	0.28
RiceFieldsCov*	-0.25	-0.24	-0.24	-0.24	-0.24	-0.31	1	0.53	0.90	0.23	-0.28	-0.28	-0.28	-0.29	-0.38	-0.30	-0.23	-0.07	-0.24	-0.24	-0.17	0.00	-0.16	-0.23	-0.01	-0.23
MASL	-0.47	-0.45	-0.45	-0.45	-0.45	-0.58	0.53	1	0.47	0.43	-0.52	-0.52	-0.52	-0.54	-0.69	-0.56	-0.43	0.01	-0.43	-0.44	-0.28	-0.13	-0.29	-0.44	0.05	-0.43
RiceFieldsMASL	-0.22	-0.22	-0.21	-0.21	-0.22	-0.28	0.90	0.47	1	0.21	-0.25	-0.25	-0.25	-0.26	-0.35	-0.27	-0.21	-0.06	-0.22	-0.22	-0.15	0.00	-0.15	-0.21	-0.01	-0.21
Bathymetry	0.00	-0.04	-0.09	-0.06	0.02	-0.03	0.23	0.43	0.21	1	0.15	0.11	0.14	0.13	-0.14	0.12	0.15	-0.25	-0.75	-0.77	-0.58	-0.44	-0.63	-0.70	0.07	-0.07
PurSeDe00-02	0.45	0.30	0.30	0.32	0.33	0.44	-0.28	-0.52	-0.25	0.15	1	0.83	0.80	0.78	0.58	0.73	0.54	0.08	-0.23	-0.19	-0.22	-0.39	-0.32	0.12	0.00	0.16
PurSeDe02-04	0.43	0.30	0.30	0.31	0.34	0.42	-0.28	-0.52	-0.25	0.11	0.83	1	0.83	0.78	0.52	0.68	0.50	0.08	-0.20	-0.16	-0.17	-0.35	-0.29	0.11	-0.02	0.13
PurSeDe04-06	0.50	0.36	0.35	0.35	0.38	0.45	-0.28	-0.52	-0.25	0.14	0.80	0.83	1	0.83	0.55	0.68	0.49	0.05	-0.21	-0.17	-0.20	-0.39	-0.31	0.10	-0.03	0.15
PurSeDe06-08	0.49	0.29	0.29	0.30	0.36	0.53	-0.29	-0.54	-0.26	0.13	0.78	0.78	0.83	1	0.65	0.71	0.56	0.01	-0.21	-0.18	-0.17	-0.41	-0.32	0.13	-0.02	0.16
PurSeDe08-10	0.36	0.26	0.26	0.28	0.30	0.50	-0.38	-0.69	-0.35	-0.14	0.58	0.52	0.55	0.65	1	0.57	0.51	-0.06	0.10	0.11	0.14	-0.13	0.00	0.31	0.00	0.28
PurSeDe22-24	0.48	0.30	0.30	0.31	0.37	0.56	-0.30	-0.56	-0.27	0.12	0.73	0.68	0.68	0.71	0.57	1	0.60	0.10	-0.17	-0.18	-0.24	-0.30	-0.25	0.17	0.01	0.29
Chl2011may	0.38	0.16	0.16	0.19	0.30	0.51	-0.23	-0.43	-0.21	0.15	0.54	0.50	0.49	0.56	0.51	0.60	1	0.04	-0.39	-0.24	-0.27	-0.41	-0.38	0.28	0.02	0.03
ColonyDist	0.19	-0.05	-0.01	-0.03	0.01	0.09	-0.07	0.01	-0.06	-0.25	0.08	0.08	0.05	0.01	-0.06	0.10	0.04	1	-0.14	-0.08	-0.37	-0.16	-0.19	0.43	0.03	0.13
ContinentDist*	-0.17	0.10	0.15	0.11	-0.05	-0.07	-0.24	-0.43	-0.22	-0.75	-0.23	-0.20	-0.21	-0.21	0.10	-0.17	-0.39	-0.14	1	0.85	0.81	0.78	0.92	0.33	-0.07	0.22
CoastDist*	-0.05	0.15	0.17	0.15	0.04	-0.05	-0.24	-0.44	-0.22	-0.77	-0.19	-0.16	-0.17	-0.18	0.11	-0.18	-0.24	-0.08	0.85	1	0.68	0.61	0.77	0.38	-0.07	0.12
TrawPortDist*	-0.42	-0.15	-0.10	-0.15	-0.33	-0.22	-0.17	-0.28	-0.15	-0.58	-0.22	-0.17	-0.20	-0.17	0.14	-0.24	-0.27	-0.37	0.81	0.68	1	0.66	0.83	0.22	-0.04	-0.07
PurSePortDist*	-0.35	-0.02	0.03	-0.02	-0.21	-0.26	0.00	-0.13	0.00	-0.44	-0.39	-0.35	-0.39	-0.41	-0.13	-0.30	-0.41	-0.16	0.78	0.61	0.66	1	0.86	0.11	-0.05	0.22
PortsDist	-0.34	0.02	0.07	0.01	-0.20	-0.22	-0.16	-0.29	-0.15	-0.63	-0.32	-0.29	-0.31	-0.32	0.00	-0.25	-0.38	-0.19	0.92	0.77	0.83	0.86	1	0.23	-0.06	0.15
Slope*	0.22	0.04	0.10	0.07	0.08	0.24	-0.23	-0.44	-0.21	-0.70	0.12	0.11	0.10	0.13	0.31	0.17	0.28	0.43	0.33	0.38	0.22	0.11	0.23	1	-0.02	0.14
PortsCov	-0.03	-0.05	-0.05	-0.05	-0.03	0.01	-0.01	0.05	-0.01	0.07	0.00	-0.02	-0.03	-0.02	0.00	0.01	0.02	0.03	-0.07	-0.07	-0.04	-0.05	-0.06	-0.02	1	0.00
SST2011may	0.30	0.26	0.24	0.26	0.26	0.28	-0.23	-0.43	-0.21	-0.07	0.16	0.13	0.15	0.16	0.28	0.29	0.03	0.13	0.22	0.12	-0.07	0.22	0.15	0.14	0.00	1

1.1. Spatio-temporal distribution (Supplementary Information)

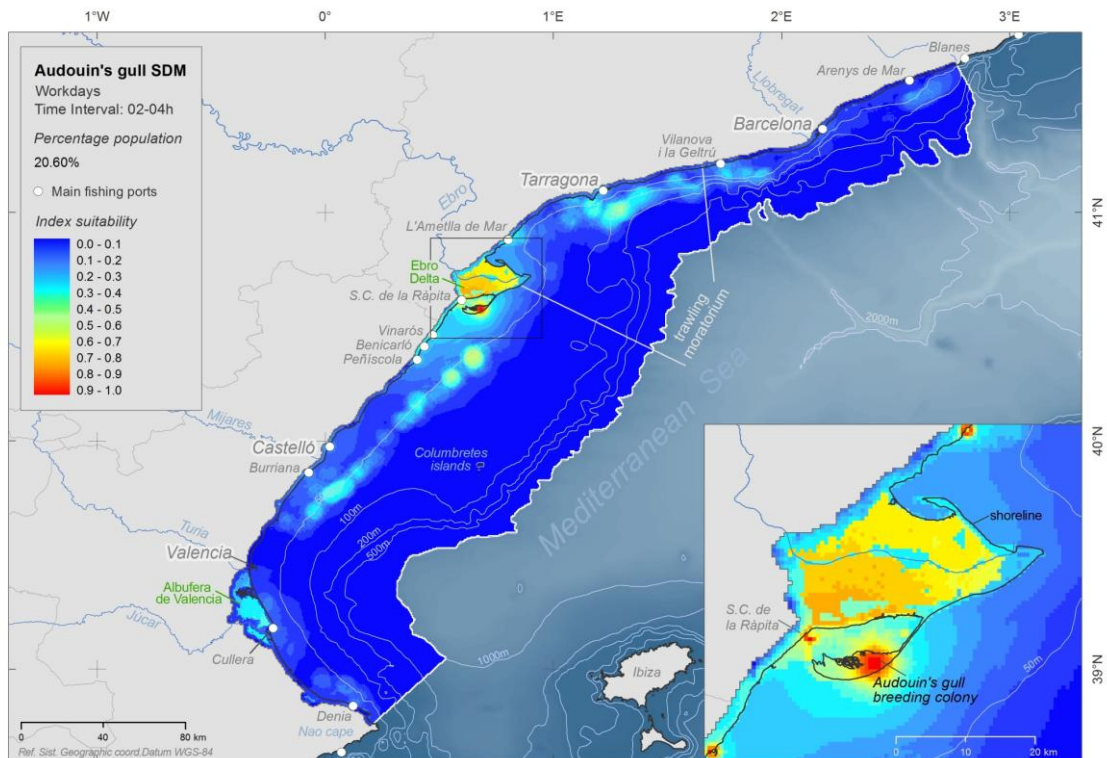
Habitat distribution models

Figures S30-S54. Habitat distribution models for each time interval

Workdays 00-02h

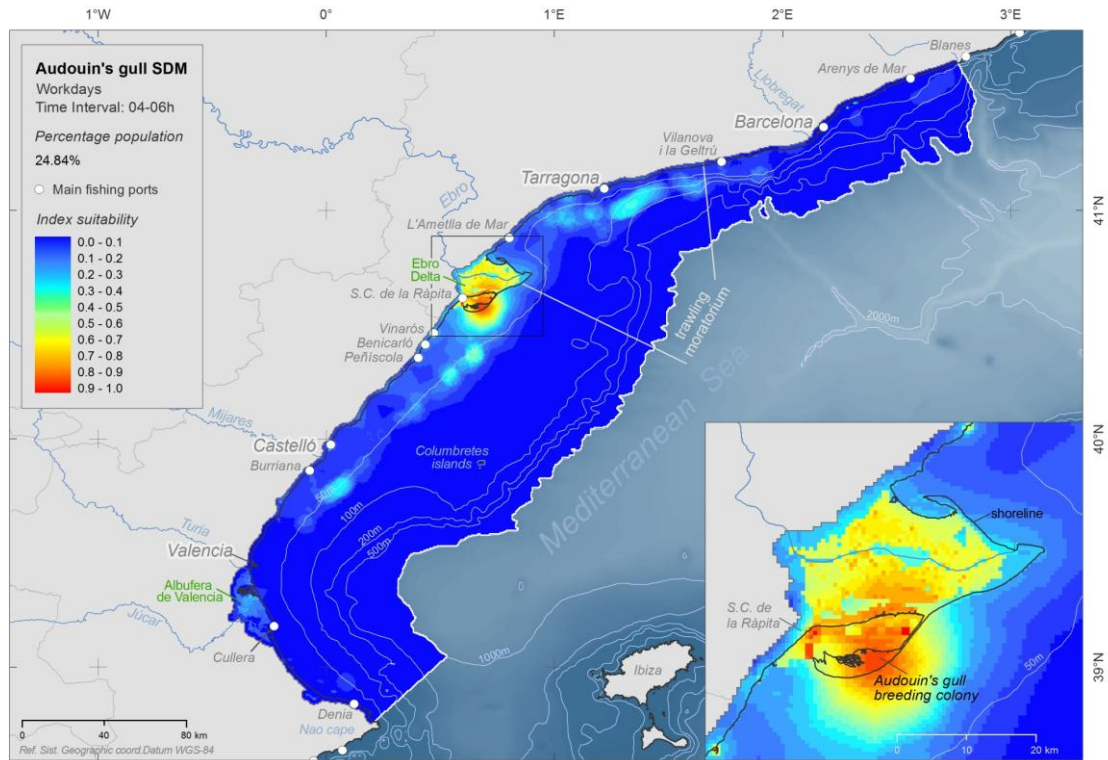


Workdays 02-04h

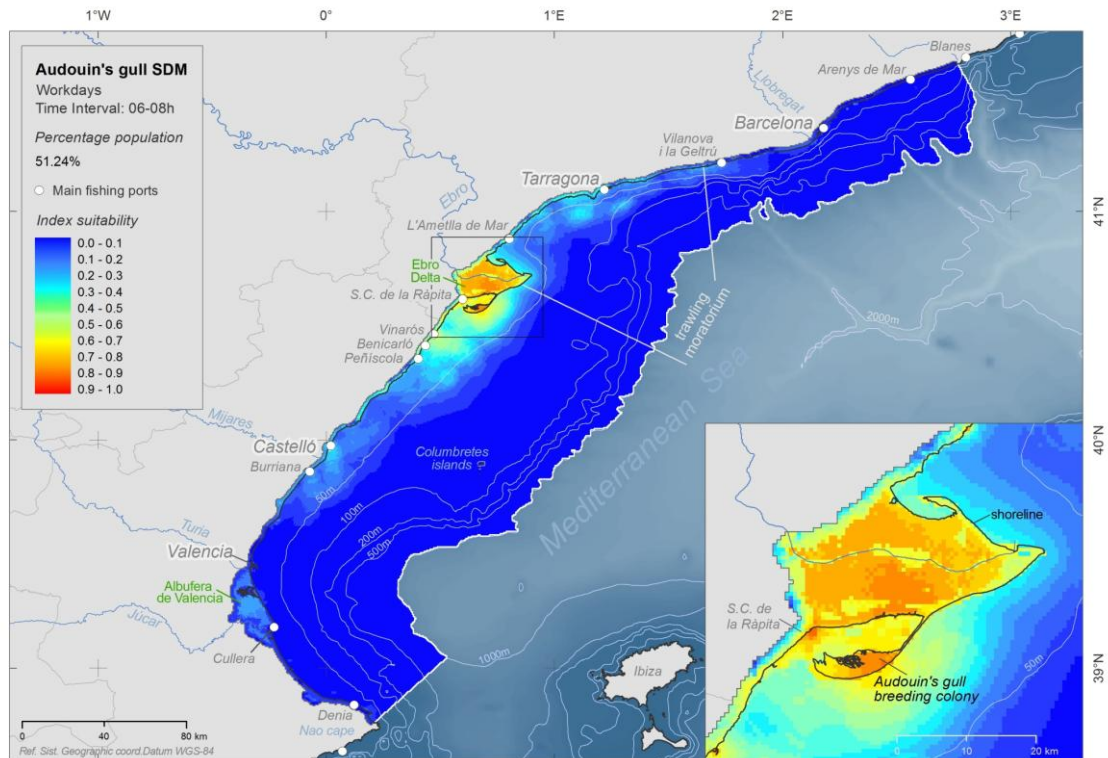


1.1. Spatio-temporal distribution (Supplementary Information)

Workdays 04-06h

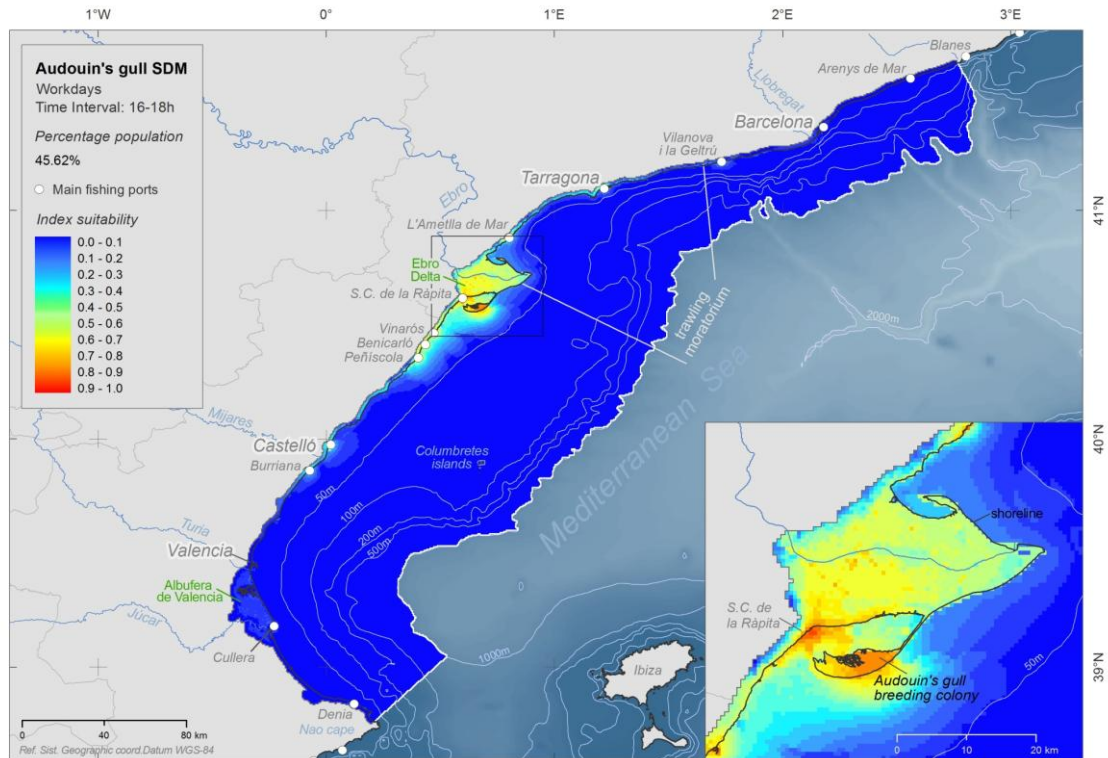


Workdays 06-08h

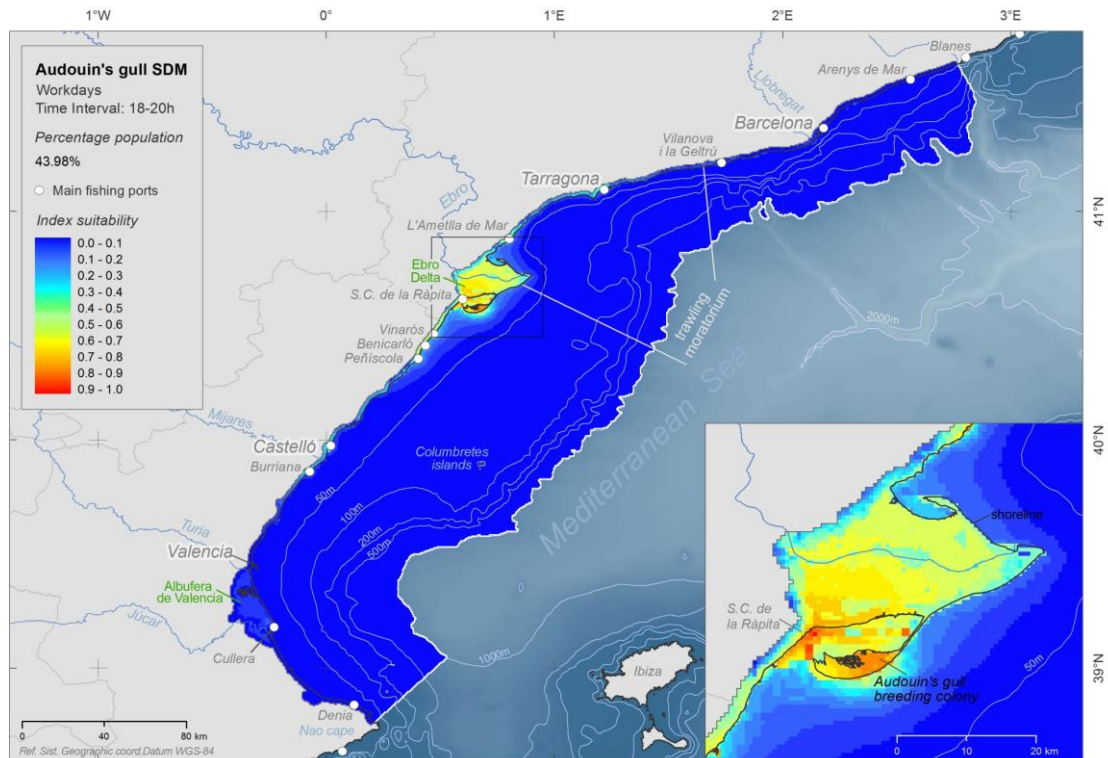


1.1. Spatio-temporal distribution (Supplementary Information)

Workdays 16-18h

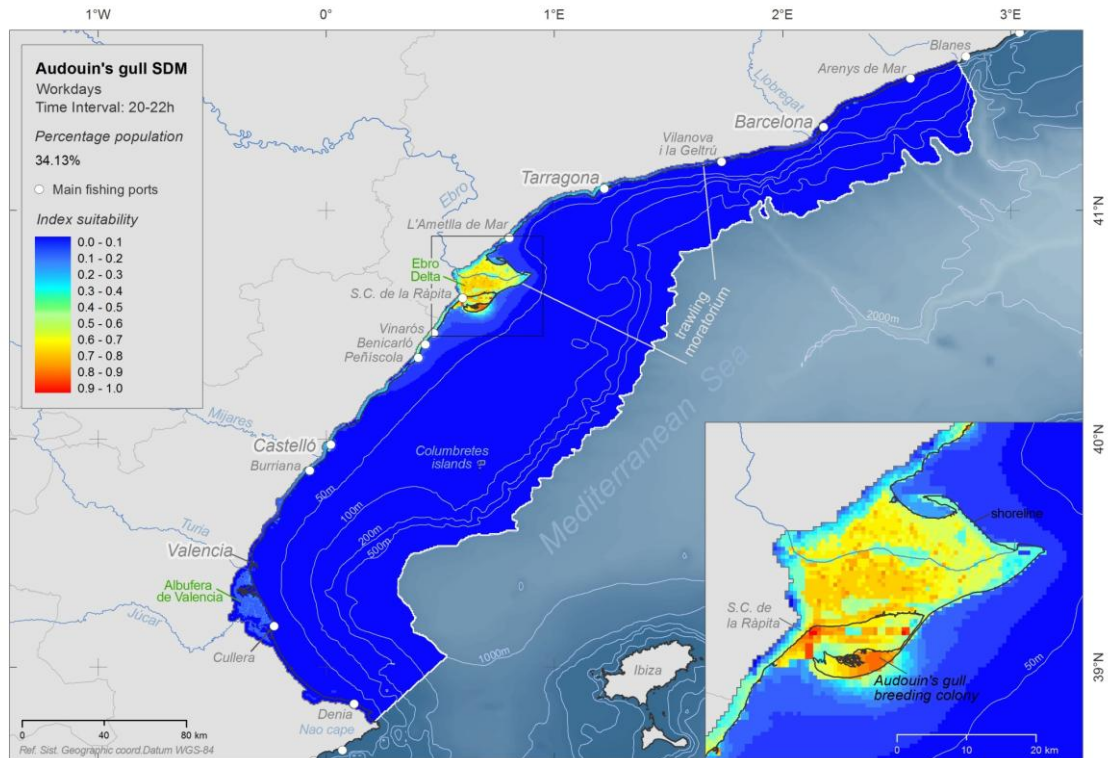


Workdays 18-20h

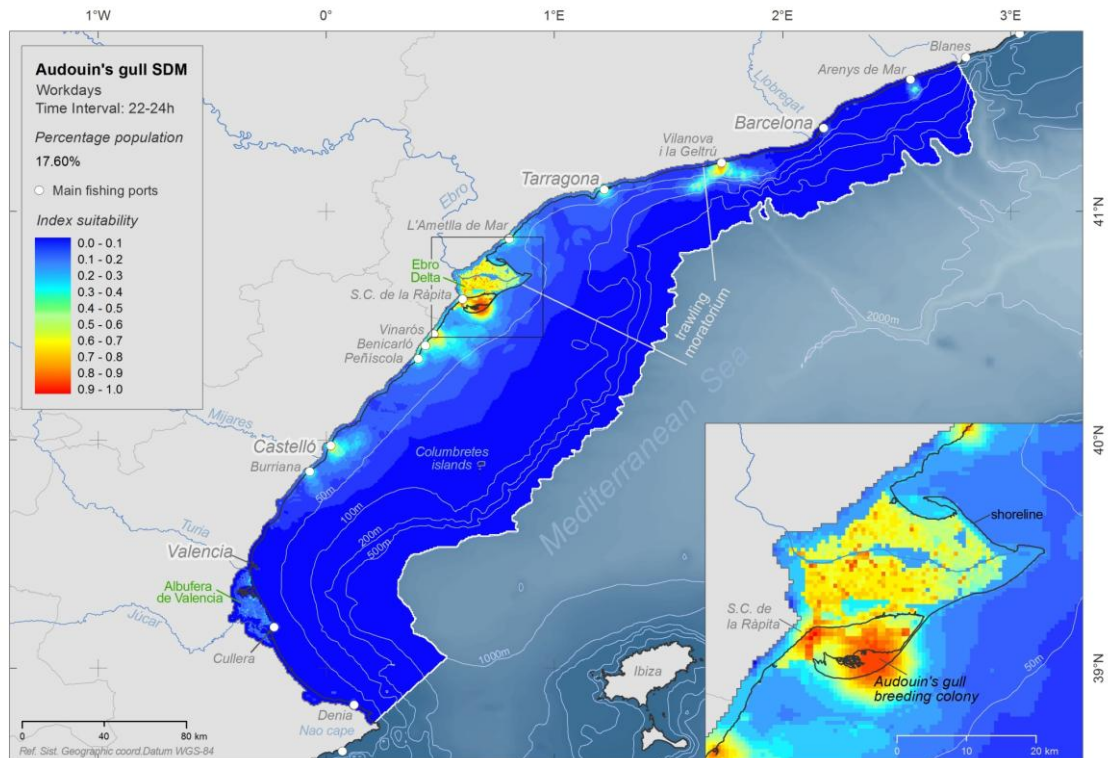


1.1. Spatio-temporal distribution (Supplementary Information)

Workdays 20-22h

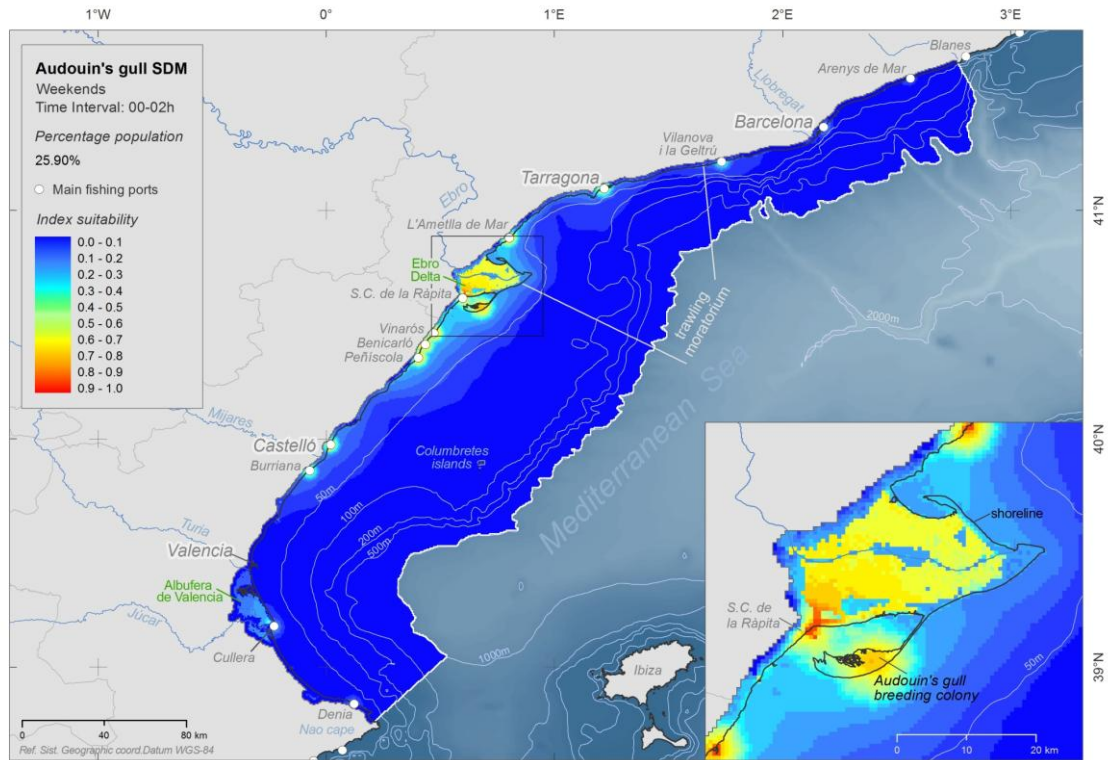


Workdays 22-24h

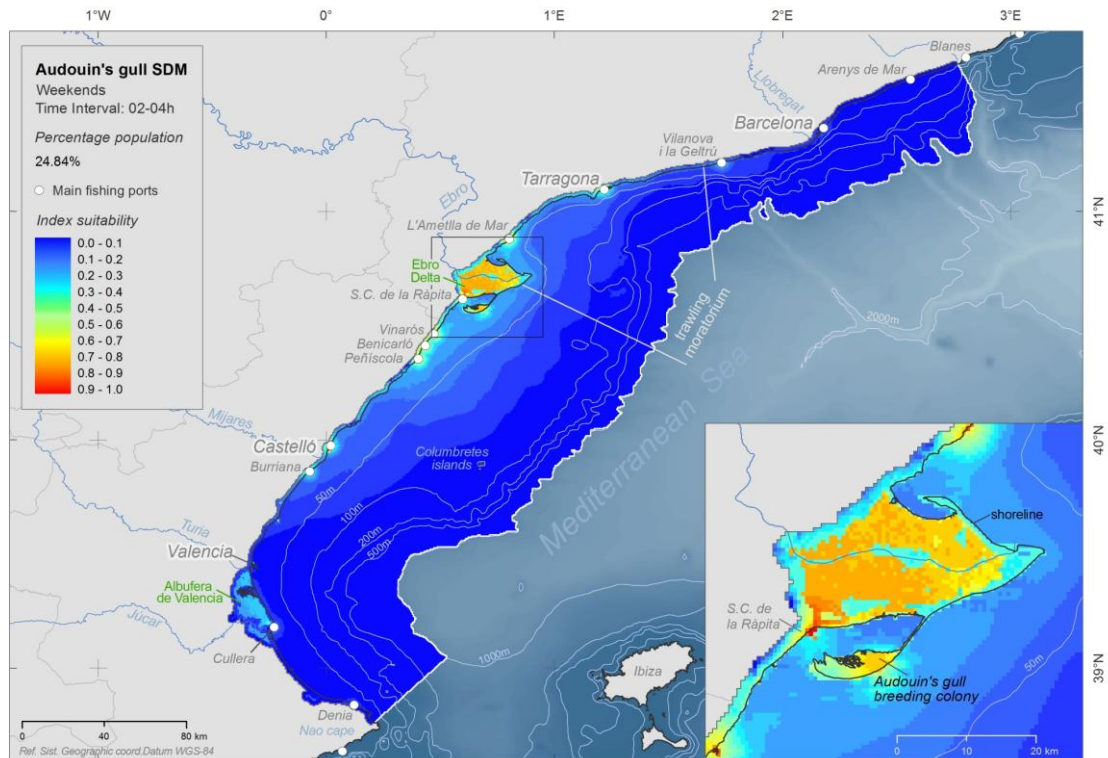


1.1. Spatio-temporal distribution (Supplementary Information)

Weekends 00-02h

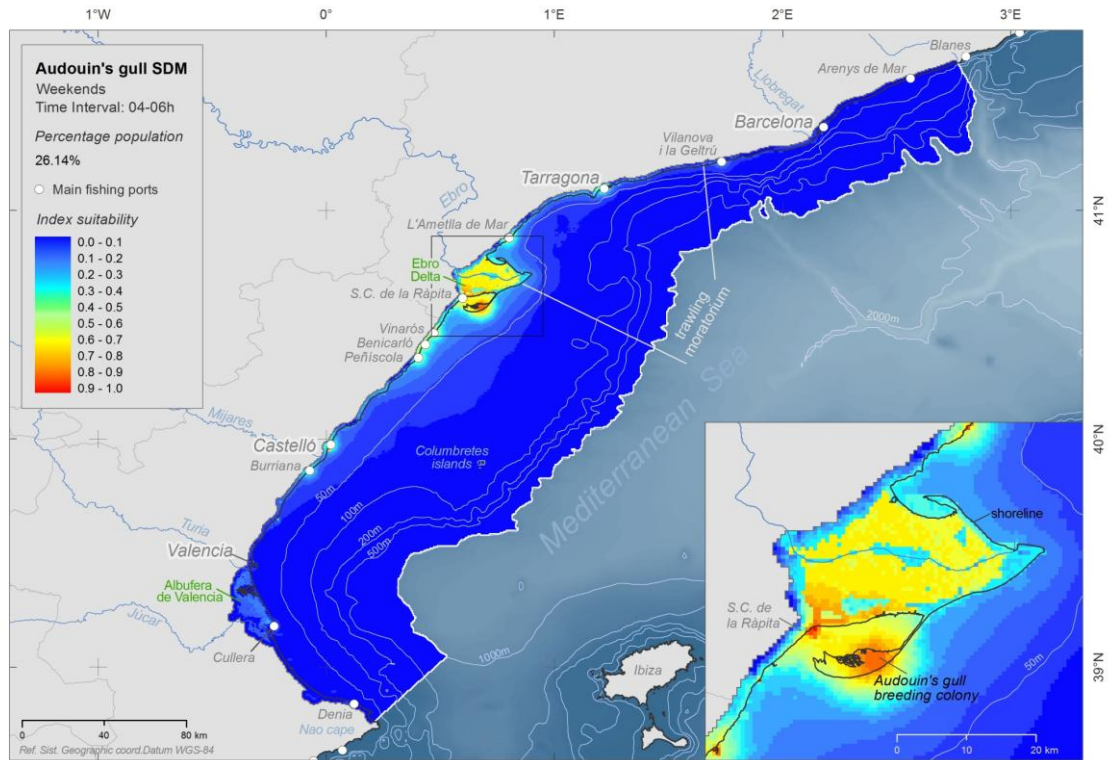


Weekends 02-04h

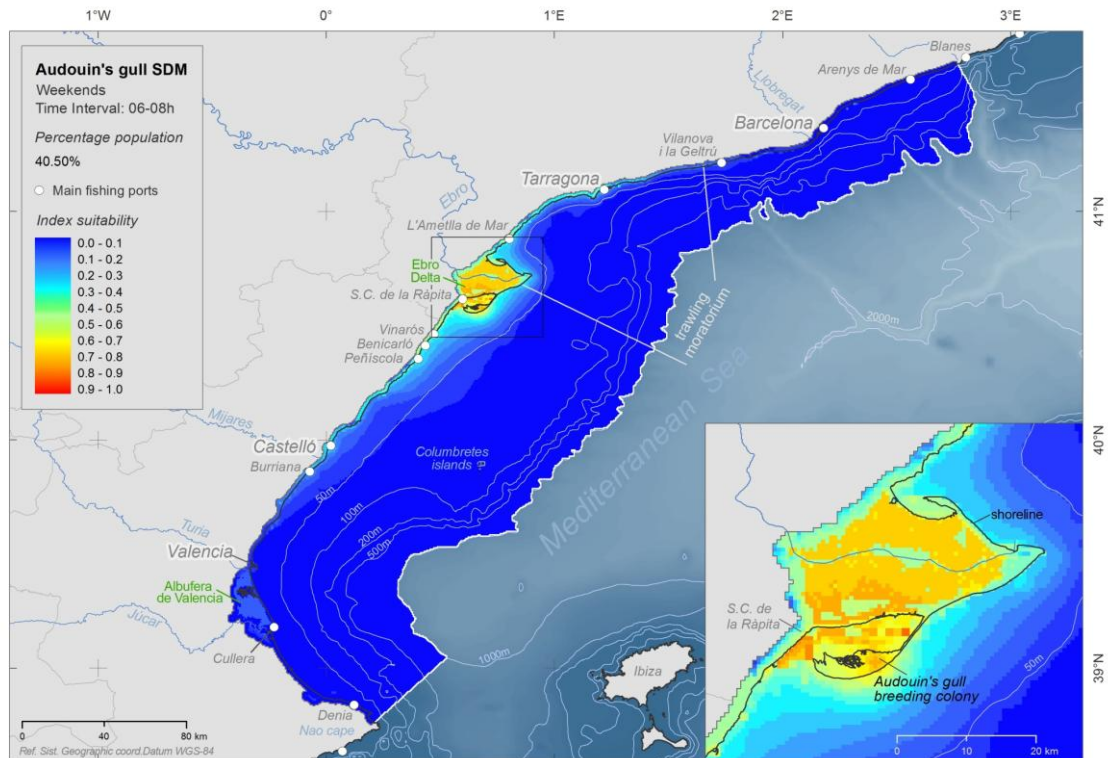


1.1. Spatio-temporal distribution (Supplementary Information)

Weekends 04-06h

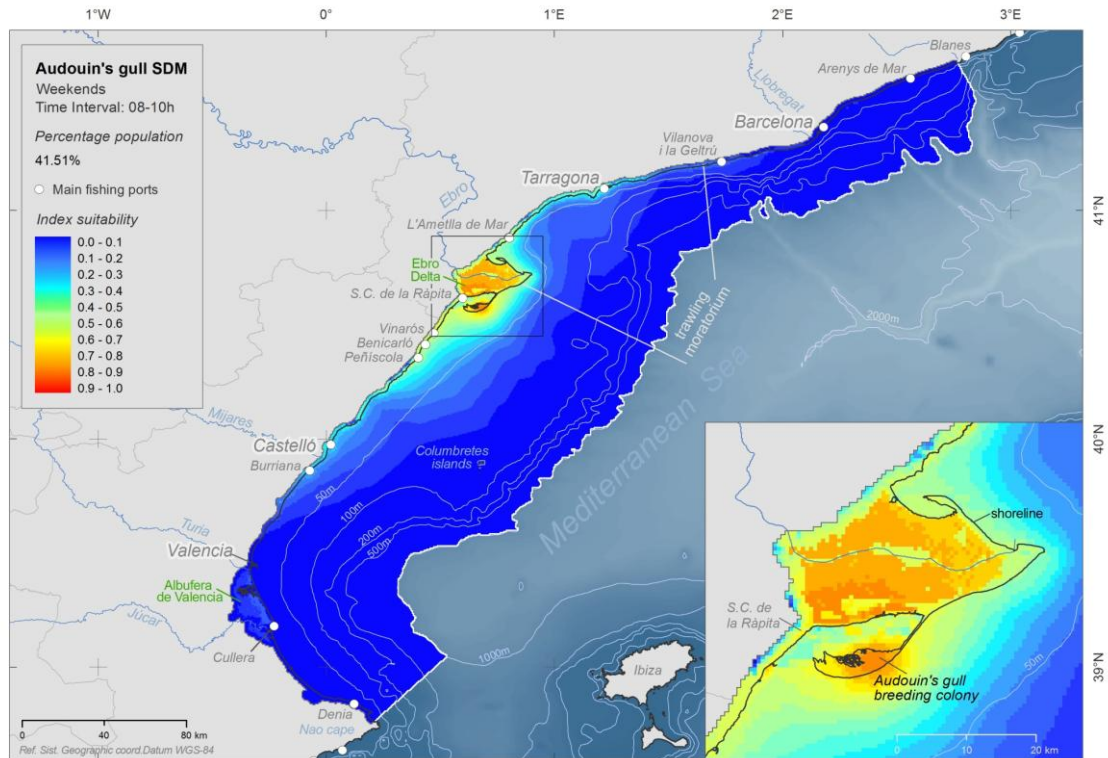


Weekends 06-08h

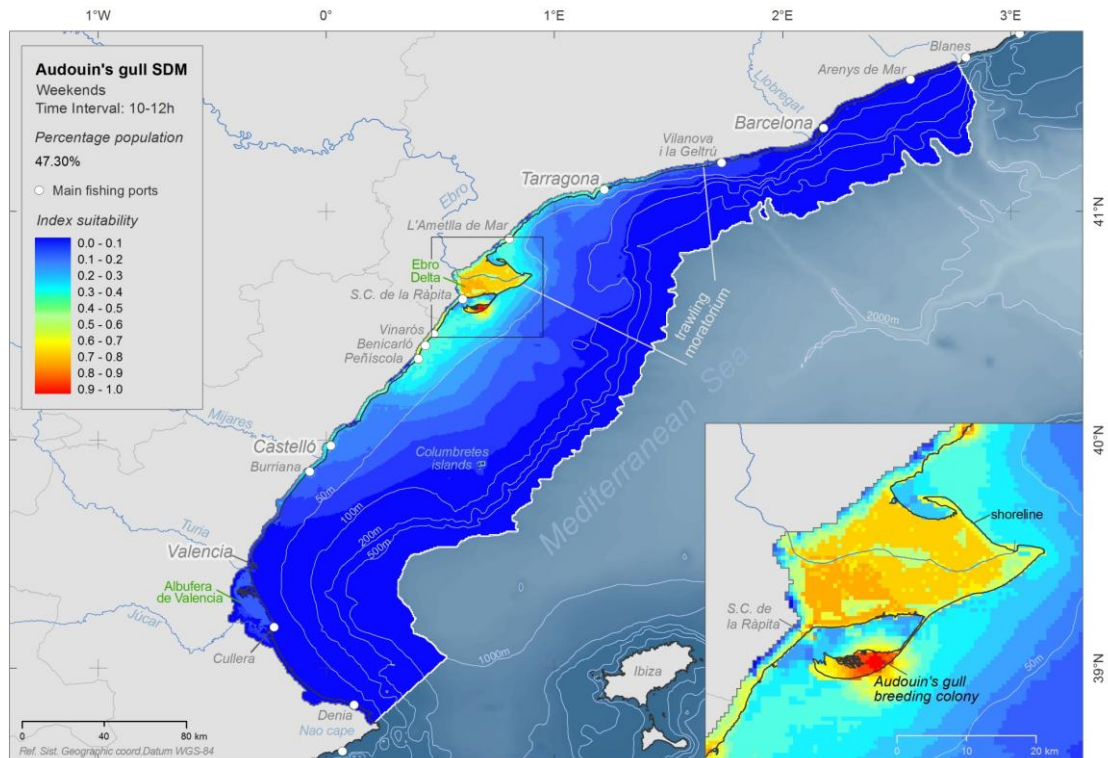


1.1. Spatio-temporal distribution (Supplementary Information)

Weekends 08-10h

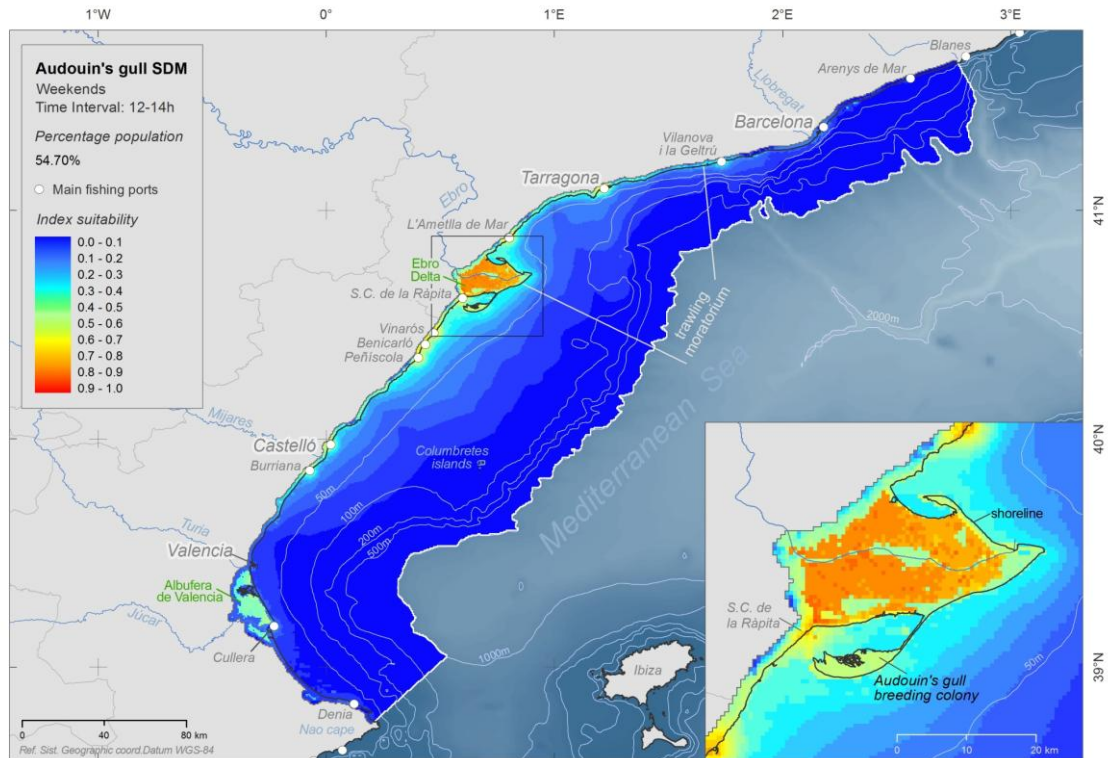


Weekends 10-12h

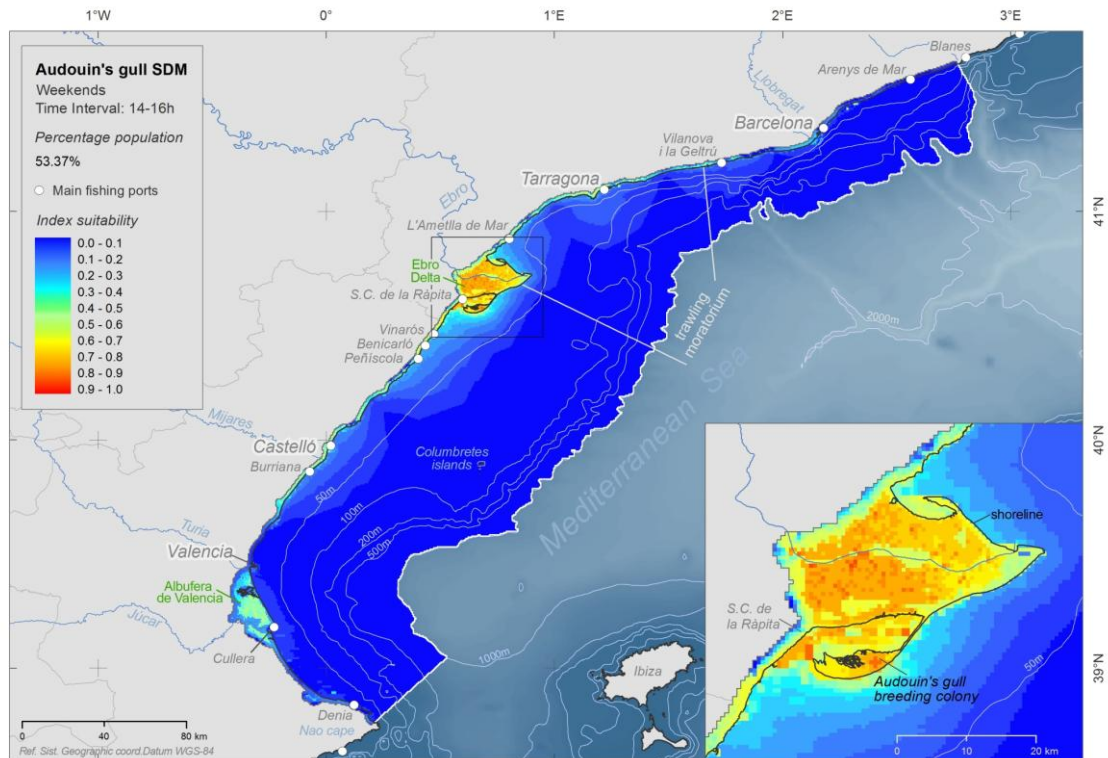


1.1. Spatio-temporal distribution (Supplementary Information)

Weekends 12-14h

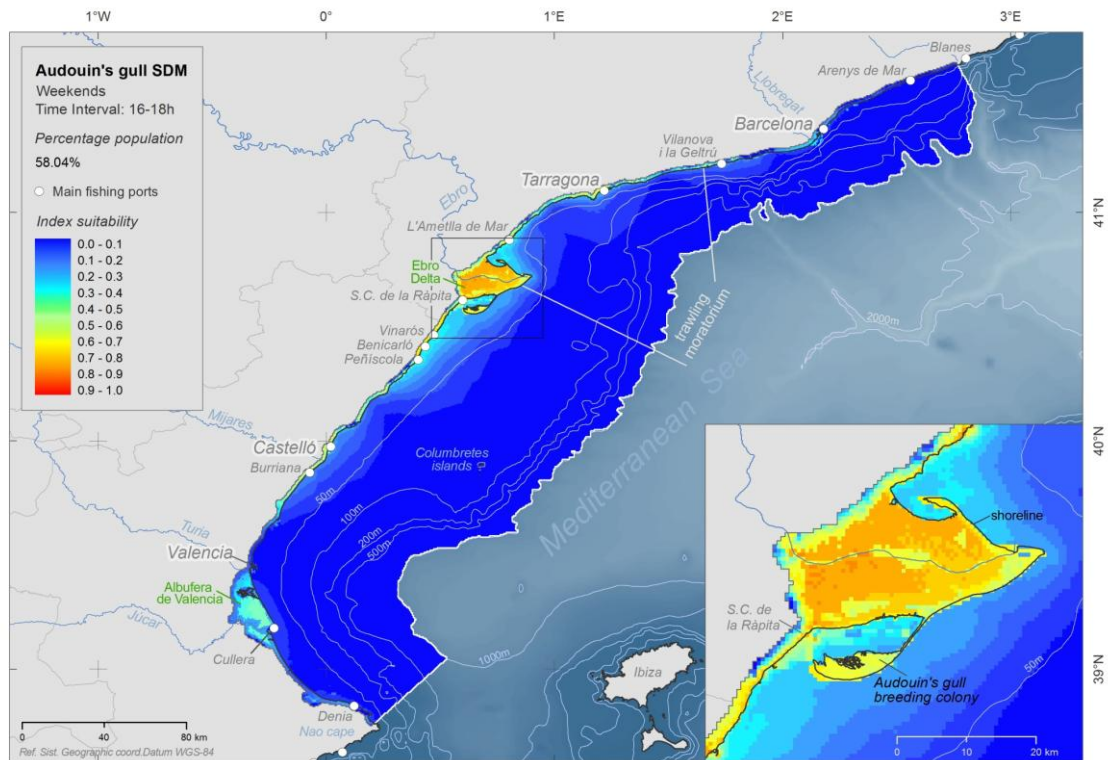


Weekends 14-16h

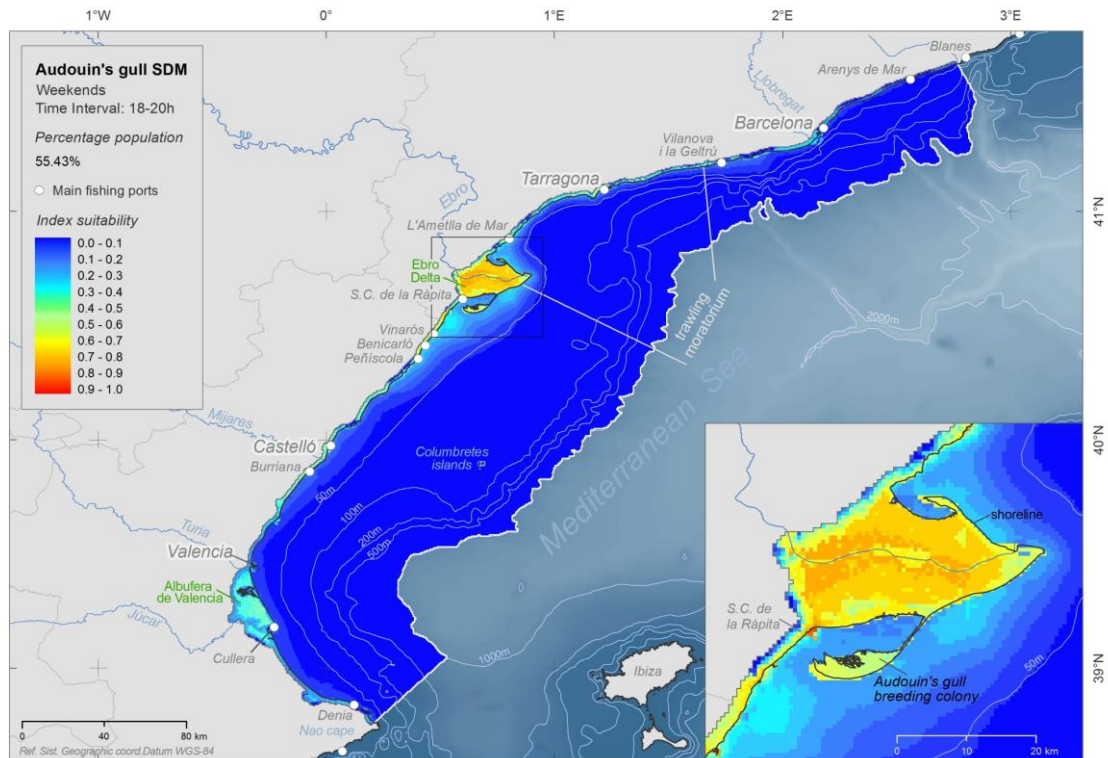


1.1. Spatio-temporal distribution (Supplementary Information)

Weekends 16-18h



Weekends 18-20h



CHAPTER 1.2. Sex-specific foraging behaviour in response to fishing activities in a threatened seabird

Título: Diferencias sexuales en el comportamiento de alimentación con relación a las actividades pesqueras en un ave marina amenazada

Resumen:

1. Algunas aves marinas han aprendido a explotar de una manera eficiente los descartes pesqueros provenientes de las actividades de la pesca de arrastre, produciendo un incremento sustancial de sus poblaciones. Sin embargo, la prohibición de los descartes pesqueros ha sido propuesta en Europa como necesaria para asegurar la sostenibilidad de nuestros mares. Por lo tanto, es de crucial importancia generar conocimiento sobre las potenciales consecuencias de una prohibición de los descartes en la ecología alimentaria de las especies amenazadas.
2. En este trabajo evaluamos la influencia de las actividades pesqueras en los hábitos de alimentación de machos y hembras de gaviota de Audouin *Larus audouinii* durante el período de cría. Los dispositivos GPS, junto al uso de los isótopos estables, nos permiten obtener una visión excepcional sobre su ecología trófica y su comportamiento de alimentación.
3. En relación a los isótopos estables, la gaviota de Audouin se mostró como una especie generalista. No obstante, una alta especialización individual enmascara esta aparente plasticidad trófica. Efectivamente, los individuos mostraron toda la gama de estrategias desde depredadores marinos a especialistas de arrozal.
4. De forma interesante, los datos GPS revelaron diferentes patrones de búsqueda de alimento entre días laborables y festivos. Durante los días laborables, los esfuerzos de vuelo fueron comparables entre sexos. Sin embargo, durante el fin de semana, las hembras llevaban a cabo viajes de alimentación más largos y más alejados que los machos.
5. Estas diferencias entre sexos en el comportamiento de alimentación, junto a la consistente repetición de la estrategia de alimentación de las hembras durante los fines de semana, indica que las hembras seleccionan preferentemente pescado marino en ausencia de actividades de arrastre. Debido a que no se encontró relación entre el hábitat de alimentación y los índices de condición

física, estas diferencias sexuales en el comportamiento de alimentación pueden estar relacionadas con requerimientos nutricionales específicos asociados a la producción de los huevos por parte de las hembras.

6. *Síntesis y aplicaciones.* La necesidad de obtener información detallada sobre la dependencia y respuesta de las aves marinas a las actividades pesqueras a nivel individual es un punto clave en el campo de la conservación. En este sentido, el comportamiento de alimentación específico de cada sexo en relación con la pesca ha pasado normalmente por alto, a pesar de que tiene implicaciones ecológicas y de conservación. Por ejemplo, esta situación puede conducir a una mortalidad diferencial por captura accidental en los palangres cuando los arrastreros no operan. Por otro lado, una aplicación gradual de cualquier política pesquera se antoja necesaria con el fin de facilitar la adaptación de los individuos especialistas a un escenario de prohibición de descartes. La combinación de los datos GPS junto a los isótopos estables se erige como una herramienta prometedora de cara a diseñar las nuevas medidas de gestión y conservación.

Title: Sex-specific foraging behaviour in response to fishing activities in a threatened seabird

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Journal: Ecology and Evolution (under review)

Sex-specific foraging behaviour in response to fishing activities in a threatened seabird

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Abstract:

1. Some seabird species have learned to exploit efficiently fishing discards from trawling activities, leading to an increase in their populations. However, a discard ban has been proposed as necessary in Europe to ensure the sustainability of the seas. Therefore, it is of crucial importance for management and conservation purposes to get knowledge about the potential consequences of a discard ban on the foraging ecology of threatened seabirds.
2. We assess the influence of fishing activities on the feeding habits of male and female Audouin's gull *Larus audouinii* during the breeding period. GPS devices, together with the use of Stable Isotope Analysis (SIA), allow an outstanding insight into their trophic ecology and foraging behaviour.
3. In relation to SIA, Audouin's gull showed as a generalist species. Nevertheless, a high individual specialization underlies this apparent trophic plasticity, displaying a full range of strategies from mainly marine to rice field predators.
4. Interestingly, GPS data revealed different foraging patterns between work days and weekends. During work days, flying efforts were comparable between sexes. However, during weekends, females undertook longer and further afield foraging trips than males.

5. Sex-differences in foraging behaviour, together with a consistent repeatability of females' foraging strategy during weekends, indicates that females target on marine fish in absence of trawling activities. Because no relationship between habitat use and body condition indices was found, this sex-specific foraging behaviour could be related to specific nutritional requirements associated to previous egg production.
6. *Synthesis and applications.* The need to get detailed information on the dependence and response of seabirds to fishing activities at the individual level is a key point in conservation sciences. In this regard, sex-specific foraging behaviour in relation to fisheries has been overlooked although it has ecological and conservation implications. For instance, this situation may lead to sex-differential bycatch mortality in longlines when trawlers don't operate. Moreover, a gradual implementation of any fisheries policy is needed in order to facilitate the adaptation of specialized individuals to a discard ban scenario. The combination of GPS data with SIA appears a promising tool to design the new management and conservation measures.

Key words: fisheries, bycatch, sex-specific, foraging behaviour, habitat use, GPS tracking, stable isotopes, Audouin's gull

1. INTRODUCTION

Industrialization of commercial fisheries over the last century has strongly impacted on the marine environment, shaping the ecology and behaviour of many species (Pauly *et al.* 1998; Bicknell *et al.* 2013; Coll *et al.* 2013). Generalist species have often taken profit of this situation, learning to exploit new food resources provided by these human activities (Oro *et al.* 2013). This is the case of many seabirds, particularly some gulls and skuas, which have learnt to exploit efficiently the increasing amounts of fishing discards as an alternative to their declining natural prey (Arcos 2001; Furness 2003). However, this situation is unsustainable in the long run due to fisheries overexploitation, and increasing efforts are being taken by fisheries managers to reduce this waste of fish (Penas 2007).

One of the major changes that will affect European marine ecosystems in a near future is the regulatory reform of the Common Fisheries Policy adopted in 2013 by the European Union (Penas 2007; <http://ec.europa.eu/fisheries/reform/>). It proposes, among others, a ban on discards to ensure that fisheries are economically and environmentally sustainable (Bicknell *et al.* 2013). Since many seabirds have learnt to exploit discards up to represent most of its diet, a discard ban will result in a food shortage and, therefore, a nutritional shortfall (Bicknell *et al.* 2013). However, some studies have shown that, when discards decrease, seabirds respond by switching their diet to alternative prey (Oro *et al.* 1996b) or moving to novel habitats (Camphuysen *et al.* 2010). Therefore, it is of crucial importance for the management of endangered seabird species and the whole marine ecosystem to anticipate environmental consequences by generating information upon which to design and implement effective conservation measures (Orians & Soulé 2001).

A representative example of a seabird that has taken advantage on fishing discards is the threatened Audouin's Gull (*Larus audouinii*), endemic to the Mediterranean. Although considered in the past a nocturnal specialist foraging on small pelagic fish and one of the most endangered seabirds in the world (Burger & Gochfeld 1996), its population significantly increased since a new colony was established in the Ebro Delta (northeast of the Iberian Peninsula) in 1981 (Genovart *et al.* 2008). This population increment in the Ebro Delta (which nowadays holds more than 50% of the total 22,000 breeding pairs) resulted from an efficient utilization of trawling discards, together with effective protection measures of the breeding grounds (Oro & Ruxton 2001, BirdLife International 2012). Actually, fish discards account for more than 75% of the energy required by this gull and represents the bulk of its marine diet (Arcos 2001;

1.2. Sex-specific foraging behaviour

Oro *et al.* 2013). Alternative foraging opportunities, which increase in relevance when trawlers do not operate, include the capture of small pelagic fish both directly and from purse-seine vessels (Arcos & Oro 2002) and the use of rice fields (Navarro *et al.* 2010). In the rice fields, its main prey is the American crayfish (*Procambarus clarkii*), an abundant invasive species (Ruiz *et al.* 1996), although depleted compared to fish in terms of nutritional content (Massias & Becker 1990; Hunner 1988). It is essential for conservation purposes to study in depth the foraging behaviour and habitat use of this species both, at population and at an individual level. For the above reasons, weekend periods (when fishing activity is negligible) stand as a suitable time-frame to study behavioural and dietary changes in a discard ban scenario (Bartumeus *et al.* 2010).

The study of foraging behaviour of seabirds has always the problem of at-sea distributions, traditionally difficult to track in detail at individual level (Votier *et al.* 2010). In breeding Audouin's gull previous works have addressed this issue at the population level (Arcos & Oro 1996; Cama *et al.* 2013), highlighting their strong dependence on fishing activities and shaping its at-sea distribution. However, the gap of knowledge is especially obvious when moving to the individual level. Other works on the same species (Mañosa *et al.* 2004; Christel *et al.* 2012) dealt with tracking devices (radio tracking and PTTs loggers, respectively) with several positioning restrictions and errors (Burger & Shafer 2008). Because the Ebro Delta holds a great diversity of habitats in a relatively small area, slight positioning errors could result in large deviations of foraging behaviour. Recently, the improvement of GPS logging devices represented a true revolution, enabling an outstanding study of free-living animal movement (Ropert-Coudert & Wilson 2005). In addition, diet tracers as stable isotopes analysis (SIA, especially carbon and nitrogen isotopes) are nowadays widely used to integrate the trophic ecology for a precise period depending on the analyzed tissue (Kelly 2000). For example, it is well known that marine habitats present enriched carbon signatures compared to freshwater ones (Cotin *et al.* 2011; García-Tarrasón *et al.* 2013). The combination of positional information and that of SIA is becoming widely used, and appears a promising tool disentangling the foraging behaviour of seabirds and designing new conservation measures.

In this study, we used the information provided by GPS loggers and plasma SIA of Audouin's gull to: 1) describe the habitat use and foraging behaviour of each sex according to the availability of fish discards (workdays vs. weekends); 2) know the degree of specialization in foraging behaviour and habitat use on an individual basis, and 3) infer the relationship between foraging strategy and body condition, if any. Due to the great dependence of Audouin's gull on commercial fisheries and considering the

new scenario that may arise from the reform of the EU fisheries policy, we hypothesize a greater foraging effort in absence of trawling activity, together with the detrimental effects of rice field resources on their body condition due to their low energy content.

2. MATERIAL & METHODS

Tagging and Sampling

The work was conducted at the Ebro Delta colony (NE Iberian Peninsula: 40° 33' N, 00° 39' E) during the 2011 breeding season. The colony is situated at Punta de la Banya, a protected sandy peninsula subject to salt works where Audouin's gull nests. From 8th to 10th of May, 60 adult Audouin's gulls with three-egg clutches were caught at the nest using cage-traps at the beginning of the incubation period. The birds were tagged with a GPS logger (CatTraqTM) fixed with harnesses by a trained technician from the Spanish Ministry of Environment (MAGRAMA). The weight of the devices once placed on the bird was about 25 grams, below the 5% of the weight of the specimen (Cochran 1980). To reduce any negative effect on the breeding performance of the birds, only one member of the pair was tagged (Mañosa *et al.* 2004). The harnesses were devised to last just a few months, thus birds that could not be retrieved would lose the device after this time. The loggers were programmed to register the position every 5 minutes. 37 birds (22 males and 15 females) were recaptured at nest from 18th to 26th of May, and the loggers were retrieved (ranging from 9 to 18 days between captures).

Adults were weighed both, at capture and recapture, to the nearest 10 g with a 1000 g Pesola spring balance. Tarsus was measured at recapture to the nearest 0.01 mm using a digital calliper. Blood samples (0.5 mL) were collected from the brachial vein in both events and placed in heparinised vials for isotopic analysis. At the first capture, 0.2 mL was preserved in a neutral vial for molecular identification of sex. Sex was determined using polymerase chain reaction (PCR) amplification of the CHD genes (Griffiths *et al.* 1998).

Positional information

Tagging provided 9 ± 5 (median $\pm$ IQR) complete days of suitable data from the individuals (max: 15 days; min: 1 day). Some devices were partially or totally unusable because of seal failure. The original sample data ($n = 89,800$ locations) were filtered to exclude incomplete days, resulting in 81,720 locations. Moreover, data from individuals

1.2. Sex-specific foraging behaviour

staying in the nest or resting in the colony were also excluded, so finally we only used the locations of foraging trips ($n = 35,154$ locations). Foraging trips are defined as all locations since the specimen leaves the colony until it returns, depicted in Fig. 1 (BirdLife 2004).

Although four different types of habitats were identified for Audouin's gull at the Ebro Delta (rice fields, sea, fishing harbours and inland), in this study only rice field and marine habitats were considered because they accounted for more than 85% of locations (Navarro *et al.* 2010; García-Tarrasón *et al.* 2013).

To estimate individual foraging behaviour we considered: 1) *habitat use* as the proportion of rice field GPS locations over the sum of marine plus rice field positions in each day. 2) *distance covered each day* (hereafter "daily distance", in km) as the total cumulative linear distance covered flying/traveled between all locations each complete day with data; 3) *maximum distance from colony each day* (hereafter "maximum daily distance", in km) as the linear distance between the furthest point of the trip and the nest for each complete day; 4) *trip length* (in km) as the total cumulative linear distance between all locations in each foraging trip and 5) *trip duration* as the time lapse (in hours) between departure and return to the colony.

"Fishing activity" (workday or weekend) was assigned to every trip/day in order to evaluate a possible fishing effect. If a foraging trip extended more than one day under different fishing activity, the assignment was made depending on whether the individual spent the most of this trip.

Diet and Stable Isotopes Analysis

The heparinised vials were kept at +4°C and centrifuged (2000 rpm for 10 min) within 6 h of collection. The supernatant plasma was pipetted off and stored in 250- μ l aliquots at -80°C. Plasma samples were lyophilised, manually homogenised and 0.3 mg were placed into tin capsules for stable carbon and nitrogen isotope ratio determination. Isotopic analyses were carried out at the Scientific and Technical Services of the Universitat de Barcelona (Spain) by means of a Thermo-Finnigan Flash 1112 elemental analyser (CE Elantech, Lakewood, NJ, USA) coupled to a Delta-C isotope ratio mass spectrometer via a CONFLOIII interface (Thermo Finnigan MAT, Bremen, Germany), with IAEA standards being applied every 12 samples to calibrate the system. Stable isotope ratios were expressed in the standard δ notation relative to Vienna Pee Dee Belemnite ($\delta^{13}\text{C}$) and atmospheric N_2 ($\delta^{15}\text{N}$). Replicate of standards

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indicated analytical measurement errors of $\pm 0.1\text{‰}$ and $\pm 0.2\text{‰}$ for $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$, respectively.

For Audouin's gull preys, we used published reference values (mean $\pm$ SD) from the Ebro Delta following García-Tarrasón *et al.* (2013). Fig. 2 shows a graphical representation of individual Audouin's gull and its potential preys. Lipids and uric acid concentrations from plasma can deplete $\delta^{13}\text{C}$ values. For that reason, plasma $\delta^{13}\text{C}$ values were corrected in Fig. 2 including $+0.5\text{‰}$ to adjust for the lack of lipid extraction in this tissue due to small quantities of plasma (Cherel *et al.* 2005). In the case of potential preys, they were represented by adding an isotopic discrimination factor of $+2.82\text{‰}$ for $\delta^{15}\text{N}$ and -0.08‰ for $\delta^{13}\text{C}$ following Caut *et al.* 2009.

Condition of adults

Body mass at the recapture, body mass variation (capture and recapture weight difference) and the ratio between body mass and tarsus length were used as condition indices.

Statistical analysis

Habitat use and foraging behaviour

Foraging behaviour, on a trip or day basis, were modelled through generalised linear mixed models (GLMMs) including individual as a random factor to account for dependence between observations made on same bird. Sex and fishing activity (workday or weekend) were considered fixed factors.

A logit link was used to model habitat use (proportion of rice field GPS locations in a day) and an identity link was used to model continuous variables (daily distance, maximum daily distance, trip length and trip duration) (Zuur *et al.* 2007). A hierarchical backward model selection, starting with a full model including both fixed factors and its interaction was used. If the interaction factor was significant, a separate analysis for each sex was carried out. Distributional assumptions about data have been checked through graphical residual analysis. Foraging behaviour variables showed clear skewed distributions which were normalized applying a logarithmic transformation.

Relationship SIA-GPS data and consistence of diet strategy

Relationship between GPS data for habitat use (% of marine and % of rice field locations) and isotopic signatures of plasma at recapture ($\delta^{13}\text{C}$ and $\delta^{15}\text{N}$) were tested

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using Pearson's correlation coefficient to assess the reliability of isotopes as indicators of foraging habitat.

We estimated consistency of individual diet strategy comparing the plasma signatures between capture and recapture (ranging from 9 to 18 days). Blood plasma has typically a half-life of around 2–5 days; therefore they inform us about diet in two non-overlapping time periods (Podlesak *et al.* 2005). Isotopic signatures were also modelled using a GLMM with identity link including sex, capture event (capture vs. recapture) and its interaction as potential explaining factors. Because capture and recapture isotopic values represent repeated measures for each individual, the repeatability of the individual feeding strategy was also estimated by calculating the ICC and its associated uncertainty (Carrasco & Jover 2003; Votier *et al.* 2010). Individual consistence of foraging behaviour (trip length and trip duration) and habitat use (rice field locations) was also evaluated using intra-class correlation coefficient (ICC) by sex and fishing activities (Stauss *et al.* 2012).

Individual condition and relation with foraging behaviour

A similar GLMM approach was used to investigate changes in body mass, which included the individual as a random factor, sex and capture event as fixed factors, and the time span (in days) between capture and recapture as covariate.

As body condition summarizes the costs (foraging effort) and rewards (food obtained) of the foraging strategy, we did an assessment of the relationship between foraging effort (log of trip distance) and habitat use (using a non GPS derived measure: the plasma $\delta^{13}\text{C}$ at recapture) through a GLMM including sex, fishing activity as well as individual random factor.

The relationship between habitat use and different condition indices (body mass at the recapture and the ratio body mass with tarsus) was assessed performing a generalised linear model (GLM) including the sex as fixed factor, and the habitat use (% of ricefield locations) and time span between capture and recapture as covariates. In the case of body mass at the recapture, the body mass at the initial capture was included as covariate. The interaction of sex and habitat was also included.

All statistical analyses were performed using SPSS 21.0 (IBM Corporation, Armonk, NY, USA).

3. RESULTS

Habitat use and foraging behaviour

When modelling the probability of rice field locations, the best model included only the fishing activity ($F_{1,31} = 7.04$, $p = 0.012$), indicating that on weekends the use of rice fields was higher than on workdays. There were no significant differences between sexes. Descriptive statistics (mean $\pm$ SD) are shown in Table 1. Parameter estimate and 95% CI of the estimated effect shown in Supplementary Material, Fig. S1.

In relation to foraging behaviour (daily distance, maximum daily distance, trip length and trip duration), all the selected final models (except the maximum distance) included the interaction between sex and fishing activity (p -values lower than 0.036). This interaction was the result of significantly longer foraging trips in space and time undertaken by the females on weekends compared to males. In the case of the maximum daily distance, females foraged significantly further than males ($F_{1,1} = 4.49$, $p = 0.043$). Descriptive statistics (mean $\pm$ SD) are shown in Table 2. Parameter estimate and 95% CI of the estimated effects are shown in Supplementary Material, Figs S2-S5.

Relationship SIA-GPS data and consistence of diet strategy

GPS locations and the plasma stable isotopes values at the recapture were highly correlated. The % of rice field locations presented a significant negative relation with the $\delta^{13}\text{C}$ ($r = -0.78$, $p < 0.001$) and positive with the $\delta^{15}\text{N}$ ($r = 0.52$, $p = 0.002$), and vice versa for the marine habitat.

No significant differences according to sex were found for $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ between capture and recapture. For nitrogen, significant differences were found between both events ($F_{1,36} = 5.43$, $p = 0.03$). Moreover, values of $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ showed a great repeatability between capture and recapture ($\delta^{13}\text{C}$ ICC = 0.78, 95%CI = 0.61 – 0.88; $\delta^{15}\text{N}$ ICC = 0.57, 95%CI = 0.31 – 0.76). Fig. 3 depicts graphical approach to the isotopic variance components.

In the case of trip distance and trip duration, males on both work days and weekends, and females on work days showed a very low repeatability (trip distance ICC ranged between 0.12 – 0.22; trip duration ICC ranged: 0.00 – 0.15). However, females on weekends showed a great repeatability in their foraging behaviour (trip distance ICC = 0.97, 95%CI = 0.91 – 0.99; trip duration ICC = 0.88, 95%CI = 0.62 – 0.97).

1.2. Sex-specific foraging behaviour

Individual condition and relation with foraging behaviour

The distance covered during the foraging trips was strongly correlated to the habitat use. The best model included only the $\delta^{13}\text{C}$ ($F_{1,28} = 17.71$, $p < 0.001$), indicating that a higher consumption of the rice field resources was related to shorter foraging trips.

Differences in body mass were only found between sexes ($F_{1,36} = 45.85$, $p < 0.001$) but no significant differences were found between both capture events. Despite the smaller flying effort required to move to neighbouring rice field habitats, the final GLM for the body mass at the recapture and the ratio “body mass/tarsus length” did not show any relationship with the habitat use from GPS data (all $p \geq 0.65$).

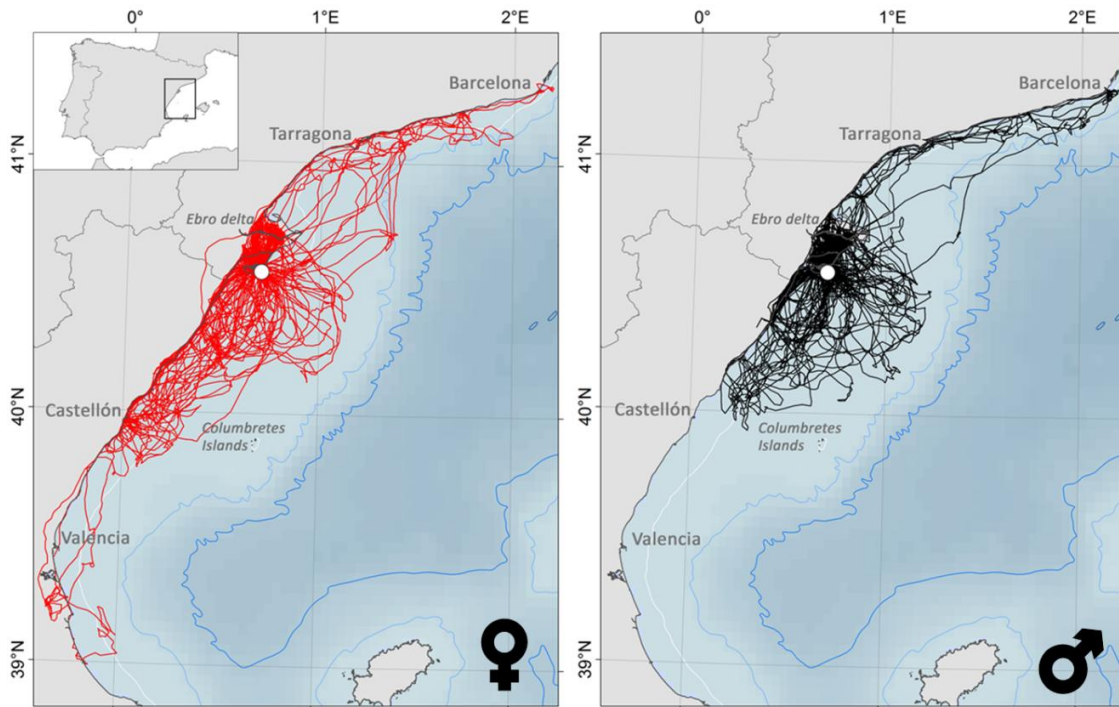


Fig. 1. Foraging tracks of tagged Audouin's gulls from the Ebro Delta (Spain) by sex.

4. DISCUSSION

Several works have addressed the population ecology and behaviour of Audouin's gull as a model seabird (Oro & Ruxton 2001; Fernández-Chacón *et al.* 2013), although spatial distribution patterns have not been studied in much detail. Thanks to the new techniques/devices, our results represent the most accurate approach to spatial patterns in foraging behaviour in Audouin's gull, so far. Our contribution, together with previous knowledge, will provide a better insight about the dependence of Audouin's gull from fishing activities and the potential effect of a ban on discards in the habitat use and the scavenging ecology for this endangered species.

SEX-SPECIFIC FORAGING BEHAVIOR IN RESPONSE TO FISHERIES

Positional data indicate that female Audouin's gulls tended to perform longer and further afield foraging trips than males. This was already suggested by Mañosa *et al.* (2004), despite the methodological and sample size constraints faced in their work. Studies on other seabird species have shown also that females usually perform longer foraging trips than males (e.g. González-Solís *et al.* 2000; Xavier & Croxall 2005). The spatial segregation by sex is widespread in seabirds, where the smaller (females in the case of Audouin's gull) usually undertakes longer trips (reviewed in Wearmouth & Sims 2008). It has been suggested that size matters probably because the smaller and lighter sex has a higher foraging and flight efficiency (Shaffer *et al.* 2001). Foraging segregation has also been attributed to a reduced intraspecific competition (Catry *et al.* 2005). However, the sexual dimorphism hypothesis is not always supported (for example Lewis *et al.* 2002; Stauss *et al.* 2012). In fact, our data indicate that these gender differences are reflected in females travelling significantly longer distances and spending longer time outside the colony during weekends, when there are no major fishing activities and therefore, no discards. The fact we did not found any relationship between the weight of individuals and descriptors of foraging trips, suggest that other, not necessarily independent, ecological/physiological factors beyond could play a role.

Table 1. Descriptive statistics for the % of habitat use (marine or rice field) of Audouin's gull by sex and fishing activities. Values are shown as mean $\pm$ standard deviation.

Sex	Fishing activity	% marine habitat	% rice field
Males	Workday	61.3 $\pm$ 39.3	38.7 $\pm$ 39.3
	Weekend	42.0 $\pm$ 40.6	58.0 $\pm$ 40.6
Females	Workday	64.5 $\pm$ 36.8	35.6 $\pm$ 36.8
	Weekend	58.5 $\pm$ 43.3	41.5 $\pm$ 43.3

Table 2. Descriptive statistics for some foraging behaviour parameters in the Audouin's gull. Values are shown as mean $\pm$ standard deviation. Maximum values are shown in parentheses.

Sex	Fishing activity	Trip length - Km	Trip duration - h	Daily distance – Km/day	Maximum distance - Km
Males	Workday	83.3 $\pm$ 71.0 (402.0)	7.2 $\pm$ 6.1 (39.6)	111.0 $\pm$ 61.7 (364.6)	35.8 $\pm$ 27.0 (145.2)
	Weekend	90.0 $\pm$ 89.2 (434.6)	9.6 $\pm$ 7.5 (32.9)	76.0 $\pm$ 51.6 (213.4)	33.2 $\pm$ 30.4 (149.8)
Females	Workday	94.3 $\pm$ 92.7 (533.5)	8.6 $\pm$ 8.5 (51.4)	126.2 $\pm$ 64.6 (282.9)	56.2 $\pm$ 43.3 (189.2)
	Weekend	160.7 $\pm$ 200.9 (771.4)	19.2 $\pm$ 20.2 (77.3)	130.5 $\pm$ 73.8 (259.8)	73.0 $\pm$ 56.7 (189.7)

NOTE: *Trip length* was calculated as the total cumulative linear distance between all locations along the foraging trip. *Trip duration* was calculated as the time lapse between departure and return from the colony. *Daily distance* stands for the total cumulative linear distance covered flying between all locations each day with data. *Maximum distance* was calculated as the linear distance between the furthest point of the trip and the nest for each day.

INDIVIDUAL SPECIALISTS IN A FLEXIBLE POPULATION

The high correlation between GPS data and stable isotopes (especially carbon, which discriminates well the use of resources from marine and freshwater habitats) indicates that the combination of both kind of data give us a good description of the trophic ecology and show that habitat use results into effective tissue assimilation. However, no significant interaction between sex and fishing activities was found for the habitat use, in contrast to what was found in the foraging trips. The possible explanation could be behind the great individual variability presented in the habitat use. Although, males and females, increased the use of rice field resources in absence of trawling activity, males during the weekends exploit this habitat to a greater extent than females. In spite of the fact that preying on rice fields increase in absence of fishing activities (Oro *et al.* 1996a), this sex-biased habitat selection (based on both, foraging behaviour and habitat use) has not been described before. However, no gender differences in plasma SIA were found, unlike what has been pointed by Navarro *et al.* 2010. This means that on a larger time-scale, traced by the stable isotopes, both males and females forage in the same habitats and consume equivalent preys during the incubation period.

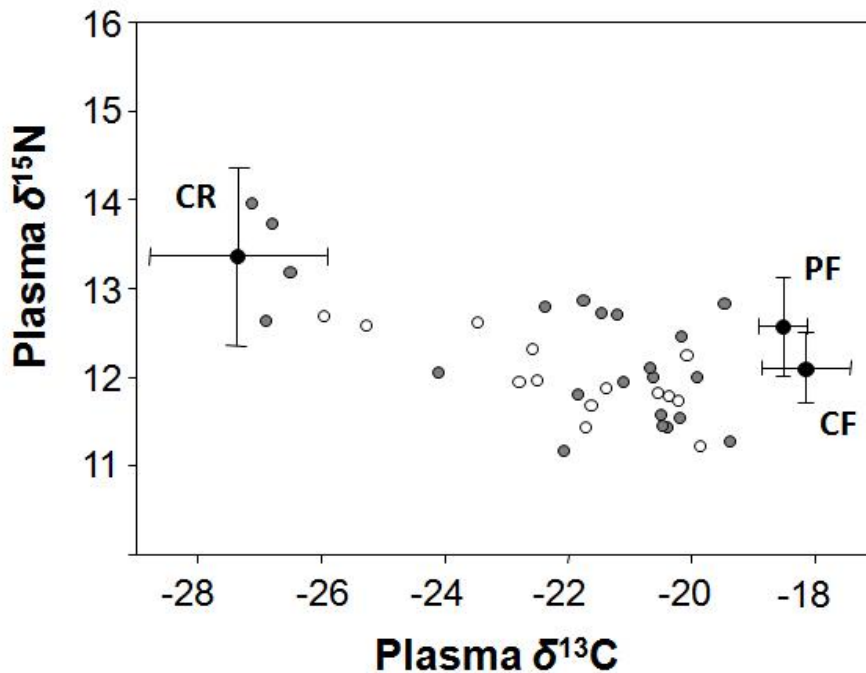


Fig. 2. Scatter plot of the dispersion between $\delta^{13}\text{C}$ / $\delta^{15}\text{N}$ plasma values of Audouin's gull at the recapture. Males are shown as grey dots, females as empty dots. The mean and standard deviation of American crayfish (CR), Perciform fish (PF) and Clupeiform fish (CF) are also shown as potential preys.

1.2. Sex-specific foraging behaviour

Both, SIA and GPS data agree that Audouin's gull exploits different habitats in the Ebro Delta. However, under this apparent trophic plasticity hides a high individual specialization. Individuals ranged from exclusively marine to rice field specialists, and all the way up to intermediate situations. Although a small decrease was found between capture events in the nitrogen signatures (ca. 0.2‰), both isotopes in plasma showed a great repeatability between capture events. The higher variation among individuals rather than within individuals suggests a great consistency in the habitat selection. Individual foraging specialisations seems to be common in seabirds (for example Votier *et al.* 2004b; Patrick *et al.* 2014) due to intra-specific aggregation and competition (Araújo *et al.* 2007), and because of the predictability of prey patches (Weimerskirch *et al.* 2007). In contrast, we found low repeatability in the foraging trips (trip length and duration); with the exception of females during weekends, which showed a great individual consistency in their foraging trips. Our results suggest that the gulls have far more foraging options at sea during work days, when trawlers are operating and large amounts of discards are available. But, during the weekends, females prefer the marine environment given the high repeatability of long foraging trips. This is consistent with the hypothesis that females need to target on specific resources after laying and they should know where and when to find these resources in situations of food shortage.

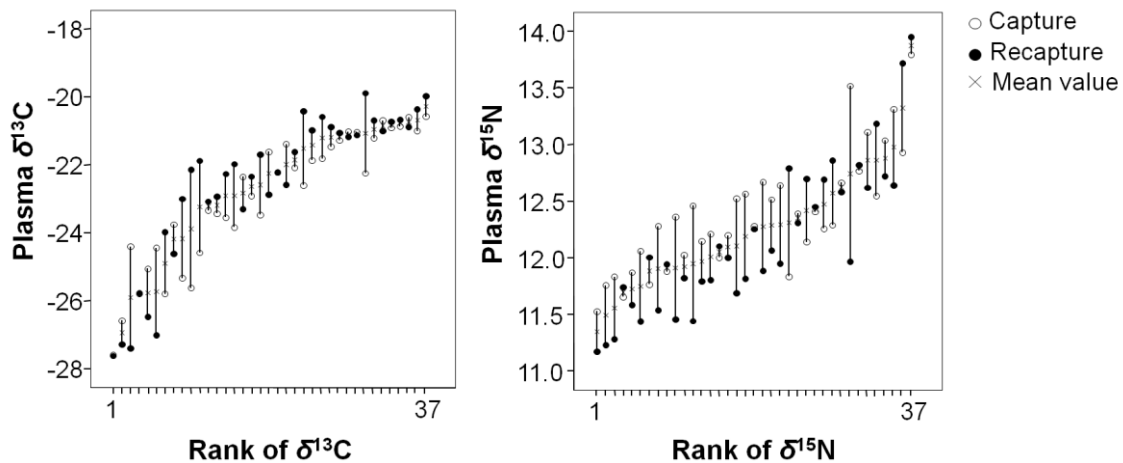


Fig. 3. Paired plots showing plasma $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ between capture events. Each bar represents capture and recapture values observed for each individual. Individuals are ranked according to its mean value.

1.2. Sex-specific foraging behaviour

IS HABITAT USE INFLUENCING THE INDIVIDUAL CONDITION?

Foraging trips descriptors suggest that flying effort is highly related to the habitat where individuals forage. Foraging effort devoted to feed in rice fields is significantly lower than that of foraging at sea, since gulls have to fly longer distances following the fishing vessels or to fish by themselves. However, and in spite of different costs, no significant relationship between the habitat use and the condition indices was detected. Tentatively, this lack of relationship could be indicating a possible trade-off between foraging effort and food quality: the larger energy cost of feeding at sea is offset by a higher nutritional quality of fish in relation to the American crayfish, and vice versa.

	Sex	Mean $\pm$ SD
$\delta^{13}\text{C}$ capture	male	-22.69 $\pm$ 1.88
	female	-23.14 $\pm$ 2.01
$\delta^{13}\text{C}$ recapture	male	-22.55 $\pm$ 2.53
	female	-22.53 $\pm$ 1.89
$\delta^{15}\text{N}$ capture	male	12.41 $\pm$ 0.54
	female	12.28 $\pm$ 0.58
$\delta^{15}\text{N}$ recapture	male	12.28 $\pm$ 0.77
	female	12.00 $\pm$ 0.44

Table 3. Descriptive statistics for the plasma stable isotopes ($\delta^{13}\text{C}$ and $\delta^{15}\text{N}$) of Audouin's gull by sex and capture event. Values are shown as mean $\pm$ standard deviation.

Why then females perform a greater use of marine habitats than males in the absence of fishing activities? Although usually overlooked, female nutritional requirements after laying could play an important role in sex-specific foraging behaviour (see for example Lewis *et al.* 2002; Xavier & Croxall 2005). It is known that egg formation is a demanding period for avian females in general (Nager *et al.* 2006) and Audouin's gull in particular (Ruiz *et al.* 2000), both in terms of energy and nutrient requirements. In this regard, some studies have shown that the need to restore calcium levels after laying could be an important explanatory factor related to sexual foraging segregation (reviewed in Wearmouth & Sims 2008). Indeed, calcium levels in females of Audouin's Gull have been reported as a limiting factor that constrains the egg synthesis (Ramirez *et al.* 2013). In this way, fish soft-bones represent a major source for absorbed calcium compared to crustaceans (Hansen *et al.* 1998). Moreover, recent work has shown that antioxidants are limiting for breeding females of Audouin's Gull, and the importance of marine diets for providing eggs a greater antioxidant capacity than rice field resources (García-Tarrasón *et al.* 2014).

CONCLUSION

Although our results agree with previous studies showing that American crayfish act as buffer for Audouin's gull in absence of trawling activities (Oro *et al.* 1996a), data presented here on foraging behaviour and habitat use point that crayfish is being differentially targeted by both sexes. Females perform longer at-sea foraging trips in situations of food shortage probably because specific nutritional requirements (energy content, micronutrients) associated to egg laying. Taking into account that Audouin's gull is more likely to approach longline vessels when trawlers are not operating (Arcos & Oro 2002), and that this species is the second most affected seabird by longline bycatch in the Mediterranean (Laneri *et al.* 2010), a ban on discards could lead to an important sex-biased bycatch mortality (Báez *et al.* 2014). Anyway, individual cognitive abilities and specializations such as learning how human activities can modify the availability and predictability of food resources may play a very important role determining the foraging behavior of the individuals (Cama *et al.* 2012; Oro *et al.* 2013). Since these individual specializations may take several years to learn (Bicknell *et al.* 2013), a gradual implementation of any policy is needed in order to facilitate the adaptation of threatened species like Audouin's gull to a discard ban scenario.

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SUPPLEMENTARY MATERIAL

Figure S1. (A) Parameter estimate $\pm$ standard error for the logistic GLMM to fit the proportion of rice field positions over the total feeding positions. Weekends were used as a reference level in the analysis. (B) 95% CI of the estimated proportion of rice field positions derived from the model.

A

	Logit Proportion of rice field positions
Intercept	0.127 ± 0.432
Work days	-0.798 ± 0.301
Weekends	Ref.
Model variances	
Individual	3.036 ± 0.869
Residual	29.697 ± 2.436

B

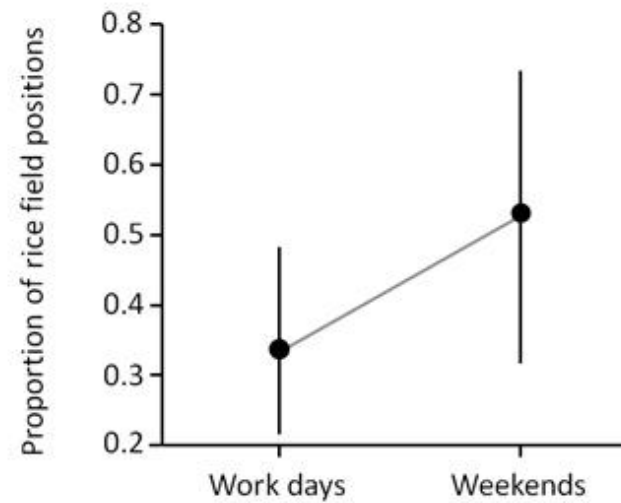


Figure S2. (A) Parameter estimate $\pm$ standard error for the selected final GLMM in the log transformed distance covered per day (daily distance, in Km). Weekends and females were used as a reference levels in the analysis. (B) 95% CI of the estimated daily distance (in Km) derived from the model.

	Log Daily Distance
Intercept	1.999 ± 0.078
Work days	0.032 ± 0.060
Weekends	Ref
Males	-0.217 ± 0.093
Females	Ref.
Work days * Males	0.160 ± 0.074
Model variances	
Individual	0.015 ± 0.006
Residual	0.052 ± 0.005

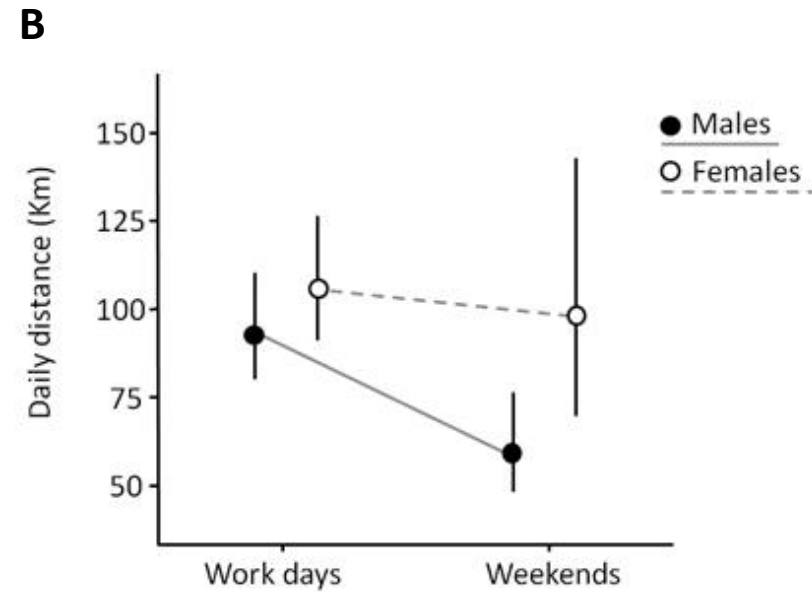


Figure S3. (A) Parameter estimate $\pm$ standard error for the selected final GLMM to fit the log transformed maximum daily distance from the colony (in Km). Females were used as a reference level in the analysis. (B) 95% CI of the estimated maximum daily distance from the colony (in Km) derived from the model.

A

	Log Maximum Daily Distance from the Colony
Intercept	1.628 ± 0.013
Males	-0.164 ± 0.077
Females	Ref.
Model variances	
Individual	0.041 ± 0.869
Residual	0.061 ± 0.005

B

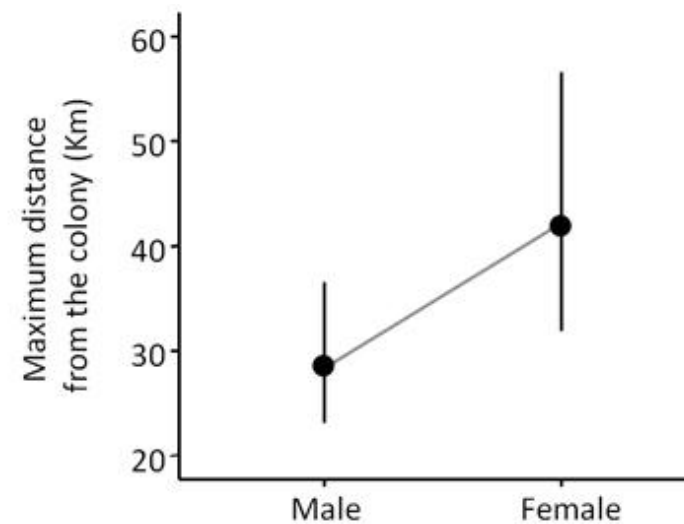


Figure S4. (A) Parameter estimate $\pm$ standard error for the selected final GLMM in the log transformed trip length (in Km). Weekends and females were used as a reference levels in the analysis. (B) 95% CI of the estimated trip distance (in Km) derived from the model (in logarithmic scale).

A

	Log Trip Length
Intercept	2.052 ± 0.129
Work days	-0.193 ± 0.093
Weekends	Ref
Males	-0.294 ± 0.142
Females	Ref.
Work days * Males	0.234 ± 0.101
Model variances	
Individual	0.029 ± 0.011
Residual	0.119 ± 0.010

B

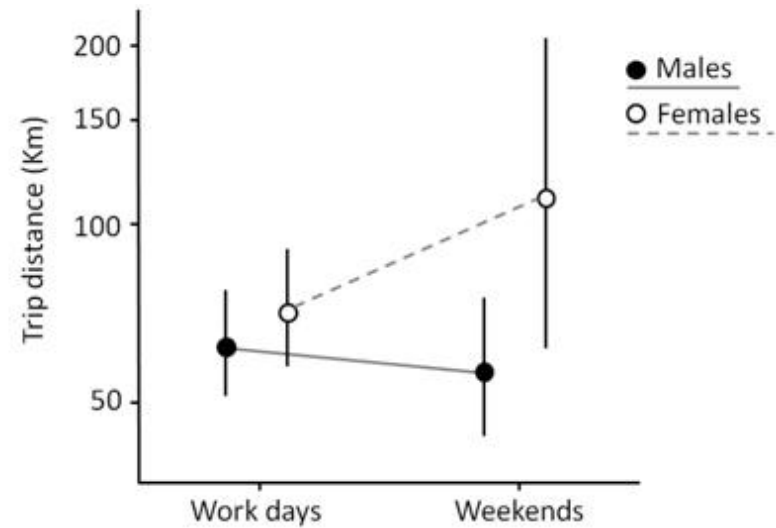
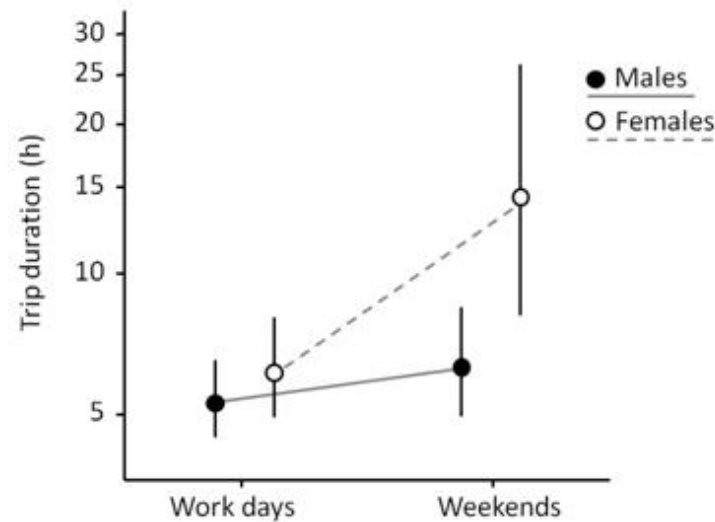


Figure S5. (A) Parameter estimate $\pm$ standard error for the selected final GLMM in the log transformed trip duration (in hours). Weekends and females were used as a reference levels in the analysis. (B) 95% CI of the estimated trip duration (in hours) derived from the model (in logarithmic scale).

A

	Log Trip Duration
Intercept	1.165 ± 0.126
Work days	-0.364 ± 0.103
Weekends	Ref
Males	-0.353 ± 0.140
Females	Ref.
Work days * Males	0.284 ± 0.117
Model variances	
Individual	0.025 ± 0.010
Residual	0.139 ± 0.011

B



CHAPTER 1.3. Anthropogenic input of heavy metals in two Audouin's gull breeding colonies

Título: Aporte antropogénico de metales pesados en dos colonias de cría de gaviota de Audouin

Resumen: Las actividades antropogénicas se han convertido en una importante fuente de metales pesados en los ecosistemas marinos. 'Centinelas biológicos' como los pollos de las aves marinas han sido ampliamente utilizados para el seguimiento de los niveles de algunos metales pesados. Debido a sus hábitos de alimentación eminentemente marinos, la gaviota de Audouin se ajusta bien para este propósito. Se midieron los niveles de mercurio y plomo en las plumas del manto de pollos de gaviota de Audouin en dos colonias del NE de la Península Ibérica: el Delta del Ebro y el Delta del Llobregat. Ambas son áreas antropizadas sujetas a un aporte diferencial de contaminantes. Los niveles de plomo fueron significativamente mayores en el Delta del Llobregat, probablemente debido al uso de combustibles con plomo en el cercano aeropuerto de Barcelona. Por otro lado, las concentraciones de mercurio fueron más altas en el Delta del Ebro, relacionado con la acumulación de sedimentos tóxicos en el embalse de Flix, arrastrados por la corriente del río Ebro. Estos niveles de mercurio detectados en los pollos del Delta del Ebro alcanzaron valores que han sido descritos como tóxicos.

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Anthropogenic input of heavy metals in two Audouin's gull breeding colonies

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Abstract: Anthropogenic activities have become an important source of heavy metals to the marine environments. Biological sentinels like seabirds' chicks have been widely used to monitorize the levels of some heavy metals. Due to its mainly marine foraging habits, Audouin's gull fits well for this purpose. Mercury and lead levels were measured in mantle feathers of Audouin's gull chicks from two colonies in NE Iberian Peninsula: the Ebro Delta and the Llobregat Delta. Both are anthropized areas subject to differential pollutant inputs. Lead levels were significantly higher in the Llobregat Delta probably due to the use of leaded fuel in the nearby Barcelona airport. On the other side, mercury concentrations were higher in the Ebro Delta, in relation with the disposal of the toxic sediments at the Flix site carried down by the Ebro River. These mercury levels in the Ebro chicks reached values that have been described as toxic.

Keywords: Audouin's gull, Stable isotopes, Mercury, Lead, Aviation gasoline

1. INTRODUCTION

Anthropogenic processes as mining, fossil fuel burning, production of chemicals and agricultural activities have become an important source of heavy metals to marine environments (Furness and Rainbow, 1990; Monteiro & Furness, 1997; Dietz *et al.* 2009). Marine species are particularly susceptible to heavy metal incorporation because oceans act as ultimate sink for the most part of pollutants and because their accumulation in the sediments of intertidal environments (Bearhop *et al.*, 2000; Burger *et al.*, 2007). For this reason, some marine animal groups have been widely used as bioindicators of pollution levels. This is the case of seabirds, particularly adequate as indicators due to the detailed knowledge about their ecological habits and their relative high trophic position in the marine food webs. Their colonial nature also allows collecting large sample sizes from a particular site in a relatively short period of time (Furness and Camphuysen, 1997). Chick feathers are ideal for this purpose because they allow a non-invasive sampling and reflect the availability of some metals of a local area during the chick rearing phase (Furness, 1993).

Mercury is one of the most studied heavy metals, emitted to the environment from both natural and anthropogenic sources (Nriagu, 1989). In the mesopelagic environment there is a major transformation from inorganic mercury to methylmercury, the most toxic and stable form (Nriagu, 1989; Monteiro *et al.*, 1998; Wright and Welbourn, 2002). In birds, dietary mercury is incorporated into the feathers from the blood, and bioaccumulates through the food chain (Nriagu, 1989; Arcos *et al.*, 2002). On the other side, lead in feather appears to originate mainly from atmospheric deposition onto feather surfaces, whereas ingested lead become firmly bound in bones and only enter feathers in trace amounts (Furness, 1993; Furness and Camphuysen, 1997). However, lead levels in feathers can also help us to elucidate the characteristics of a given area since the main part of atmospheric lead has an anthropogenic origin (Wright and Welbourn, 2002). Because both, mercury and lead can reach toxic levels when at high concentrations (Furness, 1993), it is important to monitorize the levels of these elements.

This is the possible scenario of the two wetlands that we considered in our study: the Ebro Delta and the Llobregat Delta (Fig. 1). Both are situated in the northeastern part of the Iberian Peninsula, and both are anthropized areas subject to pollutant inputs. The case of the Ebro River is particularly alarming due to decades of dumping toxic waste to its basin, mainly at Flix town (41°14'N, 00°31'E; Fig. 1) where a chemical industry has been in operation since the early 20th century. The different industrial

activities at this place and the construction of a dam close to the factory in 1960 resulted in the accumulation of 200,000–360,000 tons of industrial wastes in the riverbed. This wastes accumulated in the sediments include high concentrations of heavy metals (cadmium, arsenic, copper, chromium, lead and especially mercury), organochlorines and radioactive ^{210}Pb (Fernandez *et al.*, 1999; Bosch *et al.*, 2009). The pollutants originated at Flix site are carried downstream by the Ebro River to its Delta 90 km away, especially during floods (Vericat and Batalla, 2006). In this way, there have been many studies highlighting the effects of this problem on the biota (Quiros *et al.*, 2008; Carrasco *et al.*, 2008, 2011; Barata *et al.*, 2010; Suarez-Serrano *et al.*, 2010; Cotin *et al.*, 2012). This scenario originates one of the most important ecological problems still to be solved in western Mediterranean, not only because it affects one of the most relevant wetlands in south Europe (the Ebro Delta), but also because this is an important agricultural area and the Ebro river potentially supplies water to 2,7 million people (CHEbro 2009). On the other hand, the Llobregat Delta is a smaller wetland with a strong human alteration, located 150 km north of the Ebro Delta. Because it is located very close to the Barcelona Airport in the metropolitan area (with more than 3 million people), the anthropogenic inputs of pollutants might be also evident. The relation between lead levels and airports has been reported previously (Kessler, 2013). Aviation gasoline, commonly referred to as avgas, is a leaded fuel used to power piston-engine small aircraft (Kessler, 2013). The most commonly used avgas is 100LL. Although the “LL” stands for low lead, 100LL contains up to 0.56 g/L lead (Royal Dutch Shell, 2010). Some studies indicate that air lead levels near airports with substantial avgas use are significantly higher than background levels (U.S. EPA, 2010; Miranda *et al.*, 2011).

Both deltas hold a breeding population of Audouin's gull. In the Ebro Delta this species bred for first time in 1981, increasing rapidly their numbers and at present it accounts 11,000 pairs (approximately 60% of the world population) (Oro and Ruxton , 2001; Oro *et al.*, 2010). In the case of the Llobregat Delta, there is a small colony recently established in 2009, reaching in 2011 a population of 380 pairs (Gutiérrez and López, 2012). Due to its mainly piscivorous habits, chick feathers of Audouin's gull can be particularly appropriate to monitorize trace elements levels in these important coastal areas for biodiversity (Goutner *et al.*, 2000; Sanpera *et al.*, 2007a). Moreover, at least in the Ebro Delta these gulls can also take advantage of fresh water resources from neighbouring rice fields (Navarro *et al.*, 2010). In this way, using biogeochemical markers as the stable isotopes analysis (SIA) in feathers (mainly carbon or $\delta^{13}\text{C}$ and nitrogen or $\delta^{15}\text{N}$) we can perform better insights into the habitat use and foraging

1.3. Anthropogenic input of heavy metals

ecology of the chicks (Cherel *et al.*, 2000; Cotin *et al.*, 2012). The combination of these biomarkers (especially the nitrogen isotope, a marker of the trophic level) with heavy metal concentrations enable to check biomagnification processes (Bearhop *et al.*, 2000; Cotin *et al.*, 2011).

In this study we analyzed Audouin's gull chick feathers to assess the carbon and nitrogen stable isotope ratios (as a tracer of the chick's diet) and heavy metal levels (mercury and lead) in gulls from both localities. The main hypothesis is that mercury concentration must be higher at the Ebro Delta due to the influence of the toxic muds upstream. In the case of the Llobregat Delta, we expect to find higher lead levels in this locality due to the use of aviation fuel at the nearby Barcelona airport. The comparison of both populations of Audouin's gull will allow us to evaluate the exposure risk to which these areas are subject, and detect if there have been changes in mercury levels in recent years using previous data in the case of the Ebro Delta (Sanpera *et al.*, 2007a). Additionally, isotope ratios and metal levels were also analyzed in order to check for potential diet association with the pollutant accumulation. Finally, our study constitutes the first approach to the foraging ecology and pollutant levels for the recently established Audouin's gull colony at the Llobregat Delta.

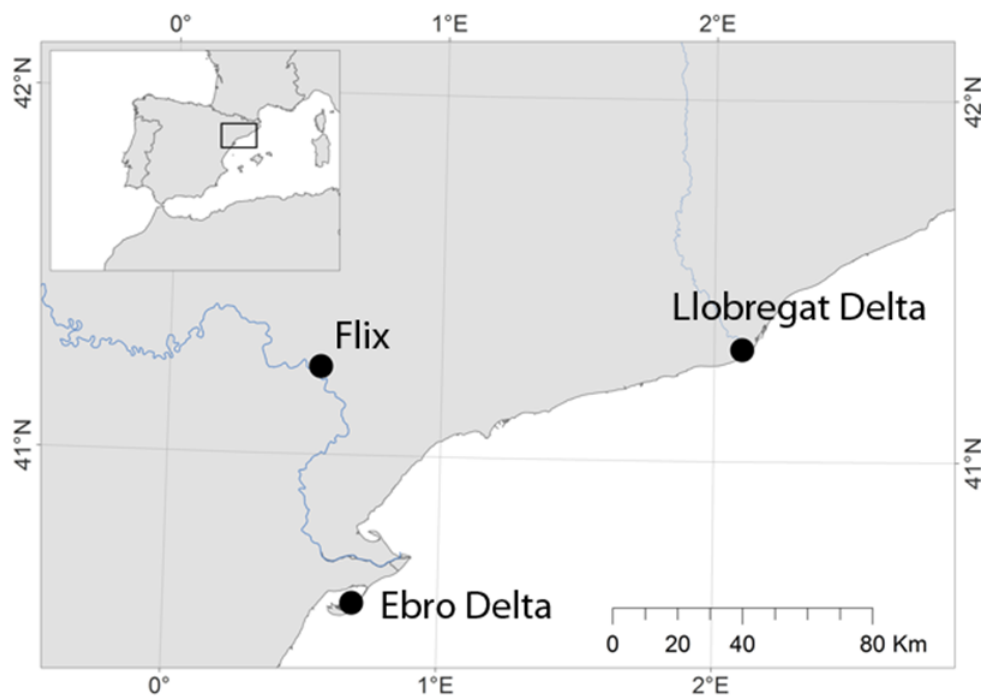


Figure 1. Map of the sampled colonies (Ebro Delta and Llobregat Delta) and the location of toxic muds accumulated at Flix dam.

2. MATERIAL & METHODS

Sampling

During the 2011 breeding season, fledging chicks of Audouin's gulls were sampled at two different locations in NE Iberian Peninsula: the Ebro Delta (N = 16) and Llobregat Delta (N = 13). Sampling was carried out taking advantage of the chick ringing in both colonies (when they were two to three weeks old). From each chick, a bunch of mantle feathers were taken, avoiding pseudo-replication by sampling only one fledging per brood. Feather samples were kept in polyethylene bags and frozen until analysis. Once in the laboratory, feathers were cleaned with 0,25M NaOH solution and dried for 24h at 60°C prior to heavy metal determination and SIA. Once cleaned and dry, in order to homogenize them, feathers were ground to powder using an impactor mill (Freezer Mill 6750, Spex CertiPrepH Inc., Metuchen, NJ, USA) operating at liquid nitrogen temperature.

Potential preys were also sampled for isotopic determination based on previous information from Audouin's gull diet at the Ebro Delta. Prey items from ricefields included American crayfish *Procambarus clarkii* (N = 15), common carp *Cyprinus carpio* (N = 10) and gambusia *Gambusia affinis* (N = 10). Items from fishing discards (the most important marine source, Oro *et al.*, 1997) were classified into two groups: clupeiform fish (sardine *Sardina pilchardus* and anchovy *Engraulis encrasicolus*, N = 20) and perciform fish (mackerel *Trachurus trachurus* and bogue *Boops boops*, N = 20). These two fish orders represent the most important ones for Audouin's gull diet (Pedrocchi *et al.*, 2002). The potential preys were treated whole and subjected to lipid extraction by successive rinsing in a chloroform and methanol solution (2:1), following the protocol of Folch *et al.* (1957), in order to reduce variability due to differences in lipid content among tissues (DeNiro and Epstein, 1978). All of the prey samples were oven-dried at 60 °C before being mechanically ground into a fine powder.

Stable isotopes analysis

Feather (ca. 0.32 to 0.35 mg) and preys (ca. 0.5 mg) subsamples were placed into tin buckets and crimped for combustion for stable carbon and nitrogen determination. Isotopic analyses were carried out at the Scientific and Technical Services of the Universitat de Barcelona (Spain) by means of a Thermo-Finnigan Flash 1112 elemental analyser (CE Elantech, Lakewood, NJ, USA) coupled to a Delta-C isotope ratio mass spectrometer via a CONFLO III interface (Thermo Finnigan MAT, Bremen, Germany). The laboratory applies international standards, which are run for

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every 12 samples: IAEA CH₇ (87 % of C), IAEA CH₆ (42 % of C) and USGS 24 (100 % of C) for ¹³C and IAEA N₁ and IAEA N₂ (with 21 % of N) and IAEA NO₃ (13.8 % of N) for ¹⁵N. Stable isotope ratios were expressed in the standard δ notation relative to Vienna Pee Dee Belemnite ($\delta^{13}\text{C}$) and atmospheric N₂ ($\delta^{15}\text{N}$). Replicate assays of standards indicated analytical measurement errors of $\pm 0.1\text{‰}$ and $\pm 0.2\text{‰}$ for $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$, respectively.

Trace elements analysis

The analysis of trace elements (Hg and Pb) in feathers was done according to the following acid digestion protocol: 0.1 g of the sample was digested with 2 ml of HNO₃ (70%) and 1 ml of H₂O₂ (30%) using a Teflon reactor for 12 h at 90°C. The digested product was then diluted in 15 ml of distilled water, and the determination of trace elements was carried out with an ICP-MS (Induction Coupled Plasma-Mass Spectrometer) Perkin Elmer Optima 6000. The accuracy of the analysis was checked by measuring CRM certified reference tissue (Human Hair CRM 397). The results for Hg and Pb gave a mean recovery of 101.9 % and 106.3 %, respectively. No corrections were applied to the original results.

Statistical analysis

Values of trace elements concentrations and stable isotope ratios were routinely checked for normal distributions with Q–Q plots. Trace elements concentrations showed skewed distributions which were normalized by applying a logarithmic transformation.

Pearson's correlation coefficient was used to test if there were relationships among the different variables of the study. ANCOVA analysis was performed in order to evaluate if the relation $\delta^{13}\text{C}$ - $\delta^{15}\text{N}$ changes between localities. A similar analysis was used for modeling heavy metal concentration using stable isotope and locality as explanatory factors. If interaction term was not significant, it was excluded from the model. Bivariate plots were used to represent the stable isotopes and heavy metal data. In the case of potential preys, they were represented by adding an isotopic discrimination factor of 2.91 ‰ for $\delta^{15}\text{N}$ and 1.15 ‰ for $\delta^{13}\text{C}$ following Caut *et al.* 2009. Statistical analyses were done using SPSS 19.0 (SPSS Inc., Chicago, USA).

3. RESULTS

Stable isotopes

Most Audouin's gull chicks from both populations showed clearly a marine diet, with characteristic enriched carbon values and depleted nitrogen ones compared to ricefield resources (Fig. 2a). However, in the case of the Ebro Delta, two chicks presented a diet including a higher proportion of ricefield resources, so these individuals were removed from subsequent analysis to reduce variability. Fig. 2b shows a detailed scatter plot only for the isotopic values of the marine-fed chicks. Taking into account both sampled colonies, a significant positive correlation between $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ was found ($r = 0.50$, $p = 0.01$, $N = 27$) (Fig. 2b). An ANCOVA analysis showed that this relationship do not vary between localities (interaction term $F_{1,23} = 0.96$, $p = 0.34$). Nevertheless mean isotopic signatures show significant shift between localities. Ebro Delta chicks presented lower $\delta^{15}\text{N}$ values ($F_{1,25} = 18.44$, $p < 0.001$) and lower $\delta^{13}\text{C}$ ones ($F_{1,25} = 5.07$, $p = 0.03$) than Llobregat Delta fledglings. Descriptive statistics for the stable carbon and nitrogen ratios of chick feathers are shown in Table 1.

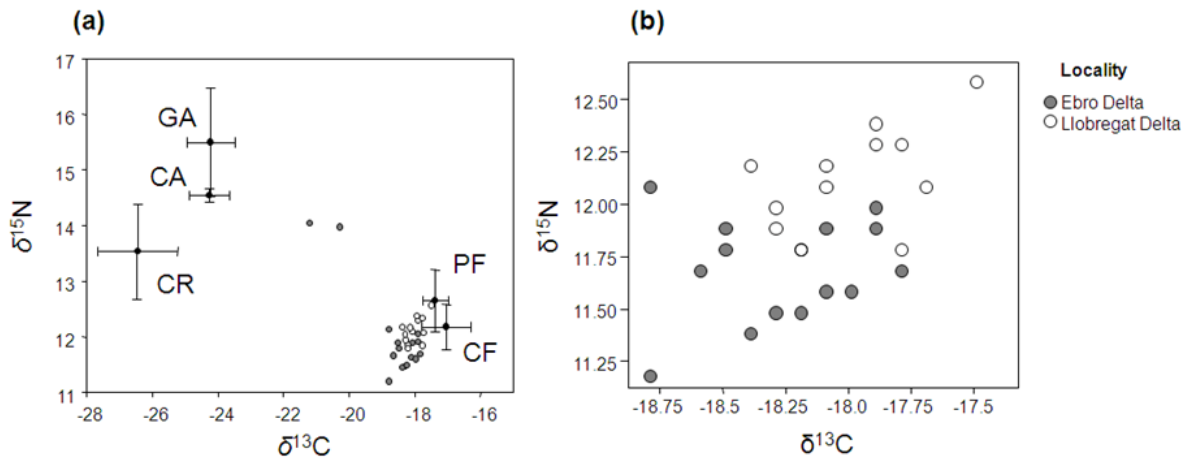


Figure 2. (a) Scatter plot of the dispersion between $\delta^{13}\text{C}$ / $\delta^{15}\text{N}$ values of Audouin's gull chicks from both localities. The mean and standard deviation of American crayfish (CR), common carp (CA) and gambusia (GA) are represented as potential preys from rice fields. Perciform fish (PF) and clupeiform fish (CF) are also shown as potential preys from the sea. (b) Detailed scatter plot of the dispersion between $\delta^{13}\text{C}$ / $\delta^{15}\text{N}$ values of marine-fed Audouin's gull chicks.

Trace elements

Bivariate plot of mercury and lead concentrations between localities (in a logarithmic scale) are shown in Fig 3.

Regarding Hg concentration, adjusted ANCOVA with the $\delta^{15}\text{N}$ and the locality factor showed that interaction term was not significant ($F_{1,23} = 0.26$, $p = 0.88$) and not significant relationship with nitrogen signatures was found ($F = 0.37$, $p = 0.55$). Final model only showed differences between localities ($F = 23.25$, $p < 0.001$), presenting the Ebro Delta higher concentrations. Adjusting a new model for mercury with the $\delta^{13}\text{C}$, the interaction term was also not significant ($F = 0.26$, $p = 0.62$) and not significant relationship with carbon signatures was found ($F = 0.12$, $p = 0.74$). Thus, only differences between localities arise.

Similar results were found in the case of Pb. Adjusting the model with the $\delta^{15}\text{N}$ and the locality, the interaction term was not significant ($F_{1,23} = 2.15$, $p = 0.16$) and not significant relationship with nitrogen signatures was found ($F = 1.4$, $p = 0.25$). In a new model for lead adjusting for the $\delta^{13}\text{C}$, the interaction term was not significant ($F = 0.22$, $p = 0.62$) and not significant relationship with carbon signatures was found ($F = 0.43$, $p = 0.52$). Final model only showed differences between localities ($F = 125.6$, $p < 0.001$).

4. DISCUSSION

Audouin's gull has been traditionally widely studied in ecological and pollution works (Pedrocchi *et al.*, 1996; 2002; Sanpera *et al.*, 2007a, b). This allows a better interpretation of results, and its comparison with previous data to detect temporal changes.

Regarding stable isotopes, our data show that chicks from both localities are fed mainly with marine piscivorous resources (Fig. 2). In the case of the Ebro Delta, a few chicks showed depleted carbon and enriched nitrogen signatures, indicating some contribution of rice field resources. Although the isotope data of Navarro *et al.* (2010) estimated that rice field consumption by the chicks was between 19-32%, the data of this study are more in agreement with those of Pedrocchi *et al.* (2002) and Sanpera *et al.* (2007a) reporting that most of this gull's diet consists of marine fish. In the case of Llobregat Delta there were no previous data about this newly formed colony. We have found a fully marine diet for these chicks, probably due to the lack of surrounding rice fields in the area unlike in the Ebro Delta. Marine-fed chicks from both localities

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presented small significant isotopic differences that could be attributed to differences in fishing activities that could influence isotopic signatures. In fact, the fishing fleet of the Llobregat Delta area consists mainly in trawlers and demersal longliners. However, in addition to a strong trawling fleet, the Ebro Delta area also holds a higher proportion of purse seiners fishing on clupeiforms (Arcos *et al.*, 2009).

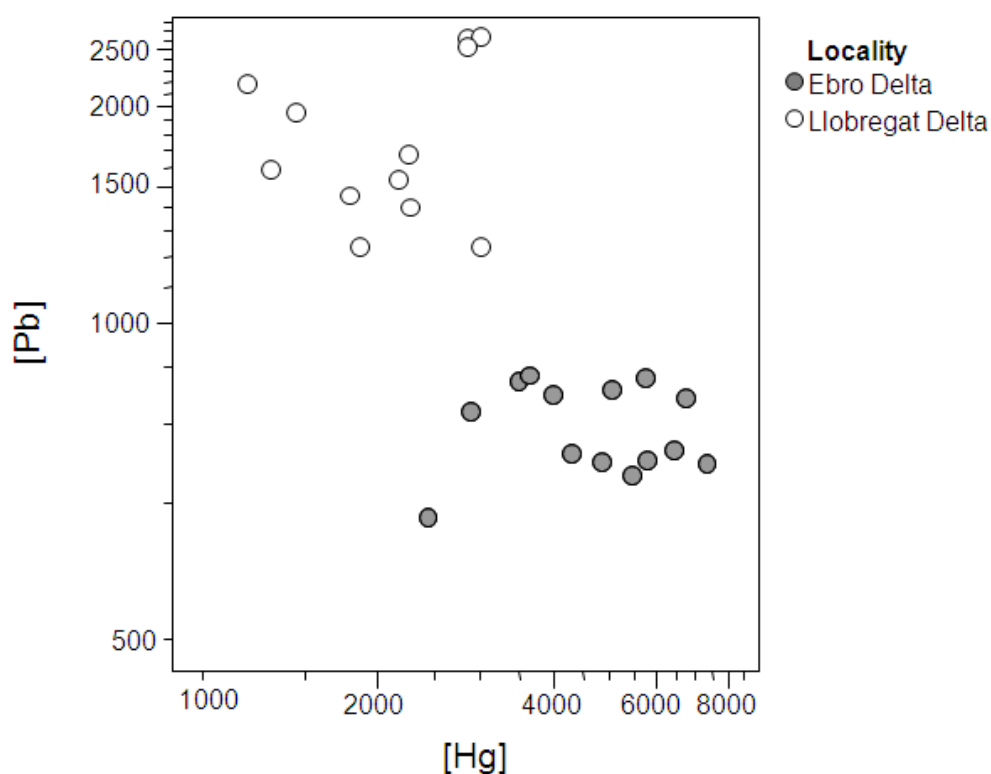


Figure 3. Scatter plot of the dispersion between the concentrations of lead and mercury (in ng·g⁻¹) of Audouin's gull chick feathers from both localities.

Given the dependence on marine resources for Audouin's gull chicks' diet, fledglings of this species seem a good candidate to monitorize both coastal areas. As we initially postulated, we have found significant higher mercury levels in the Ebro Delta than in Llobregat Delta. Indeed, although the Mediterranean Sea is already a polluted area with quite high concentrations of mercury (Cossa *et al.*, 1997), the main cause of these very high levels in the Ebro Delta must be sought in the pollutants carried by the river waters. The influence of the toxic sediments accumulated at Flix reservoir on mercury levels has even been noticeable on coastal birds some 90 km downstream (Cotin *et al.*, 2011; Cotin *et al.*, 2012). The high mercury levels

accumulated in these sediments are discharged to the sea, then converted to methylmercury and finally enter in the food web (Bryan, 1979). Since methylation processes are especially important in mesopelagic waters, the significant trawling activity in the Ebro Delta area has been put forward as a very important source of mercury pollution for seabirds (Arcos *et al.*, 2002). In this way, the conjunction of these polluted waters besides the trawling activity makes that seabird species foraging efficiently on discards (as Audouin's gull) reach important mercury levels. In many bird species, mercury concentrations in feathers above $5,000 \text{ ng}\cdot\text{g}^{-1}$ are associated with adverse reproductive effects such as lower clutch and egg size, a lower hatching success and a decreased chick survival (Eisler, 1987). All Llobregat Delta chicks are below this threshold. However, the mean concentration detected in the Ebro Delta is dangerously close to $5,000 \text{ ng}\cdot\text{g}^{-1}$. In fact, the 50% of the marine-fed chicks in this area present values above this threshold and are therefore susceptible to the sublethal effects described. The mean concentration ranged from 2.5 to 5 times that found in Audouin's gull chick feathers from Northeastern Mediterranean (Goutner *et al.*, 2000). Moreover, the levels found in our work at the Ebro Delta are very similar to those reported 7 years before in chicks of the same species in the same area (Sanpera *et al.*, 2007a), which means that this important mercury inputs discharged by the river have remained stable throughout time. However, no case of adverse reproductive effects associated with high mercury levels in eggs was observed in previous studies (Morera *et al.*, 1997). At the time this paper was written, the works to remove the toxic muds at Flix had started. It would be important to use biological sentinels as Audouin's gulls to monitorize the evolution of pollutant levels during and after removal works. Also, further research should be needed on how this mercury accumulation affects reproductive and physiological parameters in this near threatened seabird.

On the other hand, lead concentrations detected in our study showed to be significantly higher in the Llobregat Delta. Since the most part of this heavy metal present in the feathers came from atmospheric deposition, the atmospheric lead levels from both areas can give us key clues. In fact, lead atmospheric concentration is much higher in the Llobregat Delta area ($25.1 \text{ }\mu\text{g}\cdot\text{m}^{-3}$) than in the Ebro Delta ($2.5 \text{ }\mu\text{g}\cdot\text{m}^{-3}$) (IDEGCAT, 2011). As mentioned above, this situation could be a consequence of the vicinity of the Barcelona airport to the Llobregat Delta colony (just 2 Km). The use of the leaded fuel avgas by aircrafts probably represents an important input of lead to the surrounding atmosphere. In the United States, for example, lead emitted from aircraft using avgas is currently the largest source of lead to the air, constituting about 50% of lead emissions in 2005 (U.S. EPA, 2010). In a study on childhood blood by Miranda *et*

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al. (2011), lead levels decreased as we move away from the airport, with the largest impact on children living within 500 m. In birds, it has been suggested that lead levels in feathers above 4,000 ng·g⁻¹ are associated with delayed parental and sibling recognition, impaired locomotion, depth perception and feeding behavior, and lowered chick survival in gulls (reviewed in Burger & Gochfeld, 2000). In any case this threshold is exceeded, being all the chicks far from this limit and apparently safe from lead diseases. However, the Ebro Delta has traditionally suffered a very important input of lead derived from hunting activities (Mañosa *et al.*, 2001), with a lead pellet concentration ranging from <8900 to 2661000 pellets·ha⁻¹ in the first 20 cm of sediment. Further studies would be desirable on lead levels from other tissues susceptible to accumulate environmental lead.

	Locality	Mean	SD	Minimum	Maximum
$\delta^{15}N$ (‰)	Ebro Delta	11.70	0.25	11.20	12.10
	Llobregat Delta	12.12	0.25	11.80	12.60
$\delta^{13}C$ (‰)	Ebro Delta	-18.28	0.33	-18.80	-17.80
	Llobregat Delta	-18.02	0.27	-18.40	-17.50
[Hg] (ng·g ⁻¹)	Ebro Delta	4868.55	1476.64	2438.68	7333.18
	Llobregat Delta	2053.74	759.89	644.75	3006.65
[Pb] (ng·g ⁻¹)	Ebro Delta	298.35	65.40	185.90	383.27
	Llobregat Delta	1364.68	518.07	734.23	2139.66

Table 1. Descriptive statistics for the stable isotopes and heavy metal concentrations in marine-fed Audouin's gull chick feathers. Ebro Delta: N = 14, Llobregat Delta: N = 13.

Although mercury has a well known biomagnification process in living systems, we have not detected a significant relationship between heavy metal levels and the nitrogen isotope (indicating no relation with the trophic level). However, this lack or relationship may be caused by the narrow range of isotopic variation found in both localities.

In conclusion, chicks from both areas present mainly a marine diet, which makes them especially suitable to monitorize these coastal areas. Lead levels are significantly higher in the Llobregat Delta probably due to the use of leaded fuel in the nearby Barcelona airport. On the other side, mercury concentrations were much higher in the Ebro Delta, in relation with the disposal of the toxic sediments of the Flix site carried down by the Ebro river. These mercury levels in the Ebro chicks reached values that have been described as toxic.

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SECTION 2:
RESOURCE ALLOCATION TO THE
CLUTCH AND ECOPHYSIOLOGY

Audouin's gull egg hatching (Ebro Delta, Spain).
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CHAPTER 2.1. The Weekend's Hangover: intra-clutch patterns of $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ reveal the ecological footprint of fisheries in Audouin's Gull eggs

Título: La resaca del fin de semana: los patrones intra-puesta de $\delta^{13}\text{C}$ y $\delta^{15}\text{N}$ revelan la huella ecológica de las actividades pesqueras en los huevos de gaviota de Audouin

Resumen: La producción de los huevos es un proceso costoso para las aves. Sin embargo, nuestro conocimiento es escaso sobre cómo la formación del huevo está limitada energética y nutricionalmente en aves salvajes en relación al orden de puesta. Afortunadamente, hoy en día es posible trazar la asignación de nutrientes a los huevos mediante el uso de los isótopos estables. Para ello, determinamos el $\delta^{15}\text{N}$ y $\delta^{13}\text{C}$ en puestas de tres huevos de gaviota de Audouin (*Larus audouinii*) del Delta del Ebro (NE España). Dos hábitats de nidificación (dunas costeras y salinas) y dos temporadas de cría consecutivas (2009 y 2010) fueron analizados. La alta sensibilidad a cambios diarios en la disponibilidad de descartes refleja que la mayor parte del nitrógeno depositado en el albumen debe provenir de la dieta. Este factor habitualmente pasado por alto es de especial interés en especies que muestran una gran dependencia sobre las pesquerías. Sin embargo, el carbono debe tener un origen más endógeno dado que este patrón diario no es tan obvio. La contribución endógena del carbono a través de la puesta debe ser diferente dado el consistente patrón intra-puesta de empobrecimiento en el $\delta^{13}\text{C}$. Finalmente, el hábitat de nidificación y la dieta estuvieron relacionados con el tamaño del huevo, indicando que a pesar de los procesos hormonales subyacentes involucrados en generar variabilidad intra-puesta en el tamaño, la plasticidad fenotípica también juega un papel importante en el patrón final observado.

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The Weekend's Hangover: intra-clutch patterns of $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ reveal the ecological footprint of fisheries in Audouin's Gull eggs

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Summary: Egg production is a demanding process for birds. However, we have a limited understanding in how egg formation is energetically and nutritionally constrained in wild birds in relation to the laying order. Fortunately, now is possible to trace the nutrient allocation into the eggs through the stable isotopes. We determined the albumen $\delta^{15}\text{N}$ and $\delta^{13}\text{C}$ in three-egg clutches of Audouin's gull (*Larus audouinii*) from the Ebro Delta (NE Spain). Two different nesting habitats (coastal dunes and salt pans) and two consecutive breeding seasons (2009 and 2010) were analyzed. The high sensitivity to daily changes in discard availability reflects that the most part of the nitrogen deposited in the albumen must come from diet. This overlooked factor is of special interest in species that show a great dependence on fisheries. Carbon, however, must have a more endogenous origin since this daily pattern is not so obvious. The endogenous carbon contribution across the laying sequence must be different given the consistent declining $\delta^{13}\text{C}$ intra-clutch pattern. Finally, nesting habitats and diet were related to egg size, indicating that despite the underlying hormonal processes involved in generating intra-clutch variability in size, phenotypic plasticity also plays an important role in the observed final pattern.

Keywords: egg production, nutrient allocation, laying order, stable isotopes, egg size, fisheries

INTRODUCTION

Traditionally, the study of avian life-history traits has been mainly focused on the chick-rearing phase (Martin 2004). This was the consequence of the general assumption that the fitness costs of reproduction are to be found chiefly in the costs of provisioning offspring after hatching (Stearns 1992; Monaghan et al. 1998). However, actual knowledge has shown that there is little difference between daily energy expenditure of breeding females when laying, incubating and chick-rearing (Nager 2006). Thus, the costs of the phases of the breeding cycle prior to provisioning young will also significantly affect parental survival and breeding success (Monaghan & Nager 1997).

Indeed, egg formation is a demanding process for birds. Females have to acquire, synthesise and transport into the egg all the resources that are necessary for a fully functioning egg (reviewed in (Perrins 1996; Nager 2006)). Therefore, the amount and quality of resources allocated to eggs often profoundly influence the growth and survival of the progeny (Mousseau & Fox 1998). In this sense, we actually have some understanding of the major physiological and hormonal mechanisms underlying egg formation. However, the gap of knowledge is especially evident in how egg formation is nutritionally and energetically constrained in wild birds (Williams 2001; Williams 2005).

There are two main strategies of nutrient allocation into the eggs: capital and income. “Capital breeders” rely on body-stored nutrients from earlier annual cycle stages for synthesizing the eggs, while “income breeders” fuel the process with current diet from local environment (Drent & Daan 1980; Jönsson 1997). Although capital and income breeding are the two extremes of a continuum of strategies (Oppel et al. 2010), the most part of the studies conclude that females rely on local diet resources for egg formation (Hobson 2006). Moreover, diet influences not only the quality of the resources allocated, but may also be an important factor determining egg size, especially in colonial birds compared to solitary breeding birds (reviewed in (Martin 1987; Christians 2002; Nager 2006)). Although there is some controversy about it, there is evidence from a variety of taxa that egg size influences positively the developmental pattern and offspring fitness (Williams 1994; Bernardo 1996; Christians 2002; Krist 2011). Thus, females would be at the crossroads of depositing more resources in their eggs, compromising their own survival (Nager et al. 2001).

The effect of such constraints in determining the intra-clutch pattern of nutrient allocation and egg size variation has been specially overlooked (but see (Hargitai et al. 2005; Hobson et al. 2005; Ramírez et al. 2011)). The typical intra-clutch pattern of egg size in avian species is the one where egg size declines with laying sequence (Slagsvold et al. 1984; Williams 1994). This pattern has been interpreted in different ways. On one hand, it can be caused by adaptive factors as proposes the “brood reduction” hypothesis, where the intra-clutch size hierarchy is facilitated by females investing less into the last-laid egg (Lack 1954; Slagsvold et al. 1984). On the other hand, it has been also proposed a number of nonadaptive proximate factors through energetic, physiological or food restrictions that can undergo during clutch formation (Perrins 1970; Pierotti & Bellrose 1986; Leblanc 1987; Sockman et al. 2000; Ramírez et al. 2013). To shed light in the patterns of nutrient allocation to clutch formation seems essential for a better understanding in the evolution of avian life-history strategies.

Currently, some studies have addressed this issue using the stable isotopes to track the origin of nutrients allocated in the eggs, especially through the carbon ($\delta^{13}\text{C}$) and nitrogen ($\delta^{15}\text{N}$) stable isotopes (Hobson 2006). In this way, albumen seems to be the most suitable egg compartment for an intra-clutch insight since it is synthesized during non-overlapping short time periods (Ruiz et al. 2000). This technique is particularly useful when different food resources can be distinguished, for example in migratory birds or opportunist species subject to dietary changes (Hobson 2006).

The Audouin's gull (*Larus audouinii*) is not an exception the declining intra-clutch pattern in size, showing a modal clutch size of three eggs and being the last one significantly smaller than the first two (Oro, Jover, et al. 1996; Pedrocchi 1997). This medium-sized migratory species concentrates about two thirds of the world's breeding population in the Ebro Delta Natural Park, NE Spain (Arcos et al. 2009). The species shows a great dependence on human trawling activity in this area, scavenging around fishing vessels and using discards very efficiently (Oro & Ruiz 1997; Oro, Jover, et al. 1996). However, Audouin's gulls in the Ebro Delta also exploit resources from neighbouring rice fields (especially the American crayfish *Procambarus clarkii*), increasing the importance of this low-quality resource during periods without trawling activities (Oro, Genovart, et al. 1996; Oro, Ruiz, et al. 1996). The isotopic characterization of the different habitats in the Ebro Delta has been well documented, and it is recognized that rice field prey present enriched $\delta^{15}\text{N}$ signatures and depleted $\delta^{13}\text{C}$ values compared to marine fish (García-Tarrasón et al. 2013).

With all this in mind, in this study we explored the intra-clutch patterns of $\delta^{13}\text{N}$ and $\delta^{15}\text{C}$ in the albumen of three-egg clutches of Audouin's gull from the Ebro Delta, during two consecutive breeding seasons (2009 and 2010). Because diet is the main source for nutrients deposited in the eggs (Ruiz et al. 1998), any dietary change during clutch formation may be reflected in the albumen isotopic signature. For example, during weekends or trawling moratoriums periods there are no fishing activities and the amount of fish discarded is negligible (Bartumeus et al. 2010), thus increasing the contribution of rice field resources. Also, the possible relation of the diet with the egg size was tested. We hypothesize that any foraging change in response to fishing activities will be detected in the intra-clutch pattern. We also predict that rice field resources will affect negatively the egg size compared to the use of marine fish.

MATERIALS AND METHODS

Study area and sampling

The study was carried out during the 2009 and 2010 breeding season at "Punta de la Banya" colony (Ebro Delta Natural Park, NE Spain; 40° 40'N, 0° 45'E), a protected sandy peninsula with salt pans. Audouin's gull nest there in discontinuous nuclei in both natural coastal dunes with vegetation and in the salt pans.

Following a field procedure agreed with the wildlife personnel of the Natural Park, and in order to avoid excessive annoyances to this protected species, nucleus from both nesting places (dunes and salt pans) were inspected every two days during the peak egg-laying period (late April). New nests were tagged when the first egg was laid and routine inspections were conducted until clutch completion to establish the final clutch size and laying order. Fresh, new laid eggs were measured (maximum length and width) to the nearest 0.01 mm using a digital calliper. The egg size (volume, in cm^3) was estimated as " $0.485 \times \text{length} \times \text{width}^2$ " following (Ruiz et al. 1998). Three-egg clutches from both nesting places were selected and freshly laid eggs were sampled and replaced with dummy eggs. We sampled 50 clutches from the salt pans (24 in 2009 and 26 in 2010) and 48 from the coastal dunes (30 in 2009 and 18 in 2010). Eggs were marked, placed under refrigeration and transported to the laboratory.

Given the extreme dependence of Audouin's gull foraging movements on trawling activities based on GPS data (unpublished data), the day of the civil week when the egg was first detected and collected was included in order to control the

weekend effect (when the trawling fleet is not operating and, therefore, a nutritional shortfall occur). Thus, the “*Day-of-the-week*” (with seven categories, from Monday to Sunday) was assigned to each egg as a proxy of the laying day. Also, it's important to note that in 2009 a trawling moratorium started on May 1, affecting the whole Ebro Delta area. Every clutch with at least one egg collected three or more days after the beginning of this closed season was categorized as “affected by trawling moratorium” (in total 10 clutches: 7 from the salt pans and 3 from the coastal dunes) and analyzed separately. In these clutches, the “*Day-to-moratorium*” referred as the day when the egg was collected in relation to the beginning of the closed season) was noted (positive or negative, 1st of May = 0).

Ethical note

This study was authorized by the Autonomous Government of Catalonia (*Departament de Medi Ambient i Habitatge - Generalitat de Catalunya*), as well as by the wildlife personnel of the Ebro Delta Natural Park. Both agreed with the field protocol of handling and sampling of Audouin's gulls.

Laboratory procedures

Eggs were opened and the albumen was separated from the yolk manually. Each fraction was weighed, homogenised and divided into subsamples prior to storage. Albumen subsamples were frozen at -20°C and yolk ones at -80°C.

Stable isotope analysis

A subsample of homogenised albumen was lyophilised to constant mass and ground to a powder. Then, 0.35-0.38 mg of albumen from each subsample was placed into tin capsules for stable carbon and nitrogen isotope ratio determination. Isotopic analyses were carried out at the Scientific and Technical Services of the University of Barcelona (Spain) by means of a Thermo-Finnigan Flash 1112 elemental analyser (CE Elantech, Lakewood, NJ, USA) coupled to a Delta-C isotope ratio mass spectrometer via a CONFLOIII interface (Thermo Finnigan MAT, Bremen, Germany), with IAEA standards being applied every 12 samples to calibrate the system. Stable isotope ratios were expressed in the standard δ notation relative to Vienna Pee Dee Belemnite ($\delta^{13}\text{C}$) and atmospheric N_2 ($\delta^{15}\text{N}$). Replicate assays of standards indicated analytical measurement errors of $\pm 0.1\text{‰}$ and $\pm 0.2\text{‰}$ for $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$, respectively.

Statistical analysis

For the clutches not affected by the trawling moratorium, egg size and values of albumen $\delta^{15}\text{N}$, $\delta^{13}\text{C}$ were analyzed using generalised linear mixed models (GLMMs) with an identity link. Clutch (nest) was included as a random factor, and year (2009 or 2010), nesting place (coastal dunes or salt pans), laying order (1st or a-egg, 2nd or b-egg, and 3rd or c-egg) and “day-of-the-week” (from Monday to Sunday) as fixed factors. Dependence among eggs was also modelled including a first-order autoregressive structure for residual covariance matrix. Hierarchical backward model selection based on likelihood ratios was used, starting with a full model including all fixed factors and all the first order interactions between laying order and the remaining fixed factors. This procedure was carried on models fitted by maximum likelihood but once final model was chosen (details in Supplementary material), the results presented were based on restricted maximum-likelihood estimation

When modelling egg size, a possible relationship with the diet was also considered including in the initial model the values of $\delta^{15}\text{N}$ and $\delta^{13}\text{C}$ as covariates.

When an interaction term was significant, a separate analysis for each factor level was carried out. When significant, post-hoc comparisons among factor levels were made using the Šidák correction for multiplicity.

Due to limited sample size available for the clutches affected by the trawling moratorium (ten clutches, 30 eggs) we used Spearman's rank correlation coefficient to assess the presence of monotonous relationship between the albumen stable isotopes ($\delta^{13}\text{C}$, $\delta^{15}\text{N}$) and the “day-to- moratorium” in order to detect a possible diet changes related to the trawling moratorium in 2009

Statistical analyses were performed using SPSS 20.0 software (SPSS Inc., Chicago, IL, USA).

RESULTS

In relation to albumen $\delta^{15}\text{N}$, the selected model includes only the “day-of-the-week” ($F_{6,184} = 7.97$; $p < 0.001$). The Wednesday (day = 3) presented the most enriched nitrogen levels, being significantly higher than the rest of the days except the adjacent Tuesday and Thursday (Table 1, Figure 1a).

2.1. Resource allocation to the clutch

For the albumen $\delta^{13}\text{C}$, the selected final model includes the factor year ($F_{1,95} = 30.69$; $p < 0.001$), laying order ($F_{2,176} = 15.29$; $p < 0.001$) and “day-of-the-week” ($F_{6,183} = 2.19$; $p = 0.05$). Carbon values in 2009 were significantly higher than in 2010. The laying order effect showed an intra-clutch pattern with the higher values in the a-egg compared to the others ($p = 0.001$), and also presenting the b-egg higher values than c-egg ($p = 0.006$). Finally, the Thursday (day = 4) presented the most depleted carbon values, but only being significantly different from Monday ($p = 0.01$) (Table 1, Figure 1b).

When analysing the egg size, we found a model with a significant interaction between laying order and nesting place ($F_{2,132} = 7.17$; $p = 0.001$) as a result of c-egg being significantly smaller in the salt pans ($p = 0.01$), so each nesting place was analysed separately. In both places, the selected model included laying order (coastal dunes: $F_{2,62} = 61.07$, $p < 0.001$; salt pans: $F_{2,57} = 107.39$, $p < 0.001$) and $\delta^{15}\text{N}$ (coastal dunes: $F_{1,118} = 10.46$, $p = 0.002$; salt pans: $F_{1,110} = 30.16$, $p < 0.001$). The salt pans showed a more homogenous profile between a- and b-egg ($p = 0.05$), being the c-egg significantly smaller ($p < 0.001$). In the coastal dunes every egg was different in size from the others ($p < 0.001$). The parameter estimate of the $\delta^{15}\text{N}$ was always negative with the egg size (Table 2, Figure 2).

Regarding the clutches affected by the trawling moratorium, the relationship between the $\delta^{13}\text{C}$ and the “day-to-moratorium” was negative and significant ($r = -0.75$, $p < 0.001$). By contrast, in $\delta^{15}\text{N}$, the relationship was positive ($r = 0.48$, $p = 0.01$) (Figure 3).

DISCUSSION

The ecological role of fisheries in modifying the trophic ecology, behaviour, population dynamics and breeding success of seabirds has been shown in several works (Votier et al. 2010; Bicknell et al. 2013; Oro et al. 2013; Bodey et al. 2014). However, the effect of fishing activities in generating variability in intra-clutch isotopic and size patterns has not been studied in detail. Moreover, the influence of fishing activities in the diet is analyzed for the first time to elucidate the origin of resources allocated into the egg.

2.1. Resource allocation to the clutch

The selected final model for the albumen $\delta^{15}\text{N}$ only included the “day-of-the-week” as significant factor. Or what is the same: the effect of a lower availability of discards during weekends (when the availability of fishing discards is negligible and the consumption of rice field resources increase) is presumably reflected in enriched nitrogen albumen signatures reaching its peak on Wednesday. This time lag must be due to the formation time of albumen and eggshell, about 2-3 days (Nager 2006). The consistent daily pattern of $\delta^{15}\text{N}$ between years and nesting places supports the validity of this prediction, as well as shows strong dependence of Audouin’s gull on fishing activities. Moreover, the high sensitivity to daily changes in discard availability reflects that the most part of the nitrogen deposited in the albumen must come from diet. However, a synergistic enrichment in $\delta^{15}\text{N}$ due to the mobilization of body resources to egg formation in this situation of food shortage cannot be ruled out.

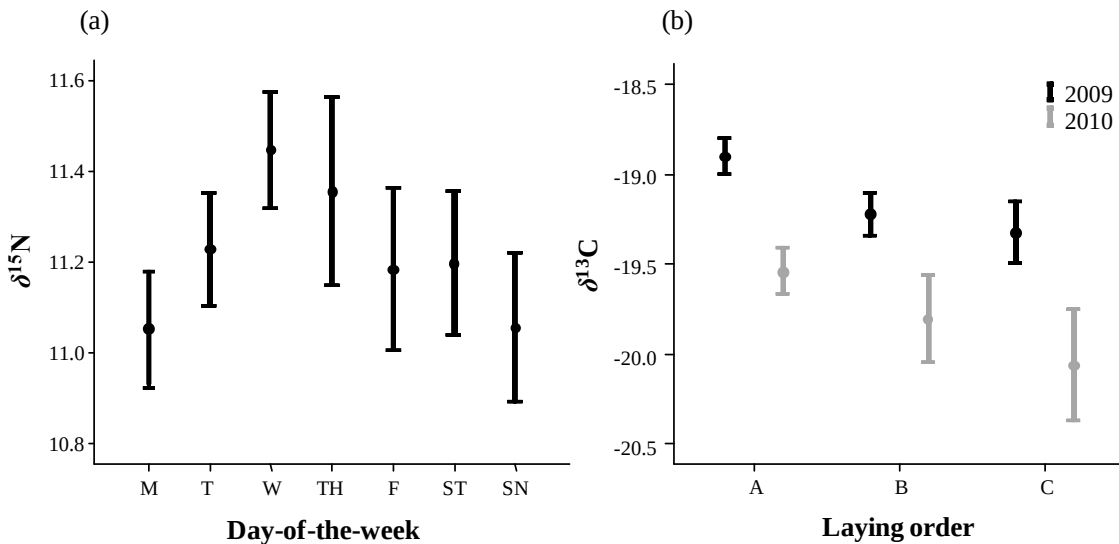


Figure 1. (a) Albumen $\delta^{15}\text{N}$ in relation to the day of the civil week when the egg was collected. M: Monday, T: Tuesday, W: Wednesday, TH: Thursday, F: Friday, ST: Saturday, SN: Sunday. (b) Intra-clutch pattern of albumen $\delta^{13}\text{C}$ by year (2009 and 2010) and laying order. All values represent observed mean and 95% confidence interval.

On the other hand, the $\delta^{13}\text{C}$ showed a consistent declining pattern through the laying sequence in both years. However, carbon values were higher in 2009 than in 2010. These differences between years may be attributable to the highly dynamic nature of the Ebro River related to weather conditions and the consequent input of particulate organic matter (POM) into the sea, mainly affecting the carbon isotope (Veyssy et al. 1998; Harmelin-Vivien et al. 2010). Indeed, the winter-spring 2008-2009 was drier than in 2009-2010 (Meteo.cat 2009; Meteo.cat 2010), and probably with less

2.1. Resource allocation to the clutch

influence of freshwater POM (with depleted $\delta^{13}\text{C}$ values) in the marine ecosystem. Carbon in the albumen must have a more endogenous origin than nitrogen since this daily pattern (although statistically significant) is not so obvious. Also, the higher residual correlation in the $\delta^{13}\text{C}$ than in $\delta^{15}\text{N}$ points in this direction. The lowest values are reached in this case on Thursday eggs, indicating a possible decoupling in the allocation routes between carbon and nitrogen. Moreover, the consistent declining intra-clutch pattern observed probably indicates differences in endogenous contribution through the laying sequence rather than a dietary switch.

	$\delta^{15}\text{N}$	$\delta^{13}\text{C}$
	Estimate $\pm$ SE	Estimate $\pm$ SE
Intercept	10.98 $\pm$ 0.09	-19.95 $\pm$ 0.13
2009		0.63 $\pm$ 0.12
2010		reference
a-egg		0.43 $\pm$ 0.08
b-egg		0.20 $\pm$ 0.07
c-egg		reference
Monday	0.079 $\pm$ 0.10	0.08 $\pm$ 0.13
Tuesday	0.23 $\pm$ 0.10	-0.06 $\pm$ 0.13
Wednesday	0.46 $\pm$ 0.10	-0.07 $\pm$ 0.13
Thursday	0.37 $\pm$ 0.11	-0.29 $\pm$ 0.15
Friday	0.19 $\pm$ 0.11	-0.05 $\pm$ 0.13
Saturday	0.22 $\pm$ 0.10	-0.06 $\pm$ 0.13
Sunday	reference	reference
Nest variance	0.51 $\pm$ 0.39	< 0.0001
Residual correlation	0.30 $\pm$ 0.17	0.65 $\pm$ 0.05
Residual variance	0.16 $\pm$ 0.04	0.40 $\pm$ 0.05

Table 1. Parameter estimate $\pm$ standard error for the selected final GLMM in the intra-clutch patterns of albumen $\delta^{15}\text{N}$ and $\delta^{13}\text{C}$.

Our results are consistent with previous works in Barrow's Goldeneye (*Bucephala islandica*) (Hobson et al. 2005), in the Harlequin Duck (*Histrionicus histrionicus*) (Bond et al. 2007) and in the King Eider (*Somateria spectabilis*) (Oppel et al. 2010). All these authors concluded that the nitrogen deposited in the eggs came primarily from the food consumed in breeding grounds in these migratory species, whereas carbon had a more endogenous contribution. Surprisingly, our results in Audouin's gull are opposite to those found in the sympatric Yellow-legged gull (*Larus*

michahellis) (Ramírez et al. 2011). In this work, the authors suggested that $\delta^{15}\text{N}$ had a more endogenous origin compared to $\delta^{13}\text{C}$, due to the increasing intra-clutch nitrogen pattern observed. They argued that this pattern was the consequence of the mobilization of stored proteins especially into the latter eggs, resulting in a greater isotopic fractionation. Since the day-of-the-week was not considered in this study, the results could be a consequence of this overlooked effect in a species that also exploits discards efficiently (Cama et al. 2012). However, differences in the allocation strategy between Yellow-legged and Audouin's gull cannot be ruled out.

Although phylogenetically distant to Audouin's gull, the ducks from these coinciding studies are also long-distance migrators as our target species. The Yellow-legged gull, on the contrary, is mostly resident (Cramp & Simmons 1983). The major source of endogenous proteins allocated to the eggs is the pectoral muscle (Kendall et al. 1973; Houston et al. 1995). However, the mobilization of protein reserves from the pectoral muscle during laying can detrimentally affect the female's flight ability (Veasey et al. 2001; Kullberg et al. 2005). Perhaps, to perform a long migration immediately after breeding limits the mobilization ability of endogenous proteins to the clutch in migratory species compared to resident ones. Further research is needed to test this hypothesis.

In relation to the intra-clutch egg size pattern, significant differences between nesting places were found regardless of the year. These differences are reflected in more homogeneous sizes between a- and b-egg in the salt pans than in the coastal dunes where. In addition to that, the last egg was significantly smaller in the salt pans than in the coastal dunes. This results are in agreement with those previously presented in Audouin's gull, where significant differences between nesting places were found in the albumen antioxidant capacity (García-Tarrasón et al. 2014). The authors argued that these differences could be associated to quality differences of the nesting place. Indeed, the salt pans seems to be a nesting place of better quality because its higher nest density and breeding success than the coastal dunes (Oro et al. 2010). Some studies in gulls suggested that higher nest density is associated to the reduced size of the third egg (Coulson et al. 1982; Pierotti & Bellrose 1986; Spaans et al. 1987). Probably, in low densities or under suboptimal situations, females can modulate the nutrient allocation by increasing investment in the final eggs, thus enhancing nestling growth and survival, and reducing hatching time (Ferrari et al. 2006). However, some studies have shown the complex nature of variations in egg size (Bonisoli-Alquati et al. 2007).

2.1. Resource allocation to the clutch

Diet is also one of the main factors that have been suggested to affect egg size (Martin 1987; Bolton et al. 1992; Williams 1994). Indeed, apart from the expected decreasing pattern in size along the laying sequence, the other main factor with a significant effect on egg size is $\delta^{15}\text{N}$. Because of the close relationship between nitrogen signature and diet discussed above, this negative relationship suggests that situations of food shortage are associated with reduced egg size. It is worth to note that if we remove $\delta^{15}\text{N}$ from the analysis, the factor “day-of-the-week” becomes significant. Further research is needed on both, how specific characteristics of the nesting place and diet affect reproductive output such as offspring viability or breeding success.

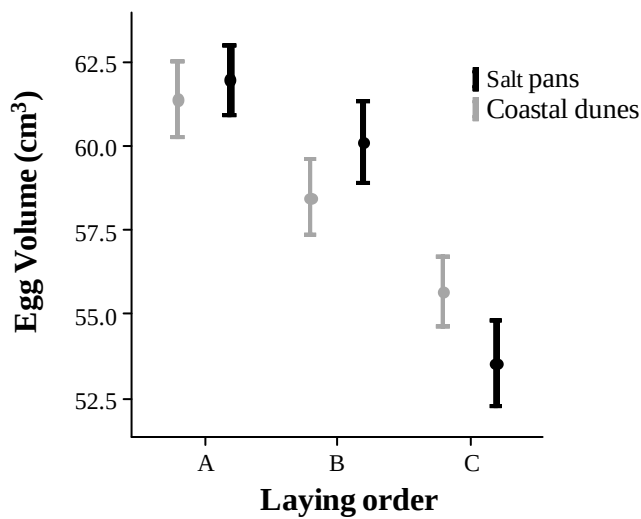


Figure 2. Intra-clutch pattern of egg size (volume, in cm^3) by nesting place (salt pans and coastal dunes) and laying order. Values represent observed mean and 95% confidence interval.

	Egg size	
	Coastal dunes	Salt pans
	Estimate $\pm$ SE	Estimate $\pm$ SE
Intercept	74.72 $\pm$ 6.02	92.57 $\pm$ 7.26
a-egg	5.76 $\pm$ 0.53	7.64 $\pm$ 0.62
b-egg	3.03 $\pm$ 0.46	6.45 $\pm$ 0.48
c-egg	reference	reference
$\delta^{15}\text{N}$	-1.71 $\pm$ 0.54	-3.45 $\pm$ 0.64
Nest variance	5.37 $\pm$ 3.08	1.15 $\pm$ 7.36
Residual correlation	7.13 $\pm$ 2.73	12.10 $\pm$ 7.62
Residual variance	0.36 $\pm$ 0.25	0.60 $\pm$ 0.26

Table 2. Parameter estimate $\pm$ standard error for the selected final GLMMs in the intra-clutch pattern of egg size (volume, in cm^3) in both nesting places.

2.1. Resource allocation to the clutch

Finally, a marked dietary switch toward rice field resources was evident in the clutches affected by the trawling moratorium. This indicates that females are, when laying eggs, closely linked to the nest and can not perform long foraging trips at sea searching for fish, so it seems that they prioritize capturing very accessible but lower quality prey. In addition, the results show that sudden changes in diet are also evident in the albumen $\delta^{13}\text{C}$, despite their apparently greater endogenous contribution.

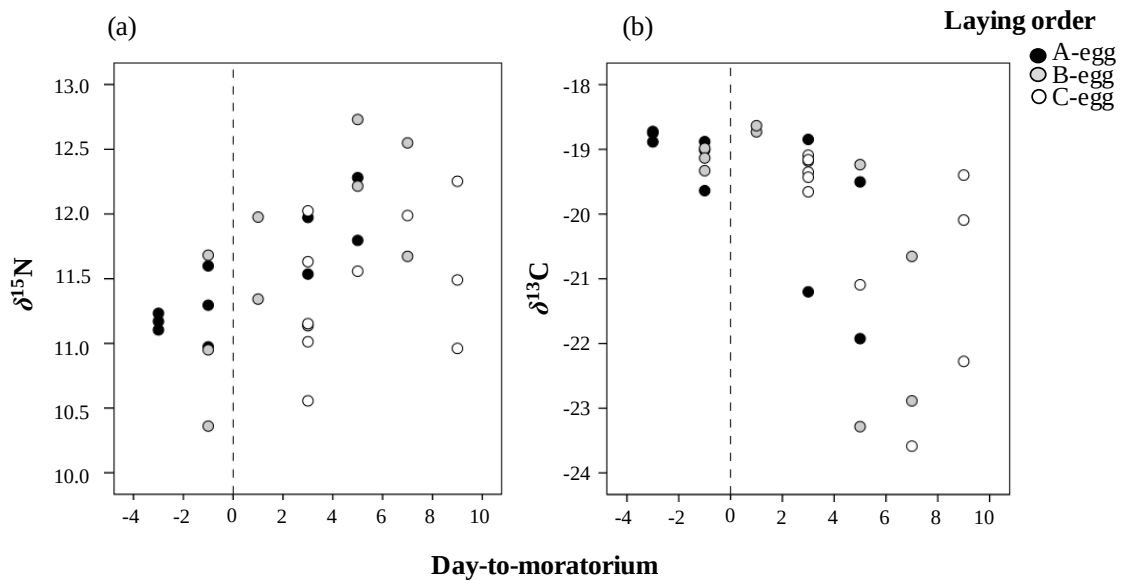


Figure 3. Bivariate plots of albumen stable isotopes vs. the day to the beginning of the trawling moratorium ("day-to-moratorium", in relation to the sampling day). The dashed line indicates the start of the moratorium (May 1, 2009). (a) albumen $\delta^{15}\text{N}$ and (b) albumen $\delta^{13}\text{C}$.

In conclusion, our study illustrates the usefulness of the stable carbon and nitrogen isotopes in determining the resource allocation to eggs. The great dependence on trawling activities was especially evident in the daily $\delta^{15}\text{N}$ pattern, an overlooked factor. The albumen $\delta^{13}\text{C}$ must have a more endogenous origin compared to $\delta^{15}\text{N}$ since this daily pattern is less marked. Finally, nesting habitats and diet were related to egg size, indicating that despite the underlying hormonal processes involved in generating intra-clutch variability in size, phenotypic plasticity also plays an important role in the observed final pattern.

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SUPPLEMENTARY INFORMATION

Table S1. Generalised linear mixed models (GLMMs) evaluated to fit albumen $\delta^{15}\text{N}$ and $\delta^{13}\text{C}$, with their corresponding Log Likelihood Function (-2 Log Likelihood or -2LL). Models were selected using a hierarchical backward model selection. Fixed factors considered were year (Y), nesting place (NP), laying order (LO) and “day-of-the-week” (DW). Clutch effect (nest) was included as a random factor. K refers to the number of parameters.

 $\delta^{15}\text{N}$

Model	-2LL	K
Y NP LO DW Y*LO NP*LO DW*LO	261.3	30
Y NP LO DW Y*LO DW*LO	261.7	28
Y NP LO DW Y*LO	273.1	16
Y NP LO DW	275.7	14
Y LO DW	276.0	13
LO DW	278.7	12
DW	284.2	10

 $\delta^{13}\text{C}$

Model	-2LL	K
Y NP LO DW Y*LO NP*LO DW*LO	380.8	30
Y NP LO DW NP*LO DW*LO	381.1	28
Y NP LO DW DW*LO	382.0	26
Y NP LO DW	399.7	14
Y LO DW	400.2	13

2.1. Resource allocation to the clutch (Supplementary Information)

Table S2. Generalised linear mixed models (GLMMs) evaluated to fit egg size (volume, cm³), and their corresponding Log Likelihood Function (-2 Log Likelihood or -2LL). Models were selected using a hierarchical backward model selection. Fixed factors considered were year (Y), nesting place (NP), laying order (LO) and “day-of-the-week” (DW). Values of $\delta^{15}\text{N}$ and $\delta^{13}\text{C}$ were included as covariates. Clutch effect (nest) was included as a random factor. A separate analysis for each nesting place level was carried out due to significant interaction. K refers to the number of parameters.

EGG VOLUME

Model	-2LL	K
Y NP LO DW Y*LO NP*LO DW*LO $\delta^{13}\text{C}$ $\delta^{15}\text{N}$	1278.7	32
Y NP LO DW Y*LO NP*LO $\delta^{13}\text{C}$ $\delta^{15}\text{N}$	1294.0	20
Y NP LO DW NP*LO $\delta^{13}\text{C}$ $\delta^{15}\text{N}$	1299.9	18

Coastal Dunes	-2LL	K
Y LO DW $\delta^{13}\text{C}$ $\delta^{15}\text{N}$	649.2	15
LO DW $\delta^{13}\text{C}$ $\delta^{15}\text{N}$	649.3	14
LO DW $\delta^{15}\text{N}$	650.8	13
LO $\delta^{15}\text{N}$	662.8	7

Salt Pans	-2LL	K
Y LO DW $\delta^{13}\text{C}$ $\delta^{15}\text{N}$	635.3	15
LO DW $\delta^{13}\text{C}$ $\delta^{15}\text{N}$	635.3	14
LO DW $\delta^{15}\text{N}$	631.0	13
LO $\delta^{15}\text{N}$	651.3	7

CHAPTER 2.2. Levels of antioxidants in breeding female Audouin's gulls and their deposition in eggs across different environments

Título: Niveles de antioxidantes en hembras reproductoras de gaviota de Audouin y su deposición en los huevos a través de diferentes ambientes

Resumen: La calidad del alimento y los antioxidantes de la dieta son factores interrelacionados que influyen en muchas características animales. Sin embargo, todavía se sabe poco sobre esta relación en aves salvajes, en especial sobre cómo la dieta puede afectar a la deposición de antioxidantes desde la hembra a los huevos durante la secuencia de puesta. En este contexto, el albumen ha sido mucho menos estudiado que la yema debido a la ausencia de carotenos, los cuales han sido el principal objeto de estudio en las investigaciones sobre los antioxidantes de la dieta. Nuestro estudio se realizó en la gaviota de Audouin (*Larus audouinii*), un ave marina que muestra una gran dependencia de los descartes de la pesca de arrastre, aunque en el Delta del Ebro también explota recursos procedentes de los campos de arroz cercanos, especialmente el cangrejo americano (*Procambarus clarkii*). Se examinó la relación entre la dieta (inferida a través del análisis de isótopos estables) y la capacidad antioxidante (no sólo de los carotenos, sino también de otros antioxidantes no enzimáticos como los tocoferoles y el retinol) en el plasma de las hembras procedentes de dos subcolonias, así como en sus puestas. En relación a los huevos, se analizaron los antioxidantes tanto en la fracción del albumen (antioxidantes hidrofílicos) como en la yema (antioxidantes hidrofílicos y lipofílicos), teniendo en cuenta la secuencia de puesta. Se encontró una disminución en la capacidad antioxidante del plasma de las hembras durante el período de incubación. En relación a los antioxidantes de la yema y del huevo entero, se encontraron escasas diferencias entre subcolonias en el patrón de deposición a través de la puesta. Sin embargo, se observó una mayor variación en los patrones intra-puesta de la capacidad antioxidante del albumen, probablemente relacionado con diferencias en la calidad de los individuos. Al tomar en cuenta la deposición total de antioxidantes en la puesta, se encontró una tendencia a la baja a través de la secuencia de puesta. Tanto la capacidad antioxidante lipofílica de la yema como la total del huevo se relacionó negativamente con el $\delta^{15}\text{N}$, lo que indica que las dietas basadas en pescado marino (con valores empobrecidos en $\delta^{15}\text{N}$) contienen una mayor cantidad de antioxidantes que las presas de arrozal.

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Levels of antioxidants in breeding female Audouin's gulls and their deposition in eggs across different environments

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Abstract: Diet quality and dietary antioxidants are interrelated factors that influence many animal traits. However, little is known about this relationship in wild birds, especially how it may affect the deposition of antioxidants from the female to the eggs over the laying sequence. In this context, albumen has been far less studied than yolk due to its lack of carotenoids, which are a common focus of dietary antioxidant research. Our study was conducted in the Audouin's Gull (*Larus audouinii*), a seabird that shows a great dependence on trawl discards, although in the Ebro Delta also exploits resources from neighbouring rice fields, especially the American crayfish (*Procambarus clarkii*). We examined the relationship between diet (traced through stable isotope analysis) and the antioxidant capacity (not only carotenoids, but also other non-enzymatic antioxidants, like tocopherols and retinol) of plasma in females from two breeding groups and their clutches. In the eggs we analyzed antioxidants in both albumen (hydrophilic antioxidants) and yolk (hydrophilic and lipophilic antioxidants) fractions, taking into account the laying sequence. A decrease in the antioxidant capacity of female plasma was found over the incubation period. We found little difference between groups in the antioxidant deposition pattern in the yolk and whole egg over the laying sequence, but a greater variation was observed in the intra-clutch patterns of albumen antioxidant capacity, probably related to quality differences. When taking into account total antioxidant deposition in the clutch, a declining tendency over the laying sequence was found. Both yolk lipophilic and whole egg antioxidant capacity were negatively related to $\delta^{15}\text{N}$, indicating that marine fish diets (with depleted $\delta^{15}\text{N}$ values) contain a higher amount of antioxidants than rice field prey.

Keywords: Albumen, Antioxidants, Laying order, Plasma, Stable isotopes, Yolk

1. INTRODUCTION

Diet quality and dietary antioxidants are interrelated factors that influence many life-history traits of animals (reviewed in Catoni *et al.*, 2008). Indeed, the diet is a source of compounds with antioxidant properties that animals can only acquire from food because they are unable to synthesise them *de novo* (Cohen *et al.* 2009). Dietary antioxidants may help protect the organism from oxidative stress arising from oxidative damage to biomolecules (Sies, 1991; Halliwell and Gutteridge, 2007; Costantini and Verhulst, 2009). This is a very important function because accumulation of such damage is thought to contribute to cellular senescence, loss of organ performance and Darwinian fitness (Kirkwood, 2005; Costantini, 2008). However, the antioxidant machinery is particularly complex and, beyond dietary antioxidants, includes many important endogenous molecules, like antioxidant enzymes or repair systems (Pamplona and Costantini, 2011).

Dietary antioxidants can be classified as hydrophilic (e.g. vitamin C and polyphenols) and lipophilic (e.g. vitamin E and carotenoids), according to their chemistry. The mechanisms of action among different classes of antioxidants vary, but their ultimate function is the same, i.e. to neutralise the damaging action of free radicals (Pamplona and Costantini, 2011). In addition, the diet contributes other substances that do not have antioxidant properties but that affect oxidative balance. For example, the polyunsaturated fatty acids in food are also important because they are substrates for lipid peroxidation (Hulbert, 2005).

The antioxidant content of the diet also influences traits other than oxidative stress, including sexual ornamentation, fertility and fecundity, and brood growth (Blount *et al.*, 2002; McGraw, 2006; de Ayala *et al.*, 2006; Biswas *et al.*, 2007). Dietary antioxidants are therefore considered important for reproduction, which represents one of the most demanding periods of animal life (Nager, 2006; Speakman, 2008), as well as for the growth and survival of progeny (Mousseau and Fox, 1998). Since these antioxidants are present in limited amounts, their deposition in the egg may reduce the capacity of the laying female to cope with reactive oxygen species, giving rise to trade-offs between self-maintenance and reproductive investment (Costantini, 2010a). Knowing how diet quality relates to the circulating non-enzymatic antioxidant levels in the breeding female and the patterns of allocation over the laying sequence will provide a better insight into how this trade-off influences life-history strategies.

Only a few experimental studies have looked at the link between diet and circulating / deposited antioxidants in wild birds (e.g. Beaulieu *et al.*, 2010; Hipfner *et*

al., 2010a; 2010b). For example, it has been shown that lower-trophic-level feeding is linked to the production of carotenoid-rich yolks (Hipfner *et al.*, 2010a; 2010b). However, recent studies have shown that carotenoids offer limited protection against oxidative stress (Costantini and Møller, 2008; Isaksson and Andersson, 2008; Simons *et al.*, 2012), highlighting the importance of looking at other egg dietary antioxidants as well. In eggs the deposition of antioxidants in albumen has received very little attention compared to their deposition in yolk (Blount *et al.*, 2002; Blount *et al.*, 2004), despite the important role of albumen in hatchling size and offspring development, behaviour and viability (Ferrari *et al.*, 2006; Bonisoli *et al.*, 2007). Therefore, there is an important need to study the relationships between diet quality and non-enzymatic antioxidant capacity (not only carotenoids) in breeding females and the albumen and yolk fractions of eggs.

To address these issues, we carried out a field study on breeding Audouin's Gulls (*Larus audouinii*), using stable isotope analysis (carbon and nitrogen isotopes) as an integrative tool for tracing the diet of gulls in the wild (Kelly, 2000; Inger and Bearhop, 2008).

The Audouin's Gull is a medium-sized migratory species categorised as "Near Threatened" at a global level by the International Union for Conservation of Nature (IUCN) (BirdLife International, 2012). About two thirds of the world's Audouin's Gull breeding population nest in the Ebro Delta Natural Park, NE Spain (Arcos *et al.*, 2009). Although originally described as a nocturnal predator of pelagic fish, especially clupeids (BWPI, 2004), the species shows a great dependence on human trawling activity in the study area, scavenging around fishing vessels and using discards very efficiently (Oro *et al.*, 1996b; Ruiz *et al.*, 1996; Oro and Ruiz, 1997). However, Audouin's Gulls in the Ebro Delta also exploit resources from neighbouring rice fields, where their main prey is the American crayfish (*Procambarus clarkii*). This invasive species seems to become a more important food resource for gulls during trawling moratorium periods (Oro *et al.*, 1996a; Oro *et al.*, 1997). The isotopic characterisation of the different habitats in the Ebro Delta has been extensively documented, and it is well known that rice field prey present enriched $\delta^{15}\text{N}$ signatures and depleted $\delta^{13}\text{C}$ values compared to marine prey (Cotin *et al.*, 2011; Garcia-Tarrason *et al.*, 2013).

Considering all the above, our main objectives were: (1) to find out the relevance of the main foraging habitats exploited by this species -marine or rice field- (Navarro *et al.*, 2010) on the circulating antioxidant capacity of breeding females (measured in the plasma of incubating females) and their clutch (measured in the albumen and yolk of a

three-egg clutch), and (2) to test the laying order effect on the antioxidant deposition, which could indicate a trade-off between antioxidant availability for females and their eggs. In fact, the laying order has been shown to be a very important factor in avian species with a brood reduction strategy such as gulls (Lack, 1954; Ramírez *et al.*, 2011; Rubolini *et al.*, 2011). We hypothesized that (1) the natural feeding resource (marine fish) would be better than the introduced American crayfish, and (2) an important laying order effect would be detected in the egg antioxidant deposition because of a limited availability of antioxidant molecules.

2. MATERIAL AND METHODS

2.1. Study area and sampling

The study was carried out during the 2010 breeding season at the Punta de la Banya (40° 40'N, 0° 45'E), a protected sandy peninsula with salt pans in the Ebro Delta Natural Park (NE Spain). Audouin's Gulls nest there in discontinuous groups in natural coastal dunes with vegetation and in salt pans subject to human activity. Two breeding groups (2.5 km apart) of similar age distribution (from plastic ring readings) were selected for comparison: one in the dunes (DG) and another in the salt pans (SG). Besides habitat, they differed markedly in parameters such as the number of pairs, population density and reproductive success (see Supplementary Information, Table S1).

During the peak egg-laying period (late April), the nests were tagged when the first egg was laid. Inspections were conducted every two days until clutch completion to establish laying order and the final clutch size. Ten three-egg clutches were selected from each group. Freshly laid eggs were sampled and replaced with dummy eggs. Eggs were marked, placed under refrigeration and transported to the laboratory. Twelve females from these nests (six from each group) were captured with nest traps during the incubation period and measured (weight, tarsus length and skull length -the distance from the supraoccipital to the bill tip-). Blood samples (2 mL) were also collected from the brachial vein; 0.5 mL was preserved in a neutral vial for molecular identification of sex and the rest was placed in heparinised vials. Blood samples were kept at +4°C until centrifugation. Sex of gulls was determined using polymerase chain reaction (PCR) amplification of the CHD genes (Ellegren, 1996; Griffiths *et al.*, 1998).

2.2. Ethical note

This study was authorized by the Autonomous Government of Catalonia (*Departament de Medi Ambient i Habitatge - Generalitat de Catalunya*), as well as by the wildlife personnel of the Ebro Delta Natural Park. Both agreed with the protocol of handling and sampling of Audouin's gulls.

2.3. Laboratory procedures

Eggs were opened and the albumen was separated from the yolk. Each fraction was weighed, homogenised and divided into subsamples prior to storage. Albumen subsamples were frozen at -20°C and yolk subsamples at -80°C.

The heparinised vials were centrifuged (2000 rpm for 10 min) within 6 h of collection. The supernatant plasma was pipetted off and stored in 250- μ l aliquots at -80°C.

2.4. Stable isotope analysis

Albumen, yolk and plasma samples were lyophilised to dryness and ground to powder. In the case of yolk, samples were lipid-extracted following the Folch method (Folch *et al.*, 1957) to remove lipid influence on carbon isotope ratios. Subsamples (0.3 mg plasma or 0.35-0.38 mg albumen and yolk) were placed in tin capsules for stable carbon and nitrogen isotope ratio determination. Isotopic analyses were carried out at the Scientific and Technical Services of the Universitat de Barcelona (Spain) by means of a Thermo-Finnigan Flash 1112 elemental analyser (CE Elantech, Lakewood, NJ, USA) coupled to a Delta-C isotope ratio mass spectrometer via a CONFLOIII interface (Thermo Finnigan MAT, Bremen, Germany), with IAEA standards being applied every 12 samples to calibrate the system. Stable isotope ratios were expressed in the standard δ notation relative to Vienna Pee Dee Belemnite ($\delta^{13}\text{C}$) and atmospheric N_2 ($\delta^{15}\text{N}$). Replicate assays of standards indicated analytical measurement errors of $\pm 0.1\text{‰}$ and $\pm 0.2\text{‰}$ for $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$, respectively.

2.5. Assays of oxidative damage and non-enzymatic antioxidant capacity

Analyses were performed at the Institute of Biodiversity, Animal Health and Comparative Medicine at the University of Glasgow (UK). Aliquot samples of both plasma and eggs were used, although only eleven individual females were available.

2.5.1. *Non-enzymatic antioxidant capacity of plasma, albumen and yolk*

The non-enzymatic antioxidant capacity was measured by colorimetric determination using the OXY-Adsorbent test (Diacron International, Grosseto, Italy). The antioxidant capacity was quantified as the ability of the lipophilic and hydrophilic antioxidants present in the biological fraction to cope with the oxidant action of hypochlorous acid (HOCl; an oxidant of pathological relevance in biological systems). The procedure for plasma and albumin was carried out in accordance with previous studies (e.g. Costantini 2010a; Costantini *et al.*, 2011). For the whole (yolkOXY) and the lipophilic (yolk lipOXY) yolk antioxidant capacity, we followed the procedure as in Costantini (2010a) with slight modifications (e.g. the yolk sample was first mixed with 0.1 ml 5% NaCl solution). The hydrophilic antioxidant capacity of yolk (yolk hydrOXY) was calculated as “whole yolkOXY” – “yolk lipOXY”. The extraction efficiency was higher than 95%. By multiplying the three antioxidant concentrations by their corresponding egg fraction mass (albumen/yolk) we obtained the corresponding total amounts (expressed as mmol of neutralised HOCl). By summing up these three quantities, we estimated the total antioxidant capacity for each egg (TOTAL Egg OXY).

The absorbance was always read with a spectrophotometer at a wavelength of 490 nm. Analyses were run in duplicate and the mean coefficients of variation were 4.4% for plasmaOXY, 6.3% for albumOXY, and 3.3% for yolkOXY. Measurements are expressed as mM of HOCl neutralised for plasmaOXY and albumOXY, and as μ mol of HOCl neutralised per mg of yolk for yolkOXY

2.5.2. *Plasma oxidative damage compounds in females*

Plasma reactive oxidative metabolites (plasmaROMs) were quantified as a measure of compounds causing oxidative damage by colorimetric determination using the d-ROMs test (Diacron International, Grosseto, Italy). Reactive oxygen metabolites (primarily hydroperoxides, ROOH) are mainly generated by the oxidation of lipids and proteins. The procedure was carried out as in previous studies (e.g. Costantini *et al.*, 2011). The absorbance was read by means of a Thermo Scientific Multiskan Spectrum spectrophotometer (Thermo Fisher, Vantaa, Finland) at a wavelength of 505 nm. Analyses were run in duplicate and the mean coefficient of variation was 6.7%. Measurements are expressed as mM of H₂O₂ equivalents.

2.6. Statistical analysis

In the eggs, concentrations of the three antioxidant fractions (albumen, and lipophilic and hydrophilic fractions of the yolk) in addition to the total antioxidant

capacity of the egg were analysed using generalised linear mixed models (GLMMs) with an identity link (Zuur *et al.*, 2007). The clutch (nest) was included as a random factor, and laying order (a-, b-, and c-egg) and breeding group (salt pan or dunes) as fixed factors. Values of $\delta^{15}\text{N}$, $\delta^{13}\text{C}$ were also included as covariates. A hierarchical backward model selection was used, starting with a full model including all fixed factors and covariates, the interaction between laying order and group and the interaction between both isotopes. When an interaction factor was significant, a separate analysis for each factor level was carried out. When significant, post-hoc comparisons within the laying order were made using the Šidák correction for multiplicity. Distributional assumptions about data have been checked through graphical residual analysis being symmetric and homocedastic in all fitted models.

Differences between females from both groups were checked using an unpaired *t*-test for the following variables: mean clutch volume, body condition (BC) index, plasma $\delta^{13}\text{C}$, plasma $\delta^{15}\text{N}$, plasmaROMs and plasmaOXY. The BC index was calculated as the residuals of the first principal component from a Principal Component Analysis including the weight, tarsus length and skull length. ANCOVA analysis (Zar, 2009) was performed to compare plasma antioxidant capacity between females from both groups, adjusting for “days of incubation”. A partial correlation was also performed between stable isotopes and plasma measurements controlling for the “days of incubation”. To assess the presence of monotonic relationships between the plasma measurements (plasmaOXY and plasmaROMs), stable isotopes ($\delta^{13}\text{C}$, $\delta^{15}\text{N}$), BC and “days of incubation” (the number of days from clutch completion to the date of capture and bleeding) we used Spearman’s rank correlation coefficient.

Statistical analyses were performed using SPSS 18.0 software (SPSS Inc., Chicago, IL, USA).

3. RESULTS

3.1. Intraclutch deposition pattern

For albumOXY, the best model (AICC = 698.6) showed a significant interaction between group and laying order ($F_{2,60} = 4.82$, $p = 0.01$), so each group was analysed separately. In the SG, the final model included only the laying order ($F_{2,20} = 4.49$, $p = 0.03$), indicating a higher concentration in the a-egg compared to the c-egg, but the differences a-b and b-c were not significant. For the DG, none of the factors were significant (Table 1, Fig. 1).

For the yolk lipOXY, the selected model (AICC = 257.4) included only $\delta^{15}\text{N}$ ($F_{1,59} = 4.62$, $p = 0.036$). The estimated parameter showed a negative relationship with $\delta^{15}\text{N}$ (Table 1, Fig. 1).

When analysing yolk hydroOXY, the final model (AICC = 284.8) only included the laying order effect ($F_{2,40} = 5.02$, $p = 0.011$), and the concentration was higher in the a-egg than the c-egg ($t = 3.17$, $p = 0.03$); the b-egg showed intermediate values but was not significantly different from the a- and c- eggs (Table 1, Fig. 1).

Finally, for the TOTAL eggOXY, the final model selected (AICC = 643.5) included laying order ($F_{2,37} = 21.44$, $p < 0.001$) and $\delta^{15}\text{N}$ ($F_{1,53} = 2.51$, $p = 0.12$). Although not significant, removing $\delta^{15}\text{N}$ from the model induced a substantial change in the estimated parameters of the remaining factors. The estimated parameter showed that the total antioxidant capacity of the c-egg was significantly lower compared to the a- and b-egg ($p < 0.001$) (Table 1, Fig. 2A).

It is worth noting that although the clutch (nest) was always included in the model as a random factor to take into account the dependence among eggs from the same clutch, its effect was never significant (p values ranging between 0.07 and 0.4). Detailed information about egg antioxidant capacity and stable isotopes according to laying order is shown in the Supplementary Information, Table S3.

3.2. Status of females

Females from the two groups did not present any significant differences in mean clutch volume, BC index, diet (plasma $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$), plasmaROMs or plasmaOXY, with p -values ranging from $p = 0.15$ to $p = 0.74$ (see Supplementary Information, Table S2), and variances being homogeneous for all variables. The relationship between “days of incubation” and plasmaOXY was negative and significant ($r = -0.68$, $p = 0.02$; Fig. 3A). ANCOVA analysis comparing the plasma antioxidant capacity of the two groups, adjusting by “days of incubation”, did not find differences for plasmaOXY ($F_{1,9} = 0.001$, $p = 0.99$). The relationship between “days of incubation” and plasmaROMs was also negative but not significant in this case ($r = -0.47$, $p = 0.14$). On the other hand, plasmaOXY and plasmaROMs showed a significant positive correlation ($r = 0.67$, $p = 0.02$; Fig. 3B). The rest of the correlations were not significant (p -values higher than 0.21, correlation coefficients lower than 0.41).

2.2. Antioxidant allocation to the clutch

Table 1. Parameter estimate $\pm$ standard error for the selected final GLMMs in egg antioxidant measures. c-egg was used as a reference level in the analysis.

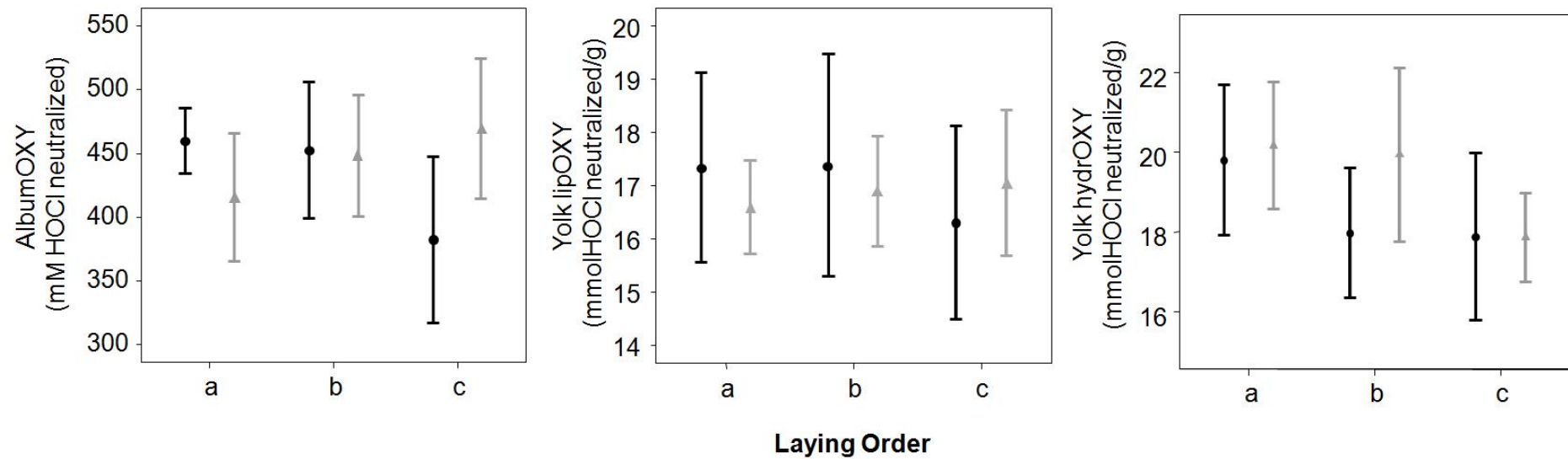
	AlbumOXY		Yolk lipOXY	Yolk hydrOXY	TOTAL eggOXY
	Salt pan group	Dune group			
Intercept	382.5 $\pm$ 21.4	444.4 $\pm$ 13.0	38.9 $\pm$ 10.2	17.9 $\pm$ 0.6	901.7 $\pm$ 212.1
a-egg	77.4 $\pm$ 28.5			2.1 $\pm$ 0.7	128.3 $\pm$ 20.0
b-egg	70.0 $\pm$ 28.5			1.1 $\pm$ 0.7	91.4 $\pm$ 20.1
c-egg	Ref.			Ref.	Ref.
$\delta^{15}\text{N}$			-1.9 $\pm$ 0.9		-29.72 $\pm$ 18.76
Nest*	513.3 $\pm$ 938.9		1.3 $\pm$ 0.7	1.5 $\pm$ 1.0	599.9 $\pm$ 714.0
Residual	4064.5 $\pm$ 1285.3	5064.5 $\pm$ 1307.6	2.8 $\pm$ 0.6	4.4 $\pm$ 1.0	3801 $\pm$ 894.3

Note. Asterisk (*) indicates estimated variances. Due to significant interaction between group and laying order in the antioxidant capacity of the albumen, each group was analysed separately.

4. DISCUSSION

The influence of nutrition on the oxidative status of birds (Blount *et al.*, 2003) can be explored by looking at the relationship between OXY variables and the stable isotope signature of tissues ($\delta^{15}\text{N}$, $\delta^{13}\text{C}$). However, our data on Audouin's gull eggs did not indicate a relationship between the $\delta^{13}\text{C}$ signature, which provides information about the carbon sources used by consumers, and the OXY variables. This result is in agreement with Hipfner *et al.* (2010a), who did not find a relationship between yolk carotenoid levels and $\delta^{13}\text{C}$ values in other seabird species. Similarly, Oppel *et al.* (2010) suggested that in the King eider (*Somateria spectabilis*), while the nitrogen deposited in the egg comes mainly from diet, carbon (especially in the yolk) has a more endogenous origin. This is also supported in our results by the strong relationship between plasma and mean egg $\delta^{15}\text{N}$ ($r = 0.57$, $p = 0.06$ for albumen; $r = 0.53$, $p = 0.08$ for yolk; $N=12$) but not for $\delta^{13}\text{C}$ ($r = -0.35$, $p = 0.26$ for albumen; $r = -0.02$, $p = 0.96$ for yolk; $N=12$).

Figure 1. Intra-clutch deposition pattern for relative egg measurements by breeding group and laying order. Black bars indicate salt pan group, grey bars indicate coastal dune group. Values are mean and 95% confidence interval. *albumOXY*: antioxidant capacity of albumen; *lipOXY*: lipophilic component of antioxidant capacity; *hydrOXY*: hydrophilic component of antioxidant capacity.



2.2. Antioxidant allocation to the clutch

In the case of albumen antioxidant concentration, there was a significant interaction between the breeding group and laying order: in the salt pan group there was a lower investment of albumen antioxidants in the third egg, while the dune group showed no variation over the laying sequence. Why did intraclutch antioxidant deposition patterns differ between salt pan and dune gulls? Since there seemed to be no obvious differences between the females in either location (Supplementary Information, Table S2), other, ecological, factors could be responsible. In fact, the dune group had a lower breeding success and lower nest density (Supplementary Information, Table S1), indicators of a poor-quality nesting site. Different antioxidant investment patterns between the two groups may relate to the “brood reduction” strategy typical of gulls (Lack, 1954). In low nest densities or sub-optimal situations, females can probably modulate albumen contribution (and molecules therein such as antioxidants) by increasing investment in the final eggs, thus enhancing nestling growth and survival, and reducing hatching time (Ferrari *et al.*, 2006). However, other studies have shown a complex relationship between albumen variation and offspring development and viability (Bonisoli *et al.*, 2007), as well as the determination of embryo sex in larger c-eggs (Rubolini *et al.*, 2009), so further research on how females modulate albumen contribution is required.

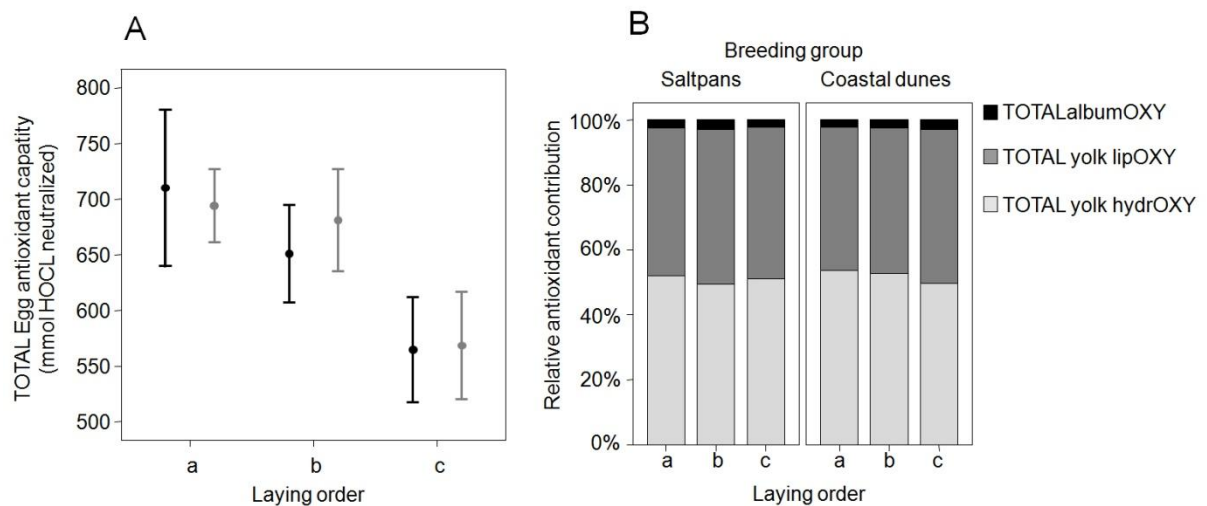


Figure 2. (A) Intra-clutch deposition pattern for total egg antioxidant capacity by breeding group and laying order. Black bars indicate salt pan group, grey bars indicate coastal dune group. Values are mean and 95% confidence interval. **(B)** Relative antioxidant contribution for each egg measurement by laying order and breeding group.

On the other hand, yolk accounted for the majority of the overall antioxidant capacity of the egg (Fig. 2B). Concentration of hydrophilic antioxidants in the yolk (hydrOXY) and the overall concentration of antioxidants in the yolk (lipOXY + hydrOXY) followed a declining pattern through the laying sequence as described in other studies on gulls (Royle *et al.*, 1999; Blount *et al.*, 2002; Groothuis *et al.*, 2006; Rubolini *et al.*, 2011). Moreover, this decline in TOTAL eggOXY was observed in both breeding groups. Such a decreasing tendency indicates that the allocation of antioxidants to the yolk is probably modulated/constrained by antioxidant resource acquisition (Royle *et al.*, 2003) as well as by competition among oocytes (Meathrel, 1991). The exception was the concentration of lipophilic antioxidants, which did not present an order effect in either of the breeding groups. This consistent pattern has probably been naturally selected to optimise the trade-off between maternal investment and reproductive success (Pedrocchi, 1997; Royle *et al.*, 1999; Hipfner *et al.*, 2010a).

Both yolk lipOXY and TOTAL eggOXY were negatively related to $\delta^{15}\text{N}$ and thus, lower $\delta^{15}\text{N}$ values were associated with higher antioxidant levels. The relation of egg lipophilic antioxidants with diet has been previously reported. Royle *et al.* (2003) found that in Zebra Finches (*Taeniopygia guttata*) over 70% of the female's daily intake of carotenoids and α -tocopherols (the two most abundant lipophilic dietary antioxidants) were deposited in the egg yolk. In the Ebro delta system lower nitrogen values derive mainly from marine diets (Cotin *et al.*, 2011; Garcia-Tarrason *et al.*, 2013), suggesting that marine resources are more beneficial in terms of antioxidant supply than rice field resources. Marine fish (especially clupeids) are the natural prey of the Audouin's Gull (BWPI, 2004), and fish are richer than crustaceans in terms of nutritional content (Massias and Becker, 1990). For example, the musculature of the American crayfish (the main prey in the rice fields) yields only between 10% and 40% of its total body weight (Hunner, 1988). Although crayfish are known to contain a high concentration of carotenoids (Negro *et al.*, 2000), mainly astaxanthin (Negro and Garrido-Fernández, 2000; Hipfner *et al.*, 2010b), the antioxidant power of astaxanthin is very low compared to other dietary antioxidants (Miller *et al.*, 1996; reviewed in Catoni *et al.*, 2008). Moreover, crayfish from the Ebro Delta rice fields have very high levels of some metals, including copper, chromium, zinc and arsenic, probably due to agricultural activity (Suárez-Serrano *et al.*, 2010). All these metals have toxic potential and may produce reactive oxygen species (ROS), especially when concentrations are high (reviewed in Koivula and Eeva, 2010). In fact, birds that feed in the Ebro Delta rice fields, such as herons and egrets, have the highest copper levels in the local bird community (Cotin, 2012). Thus, metal accumulation may be causing an increase in oxidative damage that

the antioxidant activity of carotenoids cannot cope with. Nevertheless, marine fish are also a source of metals, especially mercury (Monteiro *et al.*, 1998; Arcos *et al.*, 2002). Since marine fish constitute the natural prey of Audouin's gulls, this species has evolved in mercury-enriched environments. Although mercury levels in Audouin's gulls are known to be high (Arcos *et al.*, 2002; Sanpera *et al.*, 2007), little is known about how metal accumulation affects the oxidative status of this species.

Finally, we could not find any relationship between stable isotopes and the plasma non-enzymatic antioxidant capacity of females or their plasma ROMs. However, our results show that the female plasma antioxidant capacity decreased over the incubation period (Fig. 3A). A similar trend was found by Costantini (2010b), who reported that female pigeons show a decline in plasma non-enzymatic antioxidant capacity between the onset and end of the incubation phase. This effect can be attributed to the accumulated effort during the progress of the breeding event, which can result in depletion of female antioxidant reserves (Wiersma *et al.*, 2004). Another non-excluding hypothesis is related to hormonal processes/action. Sex hormones such as estradiol may have a positive effect on the oxidative status (Halifeoglu *et al.*, 2003; Demirbag *et al.*, 2005), as well as stimulating the production of yolk and albumen precursors (Christians and Williams, 1999). Thus, the decrease in circulating estradiol during incubation (McQueen *et al.*, 1999) could also be behind the decline in antioxidant capacity we observed.

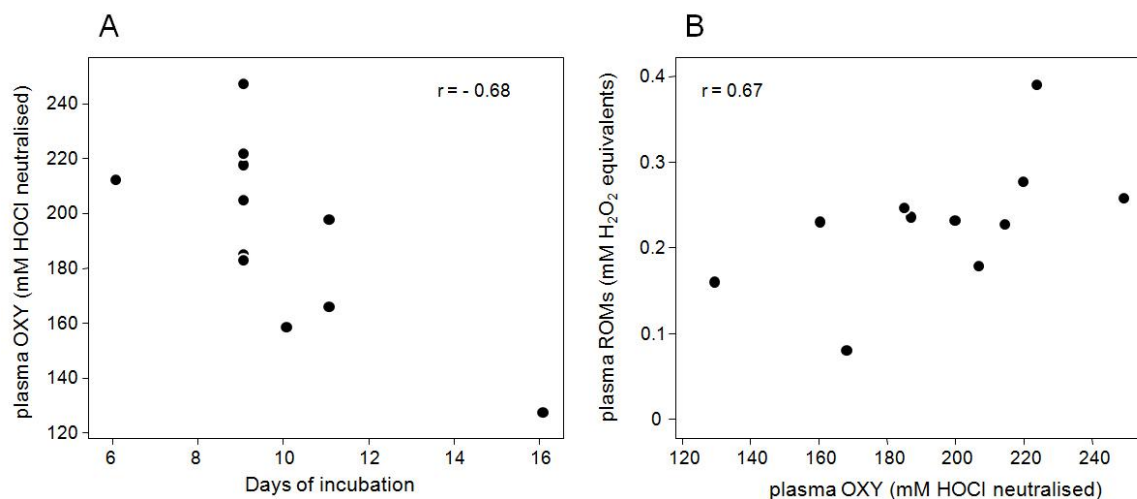


Figure 3. Breeding female bivariate plots of **(A)** “days of incubation” vs. plasma non-enzymatic antioxidant capacity, and **(B)** plasma non-enzymatic antioxidant capacity vs. plasma reactive oxygen metabolites.

A similar mechanism could explain the positive relationship between plasmaOXY and plasmaROMs (Fig. 3B). The production of lipid-rich yolk precursors might be associated with an increased production of reactive oxygen species via lipid peroxidation (Williams, 2005). In this case, plasma reactive oxygen metabolite levels would be higher during the clutch production (initial stages of incubation), when the influence of egg precursors is stronger and the antioxidant activity is higher. Indeed, plasmaROM levels and “days of incubation” showed a slightly negative, albeit not significant, pattern, probably due to the limited sample size (see Results). Although not the main focus of this study, the dual role of sex hormones is noteworthy: on the one hand, they stimulate the synthesis of egg precursors that can produce oxidative damage, and on the other hand, increase antioxidant activity. It is therefore important to take into account the “days of incubation” variable when evaluating the antioxidant capacity of breeding females. However, because of the limitations of the sample size in terms of statistical significance and biological representation, the present results on female gulls are intended as explorative and descriptive, and larger samples are needed to confirm these quantitative trends.

5. CONCLUSIONS

This study points to the potential benefit for Audouin’s gulls of a marine fish diet over rice field prey in terms of the antioxidant levels deposited in their eggs. It is well known that in the Ebro Delta this species is highly dependent on trawler discard (Oro *et al.*, 1999; Oro *et al.*, 2004), while low quality resources such as American crayfish from the rice fields are thought to act as a buffer in adverse situations such as trawling moratorium periods. Our results show that deposition of antioxidants in the eggs tended to decrease along the laying sequence, indicating their limited availability. However, further research is needed to assess how antioxidant differences during the laying period affect reproductive parameters.

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SUPPLEMENTARY INFORMATION

Table S1. Descriptive parameters for both breeding groups.

Breeding groups	Pairs in 2008*	Pairs in 2009*	Pairs in 2010*	Density in 2010 (nest / m ²)	Breeding success in 2010*	Mean age of ringed birds in 2010 (years)
Salt pans	149	670	2009	0.2715	0.52	9.2 (N = 124)
Coastal dunes	567	291	224	0.0407	0.03	9.4 (N = 56)

Note. Breeding success is expressed as “chicks per pair”. Asterisk (*) indicates data from Oro *et al.* 2010.

Table S2. Descriptive statistics of females from both breeding groups (values are shown as mean ± standard deviation).

Breeding groups	Mean clutch volume (cm ³)	BC index	plasma $\delta^{13}\text{C}$	plasma $\delta^{15}\text{N}$	plasmaROMs	plasmaOXY
Salt pans	58.06 ± 3.61	-0.13 ± 1.12	-23.21 ± 1.71	12.09 ± 0.53	0.24 ± 0.03	200.5 ± 31.0
Coastal dunes	58.70 ± 2.90	0.13 ± 0.96	-22.33 ± 1.40	11.71 ± 0.27	0.22 ± 0.11	186.2 ± 38.5
95% CI diff.	-4.85 ; 3.58	-1.60 ; 1.07	-0.17 ; 0.92	-2.88 ; 1.14	-0.09 ; 0.13	-33.04 ; 61.55

Note. *BC*: body condition. *PlasmaROMs*: plasma reactive oxygen metabolites (in mM of H₂O₂ equivalents). *PlasmaOXY*: plasma non-enzymatic antioxidant capacity (in mM of HOCl neutralised). N=11 (6 from the salt pans and 5 from the dunes) for the ROMs and OXY values, N=12 (6 from each nucleus) for the rest of the variables.

Table S3. Descriptive statistics of egg antioxidant concentration measurements and isotopic values. Values are shown as mean $\pm$ standard deviation.

Breeding groups	Laying order	AlbumOXY	Yolk lipOXY	Yolk hydroOXY	albumen $\delta^{13}\text{C}$	albumen $\delta^{15}\text{N}$	yolk $\delta^{13}\text{C}$	yolk $\delta^{15}\text{N}$
Salt pans	a	459.92 $\pm$ 36.00	17.34 $\pm$ 2.49	19.80 $\pm$ 2.64	-19.41 $\pm$ 0.31	10.99 $\pm$ 0.22	-18.99 $\pm$ 0.80	11.70 $\pm$ 0.30
	b	452.49 $\pm$ 75.10	17.38 $\pm$ 2.92	17.97 $\pm$ 2.27	-19.57 $\pm$ 0.76	10.98 $\pm$ 0.30	-19.39 $\pm$ 1.25	11.59 $\pm$ 0.38
	c	382.50 $\pm$ 91.24	16.31 $\pm$ 2.54	17.88 $\pm$ 2.94	-19.66 $\pm$ 0.49	11.27 $\pm$ 0.47	-19.70 $\pm$ 1.08	11.53 $\pm$ 0.23
Coastal dunes	a	415.51 $\pm$ 70.06	16.60 $\pm$ 1.23	20.16 $\pm$ 2.22	-19.54 $\pm$ 0.36	11.07 $\pm$ 0.35	-19.10 $\pm$ 0.42	11.72 $\pm$ 0.36
	b	448.32 $\pm$ 66.22	16.90 $\pm$ 1.45	19.93 $\pm$ 3.06	-19.83 $\pm$ 0.59	11.04 $\pm$ 0.52	-19.21 $\pm$ 0.74	11.62 $\pm$ 0.22
	c	469.41 $\pm$ 77.12	17.05 $\pm$ 1.92	17.87 $\pm$ 1.56	-20.26 $\pm$ 1.34	11.28 $\pm$ 0.83	-19.42 $\pm$ 0.72	11.66 $\pm$ 0.36

Note. *AlbumOXY*: antioxidant capacity of albumen (in mM HOCl neutralised). *Yolk lipOXY*: yolk lipophilic component of antioxidant capacity (in μmol HOCl neutralised/g yolk). *Yolk hydroOXY*: yolk hydrophilic component of antioxidant capacity (in μmol HOCl neutralised/g yolk). N = 30 eggs (10 clutches of 3 eggs) for each nucleus.

CHAPTER 2.3. The role of calcium in constraining egg synthesis in the Audouin's Gull (*Ichthyaetus audouinii*)

Título: El papel limitante del calcio en la síntesis de los huevos en la gaviota de Audouin (*Ichthyaetus audouinii*)

Resumen: La comprensión sobre cómo se asignan los recursos para la formación de los huevos es crucial para mejorar nuestro conocimiento de las estrategias reproductivas de las aves. Sin embargo, actualmente se sabe relativamente poco sobre cómo la síntesis del huevo en las aves silvestres podría verse limitada por la disponibilidad de micronutrientes específicos. En este trabajo hemos investigado el papel potencial de calcio (Ca) limitando la síntesis de los huevos en la gaviota de Audouin (*Ichthyaetus audouinii* Payraudeau 1826). En especial se evaluó la relación entre los niveles Ca (mg/dL) en el plasma de las hembras durante la incubación (como un indicador de la respuesta fisiológica de las hembras a una mayor demanda de Ca asociada con la producción de la puesta) y varios rasgos de los huevos relacionados con la eficacia biológica, como el tamaño del huevo (es decir, el volumen de huevo), la forma del huevo y el grosor de la cáscara en puestas de tres huevos. El tamaño del huevo se relacionó positivamente con los niveles de calcio circulante en el plasma de las hembras durante la incubación, siendo la pendiente de esta relación significativamente mayor para los últimos huevos de la puesta. La naturaleza observacional de este estudio impide inferencias causales, pero las relaciones observadas apoyaron el papel limitante del Ca en la síntesis de huevo, sugiriendo que el Ca también puede tener un papel en la modulación del patrón de variación intrapuesta del tamaño del huevo típico de esta especie de gaviota.

Title: The role of calcium in constraining egg synthesis in the Audouin's Gull
(*Ichthyaetus audouinii*)

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The role of calcium in constraining egg synthesis in the Audouin's Gull (*Ichthyaetus audouinii*)

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Abstract: Understanding how resources are allocated to form eggs is crucial to our better understanding of avian reproductive strategies. However, little is currently known about how egg synthesis in wild birds might be constrained by the availability of specific micronutrients. Here, we investigated the potential role of calcium (Ca) in constraining egg synthesis in the Audouin's Gull (*Ichthyaetus audouinii* Payraudeau 1826). In particular, we evaluated the relationship between plasma Ca levels (mg/dL) in incubating females (as an indicator of the physiological response of females to increased Ca demand associated with clutch production) and several fitness-related egg traits such as egg size (i.e., egg volume), egg shape, and eggshell thickness from three-egg clutches. Egg size was positively related with incubating female plasma Ca levels, with the slope of this relationship being significantly higher for later-laid eggs. The observational nature of this study and reversed timing precludes causal inferences, but observed relationships supported the constraining role of Ca in egg synthesis and suggested that Ca may also have a role in modulating the intraclutch pattern of egg-size variation typical of this gull species.

Key words: Audouin's Gull, avian reproduction, calcium, egg production, egg size, eggshell, *Ichthyaetus audouinii*.

INTRODUCTION

Avian females influence offspring quality through genetic contribution, but also through allocation of resources to their eggs, including antibodies (Hammouda *et al.* 2012), hormones (Rubolini *et al.* 2006) or macro- and micronutrients (Christians 2002; Hargitai *et al.* 2005; Rubolini *et al.* 2006; Romano *et al.* 2008). In turn, the availability and quality of those resources allocated to eggs influence the growth and survival of the progeny, thus affecting maternal fitness (Mousseau and Fox 1998; Christians 2002; Rubolini *et al.* 2006; Romano *et al.* 2008). Understanding resource acquisition and allocation to egg formation is therefore crucial for our better comprehension of avian reproductive and life-history strategies.

To date, we have some understanding of both intrinsic (physiological and hormonal mechanisms, e.g. Sockman, Schwabl and Sharp 2000; Williams, Reed and Walzem 2001) and extrinsic (e.g. resource availability, see Kilpi, Hillstrom and Lindstrom 1996; Hillstrom, Kilpi and Lindstrom 2000; Reynolds 2001; Hargitai *et al.* 2005) factors influencing avian egg production. In this latter regard, early works aimed at investigating resource allocation strategies in birds mainly focused on energy and macronutrients as main constrainers of egg synthesis (see Nager 2006, and references therein). However, specific nutrients other than proteins, lipids or carbohydrates can limit egg formation. The emphasis of more recent studies has consequently shifted to investigate the role of specific amino acids (Bolton, Houston and Monaghan 1992; Murphy 1994; Ramsay and Houston 1998), essential fatty acids (Surai *et al.* 2001a; Surai *et al.* 2001b), fat-soluble components (e.g. vitamins A and E, and carotenoids; Blount, Houston and Møller 2000), and especially calcium (hereafter Ca; Burley and Vadehra 1989; Patten 2007; Wilkin *et al.* 2009; Reynolds and Perrins 2010) in constraining egg production.

Evidences for Ca-limited avian reproduction are provided by a number of supplementary feeding experiments. Such evidences include direct changes in egg size and shell thickness (Mänd, Tilgar and Leivits 2000a), clutch/brood sizes and nestlings weights (Mänd, Tilgar and Leivits 2000b; Tilgar, Mänd and Mägi 2002; Tilgar *et al.* 2005), or occurrences of nest desertion and defective eggs (Graveland and Drent 1997). However, studies in the wild commonly suffer from practical methodological limitations associated with the difficulty of measuring Ca availability in the wild (i.e. exogenous Ca, Wilkin *et al.* 2009; Reynolds and Perrins 2010) and accounting for Ca mobilized from skeleton or resorpted from the renal tubules (i.e. endogenous Ca, Clunies, Emslie and Leeson 1992; Carey 1996). Alternatively, temporal trends in

plasma Ca levels for breeding females could be considered as a likely reflection of females' physiological response to increased Ca demand associated to clutch production. Indeed, Simkiss (1961) previously reported an hormonal-mediated increase in blood Ca levels for breeding females during the clutch production period, and a progressively decrease afterwards to reach those for males (that stand relatively constant throughout the early stages of the reproductive cycle) during incubation. Plasma Ca levels could be therefore used as an indicator of overall Ca (both exogenous and endogenous Ca) available for females during the clutch production period, thus facilitating investigations into the actual role of this micronutrient as a proximal factor constraining avian reproduction.

The constraining role of Ca for reproduction is expected to be particularly relevant for small passerines (which clutches often contain more calcium than is present in the entire skeleton of the breeding female) breeding on acidified areas, where Ca availability is particularly poor (Reynolds and Perrins 2010, and references therein). However, evidences for Ca-limited reproduction have been also reported in large birds, such as Herring Gulls (*Larus argentatus* Pontoppidan, 1763), laying a reduced number of relatively small eggs (Pierotti and Annett 1991). In addition, Ca-limited reproduction is rare, but not absent, in species that routinely consume Ca-rich foods during the non-breeding season, such as the piscivore Sandwich Tern (*Sterna sandvicensis* Latham, 1787; Brenninkmeijer, Klaassen and Stienen 1997) and Common Tern (*S. hirundo* Linnaeus, 1758; Nisbet 1997). Accordingly, we extended the application of measuring plasma Ca levels in breeding females to investigate the role of Ca in constraining clutch production in the large, long-lived, K-selected and piscivore Audouin's Gull (*Larus audouinii* Payraudeau, 1826). In particular, we investigated the relationship between female plasma Ca levels and several fitness-related egg traits, such as egg size (i.e. egg volume), egg shape and eggshell thickness (Blom and Lilja 2004; Altuntas and Sekeroglu 2008; Karlsson and Lilja 2008; Krist 2011). Owing to the potential role of Ca in constraining clutch production in this gull species, we expected that smaller and rounder eggs (which minimize the surface:volume ratio and thus optimize the use of shell material, Gosler, Higham and Reynolds 2005), along with thinner eggshells, would correspond to females with lower plasma Ca levels during incubation. We examined the constraining role of Ca at the intra-clutch level in three-egg, modal clutches. Eggshell in large gulls are synthesized in a relatively short time period (Ruiz *et al.* 2000), when per-day Ca requirements may overtake daily Ca intake (see Nisbet 1997 for calculations on the piscivore Common Tern). Accordingly ,we

expected the overall Ca availability for laying females to decrease as clutch formation progress, so that later-laid eggs would be particularly constrained by Ca shortage.

MATERIAL AND METHODS

Fieldwork procedure

Fieldwork was conducted at the Peninsula de la Banya (40°40'N, 0°45'E), a protected area of salt marshes located at the Ebro Delta Natural Park (Spain), that holds ca. 10,000 breeding pairs of Audouin's Gull. During the 2009 breeding period, nests of this gull species were tagged when the first egg was laid and inspected daily until clutch completion with the aim of determining laying order, final clutch size and the end of the laying period. Fresh, new laid eggs (< 24h after laying) were measured (maximum length and breadth) to the nearest 0.01 mm using a digital caliper. At clutch completion (ca. 4 days after the first egg was laid, Ruiz *et al.* 2000), all eggs within a clutch were sampled and replaced by dummy eggs, kept refrigerated and transported to the laboratory. To standardize individuals in the study and to prevent the potential confounding effect of different clutch sizes, egg sampling was performed during the peak laying (thus potentially including individuals of homogeneous "quality", see Parsons 1972; Sydeman *et al.* 1991) and restricted to nests with a modal clutch of three eggs ($n=43$). One bird per nest was captured using spring traps after clutch completion (due to the methodological limitation to sampling non-incubating gulls, Ramírez *et al.* 2010). Females and males are very similar by their appearance in this species, and the parental sex can be unequivocally determined from DNA samples only. Bird sampling extended throughout the entire incubation period (ca. 20 days) to minimize disturbance in the colony. Two milliliters of blood (representing ca. 0.3% of weight for a standard 600-grams individual) were taken from the tarsal vein using a 25 gauge needle; 0.5 ml were preserved in a neutral vial for molecular identification of sex and the rest was placed in heparinized vials to later obtain plasma for Ca level determinations. Blood samples were kept cool until heparinized vials were centrifugated a few hours later (max. 4 hours). Then, ca. 0.5 ml of the supernatant plasma were resampled and stored in independent tubes. Blood and plasma samples were frozen at -23 °C pending analyses.

Laboratory procedures

Once at the laboratory (<12 hours after sampling), egg contents were separated from eggshells, and shell thickness (dried shells with the membrane still intact) was measured with a micrometer to the nearest μm at two random locations about the equator of each shell. The determination of plasma Ca levels was based in the reaction between Ca in the sample and the arsenazo III forming a coloured complex that was measured at 635 nm by spectrophotometry. The measurement procedure of Ca-arsenazo was adapted to an A25[®] automated analyzer (BioSystems, Barcelona, Spain). Sex of gulls was determined using polymerase chain reaction (PCR) amplification of the CHD genes (Ellegren 1996; Griffiths *et al.* 1998). To extract DNA, blood was boiled in 100mM NaOH for 10 minutes at 100°C before being added to the PCR reaction. PCR protocols were modified from (Fridolfsson and Ellegren 1999) using the primer set 2550F-2718R, and PCR products were visualized on a 3% agarose gel stained with ethidium bromide scoring a single band in males and two bands in females.

Parameter estimations and statistical testing

Following Simkiss' reported temporal trend in plasma Ca levels for breeding birds (Simkiss 1961), a linear model was used to evaluate temporal decline of Ca plasma levels throughout incubation (i.e. number of days elapsed between clutch completion and capture of individuals; hereafter "days of incubation"). Sex and its interaction with days of incubation were included in the model to account for differences in temporal trends according to gender.

Due to the non independent nature of the data, generalized linear mixed models were subsequently used to investigate the relationship between plasma Ca levels of incubating females and several traits regarding egg size (i.e. egg volumes), egg shape (i.e. egg shape index, Anderson *et al.* 2004) and eggshell thickness (EST_{egg}). Egg volume (V_{egg} , cm^3) was estimated as $V_{\text{egg}} = 0.485 \cdot \text{length} \cdot \text{breadth}^2$ (Ruiz *et al.* 1998), whereas egg shape index (SI_{egg}) was derived following the standard method of $SI_{\text{egg}} = (\text{breadth} / \text{length}) \cdot 100$ (Anderson *et al.* 2004). Egg sequence (a-, b- and c-egg) and its interaction with plasma Ca levels were incorporated in the models so as to detect differences in these relationships according to laying order, whereas "days of incubation" was included as a covariate to account for the temporal trend in female plasma Ca levels through incubation (see Results, Table 1 and Fig. 1). Our research questions focused on whether the role of Ca in constraining egg synthesis was additive to that for several intrinsic and extrinsic individual traits such as body condition, age or

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dietary preferences. Accordingly, nest was included as a random factor to account for the above mentioned female traits along with the dependence among eggs laid by the same female. Statistical analyses were done using SPSS 19.0 (SPSS Inc., Chicago, USA).

RESULTS

Of the 43 adults analyzed, there were 19 males and 24 females. Plasma Ca levels (mg/dL) ranged 8.3-12.0 for males and 9.0-13.1 for females. Fitted model to evaluate trends of Ca levels throughout incubation included a significant interaction between sex and "days of incubation" ($F_{1,43}=4.2$, $p=0.047$), which are in agreement with the results from (Simkiss 1961) in that plasma Ca levels follow a temporal trend throughout incubation that differs according to gender. In particular, Ca levels for males held constant throughout incubation (95% CI for the slope of fitted temporal trend = -0.263, 0.282), whereas a negative trend was fitted for females (95% CI = -0.265, -0.03), with a gradual decrease until reaching similar concentrations to those for males (Table 1 and Fig. 1).

Ca (mg/dL)	
Fixed parameters ^a	Estimate (95% CI)
Intercept	11.74 (10.84, 12.64)
Male	-1.71 (-2.94, -0.46)
Days of incubation	-0.15 (-0.26, -0.03)
Male by Days of incubation	0.16 (0.02, 0.31)
MLS estimation of mean	
values	Estimate (95% CI)
Male	10.04 (7.66, 12.46)
Female	11.59 (10.58, 12.29)

^a Female has been taken as a reference category

Table 1. Parameter estimates and mean least squares (MLS) estimations of mean values derived from fitted models for plasma levels of Ca (mg/dL) in incubating Audouin's Gulls breeding at the Ebro Delta during 2009. Sex was introduced in the model as a fixed factor, whereas days of incubation (number of days elapsed between clutch completion and capture of individuals) was introduced as a covariate.

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Regarding egg characteristics, V_{egg} (cm^3) ranged 57.25-70.44, 54.13-66.72, and 48.2-59.89 for *a*-, *b*-, and *c*-eggs, respectively; Sl_{egg} ranged 66.03-74.03, 65.25-77.00 and 64.80-73.52; and EST_{egg} (μm) ranged 321-412, 313-401, and 314-386. When looking at the relationship between female Ca levels and egg/shell characteristics, we observed that plasma Ca levels were positively related with V_{egg} ($F_{1,24}=14.48$, $p=0.001$). However, such relationships differed according to laying order as indicated by the significant interactions between this three-level factor and plasma Ca levels ($F_{2,48}=3.05$, $p=0.038$), with increasing slopes throughout the laying sequence (see Table 2 and Fig. 2). Sl_{egg} did not show any relationship with female plasma Ca levels ($F_{1,24}=0.01$, $p=0.97$). However, a significant effect of the laying order was detected ($F_{2,48}=6.67$, $p=0.003$), being the Sl_{egg} values for *b*-eggs slightly higher than those obtained for *a*- and *c*-eggs (Table 2 and Fig. 3A). EST_{egg} was neither related to plasma Ca levels ($F_{1,24}=0.31$, $p=0.58$), nor to laying order ($F_{2,48}=2.91$, $p=0.064$; Fig. 3B).

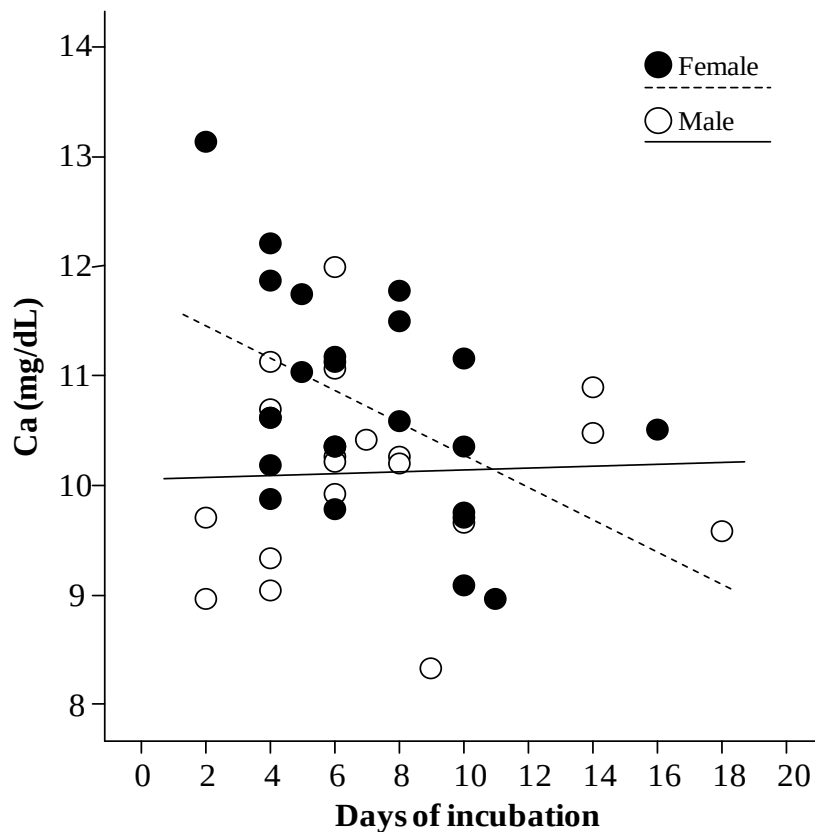


Figure 1. Temporal trends of plasma Ca (mg/dL) through incubation (Days of incubation: number of days elapsed between clutch completion and capture of individuals) according to gender for incubating Audouin's Gulls breeding at the Ebro Delta (Spain) during 2009. Solid and dashed lines represent regression lines predicted by the model for each sex (see Results and Table 1).

DISCUSSION

Our results for Audouin's Gulls indicated that higher plasma Ca levels in incubating females resulted in larger eggs. Further, this relationship varied for different eggs within the laying sequence, with increasing slopes for latter-laid eggs. Although the observational nature of this study and reversed timing precludes causal inferences, these results pointed to the constraining role of Ca for egg synthesis in this species and suggested that this limiting micronutrient may also have a role in modulating the intra-clutch pattern of egg size variation typical of this gull species.

Table 2. Parameter estimates and mean least squares (MLS) estimations of mean values derived from fitted models for egg volume (cm³) and egg-shape index (breadth/length × 100) in three-egg clutches from Audouin's Gulls (*Ichthyaeetus audouinii*) breeding at the Ebro Delta during 2009.

	Egg Volume (cm ³)	Egg Shape Index
Fixed parameters^a	estimate (95% CI)	estimate (95% CI)
Intercept	24.96 (10.69, 39.22)	69.38 (68.32, 70.43)
a-egg	27.52 (12.75, 43.30)	0.70 (-0.19, 1.58)
b-egg	11.91 (-2.86, 26.69)	1.61 (0.71, 2.49)
Ca	2.63(1.39, 3.87)	
Days of incubation	0.20 (-0.11, 0.50)	
Ca by a-egg	-1.79 (-3.16, -0.41)	
Ca by b-egg	-0.66 (-2.04, 0.7)	
MLS estimation of mean values	estimate (95% CI)	estimate (95% CI)
a-egg	62.94 (61.81, 64.06)	70.07 (69.02, 71.29)
b-egg	59.35 (58.23, 60.48)	70.98 (69.93, 72.04)
c-egg	54.57 (53.48, 55.70)	69.38 (68.32, 70.43)
Variance parameter	estimate (± SE)	estimate (± SE)
Nest	2.26 (± 1.22)	4.22 (± 1.45)
Residual	5.33 (± 1.09)	2.33 (± 0.48)

Note: Laying sequence (a-, b-, and c-egg) and nest were introduced in the models as fixed and random factors, respectively. Female plasma levels of Ca and its interaction with laying sequence were included to detect differences in these relationships according to laying order. Days of incubation (i.e., number of days elapsed between clutch completion and capture of individuals) were also included to account for observed temporal trend in female plasma Ca levels through incubation. c-egg is considered a reference category, and 95% CI and SE are the 95% confidence intervals and standard errors, respectively.

Tracing Ca availability for laying females

Avian females might adopt various strategies to meet Ca requirements for clutch formation. Dietary switches towards exploitation of Ca-rich food items during the clutch production period have been widely reported for small passerines (e.g. Wilkin *et al.* 2009; Reynolds and Perrins 2010) and several tern (e.g. Sandwich and Common Terns, Brenninkmeijer, Klaassen and Stienen 1997; Nisbet 1997) and gull species (e.g. Audouin's Gull, Navarro *et al.* 2010). Alternatively (or complementarily), avian females can show a hypercalcemic response associated to egg production, mediated by the endocrine system and consisting of increasing Ca intestinal absorption (Klasing 1998), and resorption from renal tubules and medullary bone (Clunies, Emslie and Leeson 1992; Carey 1996; Reynolds and Perrins 2010). Both dietary intake and endogenous Ca mobilization result in a dramatic increase of female plasma Ca concentrations during ovulation, to decrease progressively afterwards to reach those values for males (which do not show these changes in blood Ca levels) during incubation (Simkiss 1961; Lumeij 1994). Although our results for the Audouin's Gull referred exclusively to the incubation stage they agreed with previously reported sex-specific trends and suggested that plasma Ca levels in incubating females can be used as an indicator of plasma Ca levels during the egg formation.

The constraining role of Ca

Eggshell provides the embryo with minerals, primarily Ca, needed for the development of high-Ca consuming organs, such as the skeleton, muscle and brain (more than 80% of the Ca needed for embryonic development is derived from eggshell; Blom and Lilja 2004; Karlsson and Lilja 2008). Ca in the eggshell is exclusively mobilized from the mammillary tips, which can be viewed as functional units that provide the embryo with a limiting amount of Ca (see Karlsson and Lilja 2008) and references therein). The removal of Ca from eggshell results in decreasing mammillary densities (number of tips per unit of egg surface) and in a "natural thinning" of the eggshell throughout incubation (Karlsson and Lilja 2008; Reynolds and Perrins 2010), which likely affect shell's mechanical properties and the exchange of gas and water between the embryo and the environment. The initial mammillary density and shell thickness are therefore crucial to ensure egg feasibility and proper embryonic development. Accordingly, and in contrast to what occurred for egg size, our results indicated that eggshell thickness was independent of Ca availability, and stood relatively constant throughout the laying sequence (although slightly thinner shells were observed for last-laid eggs, as previously reported by Nisbet 1982 and Reynolds 2001).

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for Common Terns and Zebra Finches *Taeniopygia guttata* Vieillot, 1817, respectively). Egg size variations in relation to Ca availability might be therefore related to the necessity of achieving the required initial mammary density and shell thickness.

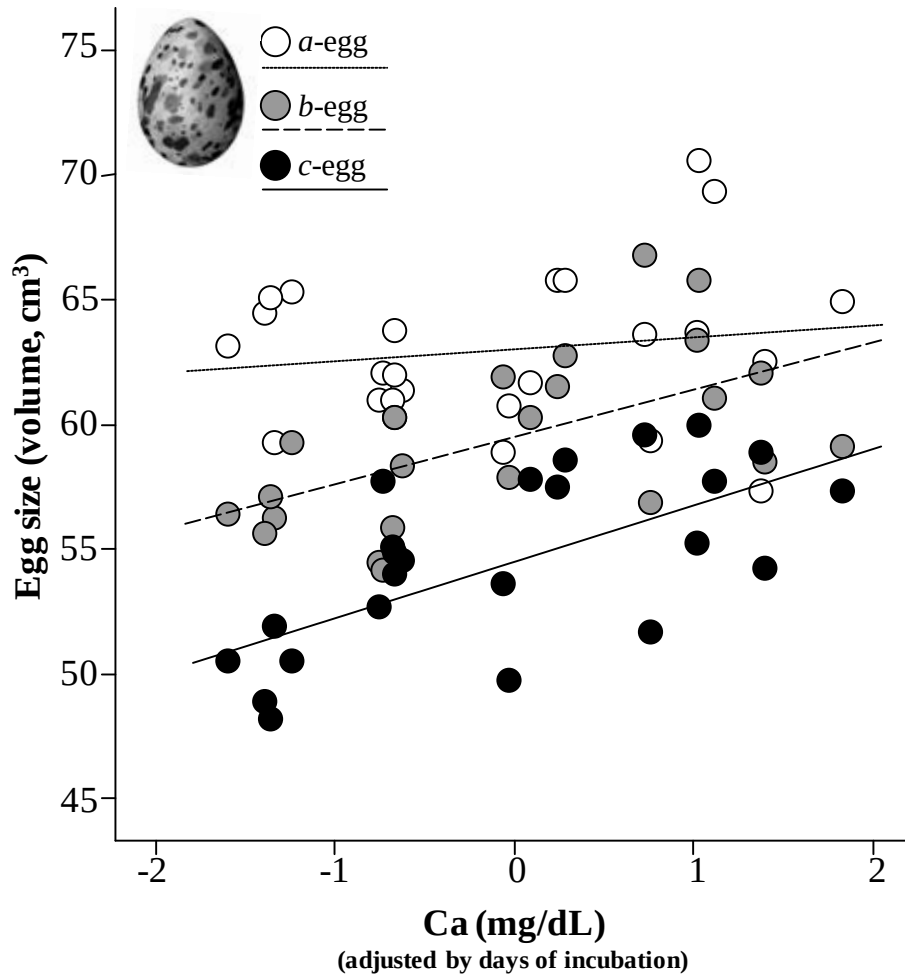


Figure 2. Bivariate plot of residuals of female plasma Ca levels (adjusted by days of incubation) in Audouin's Gulls breeding at the Ebro Delta (Spain) during 2009 vs. egg size (volume, cm³). Lines represent regression lines predicted by the model (see Results and Table 2).

Whereas the limiting role of Ca for clutch synthesis is widely accepted (Reynolds and Perrins 2010), few studies have discussed on the potential implication of this micronutrient in determining the intra-clutch pattern of egg size variation typical of many avian species (Graveland *et al.* 1994; Reynolds 2001). Indeed, the brood reduction and the hormonal hypothesis have been widely considered when explaining egg size variation throughout the laying sequence (Lack 1954; Lack 1968; Slagsvold *et al.* 1984; Sockman, Schwabl and Sharp 2000; Williams, Reed and Walzem 2001). Our results indicated that the relationship between plasma Ca levels in incubating females

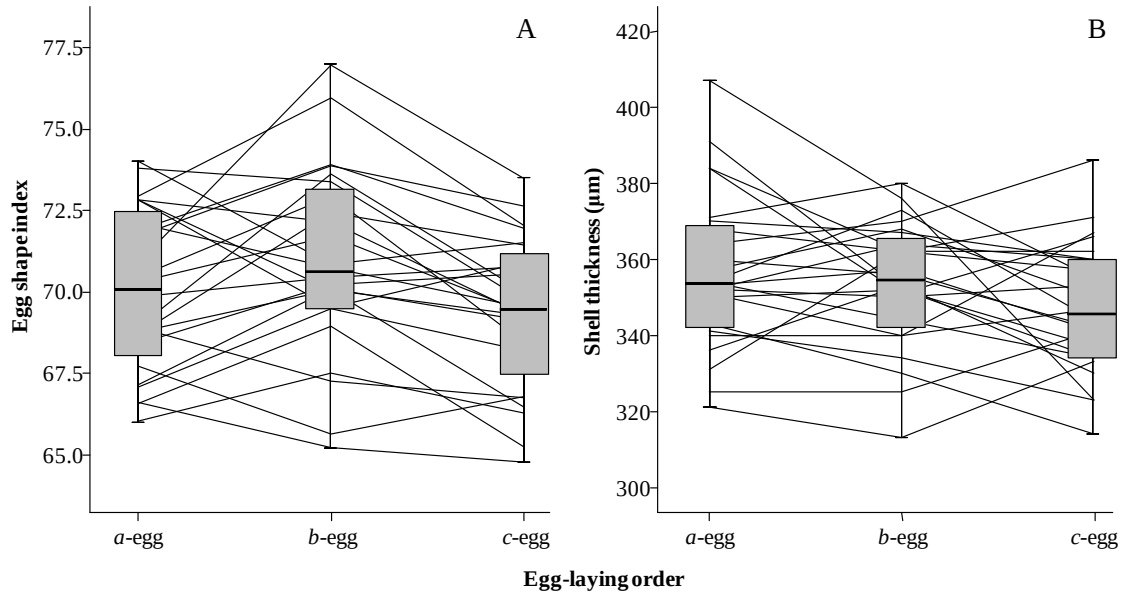
and egg size varied throughout the laying sequence, being slightly higher for latter-laid eggs (as pointed out by estimated slopes for the relationships between plasma Ca levels and estimated egg volume and surface area: $a\text{-egg} < b\text{-egg} < c\text{-egg}$). In our view, these results suggested that later-laid eggs in Audouin's Gulls were particularly sensitive to Ca availability, which could be explained by an overall decrease in Ca availability (both endogenous and exogenous) as long as this micronutrient is progressively allocated into the successive eggs (eggshells) to complete the clutch. In addition to its effect on clutch production, Ca availability may also play a relevant role in determining the intra-clutch pattern of egg size variation, with higher availabilities resulting in more homogeneous egg sizes at the intra-clutch level.

In contrast to our a priori expectations and to what Gosler, Higham and Reynolds (2005) found for Great Tits (*Parus major* Linnaeus, 1758), egg shape in the Audouin's Gull was not found to vary according to Ca availability. However, the fact that this trait significantly varied throughout the laying sequence, and that more than half of its variability was explained by the random nest factor, suggested that others, unmeasured, female traits likely operate through clutch production affecting egg shape.

Avian egg size has been previously reported to be affected by several extrinsic (e.g. food availability or ambient temperature) and intrinsic (e.g. age, endogenous protein stores, oviduct mass or rate of protein uptake by ovarian follicles) female traits (reviewed by Christians 2002). Our study focused in exploring the constraining role of Ca during reproduction and provided some evidences suggesting that, in addition to aforementioned individual traits, the availability of this micronutrient for breeding females influenced resource allocation strategies in the Audouin's Gull. In particular, higher Ca availabilities resulted in larger eggs and more homogeneous egg sizes at the intra-clutch level. We argue that proposed approach supposes an important contribution to investigate Ca-limited reproduction in the wild and we recommend its use for approaching reproductive strategies in birds.

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Figure 3. Intra-clutch pattern of variation for (A) egg shape index (breadth/length·100) and (B) shell thickness (μm). For each parameter it has been represented both individual trends and descriptive summaries using box plots (minimum, maximum -whiskers-; Q1, Q2 and Q3 -white box-).



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GLOBAL DISCUSSION

Road at “Punta de la Banya” colony (Ebro Delta Natural Park, Spain).
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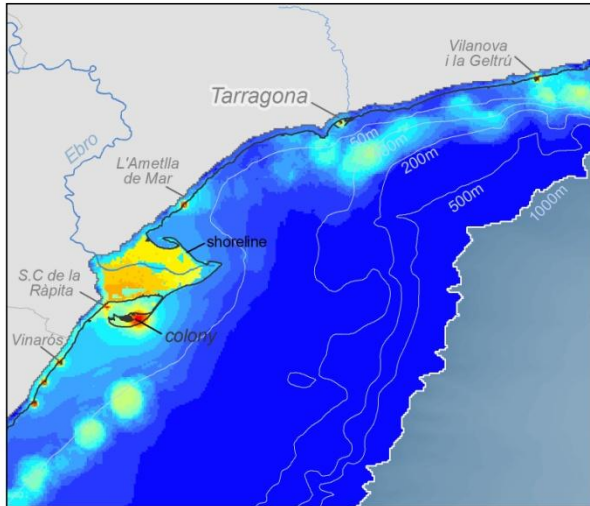
GLOBAL DISCUSSION

Advances in knowledge about spatial distribution, trophic ecology and habitat use

Although the trophic ecology and at-sea distribution of Audouin's gull have been widely treated in the past, the spatial at-sea distribution has not been treated with so much detail (see *Introduction*). The combination of accurate positional data from individuals tagged with GPS loggers, together with the information on environmental variables and vessel monitoring systems (VMS), is used for first time in this species. The combination of these powerful tools allow us to precisely model the spatial distribution patterns and foraging movements of Audouin's gull in relation to different habitats, time intervals and fishing activities. Moreover, the modelization taking into account different habitats (marine and rice field) at the same time is also novel, and represents the most accurate distributional study in the flexible Audouin's gull.

In accordance with previous studies, our results show the great dependence of Audouin's gull foraging behaviour on fishing activities. Although considered in the past a nocturnal specialist adapted to the capture of Clupeiform fish (Wallace 1969, Cramp & Simmons 1983), our results in *Chapter 1.1* show that, during work days, the probability of finding Audouin's gulls at sea during the night is much lower than during the day (compare Figure 1 and Figure 2). Indeed, the response of the models suggests that trawling activities almost completely determines the at-sea distribution of Audouin's gull, both spatially and temporally (Figure 2 and *Supplementary Information Chapter 1.1*). Moreover, most of the nocturnal activity during work days must be caused by the association with purse-seine boats, due to the concentration of individuals in front of the main purse-seine harbours (Figure 1). Although it can not be ruled that some birds would be fishing by themselves, the great association between fishing activities and the spatial distribution of Audouin's gull suggest that most of its marine diet comes from anthropogenic activities. During weekends (when there are no fishing activities) is also possible to find marine activity for the gulls, but much more dispersed. Unlike work days, the variable Chlorophyll A increases in weight, indicating that gulls target on the most productive areas to feed in the absence of fishing activities. Accordingly, the general pattern observed on weekends indicates that gulls head out to the sea early in the morning, but as they don't find fishing activities, some of them return to fishing harbours and rice fields to feed (especially after noon) (see Figure 2, Figure 3 and *Supplementary Information Chapter 1.1*).

Weekday (02-04h)



Weekend (02-04h)

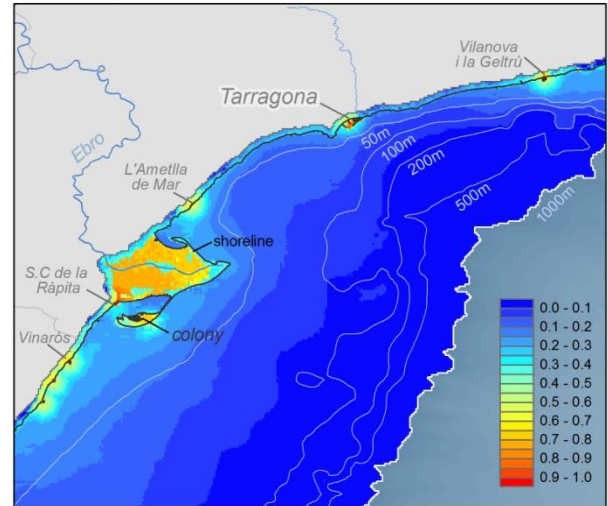
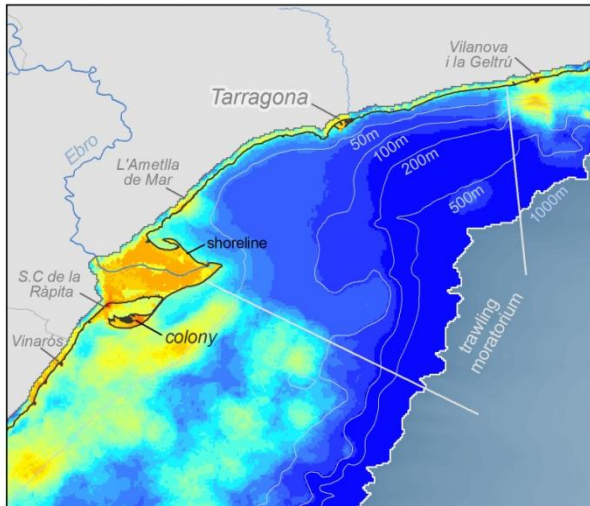


Figure 1. Audouin's gull distribution model 02-04h (purse-seine activity on weekday)

Weekday (12-14h)



Weekend (12-14h)

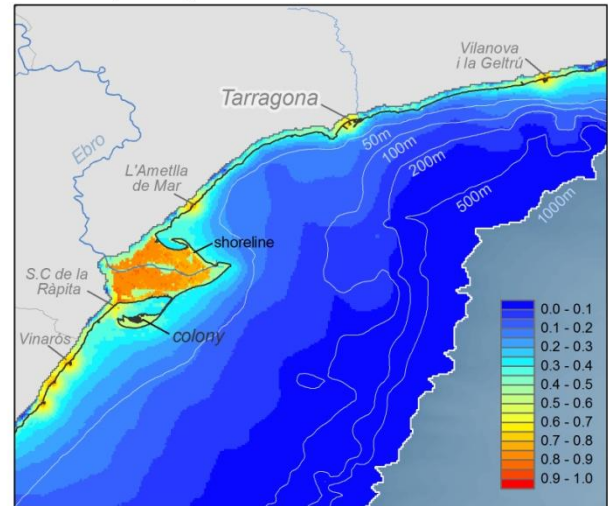
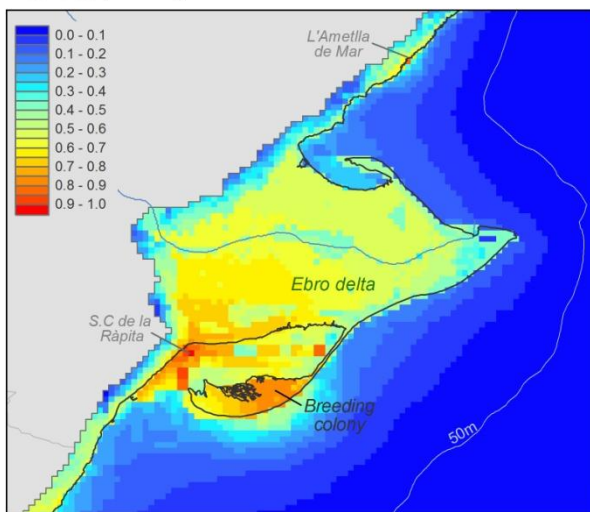


Figure 2. Audouin's gull distribution model 12-14h (trawling activity on weekday)

Weekday (18-20h)



Weekend (18-20h)

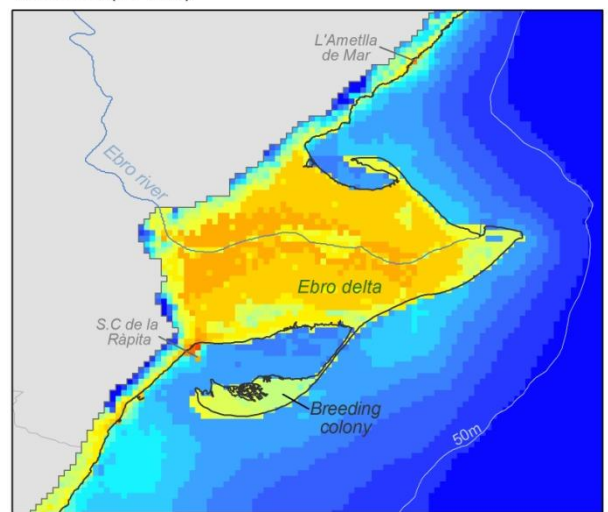


Figure 3. Distribution model 18-20h (when there is no fishing activity)

A closer approach on this feeding behaviour at the individual level (*Chapter 1.2*) shows that female Audouin's gulls tended to perform longer and further afield foraging trips than males. The spatial segregation by sex is widespread in seabirds, where the smaller sex (female in the case of Audouin's gull) usually undertakes longer trips (reviewed in Wearmouth & Sims 2008). It has been suggested that size matters probably because the smaller and lighter sex has a higher foraging and flight efficiency (Shaffer *et al.* 2001). Also, foraging segregation has been associated to a reduced intraspecific competition (Catry *et al.* 2005). However, the sexual dimorphism hypothesis is not always supported (for example Lewis *et al.* 2002; Stauss *et al.* 2012). In fact, our data indicate that these gender differences are reflected in females travelling significantly longer distances and spending longer time outside the colony during weekends, when there are no major fishing activities and therefore no discards (Figure 4).

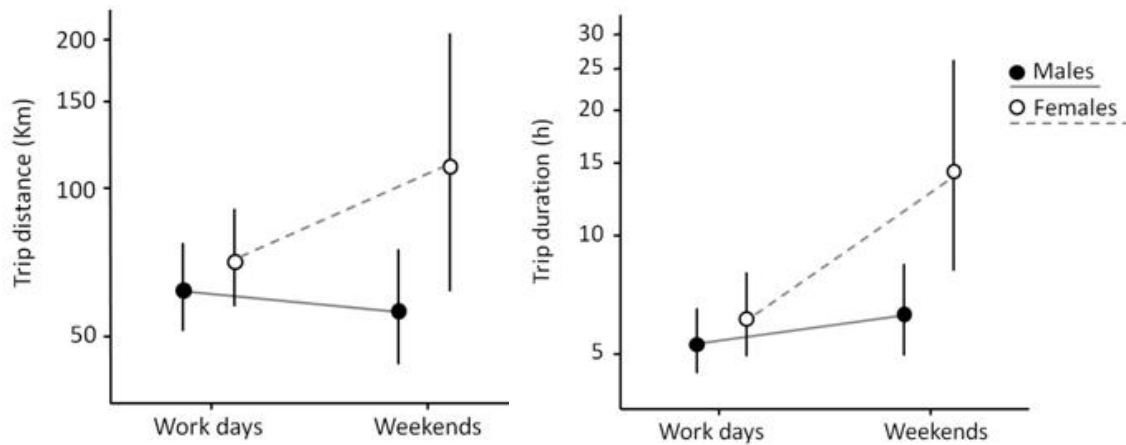


Figure 4. 95% CI of the estimated (A) trip distance (in kilometres) and (B) trip duration (in hours) derived from the selected final GLMM (in logarithmic scale).

The distance and duration of the trips are highly related to the foraging habitat, being at-sea foraging trips significantly longer than rice field ones. This means that, on weekends, females perform longer at-sea foraging trips than males. Indeed, although both sexes increased the use of rice field resources in absence of trawling activity, during the weekends males seems to exploit this habitat to a greater extent than females (Table 1). However, these gender differences were not statistically significant probably due to the great variability of strategies presented by Audouin's gull. Although it was known that this species increase the consumption of rice field prey in absence of fishing activities (Oro *et al.* 1996b), this sex-biased habitat selection presented in *Chapter 1.2* (based on both foraging behaviour and habitat use) is described for the first time.

Sex	Fishing activity	% marine habitat	% rice field
Males	Work day	61.3 ± 39.3	38.7 ± 39.3
	Weekend	42.0 ± 40.6	58.0 ± 40.6
Females	Work day	64.5 ± 36.8	35.6 ± 36.8
	Weekend	58.5 ± 43.3	41.5 ± 43.3

Table 1. Descriptive statistics for the % of habitat use (marine or rice field) of Audouin's gull by sex and fishing activities. Values are shown as mean ± standard deviation.

Combining the information provided by GPS loggers and dietary markers as the stable isotopes analysis (SIA) is also novel in disentangling the trophic ecology of this species. Both SIA and GPS data from *Chapter 1.2* showed a high correlation and agreed that Audouin's gull exploits different habitats in the Ebro Delta. Individuals ranged from exclusively marine to rice field specialists, all the way up to intermediate situations (Figure 5). However, this apparent trophic plasticity hides a high individual specialization. The higher variation among individuals rather than within individuals (there was a high individual repeatability in plasma stable isotopes between capture and recapture events) suggests a great consistency in the habitat selection. Moreover, no significant differences in foraging preferences between sexes were detected unlike what other studies have pointed (Navarro *et al.* 2010). These individual foraging specialisations seems to be common in seabirds (for example Votier *et al.* 2004; Patrick *et al.* 2014) due to intra-specific aggregation and competition (Araújo *et al.* 2007), and because of predictability of prey patches (Weimerskirch *et al.* 2007).

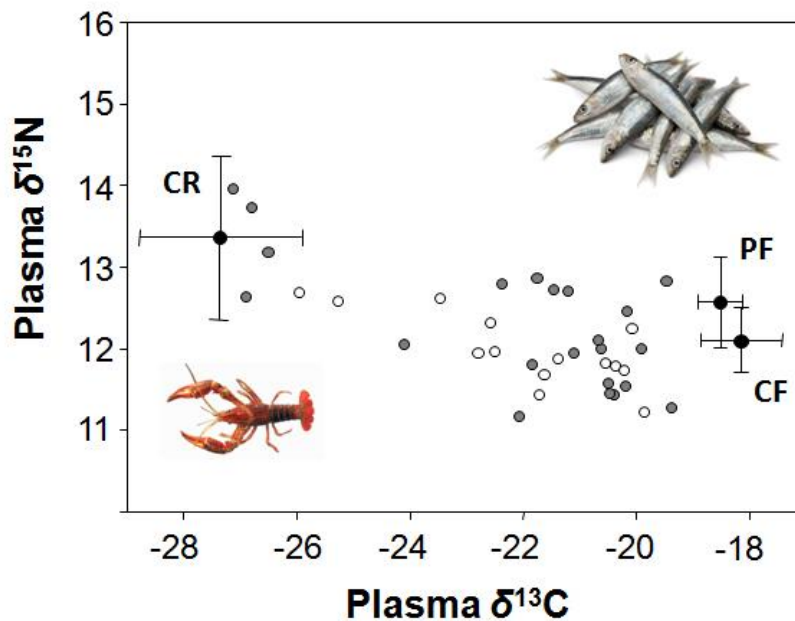


Figure 5. Scatter plot of the dispersion between $\delta^{13}\text{C}$ / $\delta^{15}\text{N}$ plasma values of Audouin's gull at the recapture moment. Males are shown as grey dots, females as empty dots. The mean and standard deviation of American crayfish (CR), Perciform fish (PF) and Clupeiform fish (CF) are also shown as potential preys.

Why females perform a greater use of marine habitats than males in the absence of fishing activities? Due to the apparent lack of relationship between the weight of individuals and descriptors of foraging trips, it seems that other, not necessarily independent, ecological/physiological factors beyond could play a role. Among these factors, the role of micronutrient requirements in creating sex-specific foraging behaviour has been sometimes overlooked (see for example Lewis *et al.* 2002, Xavier & Croxall 2005). Some studies have revealed that the need to restore calcium levels after laying could be an important explanatory factor related to sexual foraging segregation (reviewed in Wearmouth & Sims 2008). Indeed, calcium levels in females of Audouin's Gull have been shown as a limiting factor that constrains the egg synthesis (see *Chapter 2.3*). Accordingly, fish soft-bones represent a major source for absorbed calcium compared to crustaceans (Hansen *et al.* 1998). Moreover, other compounds like antioxidants molecules deposited in eggs are also present to a limited extent in females. Recent work has shown this limited abundance in female Audouin's Gull, and the importance of marine diets providing a greater antioxidant capacity to the eggs than rice field resources (see *Chapter 2.2*).

The negative effects of a trawling moratorium (and therefore, in food availability) on the breeding success of this species are widely known (Oro *et al.* 1996a, 1996b, Oro *et al.* 1999, Oro *et al.* 2004). However, this sex-specific foraging behaviour in relation to fishing activities has novel implications of conservation concern from a discard ban point of view. Audouin's gull is more likely to approach longline vessels when trawlers are not allowed to operate (Arcos & Oro 2002). Moreover, this species is the second most affected seabird by longline bycatch in the Mediterranean (Laneri *et al.* 2010). Therefore, a ban on discards could lead to important sex-biased bycatch mortality in this long-lived threatened gull (Báez *et al.* 2014). In fact, one of our tagged females was found dead in Barcelona (more than 150 kilometres north of the colony) after having swallowed nine fishing hooks (Figure 6).

Also, the great individual specialization in the habitat use observed in this species suggests that individual cognitive abilities and specializations such as learning how human activities can modify the availability and predictability of food resources may play a very important role in determining the foraging behaviour of the individuals (Cama *et al.* 2012; Oro *et al.* 2013). Since these individual specializations may take several years to learn (Bicknell *et al.* 2013), a gradual implementation of any policy is needed in order to facilitate the adaptation of threatened species like Audouin's gull to a discard ban scenario.

In conclusion, the combination of GPS data with SIA, VMS and environmental variables is becoming more and more used, and appears/constitutes as a promising tool to design the new management and conservation measures.



Figure 6. Radiography of the female Audouin's gull found dead in Barcelona in 2011. Photo © JM Arcos-SEO/BirdLife & Wildlife Centre of Torreferrussa.

Advances in knowledge about how habitat use determines pollutant accumulation

Our data on stable isotopes show that Audouin's gull chicks from both the Ebro Delta and the Llobregat Delta are fed mainly with marine piscivorous resources. Although the isotopic data of Navarro et al. (2010) estimated that rice field consumption by the chicks from the Ebro Delta was between 19-32%, the data of this study are more in agreement with those of Pedrocchi et al. (2002) and Sanpera et al. (2007a) reporting that most of the diet consists on marine fish. In the case of the newly formed colony of Llobregat Delta, there were no previous available data. We have found a fully marine diet for these chicks, probably due to the lack of surrounding rice fields in the area unlike in the Ebro Delta. Given the dependence on marine resources, and because foraging movements showed in *Chapter 1.1* and *Chapter 1.2* are mainly concentrated on surrounding waters off the colony, Audouin's gull chicks' feathers are revealed as good candidate to monitorize both coastal areas (Figure 7).

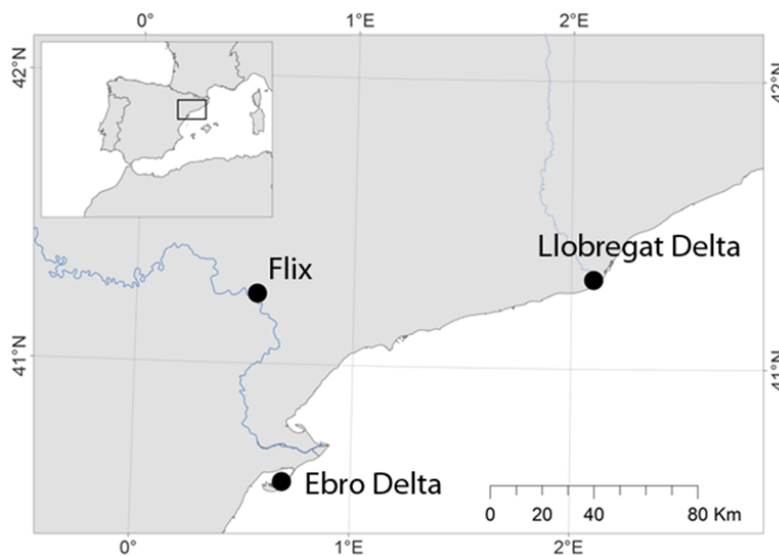


Figure 7. Map of the sampled colonies (Ebro Delta and Llobregat Delta) and the location of toxic muds accumulated at Flix dam.

We found significant higher mercury levels in the Ebro Delta than in Llobregat Delta (Figure 8). The main cause of these very high levels in the Ebro Delta must be sought in the pollutants carried by the river waters from Flix town (41°14'N, 00°31'E; Figure 7). The different industrial activities at this place due to a chemical industry that has been in operation since the early 20th century, and the construction of a dam close to the factory in 1960, resulted in the accumulation of 200,000–360,000 tons of industrial wastes in the riverbed. This wastes accumulated in the sediments include high concentrations of heavy metals (cadmium, arsenic, copper, chromium, lead and especially mercury), organochlorines and radioactive ^{210}Pb (Fernandez et al., 1999; Bosch et al., 2009). The pollutants dumped at Flix are carried downstream by the Ebro

River to its delta 90 km away, especially during floods (Vericat and Batalla, 2006). In this way, there have been many studies highlighting the effects of this problem on the biota (Quiros et al., 2008; Carrasco et al., 2008, 2011; Barata et al., 2010; Suarez-Serrano et al., 2010; Cotin et al., 2011, 2012). This scenario originates one of the most important ecological problems still to be solved in Western Mediterranean, not only because it affects one of the most relevant wetlands in south Europe (the Ebro Delta), but also because this is an important agricultural area and the Ebro river potentially supplies water to 2.7 million people (CHEbro 2009).

In many bird species, mercury concentrations in feathers above $5,000 \text{ ng}\cdot\text{g}^{-1}$ are associated with adverse reproductive effects such as lower clutch and egg size, a lower hatching success and a decreased chick survival (Eisler, 1987). The mean concentration detected in the Ebro Delta is dangerously close to $5,000 \text{ ng}\cdot\text{g}^{-1}$ (Figure 8). In fact, the 50% of the marine-fed chicks in this area present values above this threshold and are therefore susceptible to the sublethal effects described. This concentration ranged from 2.5 to 5 times that found in Audouin's gull chick feathers from North-eastern Mediterranean (Goutner et al., 2000). Further research should be carried out on how this mercury accumulation affects reproductive and physiological parameters in this near threatened seabird.

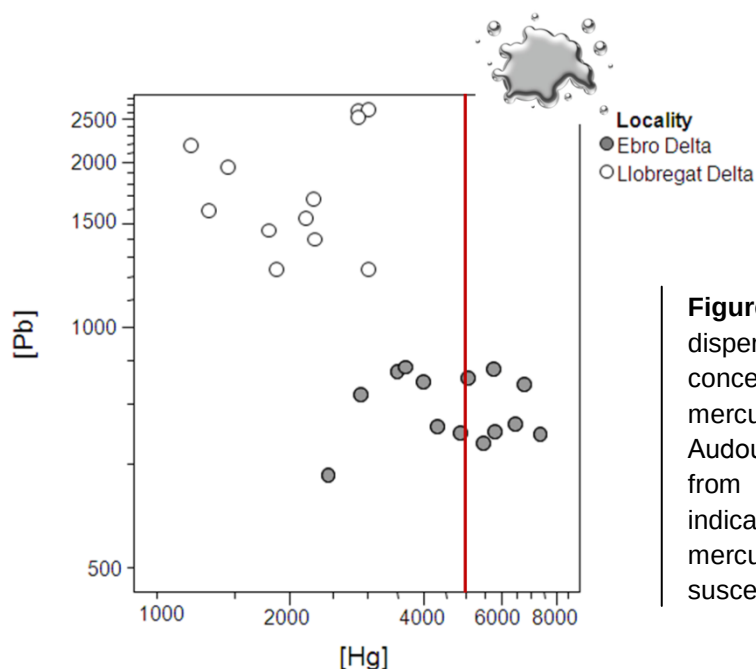


Figure 8. Scatter plot of the dispersion between the concentrations of lead and mercury (in $\text{ng}\cdot\text{g}^{-1}$) of Audouin's gull chick feathers from both localities. Red line indicates the threshold for mercury of $5,000 \text{ ng}\cdot\text{g}^{-1}$, susceptible to sublethal effects.

On the other hand, lead concentrations detected in our study showed to be significantly higher in the Llobregat Delta. Since the most part of this heavy metal present in the feathers came from atmospheric deposition, lead levels from both areas

can give us key clues. In fact, lead atmospheric concentration is much higher in the Llobregat Delta area ($25.1 \mu\text{g}\cdot\text{m}^{-3}$) than in the Ebro Delta ($2.5 \mu\text{g}\cdot\text{m}^{-3}$) (IDESCAT, 2011). This situation could be a consequence of the use of aviation gasoline in the nearby Barcelona airport (just 2 Km from the colony) as has been reported previously (Kessler, 2013). Aviation gasoline, commonly referred to as avgas, is a leaded fuel used to power piston-engine small aircraft (Kessler, 2013). In the United States, for example, lead emitted from aircraft using avgas is currently the largest source of lead to the air, constituting about 50% of lead emissions in 2005 (U.S. EPA, 2010). In birds, it has been suggested that lead levels in feathers above $4,000 \text{ ng}\cdot\text{g}^{-1}$ are associated with delayed parental and sibling recognition, impaired locomotion, depth perception and feeding behaviour, and lowered chick survival in gulls (reviewed in Burger & Gochfeld, 2000). In no case this threshold is exceeded, being all the chicks far from this limit and apparently safe from lead diseases.

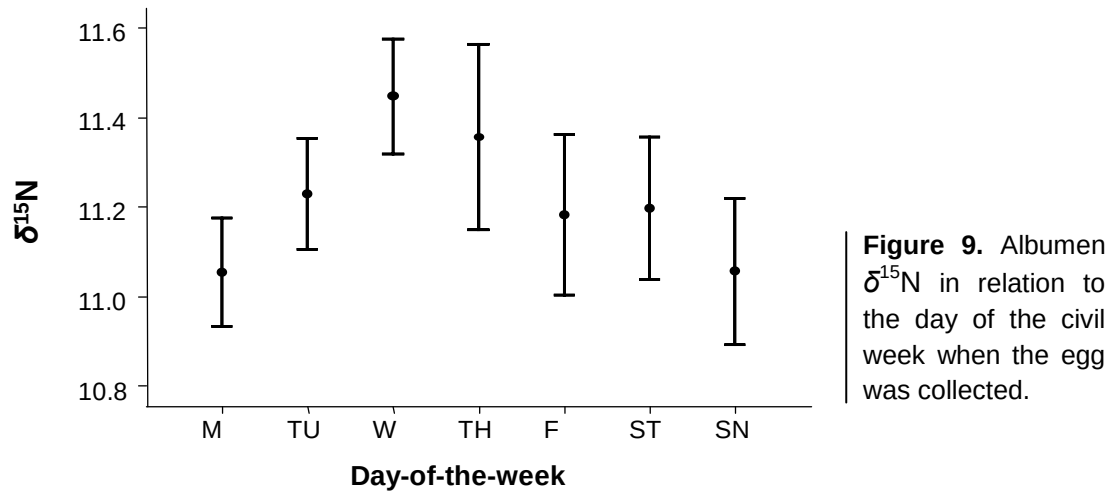
These results support the potential of Audouin's gull chicks' feathers to monitorize pollutants levels in a given coastal area. Furthermore, these results highlight how the selection of different foraging areas results in differential heavy metal accumulation. Finally, more studies are needed to determine the extent of risk for human health from the consumption of fish with high mercury levels from the Ebro Delta area.

Advances in knowledge about resource allocation into egg production

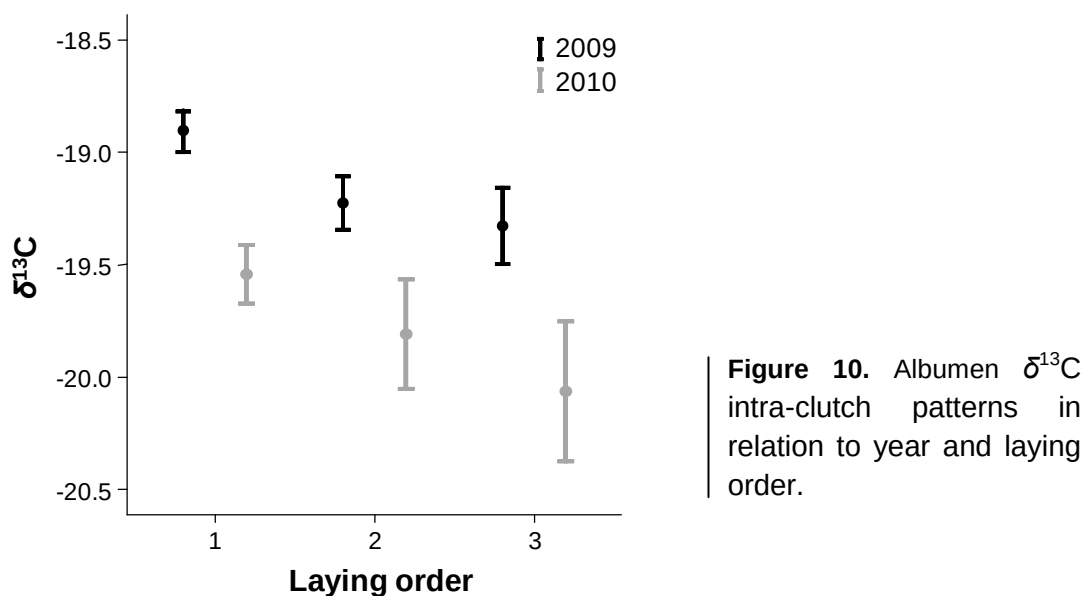
The effect of adaptive and non-adaptive constraints in determining the intra-clutch pattern of nutrient allocation and egg size variation has been traditionally overlooked. Currently, some studies have addressed this issue using the stable isotopes to track the origin of nutrients allocated into the eggs, especially through the carbon ($\delta^{13}\text{C}$) and nitrogen ($\delta^{15}\text{N}$) stable isotopes (Hobson 2006). In this way, albumen seems to be the most suitable egg compartment for an intra-clutch insight since it is synthesized during non-overlapping short time periods (Ruiz et al. 2000).

The selected final model for the albumen $\delta^{15}\text{N}$ only included the “day-of-the-week” as a significant factor (Figure 9). Or what is the same: the effects of an increased consumption of rice field resources during weekends (when the availability of trawling discards is negligible) is presumably reflected in enriched nitrogen albumen signatures reaching its peak on Wednesday. This time lag corresponds to the time needed to synthesize the albumen and eggshell, about 2-3 days (Nager 2006). The consistent daily pattern of $\delta^{15}\text{N}$ between years and nesting places supports the validity of this prediction, and points out the strong dependence of Audouin's gull on fishing

activities as shown in *Chapter 1.1* and *Chapter 1.2*. Moreover, the high sensitivity to daily changes in discard availability reflects that the most part of the nitrogen deposited in the albumen must come from diet.



On the other hand, $\delta^{13}\text{C}$ showed a consistent declining pattern through the laying sequence in both years (Figure 10). However, carbon isotopic values were higher in 2009 than in 2010. These differences between years may be attributable to the highly dynamic nature of the Ebro River related to weather conditions and the consequent input of particulate organic matter (POM) into the sea, mainly affecting the carbon isotope (Veyssy et al. 1998; Harmelin-Vivien et al. 2010). Carbon in the albumen must have a more endogenous origin than nitrogen since this daily pattern (although statistically significant) is not as obvious as in the nitrogen. Moreover, the consistent declining intra-clutch pattern observed probably indicates differences in endogenous contribution through the laying sequence rather than a dietary switch.



Our results are consistent with a number of previous studies in wild ducks showing that the nitrogen deposited in the eggs came primarily from the food consumed in the breeding grounds in these migratory species, whereas carbon had a more endogenous contribution (Hobson et al. 2005, Bond et al. 2007, Oppel et al. 2010). Surprisingly, our results in Audouin's gull are opposite to those found by Ramírez et al. (2011) in the sympatric Yellow-legged gull (*Larus michahellis*). Since the day-of-the-week was not considered in their study, the results could be a consequence of this overlooked effect in a species that also exploits discards efficiently (Cama et al. 2012). However, differences in the allocation strategy between Yellow-legged and Audouin's gull cannot be ruled out. Although phylogenetically distant to Audouin's gull, the ducks from these coinciding studies are also long-distance migrators as our target species. The Yellow-legged gull, on the contrary, is mostly resident (Cramp & Simmons 1983). The major source of endogenous proteins allocated to the eggs is the pectoral muscle (Kendall et al. 1973; Houston et al. 1995). However, the mobilization of protein reserves from the pectoral muscle during laying can detrimentally affect the female's flight ability (Veasey et al. 2001; Kullberg et al. 2005). Perhaps, to perform a long migration immediately after breeding limits the mobilization ability of endogenous proteins to the clutch in migratory species compared to resident ones. Further research is needed to test this hypothesis.

In relation to the intra-clutch egg size pattern, significant differences between nesting places were found regardless of the year (Figure 11). The salt pans seems to be a nesting place of better quality because its higher nest density and breeding success than that in the coastal dunes (Oro et al. 2010). Some studies in gulls suggested that higher nest density is associated to the reduced size of the third egg (Coulson et al. 1982; Pierotti & Bellrose 1986; Spaans et al. 1987), in agreement with our findings. Probably, in low densities or under suboptimal situations, females can modulate the nutrient allocation by increasing investment in the final eggs, thus enhancing nestling growth and survival, and reducing hatching time (Ferrari et al. 2006).

In addition to that, diet is one of the main factors that have been suggested to affect egg size (Martin 1987; Bolton et al. 1992; Williams 1994). Indeed, apart from the expected decreasing pattern in size along the laying sequence, the other main factor with a significant effect on egg size is $\delta^{15}\text{N}$. Because of the close relationship between nitrogen signature and diet discussed above, this negative relationship suggests that situations of discard shortage (increasing the consumption of rice field resources) are associated with reduced egg size. Further research is needed on both, how specific

characteristics of the nesting place and diet affect reproductive output such as offspring viability or breeding success.

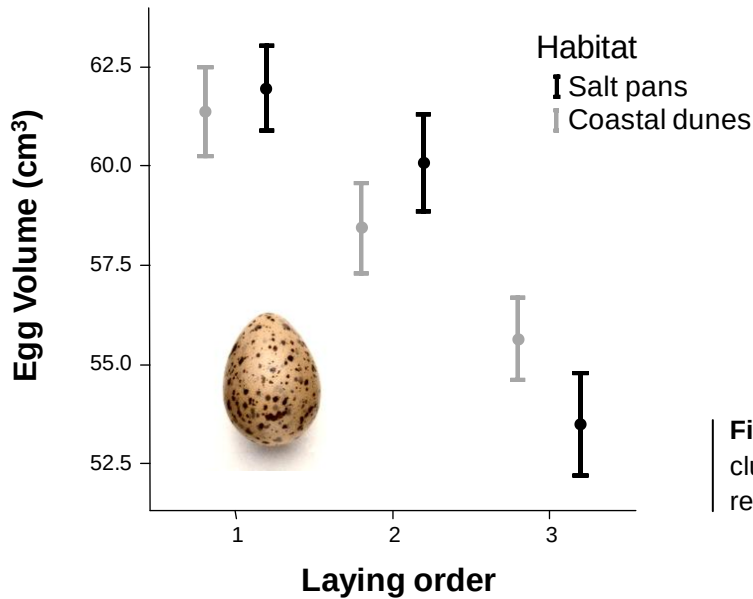


Figure 11. Audouin's gull intra-clutch patterns of egg volume in relation to nesting habitat.

Finally, it must be pointed out that a marked dietary switch toward rice field resources was evident in clutches affected by the trawling moratorium. This indicates that females are, when laying eggs, closely linked to the nest and can not perform long foraging trips at sea searching for fish, so it seems that they prioritize capturing very accessible but lower quality prey. This situation contrast with previous findings in incubating females (*Chapter 1.2*), when they performed longer at-sea foraging trips searching for fish in the absence of trawling activities probably because they are not as linked to the nest as when laying eggs. Moreover, the results show that sudden changes in diet are also evident in the albumen $\delta^{13}\text{C}$, despite their apparently greater endogenous contribution.

Advances in knowledge about antioxidant allocation to eggs

The influence of nutrition on the oxidative status of birds can be explored by looking at the relationship between OXY variables and the stable isotope signature of tissues. In our work, we assessed the relationship between stable isotopes ($\delta^{15}\text{N}$, $\delta^{13}\text{C}$) and the antioxidant capacity of the albumen and yolk in Audouin's gull clutches from two breeding groups: one breeding in the salt pans and the other in coastal dunes. However, our results did not indicate a relationship between the $\delta^{13}\text{C}$ signature, which provides information about the carbon sources used by consumers, and the OXY variables. This finding is in agreement with the previous *Chapter 2.1*, where it was noted that carbon from albumen has a more endogenous origin than nitrogen stable

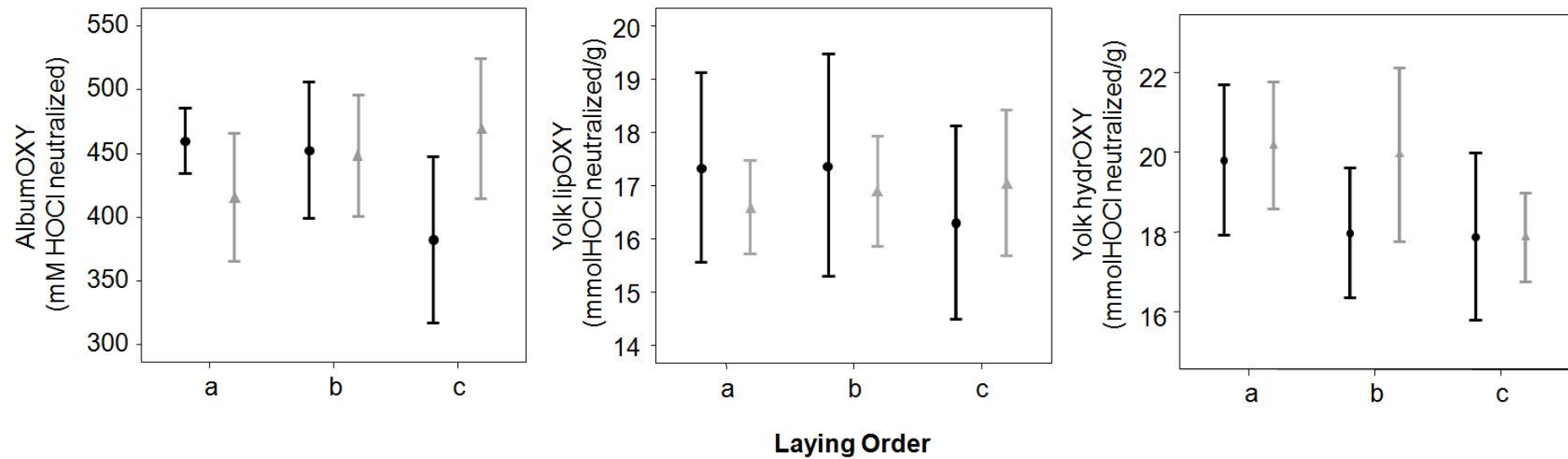
isotope. In the same direction, Hipfner et al. (2010a) did also not find a relationship between yolk carotenoid levels and $\delta^{13}\text{C}$ values in other seabird species.

Traditionally, albumen has received much less attention than yolk in antioxidant studies due to the lack of carotenoids. Thus, the gap of knowledge is especially evident in this egg compartment. When comparing the antioxidant concentration in the albumen of the two nesting groups, there was a significant interaction between the breeding group and laying order: in the salt pan group there was a lower investment of albumen antioxidants into the third egg, while the dune group showed no variation over the laying sequence (Figure 12). These albumen differences in antioxidant concentration between third-eggs are coincident with previous *Chapter 2.1*, where third-eggs from the salt pans were significantly smaller than the ones from the dune group. Nesting place quality could be playing a role as stated above. However, due to the great sensitivity of albumen deposited in the eggs to discard availability, a possible effect of the day of the week when the egg was laid may also have an influence on this variability.

On the other hand, yolk accounted for the majority of the overall antioxidant capacity of the egg (Figure 13). Concentration of hydrophilic antioxidants in the yolk (hydrOXY), as well as the overall concentration of antioxidants in the yolk (lipOXY + hydrOXY) followed a decreasing pattern through the laying sequence as described in other studies on gulls (Royle et al., 1999; Blount et al., 2002; Groothuis et al., 2006; Rubolini et al., 2011). Moreover, this decline in TOTAL eggOXY was observed in both breeding groups. Such a decreasing tendency indicates that the allocation of antioxidants to the yolk is probably modulated/constrained by antioxidant resource acquisition (Royle et al., 2003) as well as by competition among oocytes (Meathrel, 1991).

Both yolk lipOXY and TOTAL eggOXY were negatively related to $\delta^{15}\text{N}$ and thus, lower $\delta^{15}\text{N}$ values were associated with higher antioxidant levels. Royle et al. (2003) found that in Zebra Finches (*Taeniopygia guttata*) over 70% of the female's daily intake of carotenoids and α -tocopherols (the two most abundant lipophilic dietary antioxidants) were deposited in the egg yolk. As stated above, in the Ebro delta system lower nitrogen values derive mainly from marine diets, suggesting that marine resources are more beneficial in terms of antioxidant supply than rice field resources.

Figure 12. Intra-clutch deposition pattern for relative egg measurements by breeding group and laying order. Black bars indicate salt pan group, grey bars indicate coastal dune group. Values are mean and 95% confidence interval. *albumOXY*: antioxidant capacity of albumen; *lipOXY*: lipophilic component of antioxidant capacity; *hydrOXY*: hydrophilic component of antioxidant capacity.



Although crayfish are known to contain a high concentration of carotenoids (Negro et al., 2000), the antioxidant power of astaxanthin is very low compared to other dietary antioxidants (reviewed in Catoni et al., 2008). This result agree with recent studies that have shown that carotenoids offer limited protection against oxidative stress (Costantini and Møller, 2008; Isaksson and Andersson, 2008; Simons et al., 2012), highlighting the importance of looking at other egg dietary antioxidants as well.

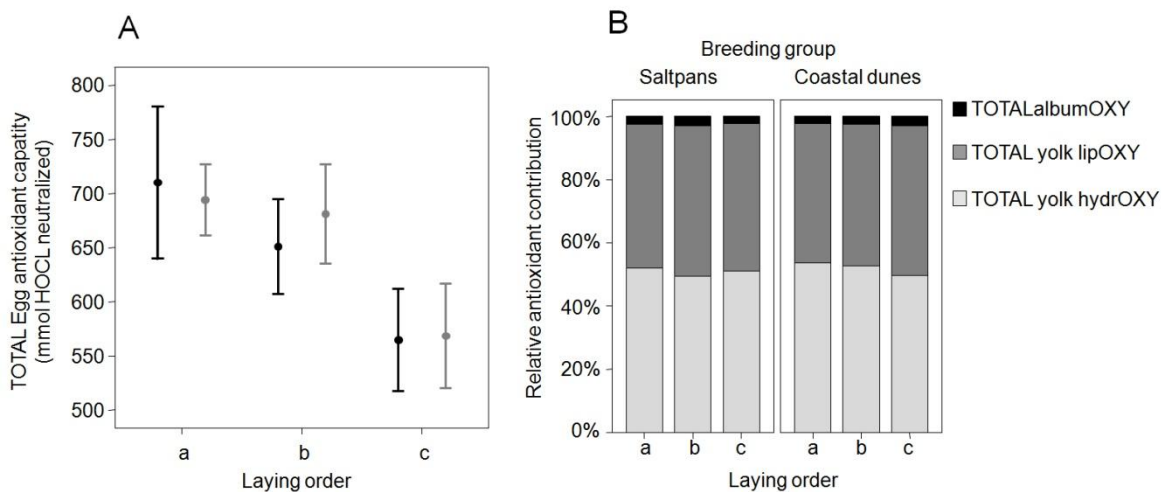


Figure 13 (A) Intra-clutch deposition pattern for total egg antioxidant capacity by breeding group and laying order. Black bars indicate salt pan group, grey bars indicate coastal dune group. Values are mean and 95% confidence interval. **(B)** Relative antioxidant contribution for each egg measurement by laying order and breeding group.

This study points to the potential benefit for Audouin's gulls of a marine fish diet over rice field prey in terms of the antioxidant levels deposited in their eggs. Also, deposition of antioxidants in the eggs tended to decrease along the laying sequence, highlighting the limited availability of antioxidant molecules.

Advances in knowledge about the role of calcium in constraining egg synthesis

There are evidences of calcium as a limiting factor in constraining egg synthesis in a number of bird species (see *Introduction*), although few studies have discussed on the potential implication of this micronutrient in determining the intra-clutch pattern of egg size variation typical of many avian species (Graveland et al. 1994; Reynolds 2001). Moreover, it has been suggested that the need for females to restore calcium levels after the clutch completion can also play an important role in generating sex-differences in foraging behavior (see *Chapter 1.2*). Therefore, we investigated the

relationship between female plasma Ca levels and several fitness-related egg traits, such as egg size (i.e. egg volume), egg shape and eggshell thickness to evaluate the limiting role of this element in the Audouin's gull.

Both dietary intake and endogenous Ca mobilization result in a dramatic increase of female plasma Ca concentrations during ovulation, to decrease progressively afterwards to reach those values for males (which do not show these changes in blood Ca levels) during incubation (Figure 14). Although our results for Audouin's Gull referred exclusively to the incubation stage, they agreed with previously reported sex-specific trends and suggested that plasma Ca levels in incubating females can be used as an indicator of plasma Ca levels during the egg formation.

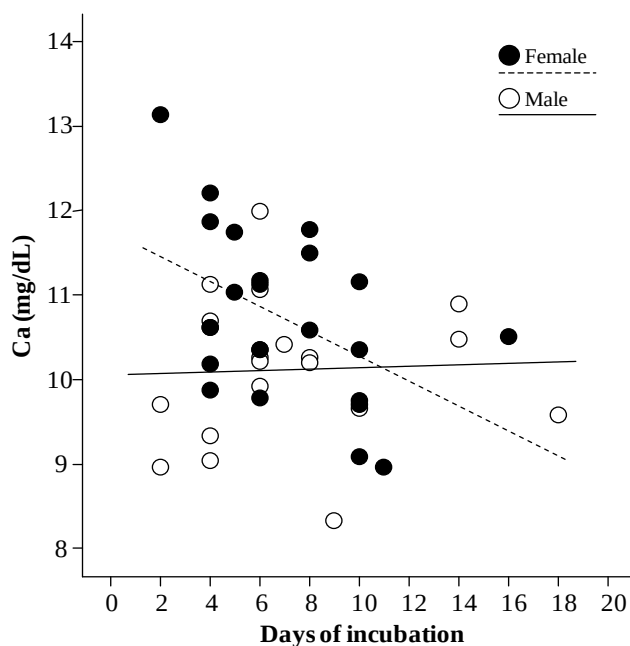


Figure 14. Temporal trends of plasma Ca (mg/dL) through incubation (Days of incubation: number of days elapsed between clutch completion and capture of individuals) according to gender for incubating Audouin's Gulls breeding at the Ebro Delta (Spain) during 2009. Solid and dashed lines represent regression lines predicted by the model for each sex.

Our results indicated that the relationship between plasma Ca levels in incubating females and egg size varied throughout the laying sequence, being slightly higher for latter-laid eggs (as pointed out by estimated slopes for the relationships between plasma Ca levels and estimated egg volume and surface area: $a\text{-egg} < b\text{-egg} < c\text{-egg}$) (**Figure 15**). In our view, these results suggested that later-laid eggs in Audouin's Gulls were particularly sensitive to Ca circulating levels, which could be explained by an overall decrease in Ca availability (both endogenous and exogenous) as long as this micronutrient is progressively allocated into the successive eggs (eggshells) to complete the clutch. In addition to its effect on clutch production, Ca availability may also play a relevant role in determining the intra-clutch pattern of egg size variation, with higher availabilities resulting in more homogeneous egg sizes at the intra-clutch level.

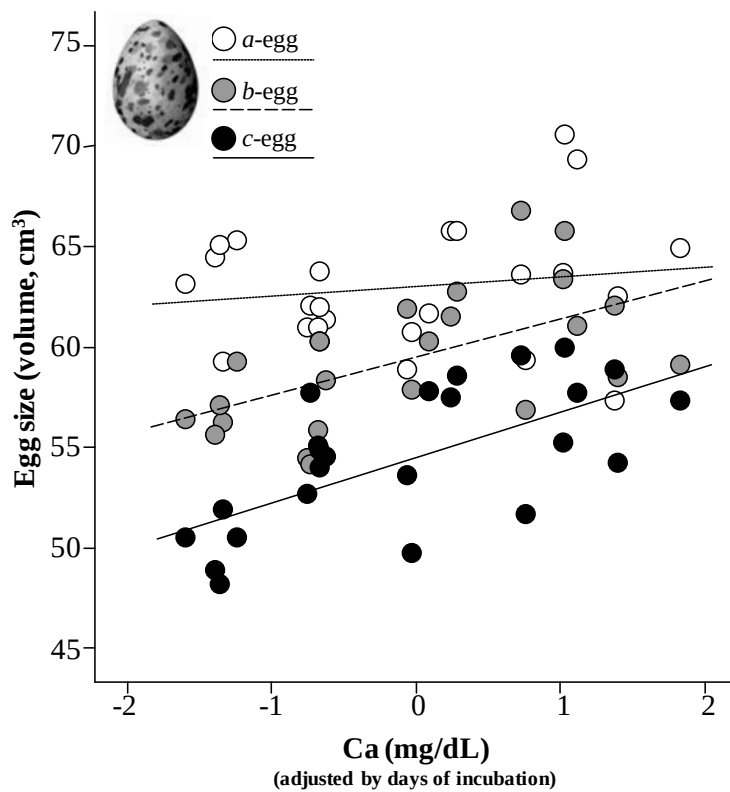


Figure 15. Bivariate plot of residuals of female plasma Ca levels (adjusted by days of incubation) in Audouin's Gulls breeding at the Ebro Delta (Spain) during 2009 vs. egg size (volume, cm³). Lines represent regression lines predicted by the model (see Results and Table 2).

In contrast, egg shape in the Audouin's Gull was not found to vary according to Ca availability. However, the fact that this trait significantly varied throughout the laying sequence, and that more than half of its variability was explained by the random nest factor, suggested that others, unmeasured, female traits likely operate through clutch production affecting egg shape.

Our study focused in exploring the constraining role of Ca during reproduction and provided some evidences suggesting that, in addition to aforementioned individual traits, calcium availability for breeding females influenced resource allocation strategies in the Audouin's Gull. In particular, higher Ca availabilities resulted in larger eggs and more homogeneous egg sizes at the intra-clutch level.

FUTURE RESEARCH DIRECTIONS

The current thesis has put forward the need of fine-scale studies when analyzing patterns of habitat use, trophic ecology and ecophysiology of seabirds. Some variables if overlooked as sex, time-intervals, day of the week, days after the trawling moratorium or days after clutch completion can decisively influence the ecology and physiology of the species. This highlights that we are still far from a complete understanding of all these patterns in Audouin's gull in particular and seabirds in general, so here we point future perspectives where studies should target.

Although weekend periods stand as a suitable approach to identify changes of feeding behavior and habitat use in the absence of discards (Bartumeus *et al.* 2010), it would be very interesting to compare with a prolonged situation of discards ban during a trawling moratorium in the whole area. A partial moratorium took place some years, when trawlers cover only either the northern or the southern part of the Ebro Delta. In 2009, however, the moratorium affected the whole waters surrounding the Ebro Delta from 1st of May, coinciding with the latter laying females. Although in the eighties-nineties some studies in Audouin's gull were done when a trawling moratorium occur across the area (see *Introduction* of this Thesis), a more detailed approach with the new techniques available would be highly desirable. Also, because of the imminent implementation of the new common fisheries policy adopted by the European Union, a monitoring program during a gradual implementation of the reform in this threatened species would be of special interest.

Another species breeding at the Ebro Delta with a great interest because its interaction with human activities is the Yellow-legged gull (*Larus michahellis*). In contrast with Audouin's gull, this species is sometimes considered as a pest-species due to the sustained use of urban habitats and rubbish dumps, although it also consumes a high proportion of discarded fish (Ramos *et al.* 2009a, 2009b, 2011). It is likely that a ban on fishing discards will also impact yellow-legged gulls by increasing the consumption of city wastes and refuse tips from dumps. Moreover, increasing taxes of interspecific kleptoparasitism (Oro & Martínez-Vilalta 1994b, González-Solís *et al.* 1997b) and higher consumption rates of other sympatric seabirds' chicks and eggs breeding at the Ebro Delta are also expected (Hernández-Matías & Ruiz 2003, Oro *et al.* 2005, Martínez-Abraín *et al.* 2003). Few works have studied in depth the patterns of distribution of Yellow-legged gull compared with the Audouin's gull (but see Cama *et al.* 2012), so a more comprehensive study to understand the impact of the new fisheries reform in this problematic species would be desirable.

The great accumulation of toxic muds at Flix site (*Chapter 1.3*) is one of the most important ecological problems still to be solved in the Western Mediterranean. The dredging of the 800,000 cubic meters of toxic muds accumulated at the bottom of the Flix reservoir began in 2013. This is the greatest work of this type undertaken in Spain to date, and for which it is planned an investment of 165 million Euros. Although it was planned to finish the action in December 2015, to date it has only been done 25% than expected. Due to the dangerous nature of these works, and given the great utility of Audouin's gull chicks' feathers for biomonitoring levels of pollutants in the marine environment (especially mercury), it is advisable to continue the monitoring of heavy metal levels in the area during and after the works of extraction of toxic muds.

Finally, an integrative and multidisciplinary meta-analysis of the different techniques used in this Thesis would be very interesting. For example, the possible interrelation of physical effort (on the basis of GPS data), diet (using SIA) and pollutant levels with the antioxidant capacity and oxidative damage levels of the individuals and their clutches may shed light on how these ecophysiological processes have evolved on seabirds and affect their life-history traits. It has been shown that some metals can cause oxidative stress by increasing the formation of reactive oxygen species (ROS), unbalancing the cellular redox status and making antioxidant compounds insufficient for defence against the growing amount of free hydrogen radicals (reviewed in Koivula & Eeva 2010). Physical activity also influences the oxidative status of birds (Costantini 2008), especially important for migratory species as Audouin's gull. The study of migratory animals can provide insights into mechanisms that evolved to cope with exhausting exercise. Moreover, high levels of metals as mercury are associated with adverse reproductive effects such as lower clutch and egg size (Eisler, 1987), an overlooked factor that probably could be generating intra-clutch variation in egg size.



CONCLUSIONS

Sunset at “Punta de la Banya” colony (Ebro Delta Natural Park, Spain).
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CONCLUSIONS

1. TROPHIC ECOLOGY AND HABITAT USE

- At the population level, our results show a great dependence of Audouin's gull foraging movements on fishing activities in the Ebro Delta. On work days, the probability of finding Audouin's gulls at sea during the day is much higher than during the night due to the great association with trawlers. Moreover, most of the nocturnal activity during work days comes from the association with purse-seine boats. On weekends, when there are no major fishing activities, Audouin's gull increases the use of rice field habitats (from an average of 35% to ca. 55% of GPS positions).
- At the individual level, both sexes showed differences in the foraging behaviour in relation to the fishing working cycles. Our data indicate that female Audouin's gulls perform significantly longer at-sea foraging trips and spend longer time outside the colony during weekends. Because these differences are presumably associated to fishing activities, and no relationship between habitat use and body condition indices was found, this sex-specific foraging behaviour could be related to distinct nutritional requirements associated to egg production (not only sexual size dimorphism).
- This last point is in contrast with the behaviour of females during the laying period, when they show a dramatic switch to a rice field diet in absence of trawling activities, probably due to the strong association to the nest when laying eggs.
- Audouin's gull diet ranged from exclusively marine to rice field specialists, all the way up to intermediate situations. However, a high individual specialization underlies this apparent trophic plasticity.
- Different foraging areas resulted also in a differential accumulation of heavy metals. Lead levels were significantly higher in the Llobregat Delta probably due to the use of leaded fuel in the nearby Barcelona airport. On the other side, mercury concentrations were higher in the Ebro Delta related to the accumulation of toxic sediments at the bottom of Flix reservoir, which are carried down by the Ebro River. These mercury levels in the Ebro chicks reached values that have been described as toxic.

2. RESOURCE ALLOCATION AND ECOPHYSIOLOGY

- The high sensitivity of albumen $\delta^{15}\text{N}$ to daily changes in diet shows that the most part of the nitrogen deposited comes from diet. $\delta^{13}\text{C}$ must have a more endogenous origin than $\delta^{15}\text{N}$ since this daily pattern is not so obvious. However, when drastic changes to rice field diets occur during trawling moratorium periods, both isotopes showed significant changes.
- Enriched albumen $\delta^{15}\text{N}$ signatures (associated to a higher use of rice field habitats or a greater mobilization of endogenous resources) were related to a smaller egg size.
- Between individuals, we found little differences through the laying sequence in the antioxidant deposition pattern in the yolk and the whole egg. A greater variation was observed in the intra-clutch patterns of albumen antioxidant capacity, probably related to quality differences or food availability.
- A declining trend over the laying sequence was found for the total antioxidant assignment to the clutch, highlighting the limiting availability of antioxidant molecules.
- Both yolk lipophilic and whole egg antioxidant capacity were negatively related to $\delta^{15}\text{N}$, indicating that a higher use of rice field resources are also associated with a lower availability of antioxidants.
- Egg size was positively related with plasma Ca levels of incubating females, being the slope of this relationship significantly higher for later-laid eggs. Also, higher Ca availabilities resulted in more homogeneous egg sizes at the intra-clutch level, modulating the egg-size profile typical of this gull species.



ECOLOGÍA TRÓFICA, USO DEL HÁBITAT
Y ECOFISIOLOGÍA DE LA GAVIOTA DE
AUDOUIN (*Larus audouinii*) EN EL
DELTA DEL EBRO

Gaviotas de Audouin en la colonia de la “Punta de la Banya”
(Delta del Ebro, Tarragona, España).

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INTRODUCCIÓN GENERAL

1. ESTUDIOS ECOLÓGICOS EN LAS AVES MARINAS: INTRODUCCIÓN Y ANTECEDENTES

"Ave marina" es un término relativamente laxo, tradicionalmente usado para cubrir aquellas especies que obtienen al menos una parte de su comida viajando por el mar. Las clasificaciones de aves marinas más usadas incluyen unas 471 especies en 4 órdenes: **Sphenisciformes** (pingüinos), **Procellariiformes** (albatros, petreles, paíños y afines), **Pelecaniformes** (pelícanos, alcatraces y piqueros, cormoranes, rabihorcados y rabijuncos) y cinco familias de **Charadriiformes** (págalos, gaviotas, charranes, rayadores y álcidos). Las aves marinas están muy extendidas por nuestro planeta, explotando un amplio espectro de hábitats marinos, desde litorales a pelágicos, y desde tropicales a polares (criando a las mayores latitudes y en los ambientes más fríos que cualquier otro vertebrado sobre la Tierra). Sin embargo, a pesar de la enorme cantidad de alimento potencialmente disponible en sus aguas, menos del 5% de las aproximadamente 10 000 especies de aves descritas explotan estos dos tercios de la superficie terrestre que representan los océanos y mares. Además, los grupos más generalistas (como las gaviotas o algunos Pelecaniformes) pueden también alimentarse de recursos terrestres y de agua dulce. El 98% de las aves marinas crían colonialmente, principalmente en islas o en zonas costeras (Furness & Monaghan 1987, Hamer *et al.* 2002, Schreiber & Burger 2002, Gaston 2004).

Aunque las aves marinas varían mucho en su forma de vida, comportamiento y fisiología, en general son especies longevas, con madurez sexual y cría retardada, además de una baja tasa anual de reproducción con extensos periodos de crecimiento de los pollos (Hamer *et al.* 2002). De acuerdo con la teoría del ciclo vital (*life-history theory*), las especies están sujetas a un balance en el que cualquier inversión en el actual evento reproductivo puede reducir su futura eficacia biológica (*fitness*), por ejemplo reduciendo la fecundidad o disminuyendo la supervivencia de los progenitores (Williams 1966, Stearns 1992). En especies longevas, como las aves marinas, la asignación de recursos entre el mantenimiento del individuo y la reproducción es de especial interés debido a que una pequeña reducción en la supervivencia adulta puede tener un gran efecto negativo en el éxito reproductor a lo largo de su vida. Los progenitores están, por lo tanto, limitados sobre cómo pueden incrementar el esfuerzo en el actual evento reproductivo con el fin de moderar el impacto en futuros intentos de

reproducción (Croxall 1982, Furness & Monaghan 1987, Russell 1999, Croxall *et al.* 1992, Gaston 2004). Por lo tanto, rastrear la asignación de recursos a su puesta durante la reproducción se convierte en clave para entender cómo han evolucionado las estrategias reproductivas de las aves marinas.

Otro punto importante que determina la dinámica de poblaciones y el éxito reproductor es la disponibilidad de alimento (Oro *et al.* 2004, 2014). En este sentido, existe una evidencia clara de que las actividades humanas en este mundo globalizado tienen un fuerte impacto sobre los ecosistemas marinos donde las aves marinas se alimentan (Halpern *et al.* 2008). En relación a la contaminación, las aves marinas están expuestas a un amplio rango de contaminantes debido a que pasan la mayor parte de su tiempo en ambientes acuáticos, donde los contaminantes se mueven más rápido que en los ambientes terrestres (Burger and Gochfeld 2002). Los contaminantes pueden tener un efecto difuso (por ejemplo la acumulación de metales pesados y organoclorados, incorporados y acumulados a través de la dieta y la ingestión de plásticos; Morera *et al.* 1997, Cotín *et al.* 2012) o un efecto concentrado con devastadoras consecuencias para las aves marinas (por ejemplo los vertidos de petróleo; Peterson *et al.* 2003, Moreno *et al.* 2013) (ver Nisbet 1994 para una revisión). La magnitud de un determinado impacto dependerá mucho del grado de asociación con el hábitat y también con el nivel de agregación de las especies, siendo especialmente vulnerables cuando están concentradas en altas densidades (Nel *et al.* 2000).

Por otro lado, la industrialización y drástico desarrollo de las pesquerías comerciales a lo largo del último siglo han ejercido una importante presión sobre nuestros mares y océanos. Esto ha resultado en un impacto significativo para los ambientes marinos al disminuir las poblaciones de algunos peces (Casey and Myers 1998, Coll *et al.* 2013), transformar la estructura de las redes tróficas (Camphuysen *et al.* 1995, Pauly *et al.* 1998) o proporcionar enormes cantidades de comida adicional en forma de descartes de la pesca de arrastre (Arcos 2001, Furness 2003). Por consiguiente, el establecimiento de las preferencias tróficas y uso del hábitat de las aves marinas parece esencial en diversas áreas clave de la ecología aplicada, como la conservación de especies amenazadas, la gestión de especies problemáticas o la interacción con actividades humanas (Tasker *et al.* 2000, Louzao *et al.* 2006, Votier *et al.* 2010, Ramos *et al.* 2011).

Esta tesis fue desarrollada a caballo entre estos dos aspectos discutidos más arriba usando la gaviota de Audouin como especie modelo: el uso del hábitat y

ecología trófica por un lado, y la asignación de recursos a la puesta durante la reproducción por otro lado. Además, ambos campos están interrelacionados debido a que un preciso conocimiento de la ecología trófica es necesario para comprender cambios en la asignación de recursos.

2. CLAVES PARA ENTENDER LA ECOLOGÍA TRÓFICA Y EL USO DEL HÁBITAT EN LAS AVES MARINAS

i. Ecología trófica: Aproximaciones isotópicas para estudios de dieta

El uso de métodos convencionales para caracterizar la dieta de una determinada población, como el análisis de contenidos estomacales (egagrópilas y regurgitados) o la observación directa, tienen ciertas limitaciones relacionadas con sesgos debidos a la diferente persistencia y visibilidad de los recursos consumidos por los individuos. En particular, mientras que los estudios basados en la observación directa tienden a sobreestimar las presas más conspicuas, su diferente digestibilidad inclina los resultados de los contenidos estomacales hacia una sobreestimación de presas con partes duras o poco digeribles (González- Solís *et al.* 1997a; Votier *et al.* 2003, Ramos *et al.* 2009a). Por otro lado, estas metodologías proporcionan una información puntual sobre las dietas individuales (Votier *et al.* 2001). Por lo tanto, obtener información a nivel poblacional requiere un exhaustivo seguimiento de los individuos a lo largo del tiempo que incrementa la dificultad y costes asociados a estos estudios (Jordan 2005). En este sentido, el uso de marcadores intrínsecos como los **isótopos estables** para estudios de la ecología trófica de poblaciones salvajes emerge con fuerza como una alternativa viable. La principal ventaja de esta aproximación reside en el hecho que esta técnica proporciona una información integrada temporalmente de la dieta asimilada mediante un muestreo puntual (Hobson *et al.* 1994, Kelly 2000). Por ello, a pesar de perder detalle en el estudio taxonómico de las presas consumidas, las aproximaciones basadas en el uso de isótopos estables permiten un amplio alcance en el que el uso de diferentes recursos puede ser identificado y cuantificado a nivel individual o poblacional (Ramos *et al.* 2009a, Moreno *et al.* 2010). Esta es la razón por la que el Análisis de Isótopos Estables (SIA, en inglés) para estudios de ecología trófica ha experimentado un incremento exponencial en las últimas décadas (Boecklen *et al.* 2011).

A una escala local, los isótopos estables más comúnmente usados para inferir el origen de la dieta y las relaciones tróficas son los isótopos del carbono ($\delta^{13}\text{C}$) y del

nitrógeno ($\delta^{15}\text{N}$) (Peterson & Fry 1987). La aplicabilidad del $\delta^{13}\text{C}$ en estudios ecológicos reside en que permite trazar las fuentes de carbono en los procesos de producción primaria y, por lo tanto, el flujo de materia a través de los ecosistemas. Esto nos permite establecer el origen de los recursos usados por el consumidor final (Chisholm *et al.* 1982, Hobson *et al.* 1994). Típicamente, los valores de $\delta^{13}\text{C}$ tienden a diferir en relación a la maquinaria fotosintética entre los diferentes productores primarios. Por ejemplo, los valores de $\delta^{13}\text{C}$ de los productores marinos son típicamente más elevados que aquellos de productores terrestres como resultado de diferentes fuentes de carbono usadas en los procesos fotosintéticos (Marshall *et al.* 2007). El $\delta^{13}\text{C}$ está representado en las tres macromoléculas tróficas (proteínas, lípidos e hidratos de carbono). Por otro lado, el $\delta^{15}\text{N}$ es generalmente usado como indicador de la posición trófica. Durante el catabolismo de las proteínas (el nitrógeno está generalmente ausente de lípidos e hidratos de carbono), la retención de aminoácidos está sesgada hacia aquellos que contienen grupos amino con el isótopo pesado del nitrógeno $^{15}\text{NH}_2$ respecto a $^{14}\text{NH}_2$, por lo tanto, con una mayor excreción del isótopo ligero (Peterson & Fry 1987). Como resultado, los tejidos de los organismos están generalmente enriquecidos en ^{15}N respecto a sus recursos alimenticios consumidos. Este enriquecimiento con cada nivel trófico es variable, pero normalmente se asumen valores próximos al 3‰ (DeNiro & Epstein 1981, Post 2002). Sin embargo, el $\delta^{15}\text{N}$ tiene que ser usado de forma cuidadosa en estudios sobre la posición trófica, siendo necesarias estimas realistas para los valores isotópicos de la base de las cadenas tróficas. Por ejemplo, es sabido que el enriquecimiento en nutrientes como resultado de las actividades antropogénicas incrementa la eutrofización de los ecosistemas, desembocando también en un incremento en el $\delta^{15}\text{N}$ no relacionado con la posición trófica (McClelland *et al.* 1997, Abdennadher *et al.* 2011, Cotín *et al.* 2012).

Finalmente, la selección del tejido adecuado es un aspecto muy importante a la hora de diseñar cualquier estudio isotópico. El marco espacio-temporal integrado por un marcador biogeoquímico como es el caso de los isótopos estables depende del crecimiento y tasa de renovación del tejido analizado (Hobson & Clark 1992, Rubenstein & Hobson 2004). Los tejidos con una alta tasa de renovación típicamente integran señales isotópicas incorporadas recientemente, mientras que tejidos con una tasa lenta de renovación integran formas isotópicas de un período de tiempo más largo (Hobson 1993). Los tejidos queratinosos de muchos organismos (no sólo aves marinas), como pueden ser pelo, uñas, plumas o escamas, son usados ampliamente en estudios ecológicos (Hobson & Clark 1992, Hobson *et al.* 1996, Bearhop *et al.*

2003, McCue *et al.* 2008) ya que permiten muestreos no invasivos y una vez formadas estas estructuras permanecen inertes integrando la información isotópica derivada de las redes tróficas locales donde fue sintetizado. Por otro lado, otros tejidos como la sangre entera integran la dieta durante un período que va desde unas pocas semanas en paseriformes pequeños hasta meses en animales de mayor tamaño, mientras que el plasma sanguíneo típicamente representa la información sobre la dieta durante un período de unos pocos días (Hobson 2005).

ii. Movimientos durante la búsqueda de alimento e interacciones con las pesquerías

Tan importante como identificar las principales fuentes de alimentación para las aves marinas, es localizar sus principales áreas de alimentación y comprender sus movimientos. Los estudios sobre los patrones de búsqueda de alimento en aves marinas han sido principalmente llevados a cabo durante la época de reproducción, cuando las aves marinas deben retornar periódicamente al lugar de nidificación para asumir las tareas de incubación o alimentación de los pollos. Debido a estas obligaciones, generalmente se asume que los rangos de búsqueda de alimento de las aves marinas se ven limitados durante la época de cría. Sin embargo, algunas especies continúan distanciándose considerablemente en busca de presas (Shealer 2002).

La distribución de las aves marinas sobre los océanos y mares no es aleatoria, debido principalmente a la concentración localizada de las presas (Shealer 2002). Se concentran en áreas donde las presas adecuadas son abundantes, como por ejemplo zonas de alta productividad donde la interacción entre las corrientes de agua y la batimetría fuerzan a las aguas ricas en nutrientes hacia la superficie (afloramiento) o zonas costeras donde los aportes de agua dulce rica en nutrientes por parte de ríos contribuyen a incrementar la productividad de manera significativa (Furness & Monaghan 1987, Shealer 2002). Normalmente, esta distribución en el mar de las aves marinas interacciona con las actividades humanas, principalmente debido a que las aguas más productivas también albergan importantes flotas pesqueras operando en la zona (Abelló *et al.* 2003). En este sentido, debido tanto a razones de gestión como de conservación, es importante estudiar las interacciones de las aves marinas con las pesquerías.

Desde el punto de vista del ave, las interacciones entre las aves marinas y las pesquerías pueden afectar en direcciones diferentes (revisado en Tasker *et al.* 2000, Montevecchi 2002, Furness 2003). Las pesquerías pueden causar mortalidad directa

de las aves marinas debido al enmallamiento de las aves en artes de pesca perdidas o debido a la captura accidental en redes de enmalle y palangres. Este último punto es de especial interés ya que los palangres son considerados hoy en día una de las mayores amenazas para las aves marinas pelágicas. Debido a los ciclos vitales que presentan las aves marinas (ver más arriba), esta fuente de mortalidad adulta está haciendo disminuir algunas poblaciones de aves marinas a unas tasas demasiado rápidas como para ser compensadas por la reproducción (Belda & Sanchez 2001, Gilman *et al.* 2005).

Paradójicamente, algunas poblaciones de aves marinas pueden sacar provecho de las actividades pesqueras. Los arrastreros demersales y otras pesquerías devuelven al mar grandes cantidades de desechos de la pesca en forma de despojos (intestinos y otros resto del pescado eviscerado) y descartes (peces, ya sea porque están por debajo del tamaño mínimo legal para desembarcarlo, porque tienen poco valor comercial, porque exceden la cuota individual para los pesqueros, o porque han sido dañados durante el proceso de pesca) (Arcos 2001). Estas prácticas permiten tener un fácil acceso a presas demersales y de mayor tamaño a las aves marinas que se alimentan en la superficie, que de otra forma permanecerían inaccesibles. Este alimento adicional se proporciona de forma abundante y es predecible en el espacio y en el tiempo (Camphuysen *et al.* 1995, Garthe *et al.* 1996, Cama *et al.* 2012). Consecuentemente, proporcionar estas cantidades enormes de esta nueva y abundante forma de alimento ha desembocado en cambios en los patrones de movimiento de las aves marinas (Bartumeus *et al.* 2010, Votier *et al.* 2010), éxito reproductor (Oro 1996), estrategias de invernada (Hüppop & Wurm 2000), dinámica de poblaciones (Oro *et al.* 2004) y composición de las comunidades (Votier *et al.* 2004a). Esta fuente adicional de alimento está considerada como un factor clave en el crecimiento que algunas poblaciones de aves marinas generalistas y oportunistas (como los págalos y algunas gaviotas grandes) han experimentado durante el último siglo debido a su eficiente explotación (Furness 2003, Oro *et al.* 2013). Sin embargo, debido a la demanda global de proteínas y a la severa amenaza sobre algunas poblaciones de peces, estas actividades arrastreras no son sostenibles para nuestros mares (Furness 2003, European Commission 2007). Por ejemplo, durante el período 1992-2001, la Organización de las Naciones Unidas para la Alimentación y la Agricultura (FAO) calculó globalmente una media de 7,3 millones de toneladas de pescado descartado cada año (Kelleher 2005). Eliminar los descartes pesqueros se antoja una tarea necesaria para asegurar que las pesquerías son económica y medioambientalmente sostenibles (Bicknell *et al.* 2013). Por esta razón, en 2011 la

Comisión Europea propuso importantes cambios en la Política Pesquera Común. Esta reforma propone, entre otras cosas, una prohibición completa de los descartes para todas las pesquerías Europeas no más tarde de 2019 (Symes 2009, Bicknell *et al.* 2013). Aunque necesario, una reducción significativa o completa de los descartes pesqueros es probable que tenga consecuencias negativas de forma directa e indirecta para estas comunidades de aves marinas carroñeras. Comprender el impacto de estas reformas en la ecología de las aves marinas es, por lo tanto, importante a efectos de gestión y conservación, pero está limitado por nuestro restringido conocimiento de la ecología de los carroñeros (Votier *et al.* 2013).

Tradicionalmente, el problema asociado cuando estudiamos los movimientos de las aves marinas y su interacción con las pesquerías, ha sido la inhabilidad de estudiar el comportamiento de búsqueda de alimento en el mar con detalle mientras, simultáneamente, se registra la actividad de los barcos pesqueros (Votier *et al.* 2010). Hasta hace poco, estos estudios eran basados principalmente en observaciones sistemáticas desde barcos, las cuales comenzaron en la década de 1970 (Wakefield *et al.* 2009). Sin embargo, en las pasadas dos décadas, los avances tecnológicos han cambiado profundamente la forma en la que estudiamos las aves marinas, proporcionando una visión sin precedentes de su biología, demografía y exposición a riesgos antropogénicos en el mar (Burger & Shafer 2008). Por ejemplo, mediante el uso de GPSs, las localizaciones pueden ser registradas cada segundo con precisión de metros, y los marcajes con GPS son hoy en día relativamente económicos y suficientemente pequeños (10-20 g) para ser usados en muchas aves marinas (Grémillet *et al.* 2004, Votier *et al.* 2011, Harris *et al.* 2012). Estos avances han permitido estudios a costes compatibles con la obtención de tamaños muestrales apropiados para llevar a cabo rigurosos análisis estadísticos. La alta resolución espacial y temporal revela incomparables detalles de la velocidad respecto al suelo, micro-movimientos y comportamientos de búsqueda de alimento en zonas restringidas (Ropert-Coudert & Wilson 2005). Además, los Sistemas de Seguimiento de Buques (*Vessel Monitoring Systems - VMS*) han sido adoptados por muchas naciones pesqueras y proporcionan datos de alta resolución espacial y temporal sobre la distribución del esfuerzo pesquero (Mills *et al.* 2007; Witt & Godley 2007). En Europa, la Comisión Europea ha introducido legislación para el seguimiento de la actividad pesquera, por lo que los barcos mayores de 15 m de eslora tienen la obligación de transmitir sus localizaciones (estimadas mediante GPS) en intervalos de 2h o menos, así que estos datos son comparables con los datos proporcionados por los sistemas de seguimiento remoto de las aves (Votier *et al.* 2010).

Estos avances, en combinación con el acceso a datos ambientales obtenidos por teledetección, forman un robusto conjunto de técnicas que permite el estudio en detalle de los comportamientos de alimentación en relación a las pesquerías.

iii. Biomonitorización de los niveles de contaminantes

Las actividades antropogénicas han ejercido desde hace mucho tiempo una enorme presión sobre los ecosistemas marinos ya sea a través de la explotación de sus recursos, así como a través de la contaminación (Furness 1993, Furness & Camphuysen 1997). Procesos como la minería, la quema de combustibles fósiles, la producción de productos químicos y las actividades agrícolas se han convertido en una importante fuente de metales pesados para el medio marino (Furness & Rainbow 1990, Monteiro & Furness 1997, Dietz *et al.* 2009). Además, los océanos son el sumidero final en los ciclos biogeoquímicos de muchos contaminantes (Clark 1992). Por lo tanto, la contaminación de las cadenas alimentarias es, hoy en día, de especial preocupación para la sociedad.

Algunos grupos de animales marinos han sido ampliamente usados como bioindicadores de los niveles de contaminantes tales como los metales pesados. Este es el caso de las aves marinas, particularmente adecuadas debido al conocimiento detallado de sus hábitos ecológicos y a su relativamente alta posición trófica en las cadenas alimentarias marinas. Su naturaleza colonial también permite colectar grandes tamaños de muestra de un sitio en particular y en un período de tiempo relativamente corto (Furness & Camphuysen 1997). Las plumas de las aves marinas, por ejemplo, proporcionan muestras no invasivas para seguimientos de contaminación por metales pesados en las redes tróficas marinas (Monteiro & Furness 1995). En particular, las plumas de pollos reflejan la disponibilidad de algunos metales en un área local durante el período de crecimiento de los pollos (Furness 1993).

Entre los metales pesados, el mercurio (Hg) y el plomo (Pb) han motivado muchas investigaciones debido a que son extremadamente tóxicos (Bryan 1979). El mercurio es el metal pesado más biodisponible en los ecosistemas acuáticos, donde se produce la principal transformación a metilmercurio (Wright & Welbourn 2002). El Hg inorgánico es biotransformado a metilmercurio y acumulado en la biota, incrementando su concentración a medida que sube su posición en las cadenas tróficas (bioacumulación) (Nriagu 1989). En los sistemas marinos, esta metilación es especialmente importante en ambientes mesopelágicos (Monteiro *et al.* 1998, Arcos *et al.* 2002). Se ha demostrado que los niveles de mercurio en plumas están significativamente relacionados con su ingesta a través de la dieta (Furness *et al.*

1986), por esta razón una aproximación trófica de la ecología del ave es necesaria para llevar a cabo una mejor interpretación de los niveles de mercurio. Afortunadamente, esto hoy en día es factible gracias a los isótopos estables (ver más arriba). Por otro lado, los niveles en pluma de otros metales pesados como el plomo, parece que se originan principalmente por deposición atmosférica en las superficies de las plumas, mientras que el Pb ingerido se une firmemente a los huesos y solo entra en las plumas en cantidades traza (Furness 1993, Furness & Camphuysen 1997). Los niveles de plomo pueden ayudarnos a elucidar las características de una determinada área de alimentación debido a que la mayor parte del plomo atmosférico tiene un origen antropogénico (Wright & Welbourn 2002). Por lo tanto, los niveles de algunos metales pesados son indicadores complementarios para determinar la dieta y hábitos de alimentación de las aves marinas.

Finalmente, las aves marinas pueden tener un papel importante como bioindicadoras, también llamados “organismos centinela” (Furness & Camphuysen 1997, Burger & Gochfeld 2004). Las especies más generalistas, como son las gaviotas, se benefician de los mismos recursos que las personas tales como los descartes pesqueros o desperdicios de basureros (Ramos *et al.* 2009b). En este sentido, el estudio de los niveles de contaminantes en estas aves puede ser también importante en campos relacionados con la salud pública, ayudándonos a identificar fuentes potenciales de contaminación ambiental y la posterior recuperación del ecosistema como han demostrado muchos estudios (Koeman 1969, Swennen 1972, Hyrenbach *et al.* 2009, Moreno *et al.* 2011).

3. CLAVES PARA ENTENDER LOS RASGOS DE LOS CICLOS VITALES (*LIFE-HISTORY TRAITS*) EN LAS AVES MARINAS

i. La asignación de recursos a la producción de huevos

Como comentábamos en la introducción, la inversión parental en un evento reproductivo particular está limitada por el efecto negativo sobre el futuro éxito reproductor (Williams 1966). Una de las principales cuestiones de la reproducción es comprender cómo los individuos asignan sus recursos dentro de un evento reproductivo para maximizar su éxito reproductor. En este contexto, los patrones intra-puesta del tamaño del huevo, representados en muchas especies por el relativo menor tamaño del último huevo de la puesta (Slagsvold *et al.* 1984, Leblanc 1987), han sido relacionados como un carácter adaptativo por tal de maximizar la eficiencia de la

inversión maternal durante la reproducción. Esta hipótesis fue postulada por primera vez por el ornitólogo británico David Lack, y se basa en la existencia de presiones selectivas que favorecen tanto la asincronía en la eclosión, como el tamaño más pequeño del último huevo (Lack 1947, 1954, 1968). Lack resumió el valor adaptativo de la siguiente forma: 'La asincronía en la eclosión...significa que en una situación de escasez de alimentos solo algunos, en lugar de todos, los pollos mueren.' (Lack 1947, p.332). De hecho, el patrón intra-puesta de tamaño del huevo da lugar a diferencias en tamaño y reservas de la progenie debido al efecto positivo del tamaño del huevo sobre el peso del pollo (Wilson 1991, Grant 1991). Si combinamos diferencias en el tamaño intra-puesta con una eclosión asincrónica, el resultado es el establecimiento de una jerarquía entre la progenie que favorece la eliminación del individuo más débil (el pollo del último huevo) bajo condiciones de baja disponibilidad alimenticia (lo que se conoce como la hipótesis de la reducción de la nidada o "**brood reduction hypothesis**"). Sin embargo, otras hipótesis adaptativas establecen la posible habilidad de las hembras para invertir recursos diferencialmente entre la secuencia de puesta con tal de asegurar la supervivencia de aquellos pollos que tienen más probabilidades de eclosionar y sobrevivir. Por ejemplo, en el ánsar nival (*Chen caerulescens caerulescens*), el mayor tamaño de los huevos situados en mitad de la secuencia de puesta ha sido interpretado como los que tienen mayor probabilidad de eclosionar: mientras que los primeros huevos están más expuestos a la depredación (la incubación empieza después), los últimos son los que tienen más probabilidad de ser abandonados (Williams *et al.* 1993).

Las variaciones de peso dentro de la puesta pueden ser también determinadas por procesos no adaptativos, como pueden ser limitaciones nutricionales o condiciones fisiológicas durante el período de puesta. Las limitaciones nutricionales pueden generar variaciones en el tamaño intra-puesta de los huevos debido a la disminución de las reservas almacenadas por las hembras (Houston *et al.* 1983, Pierotti and Bellrose 1986), por limitaciones de las opciones de alimentación de las hembras asociadas al inicio del comportamiento de incubación (Bollinger *et al.* 1990, Bollinger 1994), por cambios diarios en la disponibilidad de recursos explotados por las hembras mientras ponen los huevos (Perrins 1970) o por la calidad del hábitat (Viñuela 1997). Por otro lado, este patrón intra-puesta de variación ha sido también relacionado con factores fisiológicos que actúan tanto durante la formación del huevo como al inicio del período de incubación (Mead & Morton 1985, Sockman *et al.* 2000). El período de incubación sucede como resultado de cambios hormonales, principalmente como consecuencia del incremento en los niveles de prolactina (Sockman *et al.* 2000). Este

control hormonal está relacionado también con la regresión del sistema reproductor femenino, desembocando en el relativo menor tamaño del último huevo de la secuencia (Leblanc 1987). La asincronía en la eclosión y el menor tamaño del último huevo de la secuencia serían, por lo tanto, un subproducto de este control hormonal (Bollinger *et al.* 1990, Bollinger 1994). En este sentido, algunos autores han apuntado a la posible asignación del material absorbido durante la regresión del sistema reproductor hacia la síntesis del último huevo con tal de optimizar la inversión de las hembras en la reproducción (Ramírez *et al.* 2011). Todas estas hipótesis (adaptativas o no adaptativas) no son mutuamente excluyentes, y pueden actuar simultáneamente en la misma especie.

Trazar la adquisición de nutrientes y su posterior asignación a los huevos es hoy en día factible a través de la aplicación de aproximaciones isotópicas, principalmente enfocadas a determinar la contribución de nutrientes endógenos/exógenos en el huevo (revisado en Hobson 2006). El análisis de isótopos estables puede ayudarnos a trazar cuantitativamente la contribución de reservas en casos donde las dietas locales (durante la época de reproducción) difieren isotópicamente de las reservas endógenas típicamente adquiridas en los cuarteles de invernada o en las zonas de parada migratoria anteriores a la llegada a las zonas de cría (por ejemplo Hobson *et al.* 2005, Oppel *et al.* 2010), o en casos donde especies oportunistas están sujetas a cambios en la dieta (por ejemplo Ramírez *et al.* 2011). En este contexto, el albumen puede ser el compartimiento del huevo más idóneo para un estudio del patrón intra-puesta debido a que es sintetizado durante períodos de tiempo relativamente cortos y no superpuestos los unos con los otros (Ruiz *et al.* 2000). Sin embargo, todos estos procesos de asignación de nutrientes están lejos de ser completamente comprendidos. Las lagunas en el conocimiento son especialmente obvias a la hora de determinar la asignación diferencial de recurso a través de la secuencia de puesta. Futuras investigación en este campo son requeridas para conseguir una comprensión mejor de la asignación de recursos durante la reproducción, un aspecto de gran importancia en especies longevas como las aves marinas.

ii. Nutrientes limitantes en la producción del huevo: el Calcio y los Antioxidantes

El huevo es una compleja estructura compuesta de tres principales componentes (la yema, el albumen y la cáscara) que se desarrollan separadamente. Algunos de los recursos invertidos dentro del huevo son esenciales, y por lo tanto,

influyen en el crecimiento y supervivencia de la descendencia a través de la asignación de recursos como proteínas, lípidos, carotenos y antioxidantes, calcio, anticuerpos, hormonas y agua (revisado en Mousseau & Fox 1998, Christians 2002, Nager 2006). Además, muchos de estos recursos invertidos en el huevo tienen una disponibilidad limitada en el medio, afectando al auto-mantenimiento de las hembras. Sin embargo, el papel de nutrientes específicos (en especial los micro-nutrientes) a la hora de modular los costes de la producción de los huevos en el marco intra-puesta está poco claro (Bernardo 1996, Christians 2002). Nosotros nos centraremos en dos de estos nutrientes: el calcio y los antioxidantes.

Existen muchos estudios destacando la naturaleza limitante del calcio para las hembras durante la cría, ya que es requerido en cantidades inusualmente grandes para la formación de la cáscara del huevo (Robbins 1993). Aunque algunas especies acumulan calcio en los huesos antes de la puesta (Reynolds 1997), el calcio movilizado desde los huesos parece que contribuye relativamente poco en los requerimientos totales de calcio durante la puesta, en especial en aves pequeñas (cuyas puestas a menudo contienen más calcio que el que presentan en todo el esqueleto de la hembra) (Williams 2005). Además, efectos de una disponibilidad limitada de calcio han sido reportados en aves grandes como la gaviota argéntea (*Larus argentatus*), la cual reduce el número de huevos así como su tamaño (Pierotti & Annett 1991). Por esta razón la ingesta de calcio se incrementa marcadamente en las hembras durante la puesta, seleccionando alimentos calizos (Creutz 1953, Graveland *et al.* 1994). Sin embargo, los estudios de campo normalmente adolecen de limitaciones metodológicas asociadas a la dificultad de medir la disponibilidad de Ca en el medio salvaje (por ejemplo calcio exógeno; Wilkin *et al.* 2009, Reynolds & Perrins 2010). Alternativamente, tendencias temporales en los niveles de Ca del plasma para las hembras durante la cría pueden ser consideradas como un fiel reflejo de la respuesta fisiológica de las hembras a una demanda creciente de Ca asociada a la producción de huevos. Los niveles de Ca del plasma, por lo tanto, pueden ser usados como indicador del calcio total (tanto exógeno como endógeno) disponible para las hembras durante la producción de la puesta, facilitando las investigaciones sobre el papel de este micro-nutriente como factor proximal a la hora de limitar la reproducción de las aves.

Los compuestos antioxidantes también juegan un papel importante, influyendo en muchos rasgos del ciclo vital de los animales en general y de las aves en particular. Si echamos la vista atrás en el tiempo, hace alrededor de 2.450 millones de años tuvo lugar la *Gran Oxidación*, un incremento de los niveles de oxígeno en la atmosfera

como consecuencia de la emergencia y proliferación de los organismos fotosintéticos que fijaban carbono con la luz y liberaban oxígeno al aire (Sessions *et al.* 2009). Como consecuencia, los organismos vivos no sólo desarrollaron mecanismos fisiológicos para producir energía de una forma más eficiente usando el oxígeno resultante, sino que también tuvieron que desarrollar un conjunto de mecanismos para protegerse a sí mismos contra la toxicidad del oxígeno (una molécula altamente reactiva capaz de producir especies reactivas del oxígeno –ROS- durante el metabolismo oxidativo) y mitigar el efecto negativo de los procesos oxidativos (Halliwell & Gutteridge 2007). Por esta razón, los radicales libres del oxígeno ejercieron una importante presión selectiva durante los estadios más incipientes del metabolismo oxidativo (Costantini 2008). Sin embargo, esta presión sigue modulando los ciclos vitales de los actuales organismos vivos.

En los últimos 20 años, los estudios ecofisiológicos en aves se han centrado principalmente en pigmentos antioxidantes que subyacen a coloraciones corporales seleccionadas sexualmente (McGraw 2005). Entre ellos, los carotenos han sido el principal objetivo de esos trabajos sobre antioxidantes (por ejemplo, Blount *et al.* 2002, 2004, Hipfner *et al.* 2010a, 2010b). Los carotenos son hidrocarburos liposolubles que son sintetizados solamente por plantas y bacterias fotosintéticas. Los animales son incapaces de sintetizarlos *de novo* y deben obtenerlos a través de la dieta (Goodwin 1984). Sin embargo, estudios recientes han mostrado que los carotenos ofrecen una protección limitada contra el estrés oxidativo (Costantini & Møller 2008, Isaksson and Andersson 2008, Simons *et al.*, 2012). Por ejemplo, un reciente meta-análisis mostró que los carotenos explican < 0,002% de la capacidad antioxidante de las aves (Costantini & Møller 2008). Por lo tanto, emerge con fuerza la importancia de estudiar otros antioxidantes de la dieta también, no solamente los carotenos. Efectivamente, la calidad de la dieta y los antioxidantes de la misma son factores interrelacionados que influyen muchos rasgos de los animales (no sólo el estrés oxidativo, sino también la ornamentación sexual, la fertilidad y la fecundidad o el crecimiento de la descendencia) (revisado en Catoni *et al.* 2008). El uso de marcadores biogeoquímicos como los isótopos estables se erigen, nuevamente, como una herramienta muy útil para inferir la dieta de aves en libertad con el propósito de relacionarla con la capacidad antioxidante total (no sólo carotenos). Sólo unos pocos estudios experimentales han mirado esta relación entre la dieta y el contenido antioxidante en aves salvajes (por ejemplo, Beaulieu *et al.* 2010, Hipfner *et al.* 2010a, 2010b). Consecuentemente, todavía conocemos relativamente poco sobre cómo se produce la asignación intra-puesta de los antioxidantes, un aspecto muy importante para una

mejor interpretación sobre cómo las hembras modulan la asignación de recursos a los huevos.

4. CASO DE ESTUDIO: LA GAVIOTA DE AUDOUIN EN EL DELTA DEL EBRO

i. Área de estudio: el Delta del Ebro (Mediterráneo Occidental)

El "*Mare Nostrum*" (como lo conocían los antiguos romanos) o el "*Medi Terraneum*" ('en el medio de las tierras') es la forma latina con la que ha sido definido el mar Mediterráneo desde antiguo. Muchas importantes civilizaciones han florecido en su cuenca debido a su privilegiada situación entre los continentes de África, Asia y Europa. La cuenca mediterránea es el mayor (2 969 000 km²) y más profundo (una media de 1 460 m; máximo de 5 267 m) mar confinado sobre la Tierra (Coll *et al.* 2010). El Mediterráneo es un mar semi-cerrado, con forma alargada y con una productividad más bien baja, que se encuentra dividido en dos sub-cuencas conectadas por el Estrecho de Sicilia (Estrada 1996). La cuenca es generalmente oligotrófica, aunque con características heterogéneas que hacen aumentar la productividad en determinadas zonas costeras como por ejemplo las condiciones climáticas, termoclinas y aportes de aguas ricas en nutrientes por las corrientes, ríos y aguas residuales municipales (Coll *et al.* 2002). En verano, el régimen es oligotrófico y caracterizado por una productividad primaria baja debido a un fuerte fenómeno de estratificación. En invierno, en cambio, la productividad primaria incrementa a un régimen mesotrófico debido a procesos oceanográficos tales como la mezcla en profundidad, afloramientos y frentes oceanográficos (Allen *et al.* 2002).

A pesar de ser un área con una productividad relativamente baja, el mar Mediterráneo alberga una considerable diversidad de especies con un alto grado de endemismos (Zotier *et al.* 1999, Bianchi & Morri 2000, Coll *et al.* 2010). Nuevas estimas sobre la diversidad total de especies que habitan el Mediterráneo arrojan cifras de aproximadamente 17.000 especies, con una media de un 20% de especies endémicas dependiendo del grupo (Coll *et al.* 2010). Este es el motivo por el que a menudo el mar Mediterráneo es considerado un punto de diversidad muy importante a nivel mundial. Las aves marinas están representadas por sólo 15 especies, pero cuatro de ellas son endémicas: la pardela mediterránea (*Puffinus yelkouan*), la pardela balear (*Puffinus mauretanicus*), la pardela cenicienta mediterránea (*Calonectris diomedea*) y la gaviota de Audouin (*Larus audouinii*). El alto grado de endemismos del

Mediterráneo es principalmente el resultado de una tormentosa historia geológica, incluyendo su aislamiento de los océanos del mundo, que dio lugar una desecación casi total durante la crisis salina del Messiniense (hace 5,96 millones de años) y a drásticos cambios en el clima, nivel del mar y salinidad (Bianchi & Morri 2000, Coll *et al.* 2010). Sin embargo, la distribución de la diversidad no es aleatoria. Está principalmente asociada a un gradiente de productividad de oeste a este (Coll *et al.* 2010). Las zonas más productivas ocurren principalmente en el Mediterráneo Occidental, mostrando este área los valores más altos de riqueza de especies así como albergando la mayor parte de las especies amenazadas.

El Mediterráneo Occidental es actualmente un área altamente explotada: la mayor parte de las poblaciones de peces se encuentran totalmente explotadas o sobreexplotadas, induciendo cambios importantes en el ecosistema (Bianchi & Morri 2000, Tudela 2004, Coll *et al.* 2006, 2010). Algunos modelos ecológicos han demostrado que la pesca de arrastre es el arte de pesca con mayor impacto y menos efectividad, tanto para especies objetivo como no-objetivo (Coll *et al.* 2006). Las pesquerías de arrastre del Mediterráneo descartan entre un 20% y un 70% de las capturas totales, con una media de 17 954 toneladas de descartes cada año (Kelleher 2005). En los países mediterráneos desarrollados, la tasa de descarte de las pesquerías de arrastre puede llegar a ser de hasta el 400% de las capturas con valor comercial (Coll *et al.* 2010). En este sentido, una de las más importantes flotas de arrastre del Mediterráneo está situada precisamente en una parte de la cuenca Occidental: la plataforma del Delta del Ebro.

El Delta del Ebro (situado en el NE de la península Ibérica) ocupa un área de 320 km² y es una de las zonas húmedas más importantes del Mediterráneo Occidental, hogar de grandes colonias de aves acuáticas. Es predominantemente una zona agrícola, donde el 75% de su superficie corresponde a arrozales (aproximadamente 20 000 ha). La zona marina que lo rodea es una región con una particularmente elevada productividad en un contexto Mediterráneo. Esto es debido a los aportes de agua dulce ricos en nutrientes por parte del río Ebro, junto a los predominantes y fuertes vientos del noroeste, y a una plataforma continental relativamente ancha (Salat *et al.* 2002). Estas condiciones de productividad hacen que este área sea particularmente favorable para la comunidad biológica marina, por ejemplo para el desove de muchas especies de peces o como zona de alimentación para las aves marinas (Palomera 1992, Estrada 1996, Arcos 2009, Coll *et al.* 2010). Efectivamente, el delta del Ebro es una de las zonas más importantes de alimentación para las aves marinas en todo el Mediterráneo, especialmente durante la época de

cría. Son remarcables los números de tres especies endémicas que se alimentan en estas aguas: la pardela balear, la pardela cenicienta mediterránea y la gaviota de Audouin (Arcos 2009). En el caso de la gaviota de Audouin, sobre el 60% de la población mundial nidifica regularmente en la zona del Delta del Ebro. Por todo esto, el delta del Ebro ha sido designado recientemente como área importante para la conservación de las aves marinas (IBA marina ES409) (Arcos 2009).

ii. La especie: la gaviota de Audouin (*Larus audouinii*)

La gaviota de Audouin (*Larus audouinii* Payraudeau 1826) es una gaviota de tamaño medio endémica del Mediterráneo como reproductora. Mide entre 48-52 cm de altura, con un peso de entre 500-750 g, siendo los machos normalmente de mayor tamaño que las hembras (Ruiz *et al.* 1998, Genovart *et al.* 2003). Se considera una especie migratoria. Después de la época de cría (que termina en junio-julio), la gaviota de Audouin comienza su viaje hacia el sur desde sus zonas de cría, abandonando el Mediterráneo a través del Estrecho de Gibraltar y alcanzando sus cuarteles de invernada en el oeste de África (principalmente situados en el sur de Marruecos, Mauritania, Gambia y Senegal). A finales de invierno emprenden el viaje de vuelta, llegando a las zonas de cría de nuevo durante el mes de marzo (Cramp & Simmons 1983, Oro & Martínez-Vilalta 1994a, Pedrocchi & Oro 2005). Sin embargo, algunos individuos se quedan todo el invierno en el Mediterráneo, principalmente asociados a los puertos pesqueros (Bécares *et al.* 2012). La gaviota de Audouin está catalogada como "casi amenazada" a nivel global por la Unión Internacional para la Conservación de la Naturaleza (IUCN) (BirdLife International 2012). En España, la especie aparece como "vulnerable" (Martínez-Vilalta & Oro 2004).

Antiguamente estaba considerada como una de las aves marinas más amenazadas del mundo (Burger & Gochfeld 1996). A finales de la década de 1960, la población mundial se estimaba en solamente unas 800-1000 parejas (De Juana 1984). La situación se revirtió en 1981, cuando 36 parejas de gaviota de Audouin se establecieron en la "Punta de la Banya" (40° 35'N, 0° 42'E; una península arenosa que alberga una industria salina) en el Delta del Ebro. Desde entonces, esta nueva colonia experimentó un crecimiento espectacular, llegando a alcanzar una cifra más o menos estable de 11 000 parejas desde 1997 (Pedrocchi & Oro 2005).

La actual población mundial está estimada en 21 000 parejas (BirdLife International 2012), así que hoy en día el Delta del Ebro alberga más de la mitad de la población global. En este lugar, la gaviota de Audouin cría tanto en dunas costeras con vegetación como en las salinas. El espectacular incremento poblacional en el Delta del

Ebro se ha considerado como el resultado de un eficiente y oportunista uso de los descartes procedentes de los arrastreros, junto a efectivas medidas de protección de la zona de cría (Ruiz *et al.* 1996, Arcos *et al.* 2001, Oro & Ruxton 2001). En el pasado, esta especie estaba considerada como un gaviota piscívora adaptada a la captura de peces Clupeiformes durante la noche (Wallace 1969, Cramp & Simmons 1983). Sin embargo, desde su establecimiento, la gaviota de Audouin ha aprendido a sacar ventaja de los abundantes descartes pesqueros que arrojan los arrastreros que operan en la zona. Como resultado, se ha sugerido que, hoy en día, esta nueva fuente de alimentación supone más del 75% de la energía requerida por la especie y representa hoy en día el grueso de su dieta marina (Arcos 2001, Oro *et al.* 2013). Además de pescado marino, la otra fuente con una importante contribución a la dieta de la gaviota de Audouin son las presas de arrozal. En los arrozales, la gaviota de Audouin saca ventaja del abundante cangrejo americano (*Procambarus clarkii*) (Ruiz *et al.* 1996; Oro *et al.* 1997). Esta especie invasora está presente en el Delta del Ebro desde la década de 1980 (Gutierrez-Yurrita *et al.* 1999, Suárez-Serrano *et al.* 2010). En este sentido, se ha sugerido que el cangrejo americano incrementa su importancia para la dieta de la gaviota de Audouin durante períodos que no hay actividad arrastrera (Oro *et al.* 1996a, Ruiz *et al.* 1996, Oro *et al.* 1997).

Muchos estudios durante el pasado han enfatizado la relevancia de estas nuevas fuentes de alimentación (especialmente la disponibilidad de descartes) para esta especie, modulando sus parámetros reproductores (Pedrocchi 1997, Oro *et al.* 1996b, Oro *et al.* 1999, Oro *et al.* 2004), ecología trófica (Oro *et al.* 1996a, Ruiz *et al.* 1996, Pedrocchi *et al.* 2002) y comportamiento (Oro 1995, Arcos & Oro 1996, Mañosa *et al.* 2004). En el caso de los estudios de dieta, la mayor parte de los trabajos disponibles están basados en estudios observacionales, con el consecuente sesgo asociado a estas metodologías (González-Solís *et al.* 1997a). Pocos estudios han usado aproximaciones isotópicas para conseguir una mejor comprensión de la ecología trófica de la gaviota de Audouin, y normalmente carecen de una evaluación correcta del escenario isotópico representado por las presas (por ejemplo Sanpera *et al.* 2007a, 2007b, Navarro *et al.* 2010). También, trabajos previos sobre los movimientos de alimentación en esta especie (Mañosa *et al.* 2004, Christel *et al.* 2012) lidian con técnicas de posicionamiento (radio tracking y registradores PTT, respectivamente) las cuales tienen ciertas restricciones y errores de posicionamiento, y estos estudios han obviado mayoritariamente comparar periodos con diferentes actividades pesqueras (por ejemplo días laborables vs. fines de semana). La información sobre la ecología trófica proveniente de los isótopos estables junto a la información sobre el uso del

hábitat y movimientos de alimentación proporcionados por los nuevos y precisos registradores de posicionamiento (por ejemplo los registradores GPS) nos permitirán una aproximación muy robusta a los hábitos alimenticios de la gaviota de Audouin en el Delta del Ebro. Finalmente, una mejor comprensión de la ecología trófica (usando los isótopos estables) será de utilidad a la hora de estudiar la asignación de recursos a la puesta. En este sentido, los estudios disponibles sobre la formación de la puesta y parámetros oológicos (por ejemplo Pedrocchi 1997, Ruiz *et al.* 2000) no incorporan marcadores biogeoquímicos de la dieta para poder relacionar la ecología trófica con la asignación de diferentes nutrientes a la puesta.

5. OBJETIVOS

La presente tesis ha sido diseñada para profundizar y actualizar el conocimiento sobre la ecología trófica y reproductiva de la gaviota de Audouin. Para llevarlo a cabo, se usaron las nuevas metodologías y técnicas disponibles que permiten un estudio más preciso de la especie. Los resultados obtenidos en el presente trabajo, junto con la información generada previamente en esta especie, nos permitirán una mejor interpretación de la ecología de la especie.

La zona de estudio está centrada principalmente en la colonia del Delta del Ebro (40° 35'N, 0° 42'E), aunque la recientemente formada colonia del Delta del Llobregat (41° 18' 18"N, 2° 07' 50"E) fue también usada para comparar los niveles de metales pesados entre las dos áreas. El trabajo de campo se llevó a cabo durante las temporadas de cría de 2009 a 2011 (desde los meses de abril a junio).

Esta tesis está dividida en dos secciones. La **primera sección** está enfocada en la ecología trófica y uso del hábitat de la gaviota de Audouin, especialmente potenciales diferencias relacionadas con las actividades pesqueras. También se evaluaron diferencias geográficas en relación a los niveles de contaminantes. La **segunda sección** gira en torno a procesos ecofisiológicos y de asignación de recursos a la puesta, con especial énfasis en cómo la dieta puede influir en este proceso.

1. ECOLOGÍA TRÓFICA Y USO DEL HÁBITAT

- Estudiar la distribución espacio-temporal de la especie en el Delta del Ebro de acuerdo con las actividades pesqueras (días laborables vs. fines de semana) modelizando los datos GPS de los individuos con información procedente del VMS y variables ambientales (**Capítulo 1.1**).
- Profundizar en el conocimiento del comportamiento de alimentación, ecología trófica y uso del hábitat en la zona, combinando la información proporcionada por los registradores GPS con el análisis de isótopos estables. Se evaluaron posibles diferencias en relación al sexo y a actividades pesqueras (días laborables vs. fines de semana) (**Capítulo 1.2**).
- Evaluar diferencias geográficas en los niveles de metales pesados (mercurio y plomo) en dos colonias de gaviota de Audouin sujetas a un aporte diferencial de contaminantes de origen antropogénico (**Capítulo 1.3**).

2. ASIGNACIÓN DE RECURSOS Y ECOFISIOLOGÍA

- Explorar el patrón intra-puesta de $\delta^{13}\text{C}$ y $\delta^{15}\text{N}$ en el albumen de puestas de tres huevos durante dos temporadas de cría consecutivas, con especial énfasis en el potencial efecto de las actividades pesqueras (días laborables vs. fines de semana y periodos de veda de pesca) en esos patrones de asignación. También posibles relaciones entre la dieta y el tamaño del huevo fueron analizadas (**Capítulo 2.1**).
- Examinar la relación entre la dieta (trazada mediante el análisis de isótopos estables) y la capacidad antioxidante total (no sólo los carotenos) del plasma de las hembras durante la incubación y de sus puestas (albumen y yema) (**Capítulo 2.2**).
- Investigar el potencial papel del calcio a la hora de limitar la síntesis del huevo, evaluando la relación entre los niveles de calcio en el plasma de hembras durante la incubación con diferentes características de la puesta (tamaño del huevo, forma del huevo y grosor de la cáscara) (**Capítulo 2.3**).

DISCUSIÓN GENERAL

Avances en el conocimiento sobre la distribución espacial, ecología trófica y uso del hábitat

Aunque la ecología trófica y la distribución en el mar de la gaviota de Audouin ya han sido tratadas en el pasado, en especial la distribución espacial en el mar no ha sido tratada con mucho detalle (ver *Introducción*). La combinación de precisos datos de posicionamiento procedentes de individuos marcados con registradores GPS, junto con la información de variables ambientales y el Sistema de Vigilancia de Buques (VMS en inglés), es usado por primera vez en esta especie. La combinación de estas potentes herramientas nos permiten modelizar de una manera precisa los patrones de distribución espacial y los movimientos de alimentación de la gaviota de Audouin en relación a diferentes hábitats, intervalos horarios y actividades pesqueras. Además, la modelización teniendo en cuenta diferentes hábitats (marino y arrozal) al mismo tiempo es también novedosa, y representa el estudio de distribución más preciso para la flexible gaviota de Audouin.

De acuerdo con estudios previos, nuestros resultados muestran una gran dependencia de los movimientos de búsqueda de alimento de la gaviota de Audouin con las actividades pesqueras. Aunque considerada en el pasado una especie nocturna especializada en la captura de peces Clupeiformes (Wallace 1969, Cramp & Simmons 1983), nuestros resultados en el *Capítulo 1.1* muestran que, durante los días laborables, la probabilidad de encontrar gaviotas de Audouin en el mar durante la noche es mucho menor que durante el día. Efectivamente, la respuesta de los modelos sugiere que las actividades de pesca de arrastre determinan casi completamente la distribución en el mar de la gaviota de Audouin, tanto espacial como temporalmente (ver *Material Suplementario del Capítulo 1.1*). Además, la mayor parte de la actividad nocturna durante los días laborables debe ser debida a la asociación con las barcas de cerco debido a la concentración de individuos en frente de los principales puertos con actividad de pesca de cerco. Aunque no puede ser descartado que algunos individuos estén pescando por sí mismos, la gran asociación entre las actividades pesqueras y la distribución espacial de las gaviotas de Audouin sugiere que la mayor parte de la dieta marina proviene de actividades antropogénicas. Durante los fines de semana (cuando no hay actividades pesqueras) también encontramos actividad en el mar, pero mucho más dispersa debido a que no se concentran en torno a las barcas de pesca. En estos períodos, al contrario que los días laborables, es la

Clorofila A la variable que tiene más peso, indicando que se dirigen a las zonas más productivas para alimentarse en ausencia de actividades pesqueras. El patrón general observado durante los fines de semana indica que las gaviotas parten hacia el mar por la mañana temprano, pero al no encontrar actividades pesqueras, muchas de ellas vuelven a los puertos pesqueros y a los campos de arroz para alimentarse (especialmente después del mediodía) (ver *Material Suplementario del Capítulo 1.1*).

Una aproximación más detallada sobre este comportamiento de alimentación a nivel individual (*Capítulo 1.2*) muestra que las hembras de gaviota de Audouin tienden a realizar viajes de alimentación más largos en el espacio y en el tiempo que los machos. La segregación espacial de los sexos está extendida en las aves marinas, donde el sexo más pequeño (las hembras en el caso de la gaviota de Audouin) normalmente lleva a cabo viajes más largos (revisado en Wearmouth & Sims 2008). Se ha sugerido que el tamaño importa probablemente como consecuencia de una mayor eficiencia de vuelo y alimentación del sexo más pequeño (Shaffer *et al.* 2001). También, la segregación de la búsqueda de alimento se ha asociado a una reducción de la competencia intraespecífica (Catry *et al.* 2005). Sin embargo, la hipótesis sobre el dimorfismo sexual no ha sido siempre apoyada (por ejemplo Lewis *et al.* 2002; Stauss *et al.* 2012). De hecho, nuestros datos indican que estas diferencias de género están reflejadas en unos viajes significativamente más largos en el espacio y en el tiempo de las hembras los fines de semana, cuando no hay importantes actividades pesqueras y por lo tanto no hay descartes.

La distancia y la duración de los viajes están altamente relacionados con el hábitat de alimentación, siendo los viajes marinos significativamente más largos que los que van al arrozal. Esto significa que, durante los fines de semana, las hembras realizan viajes al mar más largos que los machos. Efectivamente, aunque ambos sexos incrementaron el uso de recursos de arrozal en ausencia de actividades de arrastre, durante los fines de semana los machos parece que explotan este hábitat en mayor medida que las hembras. Sin embargo, estas diferencias de género no fueron estadísticamente significativas probablemente debido a la gran variabilidad de estrategias que presentan las gaviotas de Audouin. Aunque era conocido que esta especie incrementa el consumo de presas de arrozal en ausencia de actividades pesqueras (Oro *et al.* 1996b), este sesgo en la selección del hábitat en relación al sexo presentado en el *Capítulo 1.2* (en base tanto al comportamiento de alimentación como al uso del hábitat) es descrito por primera vez.

La combinación de la información proveniente de los registradores GPS con marcadores de dieta (los isótopos estables) es también un aspecto nuevo para reconstruir la ecología trófica de esta especie. Los datos provenientes del Análisis de Isótopos Estables (SIA en inglés) y los datos GPS del *Capítulo 1.2* mostraron una alta correlación entre ellos, reafirmando que la gaviota de Audouin explota diferentes hábitats en el Delta del Ebro. Los individuos variaron desde exclusivamente marinos hasta especialistas de arrozal, pasando por toda la gama de estrategias intermedias. Sin embargo, esta aparente plasticidad trófica escondía una alta especialización individual. La mayor variación entre individuos que dentro de un mismo individuo (hubo una gran repetitividad en las firmas isotópicas del plasma entre la captura y la recaptura) sugiere una gran consistencia en la selección del hábitat. Además, no se observaron diferencias en la dieta entre sexos en base a los isótopos estables, al contrario de lo que otros estudios han apuntado (Navarro *et al.* 2010). Estas especializaciones individuales en la alimentación parecen comunes en aves marinas (por ejemplo Votier *et al.* 2004; Patrick *et al.* 2014) debido a la agregación y la competición intraespecífica (Araújo *et al.* 2007), además de por la predictibilidad de los parches donde se sitúan las presas (Weimerskirch *et al.* 2007).

¿Por qué las hembras utilizan en mayor medida los hábitats marinos que los machos en ausencia de actividades pesqueras? Dada la aparente ausencia de relación entre el peso de los individuos y los descriptores de los viajes de alimentación, parece que otros factores ecológicos/fisiológicos (no necesariamente independientes entre ellos) podrían estar jugando un papel importante. Entre esos factores, el papel de los requerimientos de micronutrientes a la hora de crear comportamientos sexuales de alimentación específicos ha sido a veces pasado por alto (ver por ejemplo Lewis *et al.* 2002, Xavier & Croxall 2005). Algunos estudios han mostrado que la necesidad de restablecer los niveles de calcio después de la puesta podrían ser un importante factor que explicara esta segregación sexual en la alimentación (revisado en Wearmouth & Sims 2008). Efectivamente, los niveles de calcio en las hembras de gaviota de Audouin se han mostrado como limitantes a la hora de restringir la síntesis del huevo (ver *Capítulo 2.3*). De acuerdo con esto, las espinas de los peces representan una mayor fuente de calcio absorbido comparado con los crustáceos (Hansen *et al.* 1998). Además, otros compuestos como las moléculas antioxidantes depositadas en los huevos tienen una disponibilidad limitada en las hembras. Un trabajo reciente ha mostrado su abundancia limitada en las hembras de gaviota de Audouin, y la importancia de las dietas marinas a la hora de proveer una mayor capacidad antioxidante a los huevos comparado con los recursos de arrozal (ver *Capítulo 2.2*).

Los efectos negativos de una moratoria en la pesca de arrastre (y por lo tanto, de la disponibilidad de alimento) en el éxito reproductor de esta especie son ampliamente conocidos (Oro *et al.* 1996a, 1996b, Oro *et al.* 1999, Oro *et al.* 2004). Sin embargo, este comportamiento de alimentación específico para cada sexo en relación a las actividades pesqueras tiene implicaciones novedosas de interés para la conservación desde el punto de vista de una prohibición de los descartes pesqueros. Las gaviotas de Audouin son más propensas a acercarse a los palangreros cuando los arrastreros no están operando (Arcos & Oro 2002). Además, esta especie es la segunda ave marina más afectada por la captura accidental en palangres en el Mediterráneo (Laneri *et al.* 2010). Por lo tanto, una prohibición de los descartes puede desembocar en un sesgo importante en la mortalidad accidental en palangre para esta amenazada ave marina de larga vida (Báez *et al.* 2014). De hecho, una de nuestras hembras marcadas fue encontrada muerta en Barcelona (a más de 150 kilómetros al norte de la colonia) después de haberse tragado nueve anzuelos de pesca.

También, la gran especialización en el uso del hábitat observada en esta especie, sugiere que las habilidades cognitivas y especializaciones individuales tales como el aprendizaje sobre cómo las actividades humanas pueden modificar la disponibilidad y predictibilidad de los recursos alimenticios puede jugar un papel muy importante a la hora de determinar el comportamiento de alimentación de los individuos (Cama *et al.* 2012; Oro *et al.* 2013). Dado que esas especializaciones individuales pueden tardar varios años en aprenderse (Bicknell *et al.* 2013), una implementación gradual de cualquier política sería necesario para facilitar la adaptación de las especies amenazadas como la gaviota de Audouin a un nuevo escenario de prohibición de descartes.

En conclusión, la combinación de los datos GPS con el SIA, VMS y las variables ambientales está siendo ampliamente utilizado, y se erige como una herramienta prometedora a la hora de diseñar las nuevas medidas de manejo y conservación.

Avances en el conocimiento sobre cómo el uso del hábitat determina la acumulación de contaminantes

Nuestros datos sobre isótopos estables muestran que los pollos de gaviota de Audouin tanto del Delta del Ebro como del Delta de Llobregat son alimentados principalmente con pescado marino. Aunque los datos isotópicos de Navarro *et al.*

(2010) estimaron que el consumo de arrozal por los pollos del Delta del Ebro era entre un 19-32%, los datos del presente estudio están más en concordancia con los de Pedrocchi *et al.* (2002) y Sanpera *et al.* (2007a), los cuales reportan que la mayor parte de la dieta consiste de pescado marino. En el caso de la recientemente formada colonia del Delta del Llobregat no había datos previos disponibles. Para esta colonia hemos encontrado una dieta completamente marina para los pollos, probablemente debido a la falta de arrozales en los alrededores al contrario que sucede en el Delta del Ebro. Dada la dependencia sobre los recursos marinos, y debido a que los movimientos de alimentación mostrados en el *Capítulo 1.1* y el *Capítulo 1.2* están principalmente concentrados en las aguas circundantes a la colonia, las plumas de pollos de gaviota de Audouin parecen un buen candidato para el seguimiento de los niveles de contaminantes en ambas zonas costeras.

En nuestro trabajo, encontramos niveles de mercurio significativamente más altos en el Delta del Ebro que en el Delta del Llobregat. La principal causa de esos niveles tan altos en el Delta del Ebro deben ser buscados en los contaminantes llevados por las aguas del río desde la ciudad de Flix (41°14'N, 00°31'E). Las diferentes actividades industriales que ha llevado a cabo una industria química en este lugar desde comienzos del siglo XX, unido a la construcción de una presa cerca de la factoría en 1960, resultó en la acumulación de 200.000 – 360.000 toneladas de residuos industriales en el lecho del río. Estos residuos acumulados en los sedimentos incluyen altas concentraciones de metales pesados (cadmio, arsénico, cobre, cromo, plomo y especialmente mercurio), organoclorados y ^{210}Pb radiactivo (Fernandez *et al.* 1999; Bosch *et al.* 2009). Los contaminantes vertidos en Flix son arrastrados aguas abajo por el río Ebro hasta su delta, a 90 km de distancia, especialmente durante periodos de inundación (Vericat & Batalla 2006). En este sentido, ha habido muchos estudios destacando los efectos de este problema en la biota (Quiros *et al.* 2008; Carrasco *et al.* 2008, 2011; Barata *et al.* 2010; Suarez-Serrano *et al.* 2010; Cotin *et al.* 2011, 2012). Este escenario origina uno de los problemas ecológicos más importantes todavía por resolver en el Mediterráneo Occidental, no sólo porque afecta uno de los humedales más importantes del sur de Europa (el Delta del Ebro), sino porque también es una zona agrícola importante y el río Ebro potencialmente proporciona agua a 2,7 millones de personas (CHEbro 2009).

En muchas especies de aves, las concentraciones de mercurio en pluma por encima de $5.000 \text{ ng} \cdot \text{g}^{-1}$ están asociadas con efectos reproductivos adversos como un menor tamaño de la puesta y de los huevos, un menor éxito de eclosión y una reducción en la supervivencia de los pollos (Eisler 1987). La concentración media

detectada en el Delta del Ebro está peligrosamente cercana a estos $5.000 \text{ ng}\cdot\text{g}^{-1}$. De hecho, el 50% de los pollos con alimentación marina en esta área presentan valores por encima de este umbral y por lo tanto susceptibles de estos efectos descritos. Esta concentración de mercurio osciló entre 2,5 y 5 veces la encontrada en plumas de pollos de gaviota de Audouin del noreste del Mediterráneo (Goutner *et al.* 2000). Nuevas investigaciones serían necesarias para ver el efecto de esta acumulación de mercurio sobre parámetros reproductores y fisiológicos en esta ave marina.

Por otro lado, las concentraciones de plomo detectadas en nuestro estudio fueron significativamente más altas en el Delta del Llobregat. Dado que la mayor parte de este metal pesado presente en las plumas proviene de la deposición atmosférica, los niveles de plomo de ambas zonas nos pueden dar pistas. Efectivamente, la concentración atmosférica de plomo es mucho mayor en la zona del Delta del Llobregat ($25,1 \text{ }\mu\text{g}\cdot\text{m}^{-3}$) que en el Delta del Ebro ($2,5 \text{ }\mu\text{g}\cdot\text{m}^{-3}$) (IDESCAT, 2011). Esta situación puede ser consecuencia del uso de gasolina de aviación (*aviation gasoline* en inglés) en el cercano aeropuerto de Barcelona (situado a sólo 2 km de la colonia) como ha sido reportado previamente (Kessler 2013). La gasolina de aviación, comúnmente denominada como *avgas*, es un combustible con plomo que se utiliza para alimentar motores de pistón en pequeñas aeronaves (Kessler 2013). En los Estados Unidos, por ejemplo, el plomo emitido por las avionetas que usan *avgas* es actualmente la mayor fuente de plomo atmosférico, constituyendo sobre el 50% de las emisiones de plomo en 2005 (U.S. EPA 2010). En aves, se ha sugerido que los niveles de plomo en plumas por encima de $4.000 \text{ ng}\cdot\text{g}^{-1}$ están asociados con un retraso en el reconocimiento de los progenitores y hermanos, problemas de locomoción, de la percepción de la profundidad y en el comportamiento de alimentación, así como una disminución en la supervivencia de los pollos en gaviotas (revisado en Burger & Gochfeld 2000). En ningún caso este umbral fue superado, estando todos los pollos lejos de estos límites y aparentemente a salvo de problemas provocados por el plomo.

Estos resultados apoyan el potencial de las plumas de pollos de gaviota de Audouin para el seguimiento de los niveles de contaminantes en una determinada zona costera. Además, estos resultados destacan cómo la selección de diferentes zonas de alimentación puede resultar en una acumulación diferencial de metales pesados. Finalmente, más estudios serían necesarios para determinar el grado de riesgo para la salud humana que comporta el consumo de pescado con altos niveles de mercurio de la zona del Delta del Ebro.

Avances en el conocimiento sobre la asignación de recursos a la producción de la puesta

El efecto de las limitaciones adaptativas y no adaptativas a la hora de determinar el patrón intra-puesta de asignación de nutrientes y la variación del tamaño del huevo ha sido tradicionalmente pasado por alto. Actualmente, algunos estudios han abordado este tema usando los isótopos estables para rastrear el origen de los nutrientes asignados a los huevos, especialmente a través de los isótopos estables del carbono ($\delta^{13}\text{C}$) y el nitrógeno ($\delta^{15}\text{N}$) (Hobson 2006). En este sentido, el albumen parece ser el compartimento del huevo más adecuado para un análisis intra-puesta dado que es sintetizado durante cortos períodos de tiempo que no se solapan (Ruiz *et al.* 2000).

El modelo final seleccionado para el $\delta^{15}\text{N}$ en el albumen sólo incluye el “día de la semana” como factor significativo. O lo que es lo mismo: los efectos de un incremento en el consumo de recursos de arrozal durante los fines de semana (cuando la disponibilidad de descartes de la pesca de arrastre es despreciable) se ve reflejado en un enriquecimiento de las firmas de nitrógeno en el albumen, alcanzando su máximo el miércoles. Este desfase temporal corresponde con el tiempo necesario para sintetizar el albumen y la cáscara del huevo, sobre 2-3 días (Nager 2006). El consistente patrón diario de $\delta^{15}\text{N}$ entre años y lugares de nidificación apoya la validez de esta predicción, y pone de manifiesto la fuerte dependencia de la gaviota de Audouin sobre las actividades pesqueras como muestran también los *Capítulos 1.1* y *1.2*. Además, la alta sensibilidad a los cambios diarios en la disponibilidad de descartes refleja que la mayor parte del nitrógeno depositado en el albumen debe provenir de la dieta.

Por otro lado, el $\delta^{13}\text{C}$ mostró un consistente patrón decreciente a través de la secuencia de puesta en ambos años. Sin embargo, los valores isotópicos fueron mayores en 2009 que en 2010. Estas diferencias entre años pueden ser atribuidas a la elevada naturaleza dinámica del río Ebro en relación a las condiciones meteorológicas y los consecuentes aportes de materia orgánica particulada al mar, afectando principalmente al isótopo del carbono (Veyssy *et al.* 1998; Harmelin-Vivien *et al.* 2010). El carbono en el albumen debe tener un origen más endógeno que el nitrógeno dado que este patrón diario (aunque estadísticamente significativo) no es tan obvio. Además, el decreciente patrón intra-puesta observado probablemente indica diferencias en la contribución endógena a través de la secuencia de puesta más que un cambio en la dieta.

Nuestros resultados son consistentes con varios trabajos previos en anátidas salvajes, los cuales mostraron que el nitrógeno depositado en los huevos proviene principalmente de la comida consumida en las zonas de cría para estas especies migratorias, mientras que el carbono tenía una contribución más endógena (Hobson *et al.* 2005, Bond *et al.* 2007, Oppel *et al.* 2010). Sorprendentemente, nuestros resultados en la gaviota de Audouin son opuestos a los encontrados por Ramírez *et al.* (2011) en la simpátrica gaviota patiamarilla (*Larus michahellis*). Dado que el día de la semana no fue considerado en ese estudio, los resultados podrían ser consecuencia de pasar por alto el efecto de la pesca en esa especie que también explota los descartes pesqueros eficientemente (Cama *et al.* 2012). Sin embargo, no se pueden descartar diferencias en la estrategia de asignación de recursos entre ambas especies. Aunque filogenéticamente distantes a la gaviota de Audouin, los patos de estos estudios coincidentes son también migradores de larga distancia. La gaviota patiamarilla, por el contrario, es principalmente residente (Cramp & Simmons 1983). La principal fuente de proteínas endógenas asignadas a los huevos es el músculo pectoral (Kendall *et al.* 1973; Houston *et al.* 1995). Sin embargo, la movilización de las reservas de proteínas del músculo pectoral durante la puesta puede tener efectos negativos sobre la capacidad de vuelo de las hembras (Veasey *et al.* 2001; Kullberg *et al.* 2005). Quizá, realizar una migración tan larga inmediatamente después de la cría limita la capacidad de movilizar proteínas endógenas hacia la puesta en especies migratorias comparadas con especies residentes. Más investigaciones serían recomendables para comprobar esta hipótesis.

En relación al patrón intra-puesta del tamaño del huevo, se encontraron diferencias significativas entre lugares de nidificación. Las salinas parecen ser un lugar de cría de mejor calidad debido a su alta densidad de nidos y un mayor éxito reproductor que las dunas costeras (Oro *et al.* 2010). Algunos estudios en gaviotas han sugerido que una mayor densidad está asociado a un tamaño menor del tercer huevo (Coulson *et al.* 1982; Pierotti & Bellrose 1986; Spaans *et al.* 1987), al igual que sucede en nuestro estudio. Probablemente, bajo situaciones de poca densidad o en situaciones subóptimas, las hembras pueden modular la asignación de recursos incrementando la inversión en los huevos finales, aumentando el crecimiento y supervivencia del pollo, y reduciendo el tiempo de eclosión (Ferrari *et al.* 2006).

Además de lo anterior, la dieta es otro de los principales factores sugeridos que afectan el tamaño del huevo (Martin 1987; Bolton *et al.* 1992; Williams 1994). Efectivamente, además del esperable patrón de decaimiento en el tamaño del huevo a través de la secuencia de puesta, el otro factor principal que afecta al tamaño del

huevo es el $\delta^{15}\text{N}$. Debido a la estrecha relación entre el isótopo del nitrógeno y la dieta (tal como se ha dicho anteriormente), esta relación negativa sugiere que situaciones de escasez de descartes pesqueros (incrementando el consumo de presas de arrozal) están relacionadas con menores tamaños del huevo.

Finalmente, es importante notar que un importante cambio en la dieta hacia un mayor consumo de recursos de arrozal fue evidente en las puestas afectadas por la moratoria en la pesca de arrastre. Esto indica que las hembras, mientras están poniendo los huevos, están muy ligadas al nido y no pueden realizar viajes más largos en busca de pescado, por lo que parece que priorizan la captura de presas más accesibles pero de menor calidad como el cangrejo americano. Esta situación contrasta con hallazgos previos en las hembras durante la incubación (*Capítulo 1.2*), cuando éstas realizaron viajes más largos por el mar en ausencia de actividades de arrastre probablemente debido a que no están tan ligadas al nido como cuando tienen que poner los huevos. Además, estos resultados muestran que cambios drásticos en la dieta son también evidentes en los perfiles de $\delta^{13}\text{C}$ del albumen, a pesar de su aparentemente mayor contribución endógena.

Avances en el conocimiento sobre la asignación de antioxidantes a la puesta

La influencia de la dieta en el estatus oxidativo de las aves puede ser explorada examinando la relación entre la capacidad antioxidante y la signatura isotópica de los tejidos. En nuestro trabajo, se evaluó la relación entre los isótopos estables ($\delta^{15}\text{N}$, $\delta^{13}\text{C}$) y la capacidad antioxidante del albumen y la yema de puestas de gaviota de Audouin procedentes de dos núcleos reproductores del Delta del Ebro: uno que nidifica en las salinas y otro en dunas costeras. Sin embargo, nuestros resultados no encontraron relación entre la signatura de carbono (que nos da información sobre las fuentes de carbono usadas por el consumidor) y las concentraciones de antioxidantes. Este hallazgo está en concordancia con el anterior *Capítulo 2.1*, donde se observó que el carbono del albumen tenía un origen más endógeno que el isótopo estable del nitrógeno. En la misma dirección, Hipfner *et al.* (2010a) tampoco encontraron relación entre los niveles de carotenoides en la yema y los valores de $\delta^{13}\text{C}$ en otras especies de aves marinas.

Tradicionalmente, el albumen ha recibido mucha menos atención que la yema en estudios de antioxidantes debido a la falta de carotenoides. Por lo tanto, el vacío de

conocimiento es especialmente evidente en este compartimento del huevo. A la hora de comparar la concentración de antioxidantes en el albumen de los dos grupos reproductores, hubo una interacción significativa entre el grupo reproductor y el orden de puesta: en las salinas hubo una menor inversión de antioxidantes del albumen en el tercer huevo, mientras que en el grupo que nidifica en dunas costeras no mostró variación a lo largo de la secuencia de puesta. Estas diferencias en el albumen en cuando a la concentración de antioxidantes entre los terceros huevos son coincidentes también con capítulo previo 2.1, donde los terceros huevos del grupo de las salinas fueron significativamente más pequeños que aquellos del grupo de las dunas. La calidad del lugar de nidificación podría estar jugando un papel importante como ya se ha dicho anteriormente. Sin embargo, dada la gran sensibilidad del albumen depositado en los huevos a la disponibilidad de descartes, un posible efecto del día de la semana en el que el huevo fue generado puede que también tenga influencia en esta variabilidad.

Por otro lado, la yema representó la mayor parte de la capacidad antioxidante del huevo. La concentración de antioxidantes hidrofílicos en la yema (hydrOXY), así como la concentración total de antioxidantes de la yema (lipOXY + hydrOXY) siguieron un patrón decreciente a lo largo de la secuencia de puesta como ha sido descrito en otros estudios sobre gaviotas (Royle *et al.* 1999; Blount *et al.* 2002; Groothuis *et al.* 2006; Rubolini *et al.* 2011). Además, este descenso en la capacidad antioxidante total de la yema fue observado en ambos grupos. Esta tendencia decreciente indica que la asignación de antioxidantes a la yema está probablemente modulada/limitada por la adquisición de recursos antioxidantes (Royle *et al.* 2003) así como la competición entre oocitos (Meathrel 1991).

En la yema, tanto la capacidad antioxidante lipofílica como la capacidad antioxidante total, están relacionadas negativamente con el $\delta^{15}\text{N}$, por lo tanto, signaturas menores de nitrógeno estaban asociadas a una mayor capacidad antioxidante. Royle *et al.* (2003) encontraron que en las hembras de diamante mandarín (*Taeniopygia guttata*) sobre el 70% de la ingesta diaria de carotenoides y α -tocoferoles (los dos antioxidantes lipofílicos de la dieta más abundantes) se depositaron en la yema del huevo. Como se ha dicho anteriormente, en el sistema del Delta del Ebro, valores más bajos de nitrógeno derivan principalmente de dietas marinas, sugiriendo que los recursos marinos son más beneficiosos en términos de aporte de antioxidantes que los recursos de arrozal.

Aunque el cangrejo americano es conocido por contener elevadas concentraciones de carotenoides (Negro *et al.* 2000), el poder antioxidante de la astaxantina es muy bajo comparado con otros antioxidantes de la dieta (revisado en Catoni *et al.* 2008). Este resultado está en concordancia con recientes estudios que han mostrado que los carotenoides ofrecen una protección limitada contra el estrés oxidativo (Costantini & Møller 2008; Isaksson & Andersson 2008; Simons *et al.* 2012), destacando la importancia de mirar a otros antioxidantes de la dieta también.

Este estudio apunta al potencial beneficio para la gaviota de Audouin de dietas basadas en pescado marino comparado con las presas de arrozal en términos de los niveles de antioxidantes depositados en los huevos. También, la deposición de antioxidantes en los huevos tendió a decrecer a lo largo de la secuencia de puesta, destacando la disponibilidad limitada de las moléculas antioxidantes.

Avances en el conocimiento sobre el papel limitante del calcio en la síntesis de los huevos

Existen evidencias sobre el papel limitante del calcio en la síntesis del huevo en varias especies de aves (ver *Introducción*), aunque pocos estudios han discutido sobre la potencial implicación de este micronutriente a la hora de determinar el patrón intra-puesta de tamaño del huevo típico de muchas especies de aves (Graveland *et al.* 1994; Reynolds 2001). Además, se ha sugerido para las hembras que la necesidad de restaurar los niveles de calcio después de completar la puesta puede jugar un papel importante a la hora de generar diferencias en el comportamiento de alimentación (ver *Capítulo 1.2*). Por lo tanto investigamos la relación entre los niveles de Ca en el plasma de las hembras y varios rasgos de los huevos relacionados con su eficacia biológica como el tamaño del huevo (volumen del huevo), la forma del huevo y el grosor de la cáscara del huevo para evaluar el papel limitante de este elemento en la gaviota de Audouin.

Tanto la ingesta por la dieta como la movilización de Ca endógeno resulta en un dramático incremento de las concentraciones de Ca en el plasma de las hembras durante la ovulación, para decrecer progresivamente después hasta alcanzar los valores de los machos (los cuales no muestran esos cambios en los niveles de Ca de la sangre) durante la incubación. Aunque nuestros resultados para la gaviota de Audouin se refieren exclusivamente al período de incubación, éstos concuerdan con las tendencias específicas para cada sexo reportadas anteriormente y sugieren que

los niveles de Ca en el plasma de hembras durante la incubación puede ser usado como indicador de los niveles de Ca en el plasma durante la formación del huevo.

Nuestros resultados indican que la relación entre los niveles de Ca en el plasma en las hembras durante la incubación y el tamaño del huevo varió a lo largo de la secuencia de puesta, siendo ligeramente mayor para los últimos huevos (como apuntan las pendientes estimadas para las relaciones entre los niveles de Ca en el plasma y el volumen estimado del huevo así como la superficie: huevo-a < huevo-b < huevo-c). Bajo nuestro punto de vista, estos resultados sugieren que los últimos huevos de la secuencia son particularmente sensibles a los niveles circulantes de Ca, lo que puede ser explicado por una disminución global de la disponibilidad de Ca (tanto endógeno como exógeno) siempre y cuando este micronutriente sea progresivamente asignado a los huevos sucesivos (cáscaras del huevo) para completar la puesta. Además de este efecto en la producción de la puesta, la disponibilidad de Ca puede también jugar un papel relevante a la hora de determinar el patrón intra-puesta de variación en el tamaño del huevo, con mayores disponibilidades resultando en tamaños de huevo más homogéneos a nivel intra-puesta.

Por el contrario, la forma del huevo en la gaviota de Audouin no varió de acuerdo con la disponibilidad de Ca. Sin embargo, el hecho de que este rasgo variara significativamente a lo largo de la secuencia de puesta, y que más de la mitad de esa variabilidad fuera explicada por el factor aleatorio del nido, sugiere que otros rasgos (posiblemente no medidos) probablemente estén operando a lo largo de la producción de la puesta y afectando a la forma del huevo.

Nuestro estudio se focaliza en explorar el papel limitante del Ca durante la reproducción, y proporciona varias evidencias que sugieren que, además de los ya comentados rasgos individuales, la disponibilidad de calcio para las hembras durante la cría influye en las estrategias de asignación de recursos de la gaviota de Audouin. En concreto, una elevada disponibilidad de Ca resulta en mayores huevos y un patrón más homogéneo de tamaño del huevo a nivel intra-puesta.

FUTURAS LÍNEAS DE INVESTIGACIÓN

La presente tesis ha puesto de manifiesto la necesidad de realizar estudios a una escala fina a la hora de analizar los patrones de uso del hábitat, ecología trófica y ecofisiología de las aves marinas. Si son pasadas por alto, algunas variables como el

sexo, los intervalos horarios, el día de la semana, los días desde el comienzo de la veda de arrastreros o los días desde la finalización de la puesta, pueden influir decisivamente en la ecología y fisiología de las especies. Esto pone de manifiesto que todavía estamos lejos de una completa comprensión de todos estos patrones en la gaviota de Audouin en particular y las aves marinas en general, así que aquí apuntamos algunas perspectivas de futuro hacia donde los estudios deberían apuntar.

Aunque los períodos de fin de semana se erigen como una aproximación adecuada a la hora de identificar cambios en el comportamiento de alimentación y uso del hábitat en ausencia de descartes pesqueros (Bartumeus *et al.* 2010), sería interesante comparar con períodos más prolongados donde se dé una moratoria de pesca de arrastre en toda la zona. Una veda parcial tiene lugar la mayor parte de años, cuando los arrastreros sólo operan o en la parte norte o en la parte sur del Delta del Ebro. En 2009, sin embargo, la veda de pesca de arrastre afectó todas las aguas circundantes al Delta del Ebro desde el 1 de mayo, coincidiendo con las puestas más retrasadas. Aunque en la década de los ochenta-noventa algunos estudios en la gaviota de Audouin fueron llevados a cabo bajo una veda de arrastreros en la zona (ver *Introducción* de esta Tesis), una aproximación más detallada con las nuevas técnicas disponibles sería altamente deseable. También, debido a la inminente implementación de la nueva política pesquera común adoptada por la Unión Europea, un programa de seguimiento durante la implementación gradual de esta reforma en esta especie amenazada sería de especial interés.

Otra especie que cría en el Delta del Ebro y de gran interés debido a sus interacciones con las actividades humanas es la gaviota patiamarilla (*Larus michahellis*). Al contrario que la gaviota de Audouin, esta especie es considerada a veces como una especie plaga debido al uso sostenido que hace de los hábitats urbanos y vertederos, aunque también consume una elevada proporción de descartes pesqueros (Ramos *et al.* 2009a, 2009b, 2011). Es probable que una prohibición de los descartes pesqueros también afecte a la gaviota patiamarilla incrementando su consumo de desperdicios de las ciudades y basuras de vertederos. Además, se esperarían tasas mayores de cleptoparasitismo interespecífico (Oro & Martínez-Vilalta 1994b, González-Solís *et al.* 1997b) y un mayor consumo de pollos y huevos de otras aves marinas simpátricas que crían en el Delta del Ebro (Hernández-Matías & Ruiz 2003, Oro *et al.* 2005, Martínez-Abraín *et al.* 2003). Pocos trabajos han estudiado en profundidad los patrones de distribución en el mar de la gaviota patiamarilla comparado con la gaviota de Audouin (pero ver Cama *et al.* 2012), por lo que sería

deseable un estudio más exhaustivo para comprender el impacto de esta nueva reforma pesquera en esta especie problemática.

La gran acumulación de lodos tóxicos en Flix (*Capítulo 1.3*) es uno de los problemas ecológicos más importantes que están todavía por resolver en el Mediterráneo occidental. El dragado de los 800.000 metros cúbicos de lodos tóxicos acumulados en el fondo del embalse de Flix comenzó en 2013. Esta es la mayor obra de estas características llevada a cabo en España hasta la fecha, y para la que ha sido prevista una inversión de 165 millones de euros. Aunque estaba planeado finalizarla para diciembre de 2015, hasta la fecha sólo ha sido realizado el 25% de lo esperado. Debido a la naturaleza peligrosa de estas obras, y dada la gran utilidad de las plumas de pollos de gaviota de Audouin para el seguimiento de los niveles de contaminantes en el ambiente marino (especialmente el mercurio), sería deseable continuar con el seguimiento de los niveles de metales pesados en el área durante y al finalizar las obras de extracción de los fangos tóxicos.

Finalmente, un meta-análisis integrador y multidisciplinar de las diferentes técnicas usadas en esta Tesis sería muy interesante. Por ejemplo, la posible interrelación del esfuerzo físico (en base a los datos GPS), la dieta (en base al SIA) y los niveles de contaminantes sobre la capacidad antioxidante y los niveles de daño oxidativo de los individuos y sus puestas podría arrojar luz sobre cómo estos procesos ecofisiológicos han evolucionado en las aves marinas y cómo afectan sus rasgos del ciclo biológico. Se ha demostrado que algunos metales pueden causar estrés oxidativo ya que incrementan la formación de especies reactivas del oxígeno (ROS), desequilibrando el estado redox de la célula y haciendo que los compuestos antioxidantes sean insuficientes para defender al organismo de la cantidad creciente de radicales libres (revisado en Koivula & Eeva 2010). La actividad física también influye el estado oxidativo de las aves (Costantini 2008), especialmente importante para aves migratorias como la gaviota de Audouin. El estudio de animales migratorios puede proporcionar nuevos conocimientos sobre estos mecanismos que han evolucionado para hacer frente al ejercicio exhaustivo. Además, elevados niveles de metales como el mercurio han sido asociados con efectos reproductivos adversos como un menor tamaño de la puesta y del huevo (Eisler, 1987), un factor pasado por alto que probablemente podría estar actuando también a la hora de modificar el patrón intra-puesta del tamaño del huevo.

CONCLUSIONES

1. ECOLOGÍA TRÓFICA Y USO DEL HÁBITAT

- A nivel poblacional, nuestros resultados muestran una gran relación entre los movimientos de alimentación de la gaviota de Audouin y las actividades pesqueras en la zona del Delta del Ebro. En períodos laborables, la probabilidad de encontrar gaviotas de Audouin en el mar durante el día es mucho mayor que durante la noche debido a la gran asociación con los barcos arrastreros. La mayor parte de la actividad nocturna en días laborables proviene de la asociación con barcas de cerco. Durante los fines de semana, cuando no hay actividades pesqueras importantes, el uso del arrozal incrementa (de una media de 35% a cerca de un 55% de las posiciones GPS).
- A nivel individual, ambos sexos mostraron diferencias en el comportamiento de alimentación en relación a las actividades pesqueras. Nuestros datos indican que las hembras de gaviota de Audouin realizan viajes marinos significativamente más largos y pasan más tiempo fuera de la colonia durante los fines de semana. Debido a que estas diferencias están asociadas aparentemente a las actividades pesqueras, y que no se encontró relación entre el uso del hábitat y los índices de condición de los individuos, este comportamiento de alimentación específico de cada sexo podría estar relacionado con diferentes requerimientos nutricionales asociados a la producción de los huevos (no sólo el dimorfismo sexual).
- Este último punto contrasta con el comportamiento de las hembras durante la puesta de los huevos, cuando muestran un drástico cambio de dieta hacia presas de arrozal en ausencia de actividades de arrastre, probablemente debido a la fuerte asociación con el nido.
- La alimentación de la gaviota de Audouin osciló desde individuos exclusivamente marinos a especialistas de arrozal, pasando por toda la gama intermedia de estrategias. Sin embargo, una elevada especialización individual subyace bajo esta aparente plasticidad trófica.
- Las diferentes zonas de alimentación resultaron también en una acumulación diferencial de metales pesados. Los niveles de plomo fueron significativamente más altos en el delta del Llobregat probablemente debido al uso de combustibles con plomo en el cercano aeropuerto de Barcelona. Por otro lado, las concentraciones de mercurio fueron mayores en el delta

del Ebro relacionado con la acumulación de lodos tóxicos en el fondo del embalse de Flix, los cuales son arrastrados aguas abajo por el río Ebro. Estos niveles de mercurio en las plumas de pollos alcanzaron valores que han sido descritos como tóxicos.

2. ASIGNACIÓN DE RECURSOS Y ECOFISIOLOGÍA

- La gran sensibilidad del $\delta^{15}\text{N}$ del albumen a cambios diarios en la dieta muestra que la mayor parte del nitrógeno depositado en los huevos proviene de los recursos consumidos. El $\delta^{13}\text{C}$ debe tener un origen más endógeno debido a que ese patrón diario no es tan obvio. Sin embargo, cambios drásticos en la dieta hacia un mayor consumo de presas de arrozal durante la moratoria de pesca de arrastre se vieron reflejados en ambos isótopos.
- Signaturas más elevadas de $\delta^{15}\text{N}$ en el albumen (asociadas con un mayor consumo de presas de arrozal o una mayor movilización de reservas endógenas) estuvieron relacionadas con un menor tamaño del huevo.
- Entre individuos, se encontraron pocas diferencias en el patrón de deposición de antioxidantes a través de la secuencia de puesta para la yema y para el huevo entero. Una mayor variación se observó en el patrón intra-puesta para la capacidad antioxidante del albumen probablemente relacionado con diferencias de calidad o de disponibilidad de alimento.
- Se encontró una tendencia decreciente a través de la secuencia de puesta para la capacidad antioxidante total asignada a la puesta, resaltando la disponibilidad limitada de las moléculas antioxidantes.
- Tanto la capacidad antioxidante lipofílica de la yema como la capacidad antioxidante total del huevo estuvieron relacionadas negativamente con el $\delta^{15}\text{N}$, indicando que un mayor uso de recursos de arrozal está asociado con una menor disponibilidad de antioxidantes.
- El tamaño del huevo estuvo relacionado positivamente con los niveles de calcio circulantes de las hembras, siendo la pendiente de esta relación significativamente mayor para los últimos huevos de la secuencia de puesta. También, disponibilidades mayores de Ca resultaron en tamaños de huevos más homogéneos a nivel intra-puesta, modulando el perfil de tamaño de huevo típico de esta especie de gaviota.



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Rice field at Ebro Delta Natural Park (Spain).
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APPENDICES

Audouin's gull (Ebro Delta Natural Park, Spain).
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Anthropogenic input of heavy metals in two Audouin's gull breeding colonies

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ABSTRACT

Anthropogenic activities have become an important source of heavy metals to the marine environments. Biological sentinels like seabirds' chicks have been widely used to monitorize the levels of some heavy metals. Due to its mainly marine foraging habits, Audouin's gull fits well for this purpose. Mercury and lead levels were measured in mantle feathers of Audouin's gull chicks from two colonies in NE Iberian Peninsula: the Ebro Delta and the Llobregat Delta. Both are anthropized areas subject to differential pollutant inputs. Lead levels were significantly higher in the Llobregat Delta probably due to the use of leaded fuel in the nearby Barcelona airport. On the other side, mercury concentrations were higher in the Ebro Delta, in relation with the disposal of the toxic sediments at the Flix site carried down by the Ebro River. These mercury levels in the Ebro chicks reached values that have been described as toxic.

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1. Introduction

Anthropogenic processes as mining, fossil fuel burning, production of chemicals and agricultural activities have become an important source of heavy metals to marine environments (Furness and Rainbow, 1990; Monteiro and Furness, 1997; Dietz et al., 2009). Marine species are particularly susceptible to heavy metal incorporation because oceans act as ultimate sink for the most part of pollutants and because their accumulation in the sediments of intertidal environments (Bearhop et al., 2000; Burger et al., 2007). For this reason, some marine animal groups have been widely used as bioindicators of pollution levels. This is the case of seabirds, particularly adequate as indicators due to the detailed knowledge about their ecological habits and their relative high trophic position in the marine food webs. Their colonial nature also allows collecting large sample sizes from a particular site in a relatively short period of time (Furness and Camphuysen, 1997). Chick feathers are ideal for this purpose because they allow a non-invasive sampling and reflect the availability of some metals of a local area during the chick rearing phase (Furness, 1993).

Mercury is one of the most studied heavy metals, emitted to the environment from both natural and anthropogenic sources (Nriagu, 1989). In the mesopelagic environment there is a major transformation from inorganic mercury to methylmercury, the

most toxic and stable form (Nriagu, 1989; Monteiro et al., 1998; Wright and Welbourn, 2002). In birds, dietary mercury is incorporated into the feathers from the blood, and bioaccumulates through the food chain (Nriagu, 1989; Arcos et al., 2002). On the other side, lead in feather appears to originate mainly from atmospheric deposition onto feather surfaces, whereas ingested lead become firmly bound in bones and only enter feathers in trace amounts (Furness, 1993; Furness and Camphuysen, 1997). However, lead levels in feathers can also help us to elucidate the characteristics of a given area since the main part of atmospheric lead has an anthropogenic origin (Wright and Welbourn, 2002). Because both, mercury and lead can reach toxic levels when at high concentrations (Furness, 1993), it is important to monitorize the levels of these elements.

This is the possible scenario of the two wetlands that we considered in our study: the Ebro Delta and the Llobregat Delta (Fig. 1). Both are situated in the northeastern part of the Iberian Peninsula, and both are anthropized areas subject to pollutant inputs. The case of the Ebro River is particularly alarming due to decades of dumping toxic waste to its basin, mainly at Flix town (41°14'N, 00°31'E; Fig. 1) where a chemical industry has been in operation since the early 20th century. The different industrial activities at this place and the construction of a dam close to the factory in 1960 resulted in the accumulation of 200,000–360,000 tons of industrial wastes in the riverbed. This wastes accumulated in the sediments include high concentrations of heavy metals (cadmium, arsenic, copper, chromium, lead and especially mercury), organochlorines and radioactive ²¹⁰Pb (Fernandez et al., 1999;

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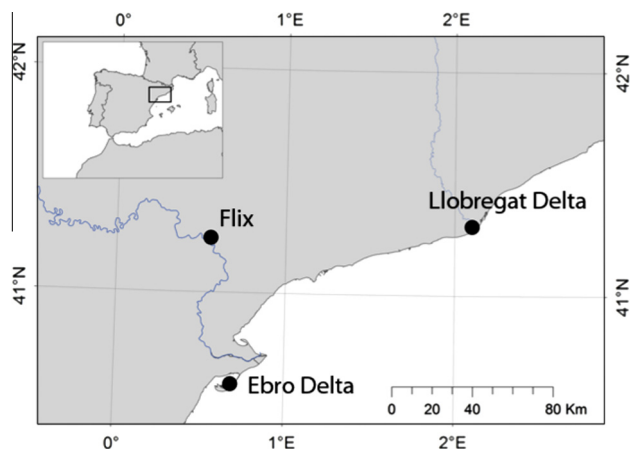


Fig. 1. Map of the sampled colonies (Ebro Delta and Llobregat Delta) and the location of toxic muds accumulated at Flix dam.

Bosch et al., 2009). The pollutants originated at Flix site are carried downstream by the Ebro River to its Delta 90 km away, especially during floods (Vericat and Batalla, 2006). In this way, there have been many studies highlighting the effects of this problem on the biota (Quirós et al., 2008; Carrasco et al., 2008, 2011; Barata et al., 2010; Suárez-Serrano et al., 2010; Cotin et al., 2012). This scenario originates one of the most important ecological problems still to be solved in western Mediterranean, not only because it affects one of the most relevant wetlands in south Europe (the Ebro Delta), but also because this is an important agricultural area and the Ebro river potentially supplies water to 2.7 million people (CHEbro, 2009). On the other hand, the Llobregat Delta is a smaller wetland with a strong human alteration, located 150 km north of the Ebro Delta. Because it is located very close to the Barcelona Airport in the metropolitan area (with more than 3 million people), the anthropogenic inputs of pollutants might be also evident. The relation between lead levels and airports has been reported previously (Kessler, 2013). Aviation gasoline, commonly referred to as avgas, is a leaded fuel used to power piston-engine small aircraft (Kessler, 2013). The most commonly used avgas is 100LL. Although the “LL” stands for low lead, 100LL contains up to 0.56 g/L lead (Royal Dutch Shell, 2010). Some studies indicate that air lead levels near airports with substantial avgas use are significantly higher than background levels (U.S. EPA, 2010; Miranda et al., 2011).

Both deltas hold a breeding population of Audouin's gull. In the Ebro Delta this species bred for first time in 1981, increasing rapidly their numbers and at present it accounts 11,000 pairs (approximately 60% of the world population) (Oro and Ruxton, 2001; Oro et al., 2010). In the case of the Llobregat Delta, there is a small colony recently established in 2009, reaching in 2011 a population of 380 pairs (Gutiérrez and Lopez, 2012). Due to its mainly piscivorous habits, chick feathers of Audouin's gull can be particularly appropriate to monitorize trace elements levels in these important coastal areas for biodiversity (Goutner et al., 2000; Sanpera et al., 2007a). Moreover, at least in the Ebro Delta these gulls can also take advantage of fresh water resources from neighboring rice fields (Navarro et al., 2010). In this way, using biogeochemical markers as the stable isotopes analysis (SIA) in feathers (mainly carbon or $\delta^{13}\text{C}$ and nitrogen or $\delta^{15}\text{N}$) we can perform better insights into the habitat use and foraging ecology of the chicks (Cherel et al., 2000; Cotin et al., 2012). The combination of these biomarkers (especially the nitrogen isotope, a marker of the trophic level) with heavy metal concentrations enable to check biomagnification processes (Bearhop et al., 2000; Cotin et al., 2011).

In this study we analyzed Audouin's gull chick feathers to assess the carbon and nitrogen stable isotope ratios (as a tracer of the

chick's diet) and heavy metal levels (mercury and lead) in gulls from both localities. The main hypothesis is that mercury concentration must be higher at the Ebro Delta due to the influence of the toxic muds upstream. In the case of the Llobregat Delta, we expect to find higher lead levels in this locality due to the use of aviation fuel at the nearby Barcelona airport. The comparison of both populations of Audouin's gull will allow us to evaluate the exposure risk to which these areas are subject, and detect if there have been changes in mercury levels in recent years using previous data in the case of the Ebro Delta (Sanpera et al., 2007a). Additionally, isotope ratios and metal levels were also analyzed in order to check for potential diet association with the pollutant accumulation. Finally, our study constitutes the first approach to the foraging ecology and pollutant levels for the recently established Audouin's gull colony at the Llobregat Delta.

2. Material and methods

2.1. Sampling

During the 2011 breeding season, fledging chicks of Audouin's gulls were sampled at two different locations in NE Iberian Peninsula: the Ebro Delta ($N = 16$) and Llobregat Delta ($N = 13$). Sampling was carried out taking advantage of the chick ringing in both colonies (when they were 2–3 weeks old). From each chick, a bunch of mantle feathers were taken, avoiding pseudo-replication by sampling only one fledging per brood. Feather samples were kept in polyethylene bags and frozen until analysis. Once in the laboratory, feathers were cleaned with 0.25 M NaOH solution and dried for 24 h at 60 °C prior to heavy metal determination and SIA. Once cleaned and dry, in order to homogenize them, feathers were ground to powder using an impactor mill (Freezer Mill 6750, Spex CertiPrepH Inc., Metuchen, NJ, USA) operating at liquid nitrogen temperature.

Potential preys were also sampled for isotopic determination based on previous information from Audouin's gull diet at the Ebro Delta. Prey items from ricefields included American crayfish *Procambarus clarkii* ($N = 15$), common carp *Cyprinus carpio* ($N = 10$) and gambusia *Gambusia affinis* ($N = 10$). Items from fishing discards (the most important marine source, Oro et al., 1997) were classified into two groups: clupeiform fish (sardine *Sardina pilchardus* and anchovy *Engraulis encrasicolus*, $N = 20$) and perciform fish (mackerel *Trachurus trachurus* and bogue *Boops boops*, $N = 20$). These two fish orders represent the most important ones for Audouin's gull diet (Pedrocchi et al., 2002). The potential preys were treated whole and subjected to lipid extraction by successive rinsing in a chloroform and methanol solution (2:1), following the protocol of Folch et al. (1957), in order to reduce variability due to differences in lipid content among tissues (DeNiro and Epstein, 1981). All of the prey samples were oven-dried at 60 °C before being mechanically ground into a fine powder.

2.2. Stable isotopes analysis

Feather (ca. 0.32–0.35 mg) and preys (ca. 0.5 mg) subsamples were placed into tin buckets and crimped for combustion for stable carbon and nitrogen determination. Isotopic analyses were carried out at the Scientific and Technical Services of the Universitat de Barcelona (Spain) by means of a Thermo-Finnigan Flash 1112 elemental analyser (CE Elantech, Lakewood, NJ, USA) coupled to a Delta-C isotope ratio mass spectrometer via a CONFLO III interface (Thermo Finnigan MAT, Bremen, Germany). The laboratory applies international standards, which are run for every 12 samples: IAEA CH₇ (87% of C), IAEA CH₆ (42% of C) and USGS 24 (100% of C) for ^{13}C and IAEA N₁ and IAEA N₂ (with 21% of N) and IAEA NO₃ (13.8% of N)

for ^{15}N . Stable isotope ratios were expressed in the standard δ notation relative to Vienna Pee Dee Belemnite ($\delta^{13}\text{C}$) and atmospheric N_2 ($\delta^{15}\text{N}$). Replicate assays of standards indicated analytical measurement errors of $\pm 0.1\text{‰}$ and $\pm 0.2\text{‰}$ for $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$, respectively.

2.3. Trace elements analysis

The analysis of trace elements (Hg and Pb) in feathers was done according to the following acid digestion protocol: 0.1 g of the sample was digested with 2 ml of HNO_3 (70%) and 1 ml of H_2O_2 (30%) using a Teflon reactor for 12 h at 90 °C. The digested product was then diluted in 15 ml of distilled water, and the determination of trace elements was carried out with an ICP-MS (Induction Coupled Plasma-Mass Spectrometer) Perkin Elmer Optima 6000. The accuracy of the analysis was checked by measuring CRM certified reference tissue (Human Hair CRM 397). The results for Hg and Pb gave a mean recovery of 101.9% and 106.3%, respectively. No corrections were applied to the original results.

2.4. Statistical analysis

Values of trace elements concentrations and stable isotope ratios were routinely checked for normal distributions with Q-Q plots. Trace elements concentrations showed skewed distributions which were normalized by applying a logarithmic transformation.

Pearson's correlation coefficient was used to test if there were relationships among the different variables of the study. ANCOVA analysis was performed in order to evaluate if the relation $\delta^{13}\text{C}$ – $\delta^{15}\text{N}$ changes between localities. A similar analysis was used for modeling heavy metal concentration using stable isotope and locality as explanatory factors. If interaction term was not significant, it was excluded from the model. Bivariate plots were used to represent the stable isotopes and heavy metal data. In the case of potential preys, they were represented by adding an isotopic discrimination factor of 2.91‰ for $\delta^{15}\text{N}$ and 1.15‰ for $\delta^{13}\text{C}$ following Caut et al., 2009. Statistical analyses were done using SPSS 19.0 (SPSS Inc., Chicago, USA).

3. Results

3.1. Stable isotopes

Most Audouin's gull chicks from both populations showed clearly a marine diet, with characteristic enriched carbon values and depleted nitrogen ones compared to ricefield resources

(Fig. 2a). However, in the case of the Ebro Delta, two chicks presented a diet including a higher proportion of ricefield resources, so these individuals were removed from subsequent analysis to reduce variability. Fig. 2b shows a detailed scatter plot only for the isotopic values of the marine-fed chicks. Taking into account both sampled colonies, a significant positive correlation between $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ was found ($r = 0.50$, $p = 0.01$, $N = 27$) (Fig. 2b). An ANCOVA analysis showed that this relationship do not vary between localities (interaction term $F_{1,23} = 0.96$, $p = 0.34$). Nevertheless mean isotopic signatures show significant shift between localities. Ebro Delta chicks presented lower $\delta^{15}\text{N}$ values ($F_{1,25} = 18.44$, $p < 0.001$) and lower $\delta^{13}\text{C}$ ones ($F_{1,25} = 5.07$, $p = 0.03$) than Llobregat Delta fledglings. Descriptive statistics for the stable carbon and nitrogen ratios of chick feathers are shown in Table 1

3.2. Trace elements

Bivariate plot of mercury and lead concentrations between localities (in a logarithmic scale) are shown in Fig. 3.

Regarding Hg concentration, adjusted ANCOVA with the $\delta^{15}\text{N}$ and the locality factor showed that interaction term was not significant ($F_{1,23} = 0.26$, $p = 0.88$) and not significant relationship with nitrogen signatures was found ($F = 0.37$, $p = 0.55$). Final model only showed differences between localities ($F = 23.25$, $p < 0.001$), presenting the Ebro Delta higher concentrations. Adjusting a new model for mercury with the $\delta^{13}\text{C}$, the interaction term was also not significant ($F = 0.26$, $p = 0.62$) and not significant relationship with carbon signatures was found ($F = 0.12$, $p = 0.74$). Thus, only differences between localities arise.

Similar results were found in the case of Pb. Adjusting the model with the $\delta^{15}\text{N}$ and the locality, the interaction term was not significant ($F_{1,23} = 2.15$, $p = 0.16$) and not significant relationship with nitrogen signatures was found ($F = 1.4$, $p = 0.25$). In a new model for lead adjusting for the $\delta^{13}\text{C}$, the interaction term was not significant ($F = 0.22$, $p = 0.62$) and not significant relationship with carbon signatures was found ($F = 0.43$, $p = 0.52$). Final model only showed differences between localities ($F = 125.6$, $p < 0.001$).

4. Discussion

Audouin's gull has been traditionally widely studied in ecological and pollution works (Pedrocchi et al., 1996, 2002; Sanpera et al., 2007a,b). This allows a better interpretation of results, and its comparison with previous data to detect temporal changes.

Regarding stable isotopes, our data show that chicks from both localities are fed mainly with marine piscivorous resources (Fig. 2).

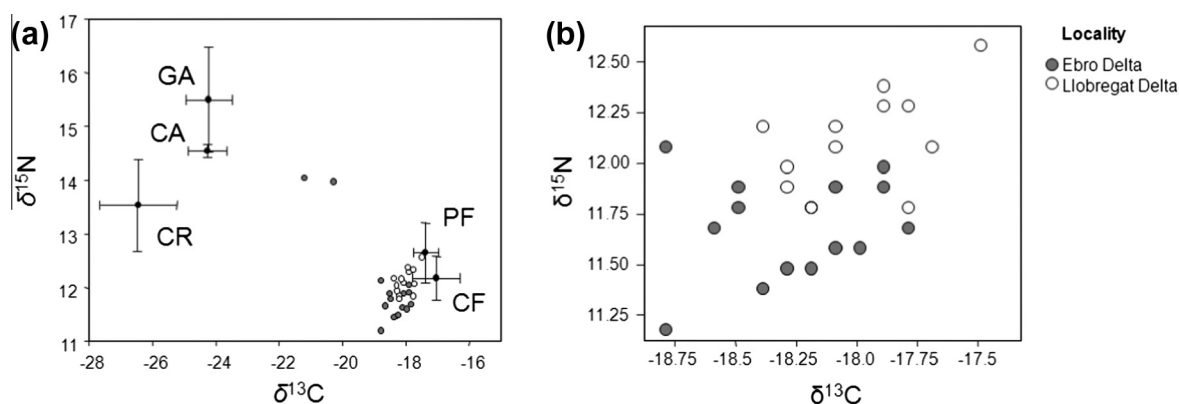


Fig. 2. (a) Scatter plot of the dispersion between $\delta^{13}\text{C}/\delta^{15}\text{N}$ values of Audouin's gull chicks from both localities. The mean and standard deviation of American crayfish (CR), common carp (CA) and gambusia (GA) are represented as potential preys from rice fields. Perciform fish (PF) and clupeiform fish (CF) are also shown as potential preys from the sea. (b) Detailed scatter plot of the dispersion between $\delta^{13}\text{C}/\delta^{15}\text{N}$ values of marine-fed Audouin's gull chicks.

Table 1

Descriptive statistics for the stable isotopes and heavy metal concentrations in marine-fed Audouin's gull chick feathers. Ebro Delta: $N = 14$, Llobregat Delta: $N = 13$.

	Locality	Mean	SD	Minimum	Maximum
$\delta^{15}\text{N}$ (‰)	Ebro Delta	11.70	0.25	11.20	12.10
	Llobregat Delta	12.12	0.25	11.80	12.60
$\delta^{13}\text{C}$ (‰)	Ebro Delta	-18.28	0.33	-18.80	-17.80
	Llobregat Delta	-18.02	0.27	-18.40	-17.50
[Hg] (ng g ⁻¹)	Ebro Delta	4868.55	1476.64	2438.68	7333.18
	Llobregat Delta	2053.74	759.89	644.75	3006.65
[Pb] (ng g ⁻¹)	Ebro Delta	298.35	65.40	185.90	383.27
	Llobregat Delta	1364.68	518.07	734.23	2139.66

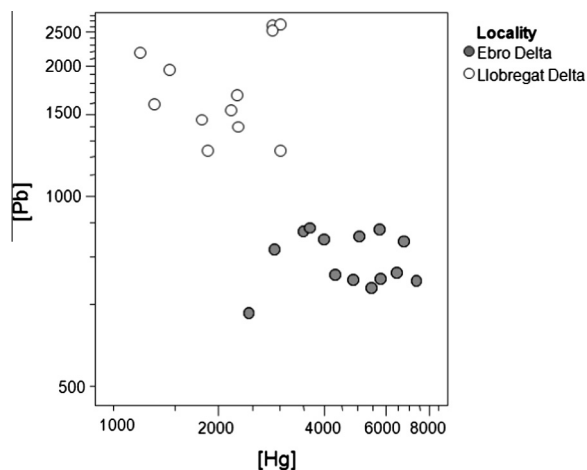


Fig. 3. Scatter plot of the dispersion between the concentrations of lead and mercury (in ng g⁻¹) of Audouin's gull chick feathers from both localities.

In the case of the Ebro Delta, a few chicks showed depleted carbon and enriched nitrogen signatures, indicating some contribution of rice field resources. Although the isotope data of Navarro et al. (2010) estimated that rice field consumption by the chicks was between 19 and 32%, the data of this study are more in agreement with those of Pedrocchi et al. (2002) and Sanpera et al. (2007a) reporting that most of this gull's diet consists of marine fish. In the case of Llobregat Delta there were no previous data about this newly formed colony. We have found a fully marine diet for these chicks, probably due to the lack of surrounding rice fields in the area unlike in the Ebro Delta. Marine-fed chicks from both localities presented small significant isotopic differences that could be attributed to differences in fishing activities that could influence isotopic signatures. In fact, the fishing fleet of the Llobregat Delta area consists mainly in trawlers and demersal longliners. However, in addition to a strong trawling fleet, the Ebro Delta area also holds a higher proportion of purse seiners fishing on clupeiforms (Arcos et al., 2009).

Given the dependence on marine resources for Audouin's gull chicks' diet, fledglings of this species seem a good candidate to monitorize both coastal areas. As we initially postulated, we have found significant higher mercury levels in the Ebro Delta than in Llobregat Delta. Indeed, although the Mediterranean Sea is already a polluted area with quite high concentrations of mercury (Cossa et al., 1997), the main cause of these very high levels in the Ebro Delta must be sought in the pollutants carried by the river waters. The influence of the toxic sediments accumulated at Flix reservoir on mercury levels has even been noticeable on coastal birds some 90 km downstream (Cotin et al., 2011, 2012). The high mercury

levels accumulated in these sediments are discharged to the sea, then converted to methylmercury and finally enter in the food web (Bryan et al., 1979). Since methylation processes are especially important in mesopelagic waters, the significant trawling activity in the Ebro Delta area has been put forward as a very important source of mercury pollution for seabirds (Arcos et al., 2002). In this way, the conjunction of these polluted waters besides the trawling activity makes that seabird species foraging efficiently on discards (as Audouin's gull) reach important mercury levels. In many bird species, mercury concentrations in feathers above 5000 ng g⁻¹ are associated with adverse reproductive effects such as lower clutch and egg size, a lower hatching success and a decreased chick survival (Eisler, 1987). All Llobregat Delta chicks are below this threshold. However, the mean concentration detected in the Ebro Delta is dangerously close to 5000 ng g⁻¹. In fact, the 50% of the marine-fed chicks in this area present values above this threshold and are therefore susceptible to the sublethal effects described. The mean concentration ranged from 2.5 to 5 times that found in Audouin's gull chick feathers from Northeastern Mediterranean (Goutner et al., 2000). Moreover, the levels found in our work at the Ebro Delta are very similar to those reported 7 years before in chicks of the same species in the same area (Sanpera et al., 2007a), which means that this important mercury inputs discharged by the river have remained stable throughout time. However, no case of adverse reproductive effects associated with high mercury levels in eggs was observed in previous studies (Morera et al., 1997). At the time this paper was written, the works to remove the toxic muds at Flix had started. It would be important to use biological sentinels as Audouin's gulls to monitorize the evolution of pollutant levels during and after removal works. Also, further research should be needed on how this mercury accumulation affects reproductive and physiological parameters in this near threatened seabird.

On the other hand, lead concentrations detected in our study showed to be significantly higher in the Llobregat Delta. Since the most part of this heavy metal present in the feathers came from atmospheric deposition, the atmospheric lead levels from both areas can give us key clues. In fact, lead atmospheric concentration is much higher in the Llobregat Delta area (25.1 µg m⁻³) than in the Ebro Delta (2.5 µg m⁻³) (IDESCAT, 2011). As mentioned above, this situation could be a consequence of the vicinity of the Barcelona airport to the Llobregat Delta colony (just 2 km). The use of the leaded fuel avgas by aircrafts probably represents an important input of lead to the surrounding atmosphere. In the United States, for example, lead emitted from aircraft using avgas is currently the largest source of lead to the air, constituting about 50% of lead emissions in 2005 (U.S. EPA, 2010). In a study on childhood blood by Miranda et al. (2011), lead levels decreased as we move away from the airport, with the largest impact on children living within 500 m. In birds, it has been suggested that lead levels in feathers above 4000 ng g⁻¹ are associated with delayed parental and sibling recognition, impaired locomotion, depth perception and feeding behavior, and lowered chick survival in gulls (reviewed in Burger and Gochfeld, 2000). In any case this threshold is exceeded, being all the chicks far from this limit and apparently safe from lead diseases. However, the Ebro Delta has traditionally suffered a very important input of lead derived from hunting activities (Mañosa et al., 2001), with a lead pellet concentration ranging from <8900 to 2,661,000 pellets ha⁻¹ in the first 20 cm of sediment. Further studies would be desirable on lead levels from other tissues susceptible to accumulate environmental lead.

Although mercury has a well known biomagnification process in living systems, we have not detected a significant relationship between heavy metal levels and the nitrogen isotope (indicating no relation with the trophic level). However, this lack or

relationship may be caused by the narrow range of isotopic variation found in both localities.

In conclusion, chicks from both areas present mainly a marine diet, which makes them especially suitable to monitorize these coastal areas. Lead levels are significantly higher in the Llobregat Delta probably due to the use of leaded fuel in the nearby Barcelona airport. On the other side, mercury concentrations were much higher in the Ebro Delta, in relation with the disposal of the toxic sediments of the Flix site carried down by the Ebro river. These mercury levels in the Ebro chicks reached values that have been described as toxic.

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Levels of antioxidants in breeding female Audouin's gulls and their deposition in eggs across different environments



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Yolk

ABSTRACT

Diet quality and dietary antioxidants are interrelated factors that influence many animal traits. However, little is known about this relationship in wild birds, especially how it may affect the deposition of antioxidants from the female to the eggs over the laying sequence. In this context, albumen has been far less studied than yolk due to its lack of carotenoids, which are a common focus of dietary antioxidant research. Our study was conducted in the Audouin's Gull (*Larus audouinii*), a seabird that shows a great dependence on trawl discards, although in the Ebro Delta also exploits resources from neighbouring rice fields, especially the American crayfish (*Procambarus clarkii*). We examined the relationship between diet (traced through stable isotope analysis) and the antioxidant capacity (not only carotenoids, but also other non-enzymatic antioxidants, like tocopherols and retinol) of plasma in females from two breeding groups and their clutches. In the eggs we analysed antioxidants in both albumen (hydrophilic antioxidants) and yolk (hydrophilic and lipophilic antioxidants) fractions, taking into account the laying sequence. A decrease in the antioxidant capacity of female plasma was found over the incubation period. We found little difference between groups in the antioxidant deposition pattern in the yolk and whole egg over the laying sequence, but a greater variation was observed in the intra-clutch patterns of albumen antioxidant capacity, probably related to quality differences. When taking into account total antioxidant deposition in the clutch, a declining tendency over the laying sequence was found. Both yolk lipophilic and whole egg antioxidant capacity were negatively related to $\delta^{15}\text{N}$, indicating that marine fish diets (with depleted $\delta^{15}\text{N}$ values) contain a higher amount of antioxidants than rice field prey.

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1. Introduction

Diet quality and dietary antioxidants are interrelated factors that influence many life-history traits of animals (reviewed in Catoni et al., 2008). Indeed, the diet is a source of compounds with antioxidant properties that animals can only acquire from food because they are unable to synthesise them de novo (Cohen et al., 2009). Dietary antioxidants may help protect the organism from oxidative stress arising from oxidative damage to biomolecules (Costantini and Verhulst, 2009; Halliwell and Gutteridge, 2007; Sies, 1991). This is a very important function because accumulation of such damage is thought to contribute to cellular senescence, loss of organ performance and Darwinian fitness (Costantini, 2008; Kirkwood, 2005). However, the antioxidant machinery is particularly complex and, beyond dietary antioxidants, includes

many important endogenous molecules, like antioxidant enzymes or repair systems (Pamplona and Costantini, 2011).

Dietary antioxidants can be classified as hydrophilic (e.g. vitamin C and polyphenols) and lipophilic (e.g. vitamin E and carotenoids), according to their chemistry. The mechanisms of action among different classes of antioxidants vary, but their ultimate function is the same, i.e. to neutralise the damaging action of free radicals (Pamplona and Costantini, 2011). In addition, the diet contributes other substances that do not have antioxidant properties but that affect oxidative balance. For example, the polyunsaturated fatty acids in food are also important because they are substrates for lipid peroxidation (Hulbert, 2005).

The antioxidant content of the diet also influences traits other than oxidative stress, including sexual ornamentation, fertility and fecundity, and brood growth (Biswas et al., 2007; Blount et al., 2002; de Ayala et al., 2006; McGraw, 2006). Dietary antioxidants are therefore considered important for reproduction, which represents one of the most demanding periods of animal life (Nager, 2006; Speakman, 2008), as well as for the growth and survival of progeny (Mousseau and Fox, 1998). Since these antioxidants are present in limited amounts, their deposition in the egg may reduce the capacity of the laying female to cope

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with reactive oxygen species, giving rise to trade-offs between self-maintenance and reproductive investment (Costantini, 2010a). Knowing how diet quality relates to the circulating non-enzymatic antioxidant levels in the breeding female and the patterns of allocation over the laying sequence will provide a better insight into how this trade-off influences life-history strategies.

Only a few experimental studies have looked at the link between diet and circulating/deposited antioxidants in wild birds (e.g. Beaulieu et al., 2010; Hipfner et al., 2010a,b). For example, it has been shown that lower-trophic-level feeding is linked to the production of carotenoid-rich yolks (Hipfner et al., 2010a,b). However, recent studies have shown that carotenoids offer limited protection against oxidative stress (Costantini and Møller, 2008; Isaksson and Andersson, 2008; Simons et al., 2012), highlighting the importance of looking at other egg dietary antioxidants as well. In eggs the deposition of antioxidants in albumen has received very little attention compared to their deposition in yolk (Blount et al., 2002, 2004), despite the important role of albumen in hatching size and offspring development, behaviour and viability (Bonisoli Alquati et al., 2007; Ferrari et al., 2006). Therefore, there is an important need to study the relationships between diet quality and non-enzymatic antioxidant capacity (not only carotenoids) in breeding females and the albumen and yolk fractions of eggs.

To address these issues, we carried out a field study on breeding Audouin's Gulls (*Larus audouinii*), using stable isotope analysis (carbon and nitrogen isotopes) as an integrative tool for tracing the diet of gulls in the wild (Inger and Bearhop, 2008; Kelly, 2000).

The Audouin's Gull is a medium-sized migratory species categorised as "Near Threatened" at a global level by the International Union for Conservation of Nature (IUCN) (BirdLife International, 2012). About two thirds of the world's Audouin's Gull breeding population nest in the Ebro Delta Natural Park, NE Spain (Arcos et al., 2009). Although originally described as a nocturnal predator of pelagic fish, especially clupeids (BWPI, 2004), the species shows a great dependence on human trawling activity in the study area, scavenging around fishing vessels and using discards very efficiently (Oro and Ruiz, 1997; Oro et al., 1996a; Ruiz et al., 1996). However, Audouin's Gulls in the Ebro Delta also exploit resources from neighbouring rice fields, where their main prey is the American crayfish (*Procambarus clarkii*). This invasive species seems to become a more important food resource for gulls during trawling moratorium periods (Oro et al., 1996b, 1997). The isotopic characterisation of the different habitats in the Ebro Delta has been extensively documented, and it is well known that rice field prey present enriched $\delta^{15}\text{N}$ signatures and depleted $\delta^{13}\text{C}$ values compared to marine prey (Cotin et al., 2011; García-Tarrasón et al., 2013).

Considering all the above, our main objectives were: (1) to find out the relevance of the main foraging habitats exploited by this species – marine or rice field – (Navarro et al., 2010) on the circulating antioxidant capacity of breeding females (measured in the plasma of incubating females) and their clutch (measured in the albumen and yolk of a three-egg clutch), and (2) to test the laying order effect on the antioxidant deposition, which could indicate a trade-off between antioxidant availability for females and their eggs. In fact, the laying order has been shown to be a very important factor in avian species with a brood reduction strategy such as gulls (Lack, 1954; Ramírez et al., 2011; Rubolini et al., 2011). We hypothesized that (1) the natural feeding resource (marine fish) would be better than the introduced American crayfish, and (2) an important laying order effect would be detected in the egg antioxidant deposition because of a limited availability of antioxidant molecules.

2. Material and methods

2.1. Study area and sampling

The study was carried out during the 2010 breeding season at the Punta de la Banya (40° 40'N, 0° 45'E), a protected sandy peninsula

with salt pans in the Ebro Delta Natural Park (NE Spain). Audouin's Gulls nest there in discontinuous groups in natural coastal dunes with vegetation and in salt pans subject to human activity. Two breeding groups (2.5 km apart) of similar age distribution (from plastic ring readings) were selected for comparison: one in the dunes (DG) and another in the salt pans (SG). Besides habitat, they differed markedly in parameters such as the number of pairs, population density and reproductive success (see Supplementary material, Table S1).

During the peak egg-laying period (late April), the nests were tagged when the first egg was laid. Inspections were conducted every two days until clutch completion to establish laying order and the final clutch size. Ten three-egg clutches were selected from each group. Freshly laid eggs were sampled and replaced with dummy eggs. Eggs were marked, placed under refrigeration and transported to the laboratory. Twelve females from these nests (six from each group) were captured with nest traps during the incubation period and measured (weight, tarsus length and skull length – the distance from the supraoccipital to the bill tip). Blood samples (2 mL) were also collected from the brachial vein; 0.5 mL was preserved in a neutral vial for molecular identification of sex and the rest was placed in heparinised vials. Blood samples were kept at +4 °C until centrifugation. Sex of gulls was determined using polymerase chain reaction (PCR) amplification of the CHD genes (Ellegren, 1996; Griffiths et al., 1998).

2.2. Ethical note

This study was authorized by the Autonomous Government of Catalonia (*Departament de Medi Ambient i Habitatge – Generalitat de Catalunya*), as well as by the wildlife personnel of the Ebro Delta Natural Park. Both agreed with the protocol of handling and sampling of Audouin's gulls.

2.3. Laboratory procedures

Eggs were opened and the albumen was separated from the yolk. Each fraction was weighed, homogenised and divided into subsamples prior to storage. Albumen subsamples were frozen at –20 °C and yolk subsamples at –80 °C.

The heparinised vials were centrifuged (2000 rpm for 10 min) within 6 h of collection. The supernatant plasma was pipetted off and stored in 250- μL aliquots at –80 °C.

2.4. Stable isotope analysis

Albumen, yolk and plasma samples were lyophilised to dryness and ground to powder. In the case of yolk, samples were lipid-extracted following the Folch method (Folch et al., 1957) to remove lipid influence on carbon isotope ratios. Subsamples (0.3 mg plasma or 0.35–0.38 mg albumen and yolk) were placed in tin capsules for stable carbon and nitrogen isotope ratio determination. Isotopic analyses were carried out at the Scientific and Technical Services of the Universitat de Barcelona (Spain) by means of a Thermo-Finnigan Flash 1112 elemental analyser (CE Elantech, Lakewood, NJ, USA) coupled to a Delta-C isotope ratio mass spectrometer via a CONFLIOIII interface (Thermo Finnigan MAT, Bremen, Germany), with IAEA standards being applied every 12 samples to calibrate the system. Stable isotope ratios were expressed in the standard δ notation relative to Vienna Pee Dee Belemnite ($\delta^{13}\text{C}$) and atmospheric N_2 ($\delta^{15}\text{N}$). Replicate assays of standards indicated analytical measurement errors of $\pm 0.1\text{‰}$ and $\pm 0.2\text{‰}$ for $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$, respectively.

2.5. Assays of oxidative damage and non-enzymatic antioxidant capacity

Analyses were performed at the Institute of Biodiversity, Animal Health and Comparative Medicine at the University of Glasgow (UK).

Aliquot samples of both plasma and eggs were used, although only eleven individual females were available.

2.5.1. Non-enzymatic antioxidant capacity of plasma, albumen and yolk

The non-enzymatic antioxidant capacity was measured by colorimetric determination using the OXY-Adsorbent test (Diacron International, Grosseto, Italy). The antioxidant capacity was quantified as the ability of the lipophilic and hydrophilic antioxidants present in the biological fraction to cope with the oxidant action of hypochlorous acid (HOCl; an oxidant of pathological relevance in biological systems). The procedure for plasma and albumin was carried out in accordance with previous studies (e.g. Costantini, 2010a; Costantini et al., 2011). For the whole (yolkOXY) and the lipophilic (yolk lipOXY) yolk antioxidant capacity, we followed the procedure as in Costantini (2010a) with slight modifications (e.g. the yolk sample was first mixed with 0.1 ml 5% NaCl solution). The hydrophilic antioxidant capacity of yolk (yolk hydrOXY) was calculated as “whole yolkOXY” – “yolk lipOXY”. The extraction efficiency was higher than 95%. By multiplying the three antioxidant concentrations by their corresponding egg fraction mass (albumen/yolk) we obtained the corresponding total amounts (expressed as mmol of neutralised HOCl). By summing up these three quantities, we estimated the total antioxidant capacity for each egg (TOTAL Egg OXY).

The absorbance was always read with a spectrophotometer at a wavelength of 490 nm. Analyses were run in duplicate and the mean coefficients of variation were 4.4% for plasmaOXY, 6.3% for albumOXY, and 3.3% for yolkOXY. Measurements are expressed as mM of HOCl neutralised for plasmaOXY and albumOXY, and as μmol of HOCl neutralised per mg of yolk for yolkOXY.

2.5.2. Plasma oxidative damage compounds in females

Plasma reactive oxidative metabolites (plasmaROMs) were quantified as a measure of compounds causing oxidative damage by colorimetric determination using the d-ROMs test (Diacron International, Grosseto, Italy). Reactive oxygen metabolites (primarily hydroperoxides, ROOH) are mainly generated by the oxidation of lipids and proteins. The procedure was carried out as in previous studies (e.g. Costantini et al., 2011). The absorbance was read by means of a Thermo Scientific Multiskan Spectrum spectrophotometer (Thermo Fisher, Vantaa, Finland) at a wavelength of 505 nm. Analyses were run in duplicate and the mean coefficient of variation was 6.7%. Measurements are expressed as mM of H_2O_2 equivalents.

2.6. Statistical analysis

In the eggs, concentrations of the three antioxidant fractions (albumen, and lipophilic and hydrophilic fractions of the yolk) in addition to the total antioxidant capacity of the egg were analysed using generalised linear mixed models (GLMMs) with an identity link (Zuur et al., 2007). The clutch (nest) was included as a random factor, and laying order (a-, b-, and c-eggs) and breeding group (salt pan or dunes) as fixed factors. Values of $\delta^{15}\text{N}$, $\delta^{13}\text{C}$ were also included as covariates. A hierarchical backward model selection was used, starting with a full model including all fixed factors and covariates, the interaction between laying order and group and the interaction between both isotopes. When an interaction factor was significant, a separate analysis for each factor level was carried out. When significant, post-hoc comparisons within the laying order were made using the Šidák correction for multiplicity. Distributional assumptions about data have been checked through graphical residual analysis being symmetric and homoscedastic in all fitted models.

Differences between females from both groups were checked using an unpaired *t*-test for the following variables: mean clutch volume, body condition (BC) index, plasma $\delta^{13}\text{C}$, plasma $\delta^{15}\text{N}$, plasmaROMs and plasmaOXY. The BC index was calculated as the residuals of the first principal component from a Principal Component Analysis

including the weight, tarsus length and skull length. ANCOVA analysis (Zar, 2009) was performed to compare plasma antioxidant capacity between females from both groups, adjusting for “days of incubation”. A partial correlation was also performed between stable isotopes and plasma measurements controlling for the “days of incubation”. To assess the presence of monotonic relationships between the plasma measurements (plasmaOXY and plasmaROMs), stable isotopes ($\delta^{13}\text{C}$, $\delta^{15}\text{N}$), BC and “days of incubation” (the number of days from clutch completion to the date of capture and bleeding), we used Spearman’s rank correlation coefficient.

Statistical analyses were performed using SPSS 18.0 software (SPSS Inc., Chicago, IL, USA).

3. Results

3.1. Intr clutch deposition pattern

For albumOXY, the best model (AICC = 698.6) showed a significant interaction between group and laying order ($F_{2,60} = 4.82$, $p = 0.01$), so each group was analysed separately. In the SG, the final model included only the laying order ($F_{2,20} = 4.49$, $p = 0.03$), indicating a higher concentration in the a-egg compared to the c-egg, but the differences a–b and b–c were not significant. For the DG, none of the factors were significant (Table 1, Fig. 1).

For the yolk lipOXY, the selected model (AICC = 257.4) included only $\delta^{15}\text{N}$ ($F_{1,59} = 4.62$, $p = 0.036$). The estimated parameter showed a negative relationship with $\delta^{15}\text{N}$ (Table 1, Fig. 1).

When analysing yolk hydrOXY, the final model (AICC = 284.8) only included the laying order effect ($F_{2,40} = 5.02$, $p = 0.011$), and the concentration was higher in the a-egg than the c-egg ($t = 3.17$, $p = 0.03$); the b-egg showed intermediate values but was not significantly different from the a- and c-eggs (Table 1, Fig. 1).

Finally, for the TOTAL eggOXY, the final model selected (AICC = 643.5) included laying order ($F_{2,37} = 21.44$, $p < 0.001$) and $\delta^{15}\text{N}$ ($F_{1,53} = 2.51$, $p = 0.12$). Although not significant, removing $\delta^{15}\text{N}$ from the model induced a substantial change in the estimated parameters of the remaining factors. The estimated parameter showed that the total antioxidant capacity of the c-egg was significantly lower compared to the a- and b-eggs ($p < 0.001$) (Table 1, Fig. 2A).

It is worth noting that although the clutch (nest) was always included in the model as a random factor to take into account the dependence among eggs from the same clutch, its effect was never significant (*p* values ranging between 0.07 and 0.4). Detailed information about egg antioxidant capacity and stable isotopes according to laying order is shown in the Supplementary material, Table S3.

3.2. Status of females

Females from the two groups did not present any significant differences in mean clutch volume, BC index, diet (plasma $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$), plasmaROMs or plasmaOXY, with *p*-values ranging from $p = 0.15$ to $p = 0.74$ (see Supplementary material, Table S2), and variances being homogeneous for all variables. The relationship between “days of incubation” and plasmaOXY was negative and significant ($r = -0.68$, $p = 0.02$; Fig. 3A). ANCOVA analysis comparing the plasma antioxidant capacity of the two groups, adjusting by “days of incubation”, did not find differences for plasmaOXY ($F_{1,9} = 0.001$, $p = 0.99$). The relationship between “days of incubation” and plasmaROMs was also negative but not significant in this case ($r = -0.47$, $p = 0.14$). On the other hand, plasmaOXY and plasmaROMs showed a significant positive correlation ($r = 0.67$, $p = 0.02$; Fig. 3B). The rest of the correlations were not significant (*p*-values higher than 0.21, correlation coefficients lower than 0.41).

Table 1Parameter estimate $\pm$ standard error for the selected final GLMMs in egg antioxidant measures. c-egg was used as a reference level in the analysis.

	AlbumOXY		Yolk lipOXY	Yolk hydrOXY	Total eggOXY
	Salt pan group	Dune group			
Intercept	382.5 $\pm$ 21.4	444.4 $\pm$ 13.0	38.9 $\pm$ 10.2	17.9 $\pm$ 0.55	901.66 $\pm$ 212.09
a-egg	77.4 $\pm$ 28.5			2.1 $\pm$ 0.7	128.26 $\pm$ 20.03
b-egg	70.0 $\pm$ 28.5			1.1 $\pm$ 0.7	91.43 $\pm$ 20.13
c-egg	Ref.			Ref.	Ref.
$\delta^{15}\text{N}$			−1.9 $\pm$ 0.9		−29.72 $\pm$ 18.76
Nest*	513.3 $\pm$ 938.9	5064.5 $\pm$ 1307.6	1.3 $\pm$ 0.7	1.5 $\pm$ 1.0	599.9 $\pm$ 714.0
Residual	4064.5 $\pm$ 1285.3		2.8 $\pm$ 0.6	4.4 $\pm$ 1.0	3801 $\pm$ 894.3

Note. Asterisk (*) indicates estimated variances. Due to significant interaction between group and laying order in the antioxidant capacity of the albumen, each group was analysed separately.

4. Discussion

The influence of nutrition on the oxidative status of birds (Blount et al., 2003) can be explored by looking at the relationship between OXY variables and the stable isotope signature of tissues ($\delta^{15}\text{N}$, $\delta^{13}\text{C}$). However, our data on Adouin's gull eggs did not indicate a relationship between the $\delta^{13}\text{C}$ signature, which provides information about the carbon sources used by consumers, and the OXY variables. This result is in agreement with Hipfner et al. (2010a), who did not find a relationship between yolk carotenoid levels and $\delta^{13}\text{C}$ values in other seabird species. Similarly, Oppel et al. (2010) suggested that in the King eider (*Somateria spectabilis*), while the nitrogen deposited in the egg comes mainly from diet, carbon (especially in the yolk) has a more endogenous origin. This is also supported in our results by the strong relationship between plasma and mean egg $\delta^{15}\text{N}$ ($r = 0.57$, $p = 0.06$ for albumen; $r = 0.53$, $p = 0.08$ for yolk; $N = 12$) but not for $\delta^{13}\text{C}$ ($r = -0.35$, $p = 0.26$ for albumen; $r = -0.02$, $p = 0.96$ for yolk; $N = 12$).

In the case of albumen antioxidant concentration, there was a significant interaction between the breeding group and laying order: in the salt pan group there was a lower investment of albumen antioxidants in the third egg, while the dune group showed no variation over the laying sequence. Why did intraclutch antioxidant deposition patterns differ between salt pan and dune gulls? Since there seemed to be no obvious differences between the females in either location (Supplementary material, Table S2), other, ecological, factors could be responsible. In fact, the dune group had a lower breeding success and lower nest density (Supplementary material, Table S1), indicators of a poor-quality nesting site. Different antioxidant investment patterns between the two groups may relate to the “brood reduction” strategy typical of gulls (Lack, 1954). In low nest densities or sub-optimal situations, females can probably modulate albumen contribution (and molecules therein such as antioxidants) by increasing investment in the final eggs, thus enhancing nestling growth and survival, and reducing hatching time (Ferrari et al., 2006). However, other studies have shown a complex relationship between albumen variation and offspring development and viability (Bonisoli Alquati et al., 2007), as well as the

determination of embryo sex in larger c-eggs (Rubolini et al., 2009), so further research on how females modulate albumen contribution is required.

On the other hand, yolk accounted for the majority of the overall antioxidant capacity of the egg (Fig. 2B). Concentration of hydrophilic antioxidants in the yolk (hydrOXY) and the overall concentration of antioxidants in the yolk (lipOXY + hydrOXY) followed a declining pattern through the laying sequence as described in other studies on gulls (Blount et al., 2002; Groothuis et al., 2006; Royle et al., 1999; Rubolini et al., 2011). Moreover, this decline in TOTAL eggOXY was observed in both breeding groups. Such a decreasing tendency indicates that the allocation of antioxidants to the yolk is probably modulated/constrained by antioxidant resource acquisition (Royle et al., 2003) as well as by competition among oocytes (Meathrel, 1991). The exception was the concentration of lipophilic antioxidants, which did not present an order effect in either of the breeding groups. This consistent pattern has probably been naturally selected to optimise the trade-off between maternal investment and reproductive success (Hipfner et al., 2010a; Pedrocchi, 1997; Royle et al., 1999).

Both yolk lipOXY and TOTAL eggOXY were negatively related to $\delta^{15}\text{N}$ and thus, lower $\delta^{15}\text{N}$ values were associated with higher antioxidant levels. The relation of egg lipophilic antioxidants with diet has been previously reported. Royle et al. (2003) found that in Zebra Finches (*Taeniopygia guttata*) over 70% of the female's daily intake of carotenoids and α -tocopherols (the two most abundant lipophilic dietary antioxidants) were deposited in the egg yolk. In the Ebro delta system lower nitrogen values derive mainly from marine diets (Cotin et al., 2011; García-Tarrasón et al., 2013), suggesting that marine resources are more beneficial in terms of antioxidant supply than rice field resources. Marine fish (especially clupeids) are the natural prey of the Audouin's Gull (BWPI, 2004), and fish are richer than crustaceans in terms of nutritional content (Massias and Becker, 1990). For example, the musculature of the American crayfish (the main prey in the rice fields) yields only between 10% and 40% of its total body weight (Hunner, 1988). Although crayfish are known to contain a high concentration of carotenoids (Negro et al., 2000), mainly astaxanthin (Hipfner et al., 2010b;



Fig. 1. Intra-clutch deposition pattern for relative egg measurements by breeding group and laying order. Black bars indicate salt pan group, grey bars indicate coastal dune group. Values are mean and 95% confidence interval. albumOXY: antioxidant capacity of albumen; lipOXY: lipophilic component of antioxidant capacity; hydrOXY: hydrophilic component of antioxidant capacity.

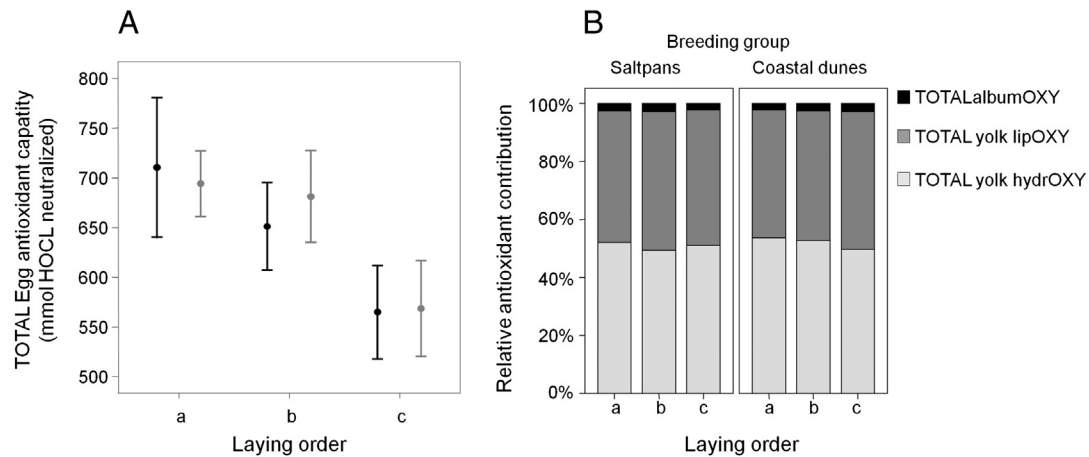


Fig. 2. (A) Intra-clutch deposition pattern for total egg antioxidant capacity by breeding group and laying order. Black bars indicate salt pan group, grey bars indicate coastal dune group. Values are mean and 95% confidence interval. (B) Relative antioxidant contribution for each egg measurement by laying order and breeding group.

Negro and Garrido-Fernandez, 2000), the antioxidant power of astaxanthin is very low compared to other dietary antioxidants (Miller et al., 1996; reviewed in Catoni et al., 2008). Moreover, crayfish from the Ebro Delta rice fields have very high levels of some metals, including copper, chromium, zinc and arsenic, probably due to agricultural activity (Suárez-Serrano et al., 2010). All these metals have toxic potential and may produce reactive oxygen species (ROS), especially when concentrations are high (reviewed in Koivula and Eeva, 2010). In fact, birds that feed in the Ebro Delta rice fields, such as herons and egrets, have the highest copper levels in the local bird community (Cotin, 2012). Thus, metal accumulation may be causing an increase in oxidative damage that the antioxidant activity of carotenoids cannot cope with. Nevertheless, marine fish are also a source of metals, especially mercury (Arcos et al., 2002; Monteiro et al., 1998). Since marine fish constitute the natural prey of Audouin's gulls, this species has evolved in mercury-enriched environments. Although mercury levels in Audouin's gulls are known to be high (Arcos et al., 2002; Sanpera et al., 2007), little is known about how metal accumulation affects the oxidative status of this species.

Finally, we could not find any relationship between stable isotopes and the plasma non-enzymatic antioxidant capacity of females or their plasma ROMs. However, our results show that the female plasma antioxidant capacity decreased over the incubation period (Fig. 3A). A similar trend was found by Costantini (2010b), who reported that female pigeons show a decline in plasma non-enzymatic antioxidant

capacity between the onset and end of the incubation phase. This effect can be attributed to the accumulated effort during the progress of the breeding event, which can result in depletion of female antioxidant reserves (Wiersma et al., 2004). Another non-excluding hypothesis is related to hormonal processes/action. Sex hormones such as estradiol may have a positive effect on the oxidative status (Demirbag et al., 2005; Halifeoglu et al., 2003), as well as stimulating the production of yolk and albumen precursors (Christians and Williams, 1999). Thus, the decrease in circulating estradiol during incubation (McQueen et al., 1999) could also be behind the decline in antioxidant capacity we observed.

A similar mechanism could explain the positive relationship between plasmaOXY and plasmaROMs (Fig. 3B). The production of lipid-rich yolk precursors might be associated with an increased production of reactive oxygen species via lipid peroxidation (Williams, 2005). In this case, plasma reactive oxygen metabolite levels would be higher during the clutch production (initial stages of incubation), when the influence of egg precursors is stronger and the antioxidant activity is higher. Indeed, plasmaROM levels and “days of incubation” showed a slightly negative, albeit not significant, pattern, probably due to the limited sample size (see Results). Although not the main focus of this study, the dual role of sex hormones is noteworthy: on the one hand, they stimulate the synthesis of egg precursors that can produce oxidative damage, and on the other hand, increase antioxidant activity. It is therefore important to take into account the “days of incubation” variable

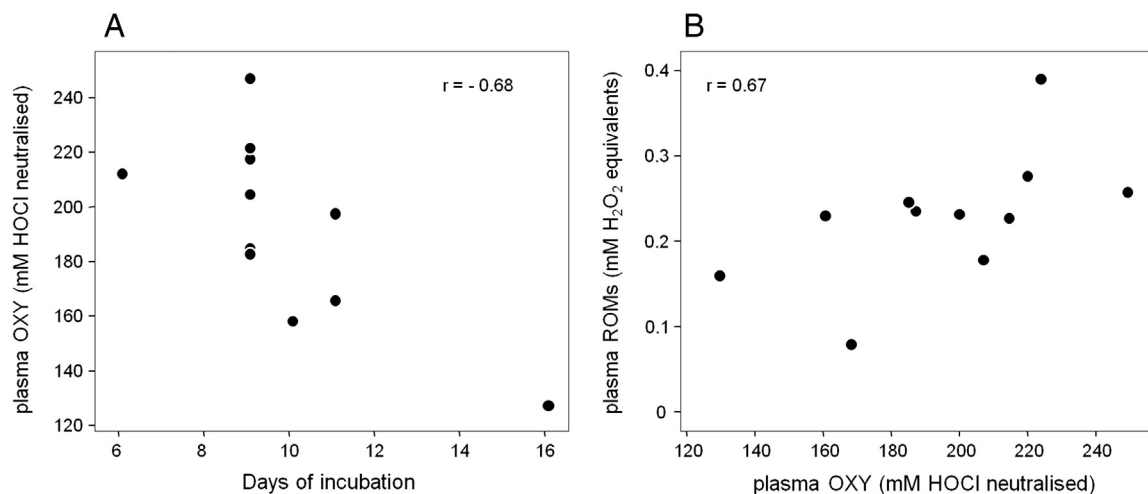


Fig. 3. Breeding female bivariate plots of (A) “days of incubation” vs. plasma non-enzymatic antioxidant capacity, and (B) plasma non-enzymatic antioxidant capacity vs. plasma reactive oxygen metabolites.

when evaluating the antioxidant capacity of breeding females. However, because of the limitations of the sample size in terms of statistical significance and biological representation, the present results on female gulls are intended as explorative and descriptive, and larger samples are needed to confirm these quantitative trends.

5. Conclusions

This study points to the potential benefit for Audouin's gulls of a marine fish diet over rice field prey in terms of the antioxidant levels deposited in their eggs. It is well known that in the Ebro Delta this species is highly dependent on trawler discard (Oro et al., 1999, 2004), while low quality resources such as American crayfish from the rice fields are thought to act as a buffer in adverse situations such as trawling moratorium periods. Our results show that deposition of antioxidants in the eggs tended to decrease along the laying sequence, indicating their limited availability. However, further research is needed to assess how antioxidant differences during the laying period affect reproductive parameters.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <http://dx.doi.org/10.1016/j.jembe.2014.01.012>.

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The role of calcium in constraining egg synthesis in the Audouin's Gull (*Ichthyaeetus audouinii*)

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Abstract: Understanding how resources are allocated to form eggs is crucial to our better understanding of avian reproductive strategies. However, little is currently known about how egg synthesis in wild birds might be constrained by the availability of specific micronutrients. Here, we investigated the potential role of calcium (Ca) in constraining egg synthesis in the Audouin's Gull (*Ichthyaeetus audouinii* (Payraudeau, 1826)). In particular, we evaluated the relationship between plasma Ca levels (mg/dL) in incubating females (as an indicator of the physiological response of females to increased Ca demand associated with clutch production) and several fitness-related egg traits such as egg size (i.e., egg volume), egg shape, and eggshell thickness from three-egg clutches. Egg size was positively related with incubating female plasma Ca levels, with the slope of this relationship being significantly higher for later-laid eggs. The observational nature of this study and reversed timing precludes causal inferences, but observed relationships supported the constraining role of Ca in egg synthesis and suggested that Ca may also have a role in modulating the intraclutch pattern of egg-size variation typical of this gull species.

Key words: Audouin's Gull, avian reproduction, calcium, egg production, egg size, eggshell, *Ichthyaeetus audouinii*.

Résumé : La compréhension de l'affectation de ressources sont allouées pour former des œufs est essentielle à une meilleure compréhension des stratégies de reproduction des oiseaux. Pourtant, l'incidence de la disponibilité de micronutriments sur la synthèse des œufs chez les oiseaux sauvages demeure méconnue. Nous avons examiné le rôle possible du calcium (Ca) dans la synthèse des œufs chez le goéland d'Audouin (*Ichthyaeetus audouinii* (Payraudeau, 1826)). Nous avons plus particulièrement évalué le lien entre les concentrations plasmiqes en Ca (mg/dL) de femelles couveuses (en tant qu'indicateur de la réponse physiologique de ces femelles à la demande accrue en Ca associée à la production de la couvée) et plusieurs caractères des œufs associés à l'aptitude tels que leur taille (c.-à-d. le volume des œufs), leur forme et l'épaisseur de leur coquille, pour trois couvées. La taille des œufs présentait une corrélation positive avec les concentrations en Ca du plasma des femelles couveuses, la pente de cette relation étant significativement plus grande pour les œufs pondus plus tardivement. Étant donné le caractère empirique de l'étude et sa chronologie inversée, l'établissement de liens causaux n'est pas possible, mais les relations observées appuient l'hypothèse selon laquelle le Ca contrôle en partie la synthèse des œufs et semblent indiquer qu'il pourrait également jouer un rôle dans la modulation de la variation de la taille des œufs d'une même couvée, un aspect typique de cette espèce de goéland. [Traduit par la Rédaction]

Mots-clés : goéland d'Audouin, reproduction des oiseaux, calcium, production d'œufs, taille des œufs, coquille d'œuf, *Ichthyaeetus audouinii*.

Introduction

Avian females influence offspring quality through genetic contribution, but also through allocation of resources to their eggs, including antibodies (Hammouda et al. 2012), hormones (Rubolini et al. 2006), or macro- and micro-nutrients (Christians 2002; Hargitai et al. 2005; Rubolini et al. 2006; Romano et al. 2008). In turn, the availability and quality of those resources allocated to eggs influence the growth and survival of the progeny, thus affecting maternal fitness (Mousseau and Fox 1998; Christians 2002; Rubolini et al. 2006; Romano et al. 2008). Understanding resource acquisition and allocation to egg formation is therefore crucial to our better understanding of avian reproductive and life-history strategies.

To date, we have some understanding of both intrinsic (physiological and hormonal mechanisms: Sockman et al. 2000; Williams et al. 2001) and extrinsic (e.g., resource availability: Kilpi et al. 1996; Hillstrom et al. 2000; Reynolds 2001; Hargitai et al.

2005) factors that influence avian egg production. Regarding the latter, early works aimed at investigating resource-allocation strategies in birds mainly focused on energy and macronutrients as main constraints in egg synthesis (see Nager 2006 and references therein). However, specific nutrients other than proteins, lipids, or carbohydrates can limit egg formation. The emphasis of more recent studies has consequently shifted to investigate the role of specific amino acids (Bolton et al. 1992; Murphy 1994; Ramsay and Houston 1998), essential fatty acids (Surai et al. 2001a, 2001b), fat-soluble components (e.g., vitamins A and E, and carotenoids: Blount et al. 2000), and especially calcium (hereafter Ca) (Burley and Vadehra 1989; Patten 2007; Wilkin et al. 2009; Reynolds and Perrins 2010) in constraining egg production.

Evidences for Ca-limited avian reproduction are provided by a number of supplementary feeding experiments. Such evidences include direct changes in egg size and eggshell thickness (Mänd et al. 2000a), clutch and brood sizes and nestling masses (Mänd

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et al. 2000b; Tilgar et al. 2002, 2005), or occurrences of nest desertion and defective eggs (Graveland and Drent 1997). However, field studies commonly suffer from practical methodological limitations associated with difficulty in measuring Ca availability in the wild (i.e., exogenous Ca: Wilkin et al. 2009; Reynolds and Perrins 2010) and accounting for Ca mobilized from skeleton or resorbed by the renal tubules (i.e., endogenous Ca: Clunies et al. 1992; Carey 1996). Alternatively, temporal trends in plasma Ca levels for breeding females could be considered a likely reflection of females' physiological response to increased Ca demand associated with clutch production. Indeed, Simkiss (1961) reported an hormonal-mediated increase in blood Ca levels in breeding females during the clutch-production period, followed by a progressive decrease in blood Ca levels to reach that of males (which remained relatively constant throughout the early stages of the reproductive cycle) during incubation. Plasma Ca levels, therefore, could be used as an indicator of overall Ca (both exogenous and endogenous) available to females during the clutch-production period, thus facilitating investigations into the actual role of this micronutrient as a proximal factor constraining avian reproduction.

The limiting role of Ca in reproduction is expected to be particularly relevant for small passerines (whose clutches often contain more Ca than is present in the entire skeleton of the breeding female) breeding in acidified areas where Ca availability is particularly poor (Reynolds and Perrins 2010 and references therein). However, evidences of Ca-limited reproduction have been also reported in large birds, such as the European Herring Gull (*Larus argentatus* Pontoppidan, 1763), laying a reduced number of relatively small eggs (Pierotti and Annett 1991). Additionally, Ca-limited reproduction is rare, but not absent, in species that routinely consume Ca-rich foods during the nonbreeding season, such as the piscivore Sandwich Tern (*Thalasseus sandvicensis* Latham, 1787) (Brenninkmeijer et al. 1997) and Common Tern (*Sterna hirundo* L., 1758) (Nisbet 1997). Accordingly, we extended the application of measuring plasma Ca levels in breeding females to investigate the role of Ca in constraining clutch production in the large, long-lived, K-selected, and piscivore Audouin's Gull (*Ichthyaeus audouinii* (Payraudeau, 1826)). In particular, we investigated the relationship between female plasma Ca levels and several fitness-related egg traits such as egg size (i.e., egg volume), egg shape, and eggshell thickness (Blom and Lilja 2004; Altuntas and Sekeroglu 2008; Karlsson and Lilja 2008; Krist 2011). Owing to the potential role of Ca in constraining clutch production in this gull species, we expected that smaller and rounder eggs (which minimize the surface to volume ratio, and thus optimize the use of shell material; Gosler et al. 2005), along with thinner eggshells, would correspond to females with lower plasma Ca levels during incubation. We examined the constraining role of Ca at the intra-clutch level in three-egg, modal clutches. Eggshell in large gulls are synthesized in a relatively short time period (Ruiz et al. 2000), when per-day Ca requirements may overtake daily Ca intake (for calculations on the piscivore Common Tern see Nisbet 1997). Accordingly, we expected the overall Ca availability for laying females to decrease as clutch formation progresses, so that later-laid eggs would be particularly constrained by Ca shortage.

Materials and methods

Fieldwork procedure

Fieldwork was conducted at the Peninsula de la Banya (40°40'N, 0°45'E), a protected area of salt marshes located at the Ebro Delta Natural Park (Spain), that supports approximately 10 000 breeding pairs of Audouin's Gulls. During the 2009 breeding period, nests of this gull species were tagged when the first egg was laid and inspected daily until clutch completion to determine laying order, final clutch size, and the end of the laying period. Fresh, newly laid eggs (<24 h after laying) were measured (maximum length and breadth) to the nearest 0.01 mm using a digital caliper.

At clutch completion (approximately 4 days after the first egg was laid; Ruiz et al. 2000), all eggs within a clutch were sampled and replaced with dummy eggs; the eggs were kept refrigerated and transported to the laboratory. To standardize individuals in the study and to prevent the potential confounding effect of different clutch sizes, egg sampling was performed during the peak laying (thus potentially including individuals of homogeneous "quality", see Parsons 1972; Sydeman et al. 1991) and restricted to nests with a modal clutch of three eggs ($n = 43$). One bird per nest was captured using spring traps after clutch completion because of the methodological limitation in sampling nonincubating gulls (Ramírez et al. 2010). Female and male Audouin's Gulls are very similar in appearance, thus parental sex can only be unequivocally determined from DNA samples. Bird sampling extended throughout the entire incubation period (approximately 20 days) to minimize disturbing in the colony. Two millilitres of blood (representing approximately 0.3% of mass of a standard 600 g individual) were taken from the tarsal vein using a 25-gauge needle; 0.5 mL were preserved in a neutral vial for molecular identification of sex and the rest was placed in heparinized vials to later obtain plasma for Ca-level determinations. Blood samples were kept cool until heparinized vials were centrifugated a few hours later (maximum 4 h). Then, approximately 0.5 mL of the supernatant plasma were resampled and stored in independent tubes. Blood and plasma samples were frozen at -23°C pending analyses.

Laboratory procedures

Once at the laboratory (<12 h after sampling), egg contents were separated from eggshells and eggshell thickness (dried shells with the membrane still intact) was measured with a micrometer to the nearest micrometre at two random locations about the equator of each shell. The determination of plasma Ca levels was based on the reaction between Ca in the sample and arsenazo III forming a coloured complex that was measured at 635 nm by spectrophotometry. The measurement procedure of Ca-arsenazo was adapted to an A25[®] automated analyzer (BioSystems, Barcelona, Spain). Sex of gulls was determined using polymerase chain reaction (PCR) amplification of the *CHD* genes (Ellegren 1996; Griffiths et al. 1998). To extract DNA, blood was boiled in 100 mmol/L NaOH for 10 min at 100°C before being added to the PCR reaction. PCR protocols were modified from Fridolfsson and Ellegren (1999) using the primer set 2550F–2718R, and PCR products were visualized on a 3% agarose gel stained with ethidium bromide scoring a single band in males and two bands in females.

Parameter estimations and statistical testing

Following Simkiss' reported temporal trend in plasma Ca levels for breeding birds (Simkiss 1961), a linear model was used to evaluate the temporal decline in plasma Ca levels throughout incubation (i.e., number of days elapsed between clutch completion and capture of individuals; hereafter "days of incubation"). Sex and its interaction with days of incubation were included in the model to account for differences in temporal trends based on gender.

Because of the nonindependent nature of the data, generalized linear mixed models were subsequently used to investigate the relationship between plasma Ca levels of incubating females and the traits egg size (i.e., egg volumes), egg shape (i.e., egg-shape index) (Anderson et al. 2004), and eggshell thickness (EST_{egg}). Egg volume (V_{egg} , cm^3) was estimated as $V_{\text{egg}} = 0.485 \times \text{length} \times \text{breadth}^2$ (Ruiz et al. 1998), whereas the egg-shape index (SI_{egg}) was derived following the standard method of $SI_{\text{egg}} = \text{breadth}/\text{length} \times 100$ (Anderson et al. 2004). Egg sequence (a -, b -, and c -egg) and its interaction with plasma Ca levels were incorporated in the models to detect differences in these relationships based on laying order, whereas days of incubation was included as a covariate to account for the temporal trend in female plasma Ca levels through incubation (Table 1, Fig. 1). Our research questions focused on whether or not the role of Ca in constraining egg synthesis was additive to that of

Table 1. Parameter estimates and mean least squares (MLS) estimations of mean values derived from fitted models for plasma levels of Ca (mg/dL) in incubating Audouin's Gulls (*Ichthyaeus audouinii*) breeding at the Ebro Delta during 2009.

Ca (mg/dL)	Estimate	95% CI
Fixed parameters		
Intercept	11.74	10.84, 12.64
Male	-1.71	-2.94, -0.46
Days of incubation	-0.15	-0.26, -0.03
Male × days of incubation	0.16	0.02, 0.31
MLS estimation of mean values		
Male	10.04	7.66, 12.46
Female	11.59	10.58, 12.29

Note: Sex was introduced in the model as a fixed factor, whereas days of incubation (i.e., number of days elapsed between clutch completion and capture of individuals) was introduced as a covariate. "Female" is considered a reference category and 95% CI is the 95% confidence intervals.

several intrinsic and extrinsic individual traits such as body condition, age, or dietary preferences. Accordingly, nest was included as a random factor to account for the above-mentioned female traits along with the dependence among eggs laid by the same female. Statistical analyses were completed using SPSS version 19.0 (SPSS Inc., Chicago, Illinois, USA).

Results

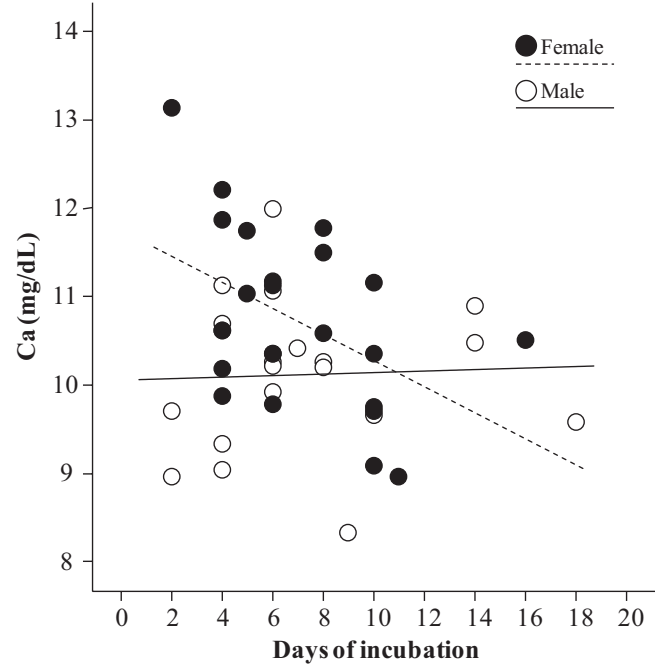
Of the 43 adults analyzed, 19 were males and 24 were females. Plasma Ca levels ranged 8.3–12.0 mg/dL for males and 9.0–13.1 mg/dL for females. Fitted model to evaluate trends in Ca levels throughout incubation included a significant interaction between sex and days of incubation ($F_{[1,43]} = 4.2, p = 0.047$), which is in agreement with the results from Simkiss (1961) in that plasma Ca levels follow a temporal trend throughout incubation that differs according to gender. In particular, Ca levels for males were constant throughout incubation (95% confidence intervals (CI) for the slope of fitted temporal trend = -0.263, 0.282), whereas a negative trend was fitted for females (95% CI = -0.265, -0.03), with a gradual decrease until reaching similar concentrations to that of males (Table 1, Fig. 1).

Regarding egg characteristics, V_{egg} ranged 57.25–70.44, 54.13–66.72, and 48.2–59.89 cm³ for *a*-, *b*-, and *c*-eggs, respectively; SI_{egg} ranged 66.03–74.03, 65.25–77.00, and 64.80–73.52 for *a*-, *b*-, and *c*-eggs, respectively; and EST_{egg} ranged 321–412, 313–401, and 314–386 μm for *a*-, *b*-, and *c*-eggs, respectively. When looking at the relationship between female Ca levels and egg and eggshell characteristics, we observed that plasma Ca levels were positively related with V_{egg} ($F_{[1,24]} = 14.48, p = 0.001$). However, such relationships differed according to laying order as indicated by the significant interactions between this three-level factor and plasma Ca levels ($F_{[2,48]} = 3.05, p = 0.038$), with increasing slopes throughout the laying sequence (Table 2, Fig. 2). SI_{egg} did not show any relationship with female plasma Ca levels ($F_{[1,24]} = 0.01, p = 0.97$). However, a significant effect of the laying order was detected ($F_{[2,48]} = 6.67, p = 0.003$), with the SI_{egg} values for *b*-eggs being slightly higher than those obtained for *a*- and *c*-eggs (Table 2, Fig. 3A). EST_{egg} was neither related to plasma Ca levels ($F_{[1,24]} = 0.31, p = 0.58$) nor to laying order ($F_{[2,48]} = 2.91, p = 0.064$) (Fig. 3B).

Discussion

Our results for Audouin's Gulls indicated that higher plasma Ca levels in incubating females were associated to larger eggs. Furthermore, this relationship varied for different eggs within the laying sequence, with increasing slopes for later-laid eggs. Although the observational nature of this study and reversed timing precludes causal inferences, these results pointed to the constraining role of Ca for egg synthesis in this species and suggested

Fig. 1. Temporal trends of plasma Ca (mg/dL) through incubation (days of incubation is the number of days elapsed between clutch completion and capture of individuals) according to gender for incubating Audouin's Gulls (*Ichthyaeus audouinii*) breeding at the Ebro Delta (Spain) during 2009. Solid and broken lines represent regression lines predicted by the model for each sex (see Results and Table 1).



that this limiting micronutrient may have a role in modulating the intraclutch pattern of egg-size variation typical of this gull species.

Tracing Ca availability for laying females

Avian females might adopt various strategies to meet Ca requirements for clutch formation. Switching diets to exploit Ca-rich food items during the clutch production period have been widely reported for small passerines (e.g., Wilkin et al. 2009; Reynolds and Perrins 2010) and several tern species (e.g., Sandwich Terns and Common Terns: Brenninkmeijer et al. 1997; Nisbet 1997) and gull species (e.g., Audouin's Gull: Navarro et al. 2010). Alternatively (or complementarily), avian females can show a hypercalcemic response associated with egg production, mediated by the endocrine system, and consisting of increasing Ca intestinal absorption (Klasing 1998) and resorption by renal tubules and medullary bone (Clunies et al. 1992; Carey 1996; Reynolds and Perrins 2010). Both dietary intake and endogenous Ca mobilization result in a dramatic increase in female plasma Ca concentrations during ovulation that decrease progressively after ovulation to reach concentrations similar to that of males (which remained relatively constant) during incubation (Simkiss 1961; Lumeij 1994). Although our results for the Audouin's Gull referred exclusively to the incubation stage, they agree with previously reported sex-specific trends and suggest that plasma Ca levels in incubating females can be used as an indicator of plasma Ca levels during egg formation.

The constraining role of Ca

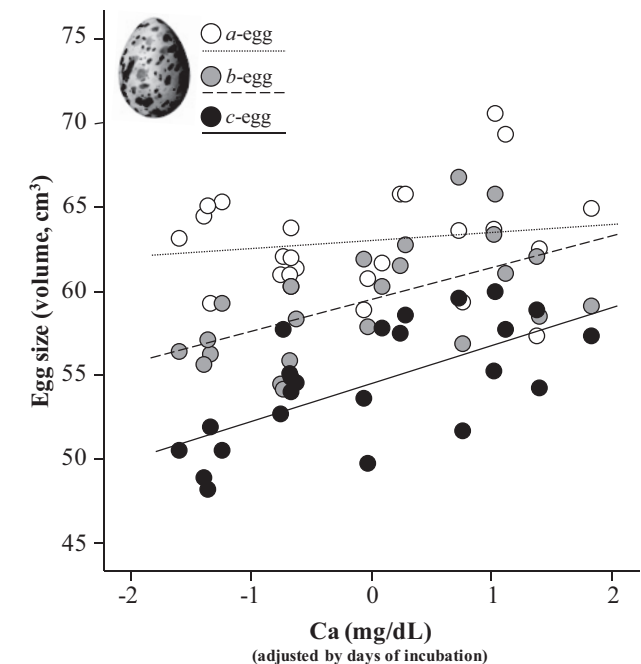
Eggshell provides the embryo with minerals, primarily Ca, needed for the development of high Ca-consuming tissues, such as the skeleton, muscle, and brain (more than 80% of the Ca needed for embryonic development is derived from the eggshell; Blom and Lilja 2004; Karlsson and Lilja 2008). Ca in the eggshell is

Table 2. Parameter estimates and mean least squares (MLS) estimations of mean values derived from fitted models for egg volume (cm³) and egg-shape index (breadth/length × 100) in three-egg clutches from Audouin's Gulls (*Ichthyaeetus audouinii*) breeding at the Ebro Delta during 2009.

	Egg volume (cm ³)			Egg-shape index		
	Estimate	95% CI	SE	Estimate	95% CI	SE
Fixed parameters						
Intercept	24.96	10.69, 39.22		69.38	68.32, 70.43	
a-egg	27.52	12.75, 43.30		0.70	−0.19, 1.58	
b-egg	11.91	−2.86, 26.69		1.61	0.71, 2.49	
Ca	2.63	1.39, 3.87				
Days of incubation	0.20	−0.11, 0.50				
Ca × a-egg	−1.79	−3.16, −0.41				
Ca × b-egg	−0.66	−2.04, 0.7				
MLS estimation of mean values						
a-egg	62.94	61.81, 64.06		70.07	69.02, 71.29	
b-egg	59.35	58.23, 60.48		70.98	69.93, 72.04	
c-egg	54.57	53.48, 55.70		69.38	68.32, 70.43	
Variance parameter						
Nest	2.26		±1.22	4.22		±1.45
Residual	5.33		±1.09	2.33		±0.48

Note: Laying sequence (a-, b-, and c-egg) and nest were introduced in the models as fixed and random factors, respectively. Female plasma levels of Ca and its interaction with laying sequence were included to detect differences in these relationships according to laying order. Days of incubation (i.e., number of days elapsed between clutch completion and capture of individuals) were also included to account for observed temporal trend in female plasma Ca levels through incubation. c-egg is considered a reference category, and 95% CI and SE are the 95% confidence intervals and standard errors, respectively.

Fig. 2. Bivariate plot of residuals of female plasma Ca levels (adjusted by days of incubation) in Audouin's Gulls (*Ichthyaeetus audouinii*) breeding at the Ebro Delta (Spain) during 2009 versus egg size. Lines represent regression lines predicted by the model (see Results and Table 2).



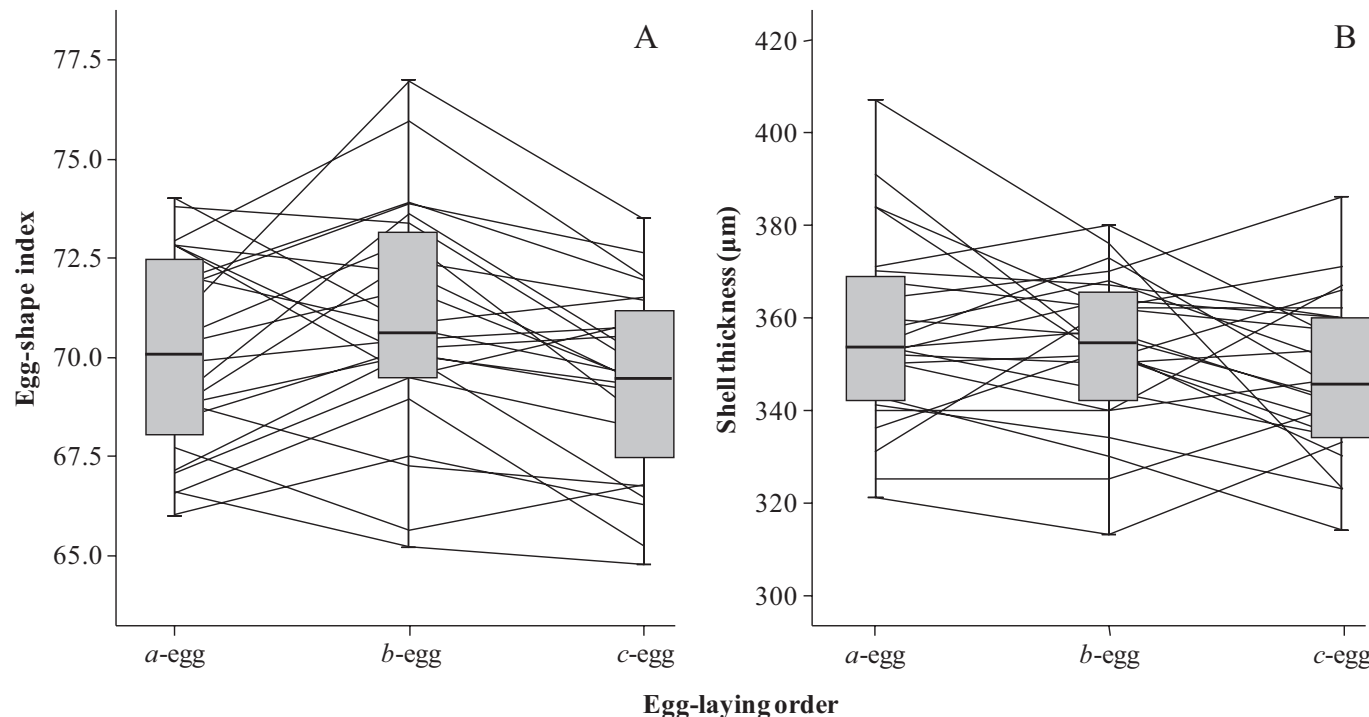
exclusively mobilized from the mammillary tips, which can be viewed as functional units that provide the embryo with a limiting amount of Ca (see Karlsson and Lilja 2008 and references therein). The removal of Ca from the eggshell results in decreasing mammillary densities (number of tips per unit of egg surface) and in a “natural thinning” of the eggshell throughout incubation (Karlsson and Lilja 2008; Reynolds and Perrins 2010), which likely affect the eggshell’s mechanical properties and the exchange of gas and water between the embryo and the environment. The

initial mammillary density and eggshell thickness are therefore crucial in ensuring egg feasibility and proper embryonic development. Accordingly, and in contrast with what occurred for egg size, our results indicated that eggshell thickness was not significantly affected by Ca availability and remained relatively constant throughout the laying sequence, although slightly thinner eggshells were observed for last-laid eggs, as previously reported by Nisbet (1982) and Reynolds (2001) for Common Terns and Zebra Finches (*Taeniopygia guttata* (Vieillot, 1817)), respectively. Egg-size variations in relation to Ca availability might therefore be related to the necessity of achieving the required initial mammillary density and eggshell thickness.

Whereas the limiting role of Ca for clutch synthesis is widely accepted (Reynolds and Perrins 2010), few studies have discussed the potential implication of this micronutrient in determining the intraclutch pattern of egg-size variation typical in many avian species (Graveland et al. 1994; Reynolds 2001). Indeed, the brood reduction and the hormonal hypothesis have been widely considered when explaining egg-size variation throughout the laying sequence (Lack 1954; Lack 1968; Slagsvold et al. 1984; Sockman et al. 2000; Williams et al. 2001). Our results indicated that the relationship between plasma Ca levels in incubating females and egg size varied throughout the laying sequence, being slightly higher for later-laid eggs (as noted by the estimated slopes for the relationships between plasma Ca levels and estimated egg volume and surface area: a-egg < b-egg < c-egg). In our view, these results suggested that later-laid eggs in Audouin's Gulls were particularly sensitive to Ca availability, which could be explained by an overall decrease in Ca availability (both endogenous and exogenous) as long as this micronutrient is progressively allocated into the successive eggs (eggshells) to complete the clutch. In addition to its effect on clutch production, Ca availability may also play a relevant role in determining the intraclutch pattern of egg-size variation, with higher availabilities resulting in more homogeneous egg sizes at the intraclutch level.

In contrast to our a priori expectations and to what Gosler et al. (2005) found in Great Tits (*Parus major* L., 1758), egg shape in the Audouin's Gull did not vary with Ca availability. However, the fact that this trait significantly varied throughout the laying sequence, and that more than half of its variability was explained by the

Fig. 3. Intracutch pattern of variation in Audouin's Gull (*Ichthyaeetus audouinii*) (A) egg-shape index (breadth/length $\times$ 100) and (B) eggshell thickness (μm). For each parameter, we represent individual nest trends (solid black lines) and descriptive summaries using box plots (whiskers represent minimum and maximum values; grey boxes represent first, second, and third quartiles) according to laying order.



random nest factor, suggests that other unmeasured female traits likely operating throughout clutch production affect egg shape.

Avian egg size has been previously reported to be affected by several extrinsic (e.g., food availability or ambient temperature) and intrinsic (e.g., age, endogenous protein stores, oviduct mass, or rate of protein uptake by ovarian follicles) female traits (reviewed in Christians 2002). Our study focused on the constraining role of Ca during reproduction and provided some evidences suggesting that, in addition to the aforementioned individual traits, the availability of this micronutrient to breeding female Audouin's Gulls influenced their resource allocation strategies. In particular, higher Ca availabilities resulted in larger eggs and more homogeneous egg sizes at the intracutch level. We highlight the usefulness of our approach (i.e., measuring plasma Ca levels) to investigate the role of Ca in constraining or limiting egg production in birds.

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