Ecología y genética de ascidias invasoras en el Mediterráneo occidental

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ECOLOGÍA Y GENÉTICA DE ASCIDIAS INVASORAS EN EL MEDITERRÁNEO OCCIDENTAL

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CONTENIDOS

Introducción general

INTRODUCCIÓN GENERAL

Las Invasiones Biológicas

Las invasiones biológicas son un tema de especial importancia en la comunidad científica de todo el mundo, tanto por sus repercusiones a nivel ecológico como a nivel económico. Existen diversas definiciones de especies invasoras, pero en general implican (a) introducción en una nueva zona y (b) proliferación en ella. Así, para Elton (1958) una invasión biológica ocurre cuando un organismo es transportado a una nueva región, normalmente distante, donde sus descendientes proliferan, se expanden y persisten. Según Williamson & Fitter (1996), una especie invasora es una especie introducida que resulta ecológica y/o económicamente perjudicial, es decir, una plaga. Estas invasiones pueden darse de manera natural sin la participación del ser humano. Sin embargo, el alcance geográfico de estas invasiones, así como la frecuencia y el número de especies involucradas ha crecido enormemente como consecuencia directa de un transporte y comercio en expansión (sensu Mack *et al.* 2000) y por lo tanto como consecuencia del hombre, convirtiéndolo en principal responsable de las invasiones.

No todas las especies introducidas logran establecerse en su nuevo hábitat y, de hecho, muchas no lo hacen (Griffiths *et al.* 1992, Kareiva 1996). Aun así, resulta alarmante la cantidad de hábitats diferentes afectados por especies introducidas por el hombre: hábitats terrestres continentales, islas, ríos, lagos, humedales, ambientes marinos y estuáricos (Ruiz *et al.* 1997, Mack *et al.* 2000, Lowe *et al.* 2000, Wonham & Carlton 2005, Strayer 2010). Pero más preocupante es el número de especies involucradas (miles de ellas) pertenecientes a un abanico sorprendentemente amplio de grupos taxonómicos diferentes: mamíferos, aves, reptiles, anfibios, peces, ascidias, briozoos, equinodermos, crustáceos, insectos, moluscos, anélidos, helmintos, nematodos, cnidarios, ctenóforos, poríferos, protozoos, cormófitos, pteridófitos, diferentes tipos de algas, hongos (Carlton & Geller 1993, Ruiz *et al.* 1997, 2000, Lowe *et al.* 2000, Alcaraz *et al.* 2005, Real *et al.* 2008, Galil 2009, Strayer 2010, Courchamp 2013). Estas introducciones asociadas con las actividades humanas pueden darse involuntariamente, pero otras son intencionadas y asociadas a intereses comerciales (Pimentel *et al.* 2000). Un ejemplo es el caso de la introducción del mejillón mediterráneo *Mytilus galloprovincialis* en Sudáfrica (Branch & Steffani 2004, Griffiths *et al.* 2005). Este bivalvo fue introducido para su explotación comercial en cultivos de acuacultura. Sin embargo, la especie se expandió con gran éxito convirtiéndose en una plaga por toda la costa sudafricana (Bownes & McQuaid 2006). La liberación accidental o irresponsablemente deliberada de mascotas o animales o plantas de acuario también representa un serio problema (Bomford *et al.* 2009). Otras veces es más grave, ya que se introducen intencionadamente especies exóticas para el control biológico de otras introducidas anteriormente, pero sin obtener el resultado esperado, sumando así otro espécimen más a la lista de plagas introducidas por el hombre. Este es el caso, por ejemplo, del gasterópodo pulmonado *Euglandina rosea* introducido en la isla de Moorea, en la Polinesia Francesa (Clarke *et al.* 1984).

Es importante tener en cuenta que para que tenga lugar una invasión biológica, la especie introducida ha de superar toda una serie de obstáculos (Blackburn *et al.* 2011). De este modo, ésta ha de ser capaz de formar propágulos que sean transportados a la nueva región, sobrevivir al proceso y ser capaz de establecerse y de expandirse en el nuevo hábitat, siendo cada paso un desafío y un nuevo tipo de selección para la especie (Sakai et la 2001, Suarez & Tsutsui 2008). Además, algunas especies exóticas no causan invasiones justo después de su introducción, sino que mantienen un crecimiento discreto en una fase de latencia (o “lag-phase”) en la que se adaptan a las nuevas condiciones ambientales y a la comunidad residente, creciendo luego exponencialmente llegando a ser ampliamente invasivas (Crooks 2005). En este sentido, la presión de propágulos tiene especial importancia, ya que cuanto mayor son el tamaño y la recurrencia de la introducción, más facilidad para la invasión, además de poder aportar (en principio) mayor diversidad genética (Sakai *et al.* 2001, Simberloff 2009).

En consecuencia, las características ambientales de la nueva área serían cruciales para el éxito en el establecimiento de las especies invasoras, así como la tolerancia fisiológica de éstas (Ashton *et al.* 2007, Bomford *et al.* 2009, Bomford *et al.* 2010). Además, aquellas especies que se establezcan con éxito pueden interactuar con las especies residentes (sean nativas o no), como por ejemplo en competencia, depredación o parasitismo (Torchin *et al.* 2002, Bando 2006), de manera que habrá especies que se beneficien de ello mientras que otras se verán perjudicadas (Rodriguez 2006).

Consecuencias y gestión de las Invasiones Biológicas

Las especies alóctonas pueden modificar las comunidades nativas, como por ejemplo provocando cambios en la composición y distribución de las especies, desplazamiento competitivo de las especies nativas, transmisión de enfermedades, hibridaciones con las especies nativas, incluso la extinción de éstas (Grosholz 2002, Orensanz *et al.* 2002, Clavero & García-Berthou 2005, Didham *et al.* 2005, Bomford *et al.* 2009). Además, hay todo un gasto económico asociado a estas invasiones, incluyendo pérdidas por daños causados por la plaga, gastos de control y de erradicación (Pimentel *et al.* 2005, Williams *et al.* 2010). Como ejemplo mencionar el gasto de casi 120 billones de dólares por año sólo en Estados Unidos como consecuencia de las invasiones (Pimentel *et al.* 2005). Normalmente se recurre a la erradicación como solución de estas plagas, pero los costes de ésta incrementan exponencialmente a medida que la extensión de la invasión progresa (Williams *et al.* 2010). Además, muchas veces las medidas no llegan lo suficientemente rápido, o simplemente no resultan efectivas. No obstante existe una estrategia más eficiente en el control de las invasiones biológicas que ha demostrado ser incluso mucho más económica que cualquier tipo de erradicación: la prevención (Myers *et al.* 2000, Strayer 2010, Williams *et al.* 2010).

Lamentablemente, aun existiendo la posibilidad de evitarlo, hay evidencias de que la introducción de especies no indígenas está incrementando (Simberloff 2004, Gittenberger 2009).

Herramientas para la gestión de las Invasiones Biológicas

El conocimiento de la biología de las especies no indígenas resulta esencial para prevenir nuevas introducciones, disminuir su efecto nocivo o para su erradicación (Myers *et al.* 2000). Conocer las respuestas a las condiciones ambientales de la especie, su capacidad de dispersión o su ciclo biológico, son decisivos para entender de una manera más eficiente su potencial invasivo. Además, la correcta identificación de las especies invasoras es un paso inicial importante para su apropiada gestión (Geller *et al.* 1997). Sin embargo, el estudio de estas especies suele encontrarse con confusiones taxonómicas y pueden no llegar a ser reconocidas como introducidas en el nuevo área colonizada. Por ejemplo, la ascidia colonial *Didemnum vexillum* Kott, 2002 fue identificada en Nueva Zelanda como una nueva especie nativa de la región (Kott 2002). Posteriormente se demostró morfológica y genéticamente que era en realidad una introducción proveniente del Japón (Stefaniak *et al.* 2009, 2012, Lambert 2009a). La genética ha demostrado ser un método muy útil en la identificación de organismos foráneos, como por ejemplo en las invasiones crípticas (Geller *et al.* 1997, McGlashan *et al.* 2008). Además, la genética nos permite hacer estudios poblacionales: podemos analizar la conectividad entre poblaciones (García-Merchán *et al.* 2012), estudiar la capacidad de dispersión de una especie (Calderón *et al.* 2007), conocer el área nativa de una especie introducida (Rius *et al.* 2008, Stefaniak *et al.* 2009), así como averiguar la historia de su introducción (Rius *et al.* 2012).

El medio marino y las Invasiones Biológicas

Las especies no indígenas son una gran amenaza para el mantenimiento de la biodiversidad y el funcionamiento de los ecosistemas marinos costeros (Ruiz *et al.* 1999, Harris & Tyrrell 2001), y el cambio climático está resultando ser un gran aliado para estos organismos (Hawkins 2012). Las actividades humanas (Panel 1) relacionadas con el intenso tráfico marítimo (tanto transoceánico como trayectos de corta distancia) o la náutica recreacional, han facilitado la introducción de especies no indígenas de invertebrados de un sitio al otro del globo, mediante las aguas de lastre o como recubrimiento sobre los cascos de los barcos (Carlton & Geller 1993, Ruiz *et al.* 1997, Lambert 2002, Godwin 2003). Estos procesos de introducción artificial de las especies se han denominado procesos pre-frontera (o "pre-border", Forrest *et al.* 2009). Algunos de estos organismos no indígenas proliferan y establecen poblaciones recluidas en los puertos donde han sido transportados. Otras veces, son capaces de expandir su distribución fuera de estas estructura artificiales (Simkanin *et al.* 2012), un proceso que se conoce como post-frontera (o "post-border", Forrest *et al.* 2009). De hecho, esta capacidad de infiltración en hábitats naturales a veces se considera como diferenciador entre una especie "introducida" y una "invasiva" (Colautti & MacIsaac 2004). Además, los procesos post-frontera abren valiosas oportunidades para la gestión y prevención de las especies invasivas (Wotton & Hewitt 2004, Forrest *et al.* 2009, Airoldi & Bulleri 2011). Supervivencia, reproducción, dispersión y las condiciones ambientales locales son los principales factores que determinan el éxito de una población introducida durante el estado de post-frontera (Blackburn *et al.* 2011). Un proceso clave es la dispersión regional de la especie que, contrariamente a lo que pasa en la dispersión pre-frontera, recae sobre todo en sus habilidades naturales de dispersión, así como también en transportes pasivos por el hombre a escala local (Wasson *et al.* 2001, Branch & Steffani 2004)

A parte de los ya mencionados, hay otros vectores involucrados en la introducción de especies no indígenas en el medio marino, como aquellos asociados a la industria de la maricultura o acuacultura. En efecto, la acuacultura se ha convertido en un vector destacado de introducción de especies invasoras por todo el mundo (Naylor *et al.* 2001, Minchin 2007). Y no sólo por la introducción intencionada de especies comerciales, como sería el caso de *Mytilus galloprovincialis* comentado anteriormente, sino también de manera involuntaria mediante la epibiota asociada al stock (Gollasch 2006).

Sea cual sea el causante de su introducción, una característica común de muchos invasores marinos es que sus historias de colonización suelen involucrar múltiples introducciones provenientes de múltiples fuentes (Sakai *et al.* 2001, Geller *et al.* 2010), lo que a menudo se cumple a través de un transporte repetido tanto por el tráfico marítimo como por la acuacultura (Roman & Darling 2007). Esto suele llevar a una diversidad genética elevada de las poblaciones introducidas a través de la mezcla de fuentes genéticamente diferenciadas (Geller *et al.* 2010, Rius *et al.* 2012). Además, la interacción entre los diferentes acervos genéticos (McIvor *et al.* 2001, Meusnier *et al.* 2002, Kelly *et al.* 2006, Pineda *et al.* 2011) de diferentes orígenes y, potencialmente, de diferentes características ecofisiológicas puede afectar significativamente al éxito de las poblaciones introducidas, en una manera que aún permanece inexplorada.

En las costas altamente urbanizadas, la dispersión es también favorecida por la existencia de marinas y puertos, con sus asociadas actividades de transporte. Mientras que las introducciones primarias dependerían del tráfico entre los principales puertos, la red de marinas con actividades náuticas recreacionales contribuiría a una dispersión secundaria (Lambert & Lambert 1998, Wasson *et al.* 2001, Lacoursière-Roussel *et al.* 2012). La dispersión natural entre hábitats adecuados puede llevar a un modelo de dispersión de piedras de paso ("stepping-stone"), mientras que la dispersión mediada por la náutica recreacional puede romper las relaciones entre distancia geográfica y conectividad natural en escalas locales (Lacoursière-Roussel *et al.* 2012).

Los sustratos artificiales pueden actuar como corredores de dispersión para las especies exóticas (Bulleri & Airoldi 2005, Glasby *et al.* 2007, Vaselli *et al.* 2008, Bulleri & Chapman 2010, Airoldi & Bulleri 2011) y pueden representar una fuente de infiltración al bentos natural por especies no indígenas (Simkanin *et al.* 2012). Estas estructuras no sustituyen a los sustratos naturales, ya que la estructura y la dinámica de las comunidades sobre sustrato duro artificial son diferentes de los fondos rocosos naturales adyacentes (por ejemplo, Pinn *et al.* 2005, Tyrrell & Byers 2007, Bulleri & Chapman 2010, Airoldi & Bulleri 2011). En general, la riqueza alélica es menor en comunidades establecidas sobre sustrato artificial que en las comunidades naturales (por ejemplo, Bacchiocchi & Airoldi 2003), y muchos procesos ecológicos como son el reclutamiento, competencia o depredación pueden diferir entre hábitats naturales y artificiales (Bulleri & Chapman 2010, Dumont *et al.* 2011). En general, se espera que las comunidades naturales sean capaces de influenciar el asentamiento de los propágulos más que las artificiales, siendo así menos propensas a las invasiones (Stachowicz & Tilman 2005, Tyrrell & Byers 2007, Dumont *et al.* 2011, en cambio ver Osman & Whitlatch 2007).

Panel 1. Introducción de especies exóticas en el medio marino

A

(Foto: http://massbay.mit.edu/exoticspecies/ballast)

Las aguas de lastre (A), así como el recubrimiento del casco de los barcos por una gran variedad de especies (B y C), ha facilitado el transporte y la introducción de especies exóticas por todo el mundo debido al gran tráfico marítimo. Una vez establecidas en las nuevas regiones, suelen desarrollar una comunidad sobre las estructuras artificiales de los puertos donde fueron introducidos, como son los cabos de amarre (D).

B

(Foto: http://www.cleanaboatantifouling.com.au/)

C

(Foto: http://www.yachtandboat.com)

D

(Foto: Víctor Ordóñez)

El stock comercializado en acuacultura y su epibiota (E) también representan un peligro de introducción de especies. Las instalaciones de acuacultura son otro sustrato donde estas especies pueden instalarse (F).

E

(Foto: Víctor Ordóñez)

F

(Foto: Víctor Ordóñez)

Interacciones Biológicas en el medio marino

El mantenimiento de la diversidad en el mar se atribuye a la interacción entre la llegada de propágulos y la competición, depredación y perturbaciones (Dayton 1971, Paine 1984). Para los invertebrados sésiles que crecen sobre sustrato duro en las comunidades bentónicas, el espacio disponible resulta el recurso limitante principal (Sebens 1982) y se desarrollan una serie de interacciones entre estas especies con el fin de hacerse con este recurso tan preciado (Panel 2). Entre los mecanismos que intervienen en la competencia por el espacio se encuentran: éxito en la reproducción y reclutamiento, desarrollo de estructuras y patrones de crecimiento que reducen u obstaculizan el crecimiento excesivo de otros competidores, baja susceptibilidad al recubrimiento por otras especies (epibiota), comportamiento agresivo o diferente susceptibilidad a perturbaciones (Jackson & Buss 1975, Sebens 1982).

El medio marino es particularmente vulnerable al problema de las invasiones, y sus hábitats costeros se encuentran entre los sistemas de la Tierra más gravemente afectados por organismos invasores (Grosholz 2002). Los estudios de introducciones biológicas marinas se suelen focalizar en los efectos negativos de éstas sobre las especies nativas (Caro *et al.* 2011), aunque se ha sugerido la facilitación interespecífica entre especies invasoras que puede derivar en lo que se llama una crisis por invasión (o “invasional meltdown”) (Simberloff & Von Holle 1999, Grosholz 2005). Aunque todavía no hay una evidencia clara de la naturaleza generalizada de este efecto (Simberloff 2006), la intensidad de los eventos recurrentes de introducción suelen llevar a un incremento en la heterogeneidad de la comunidad a través de la coexistencia de diversas especies no indígenas que interaccionan tanto entre ellas como con la comunidad residente (Byrnes & Stachowicz 2009, Dijkstra & Harris 2009). En efecto el control sobre especies exóticas recién llegadas a una comunidad puede ejercerse por otras especies ya introducidas más que por las nativas. Por ejemplo, hábitats dominados por especies introducidas serían más resistentes a futuras invasiones que las comunidades nativas (Osman & Whitlatch 2007). Por eso es importante entender las interacciones entre las especies introducidas dominantes, que en el medio marino suelen competir agresivamente por el espacio.

Además, los primeros estadios ontogenéticos de los organismos marinos (por ejemplo, fertilización, asentamiento y supervivencia larvaria) son particularmente vulnerables a las interacciones de competencia, las cuales pueden influir fuertemente en la eficacia biológica de la especie, y son la clave del establecimiento de los organismos marinos (Grosberg 1981, Bingham & Walters 1989, Osman & Whitlatch 1995a,b, Lambert 2000, Porri *et al.* 2008).

Panel 2. Interacciones biológicas

Las interacciones biológicas pueden ocurrir no solo entre las especies introducidas y las residentes, sino también entre especies no indígenas en el mismo hábitat. Hay un amplio rango de potenciales efectos de competencia tanto directos como indirectos.

La competencia por el espacio puede llegar a ser muy fuerte entre los invertebrados sésiles marinos. El más mínimo espacio disponible es suficiente para desencadenar una agresiva lucha por éste. En la foto vemos a varios invertebrados marinos sobre una estructura artificial en una instalación de acuacultura: poliquetos sabélidos y serpúlidos, briozoos, algas y ascidias, destacando la ascidia solitaria introducida *Styela plicata* (flecha negra).

(Foto: Víctor Ordóñez)

Para los organismos marinos que coinciden en la época de desove, puede darse competencia de interferencia en la fertilización debido a la competencia espermática (Lambert 2000).

(Foto: Víctor Ordóñez)

Algunas larvas de invertebrados marinos eligen el lugar de asentamiento teniendo en cuenta futuros riesgos como la presencia de depredadores (Johnson & Strathmann 1989, Stoner 1994) o competidores dominantes (Grosberg 1981). De este modo, el comportamiento larvario durante el asentamiento es crucial para el éxito del reclutamiento (Young & Chia 1984, Young & Cameron 1989, Stoner 1994). En la foto, larva de la ascidia *Microcosmus squamiger*.

La depredación de las larvas y juveniles también es importante para determinar el reclutamiento y la conectividad de la población (Bingham & Walters 1989, Young & Cameron 1989, André *et al.* 1993) controlando el establecimiento de los recién llegados (Osman & Whitlatch 1998).
En la foto, reclutas recién asentados de las ascidias *Microcosmus squamiger* (izquierda) y *Styela plicata* (derecha).

(Foto: Víctor Ordóñez)

El Mar Mediterráneo

El Mar Mediterráneo, con sus 2,5 millones de kilómetros cuadrados (el 0,82% del área total de los océanos), es uno de los ecosistemas marinos con más biodiversidad de todo el globo: representa entre el 4 y el 18% de la biodiversidad marina total (Bianchi & Morri 2000, Lejeusne *et al.* 2010). Es una región altamente vulnerable para la introducción de especies de diferentes orígenes, debido a su gran gradiente de temperaturas, tanto espacialmente (desde el Mar Alborán en el oeste, hasta el Mar Levantino en el este) como estacionalmente (cálido en verano y templado en invierno) (Izquierdo-Muñoz *et al.* 2009). Es uno de los mares más castigados y contaminados por la gran actividad humana costera y por la explotación de sus recursos (Coll *et al.* 2010). Históricamente ha tenido una gran afluencia de tráfico marítimo, tanto entre las diferentes zonas interiores del mar, como con otros mares sobre todo a través del Estrecho de Gibraltar (Panel 3). Este tráfico marítimo (mediante las aguas de lastre o el recubrimiento de los barcos) y la acuacultura han representado una importante vía de entrada de especies no indígenas en el Mediterráneo occidental (Galil 2009, Izquierdo-Muñoz 2009, Zenetos *et al.* 2012). La apertura del canal de Suez en 1869, aparte de conectar el Mar Mediterráneo con el Mar Rojo para fines comerciales, permitió la entrada al Mediterráneo oriental de todo un abanico de organismos marinos proveniente del Mar Rojo, en un proceso de entrada conocido como migración Lessepsiana (por Ferdinand de Lesseps, el francés que dirigió la construcción del canal). La migración Lessepsiana ha representado la introducción de cientos de especies no indígenas (más de 300, Galil 2009), ya sea por dispersión natural de las especies o asociado al tráfico marítimo del canal, convirtiéndose en el principal responsable de introducciones en el Mediterráneo oriental (Galil 2009, Zenetos *et al.* 2012).

Panel 3. Invasiones biológicas en el Mar Mediterráneo (I)

El abanico de distintos grupos de organismos introducidos en el Mar Mediterráneo es muy amplio: cordados, ascidias, equinodermos, moluscos, artrópodos, anélidos, ectoprocta, cnidarios, platihelmintos, ctenóforos, diferentes grupos de algas…
Del mismo modo, las especies introducidas provienen de todas partes del globo, incluyendo el Océano Índico, el Atlántico, de varias zonas del Océano Pacífico o del Mar Rojo.
El gran tráfico marítimo ha introducido un gran número de especies mediante las aguas de lastre o como recubrimiento del casco de los barcos. Por otro lado, la acuacultura es una industria en crecimiento en todo el mundo pero también en el Mar Maditerráneo (Basurco & Lovatelli 2003, Galil 2009, Theodorou *et al.* 2011), lo que ha representado otra importante vía de entrada de especies no indígenas.

Botrylloides violaceus (introducida vía acuacultura, originario del Japón) (Foto: Judith Oakley)

Diadema setosum (varias propuestas de vía de introducción: migración Lessepsiana, aguas de lastre, acuarios, origen Indo-Pacífico) (Foto: Wikipedia)

Mnemiopsis leidyi (introducido vía aguas de lastre, originaria del oeste del Atlántico) (Foto: Lars Johan Hansson)

Polyandrocarpa zorritensis (originaria del Perú) (Foto: Víctor Ordóñez)

Crassostrea gigas (introducida vía acuacultura, originario del Japón) (Foto: Víctor Ordóñez)

Caulerpa taxifolia (introducida a través de acuarios) (Foto: http://www.sardi.sa.gov.au)

Callinectes sapidus (cangrejo azúl Americano, introducido vía aguas de lastre) (Foto: http://www.jaxshells.org/bcrab.htm)

Phallusia nigra (introducción desde el Mar Rojo) (Foto: Nadia Bonnet)

Independientemente de su vector, zona de introducción o procedencia, el Mar Mediterráneo cuenta ya con cerca de 1000 especies exóticas, contando tanto metazoos como macrófitos (Boudouresque & Verlaque 2002, Zenetos *et al.* 2010, 2012), y la tendencia es a ir en aumento (Galil 2008, 2009, Zenetos *et al.* 2012). Además, el progresivo calentamiento del Mar Mediterráneo debido al cambio climático, está favoreciendo la continua introducción de especies tropicales exóticas (Raitsos *et al.* 2010).

Las ascidias

Las ascidias (Panel 4) son un grupo de invertebrados marinos importante en las comunidades bentónicas litorales por la diversidad que poseen y la biomasa que presentan en determinados hábitats (Monniot *et al.* 2001). Son hermafroditas y presentan una larva planctónica de vida libre: la larva renacuajo. Estas larvas son de vida corta (unas pocas horas) y lecitotróficas, se valen de sus reservas para subsistir hasta que se asientan en el substrato y realizan la metamorfosis (Svane & Young 1989). Esto implica, en principio, que no pueden recorrer grandes distancias y que su dispersión es limitada. Por lo tanto, el trasporte antropogénico es importante para su dispersión a largas distancias (López-Legentil *et al.* 2006). No obstante, se ha visto que algunas ascidias tienen una capacidad de dispersión natural relativamente alta dependiendo de las condiciones hidrológicas locales (Fletcher *et al.* 2013).

De entre los organismos marinos invasores, las ascidias se han convertido en un problema global (Lambert & Lambert 1998, 2003, Dijkstra *et al.* 2007, Lambert 2007). Tienen una gran capacidad de colonización de estructuras artificiales (Tyrrell & Byers 2007). Puertos, marinas, instalaciones de acuacultura y otras estructuras artificiales suelen ser los hábitats donde son introducidas y proliferan (Lambert & Lambert 2003, Lambert 2007, Locke *et al.* 2009). Desde estos sustratos artificiales, algunas especies de ascidias son capaces de colonizar los hábitats naturales adyacentes (Rius *et al.* 2009a, Simkanin *et al.* 2012). Por otro lado, la acuacultura de bivalvos es una industria comúnmente afectada por ascidias introducidas en todo el mundo (Gittenberger 2009, Arens *et al.* 2011) incluso causando grandes pérdidas económicas (Coutts & Forrest 2007, Daigle & Herbinger 2009). Las ascidias no indígenas cubren los bivalvos añadiendo peso y restringiendo el intercambio de agua y nutrientes, decreciendo la productividad del bivalvo (LeBlanc *et al.* 2002, Gittenberger 2009, Daigle & Herbinger 2009) o incluso ahogándolo por completo.

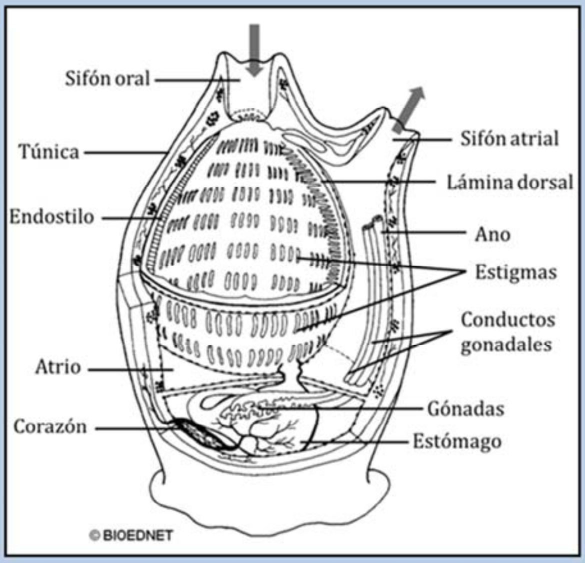
La eficiente capacidad de filtración de las ascidias podría ser la clave de las recientes explosiones poblacionales en regiones cercanas a zonas explotadas por el hombre, debido al aumento de bacterias y partículas en suspensión de las cuales se alimentan (Lambert 2005). De hecho, se conocen algunas ascidias tolerantes al estrés abiótico producido por contaminantes derivados de la actividad humana (Naranjo *et al.* 1996, Pineda *et al.* 2012a). Además, tienen una gran capacidad competitiva, desplazando a otros organismos sésiles y alterando la comunidad bentónica donde habitan (Castilla *et al.* 2004, Bullard *et al.* 2007a).

A parte de las posibles repercusiones ecológicas, la problemática que presentan globalmente las ascidias en las estructuras artificiales en puertos y marinas, pero sobretodo en las instalaciones de acuacultura de bivalvos, incluyendo el stock comercial, ha desencadenado el desarrollo de toda una serie de medidas para evitar los daños económicos que causan estos tunicados (Coutts & Forrest 2007, Locke *et al.* 2009). De esta manera, se han sugerido diferentes métodos para el control y erradicación de ascidias invasoras en el cultivo de bivalvos, como por ejemplo el uso de agua a alta presión (Arens *et al.* 2011), ácido acético (Page *et al.* 2011) o incluso lejía (Denny 2008), así como medidas de prevención intentando evitar su proliferación en estructuras artificiales que funcionan como una fuente de propágulos para estas especies (Coutts & Forrest 2007). Por otro lado y como hemos comentado anteriormente, los procesos post-frontera también abren oportunidades para la gestión de especies introducidas. En este sentido, una correcta identificación de las especies y el conocimiento de la biología de éstas (capacidad de dispersión, interacción con otras especies residentes, ciclo biológico, composición genética y conectividad entre poblaciones) son cruciales para poder desarrollar tales gestiones.

En esta tesis estudiamos aspectos ecológicos y genéticos de la biología de cuatro ascidias invasoras en el Mediterráneo occidental: *Microcosmus squamiger* y *Styela plicata* son dos ascidias solitarias introducidas en la región hace ya décadas, en las que se habían estudiado sus procesos pre-frontera así como otros aspectos de su biología (*M. squamiger*: Rius *et al.* 2008, 2009a,b, 2010, 2012; y *S. plicata*: Pineda *et al.* 2011, 2012a, b, 2013). Las otras dos especies son *Didemnum vexillum* y *Clavelina oblonga*.

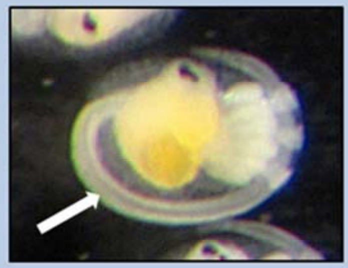
Estas cuatro especies están introducidas en varias partes del globo y son importantes por sus repercusiones ecológicas y/o económicas. A continuación proporcionaremos información más detallada de cada una de ellas.

Panel 4. Las Ascidias



Las ascidias son un grupo de invertebrados marinos, sésiles en su fase adulta, pertenecientes a los tunicados, llamados así por la característica túnica que les recubre el cuerpo. Los adultos pueden ser formas solitarias o formas coloniales. Son eficientes filtradores de plancton y materia orgánica particulada presentes en la columna de agua. Son hemafroditas. Las formas solitarias se reproducen sólo sexualmente, mientras que las coloniales lo hacen sexual y asexualmente.

En su fase larvaria presentan notocorda y tubo neural dorsal en la cola (flecha). La larva (o larva renacuajo) es planctónica, lecitotrófica (no se alimenta), nadadora y de vida corta (Foto: Víctor Ordóñez).



Microcosmus squamiger
(Foto: Víctor Ordóñez)

Styela plicata
(Foto: Víctor Ordóñez)

Didemnum vexillum
(Foto: Víctor Ordóñez)

Clavelina oblonga
(Foto: Carles Bori)

Microcosmus squamiger

La ascidia solitaria *Microcosmus squamiger* Michaelsen 1927 es nativa de Australia (Rius *et al.* 2008) y ha sido introducida en muchas partes templadas del globo, como el Atlántico este, el Mediterráneo occidental, California, Sudáfrica, la India y Nueva Zelanda (Rius *et al.* 2012 y referencias allí incluidas). *M. squamiger* se ha introducido como polizón en puertos y marinas a través de las aguas de lastre o como recubrimiento de los cascos de los barcos (Rius *et al.* 2008) y tiene la capacidad de colonizar sustratos naturales (Turon *et al.* 2007) donde forma poblaciones densas (Rius *et al.* 2009a). La ascidia puede tolerar aguas contaminadas por la actividad humana (Naranjo *et al.* 1996). Su ciclo de vida incluye un largo período de reproducción durante todo el verano (Rius *et al.* 2009a). Además, *M. squamiger* coexiste con otros invertebrados con los que compite por el espacio tanto en estructuras artificiales de puertos o marinas (como por ejemplo con los mejillones *Mytilus galloprovincialis* y *Perna perna* o la ascidia solitaria *Styela plicata*), como en sustratos rocosos naturales (con *M. galloprovincialis*). En consecuencia, esta especie representa un valioso modelo para estudiar el comportamiento y las capacidades de dispersión de poblaciones invasoras de ascidias, así como para estudiar las distintas interacciones que establece con otros organismos bentónicos.

Styela plicata

Styela plicata (Lesueur, 1823) es otra ascidia solitaria que goza de amplia distribución por los puertos y marinas de los mares cálidos y templados de todo el globo (Pineda *et al.* 2011). Es probablemente nativa del noroeste del Océano Pacífico, aunque las introducciones recurrentes y aleatorias por un largo período de tiempo hacen difícil concretar su fuente de origen (Pineda *et al.* 2011). La ascidia se suele encontrar sobretodo en estructuras artificiales hechas por el hombre en puertos y marinas, y sólo se sabe de algunas poblaciones establecidas en sustrato natural (Pineda 2012). Su gran éxito de introducción se ha atribuido a su gran tolerancia a aguas contaminadas (Naranjo *et al.* 1996) y a un amplio rango de salinidad y temperatura (Pineda *et al.* 2012a, b). Además, *S. plicata* se encuentra fértil prácticamente durante todo el año (Pineda *et al.* 2013) lo que le confiere una gran capacidad de producción de propágulos para su dispersión y colonización, compitiendo con otros organismos sésiles por el espacio, incluso desplazando especies nativas (Rius *et al.* 2009b). Por lo tanto, representa un excelente modelo para el estudio de interacciones biológicas con otros organismos sésiles de las comunidades artificiales establecidas en puertos y marinas de todo el mundo.

Didemnum vexillum

La ascidia colonial *Didemnum vexillum* Kott 2002, es nativa del noroeste del Océano Pacífico (Lambert 2009a; Stefaniak *et al.* 2009, 2012). Ha resultado ser un invasor muy exitoso en las aguas templadas de todo el mundo, incluyendo Nueva Zelanda, ambas costas de Norte América, Holanda, Gran Bretaña, Francia, Irlanda (Lambert 2009a, Stefaniak *et al.* 2009, 2012) así como en la costa oeste de la península Ibérica (El Nagar *et al.* 2010) y más recientemente en el Mar Adriático (Tagliapietra *et al.* 2012). *D. vexillum* ha resultado ser un gran problema mundial, tanto ecológico como económico, por su gran capacidad invasora tanto de sustratos artificiales, como de instalaciones y stocks de acuacultura, e incluso de fondos naturales llegando a alterar fondos de pesca (Coutts & Forrest 2007, Bullard *et al.* 2007a, Valentine *et al.* 2007a). La ascidia tiene una gran capacidad de excluir por competencia a otros organismos dominantes de fondos duros incluyendo otras ascidias (Dijkstra & Harris 2009, Dijkstra *et al.* 2007). Se han identificado varios vectores de introducción y dispersión a larga distancia de *D. vexillum*, incluyendo el recubrimiento de barcos (Herbog *et al.* 2009) o como epibiota del stock en acuacultura (Dijkstra *et al.* 2007, Denny 2008). Además, la fragmentación de la colonia representa otro mecanismo de dispersión, ya que los fragmentos pueden ser dispersados por corrientes, mareas o en aguas de lastre de los barcos y ser capaces de volver a unirse al sustrato y desarrollarse (McCarthy *et al.* 2007, Bullard *et al.* 2007b, Valentine *et al.* 2007a, Morris & Carman 2012).

La especie fue documentada por primera vez en el Mar Mediterráneo en 2012, en la Laguna de Venecia, Italia (Tagliapietra *et al.* 2012). Sin embargo, según observaciones de estos autores, es posible que la especie lleve más años establecida en la Laguna (al menos desde el 2007). *D. vexillum* se encontró en las instalaciones de acuacultura del Delta del Ebro, Tarragona, en la primavera del 2012, aunque según los trabajadores de las instalaciones podría haber estado presente, aunque más restringida, desde los últimos diez años. Es probable que la ascidia haya sido introducida a través del stock comercial del ostrón *Crassostrea gigas*, y se sospecha que provenga de Francia. Además, se ha observado que *D. vexillum* presenta una gran estacionalidad en su crecimiento y abundancia, pero antes del inicio de esta tesis aún no se sabía con exactitud cómo evolucionaba su ciclo de vida en aguas templadas, como por ejemplo su estado reproductor o su patrón de crecimiento a lo largo de las estaciones del año. Teniendo en cuenta la magnitud de la gravedad de las invasiones por *D. vexillum* y su complicada gestión (Coutts & Forrest 2007, Locke & Hanson 2009, Holt & Cordingley 2011, Switzer *et al.* 2011), aparte de conocer su procedencia, esta información biológica sería de especial valor para el conocimiento de la especie y el desarrollo de medidas preventivas eficientes para evitar dilatar más el rango de expansión de esta ascidia colonial y las posibles pérdidas económicas asociadas.

Clavelina oblonga

Clavelina oblonga Herdman, 1880 es una ascidia colonial nativa del caribe y de la costa sureste de Norte América, y ha sido introducida desde hace muchos años en Brasil, Islas Azores, Cabo Verde y Senegal (Rocha *et al.* 2012 y referencias incluidas). No obstante, la ascidia fue tratada como una especie más en la comunidad natural bentónica brasileña antes de averiguar su origen introducido (Rocha 1991, Rocha *et al.* 2009). A finales del verano de 2011, una ascidia creció sobre los mejillones cultivados en las instalaciones de acuacultura del Delta del Ebro, ahogándolos y provocando grandes pérdidas para el sector. La ascidia fue identificada morfológicamente como *Clavelina* sp. por X. Turon. Por otro lado, otra ascidia morfológicamente muy parecida había sido descrita en el Lago Fusaro, Italia en el 1929 como *C. phlegraea* Salfi, 1929. La similitud morfológica entre *C. oblonga* y *C. phlegraea* dejaba abierta la posibilidad de que la especie encontrada en el Delta del Ebro pudiera ser tanto una como la otra. Como veremos en el cuarto capítulo, *C. phlegraea* no es una especie válida y se trata en realidad de *C. oblonga*. Conocer el ciclo de vida de la ascidia (patrones de abundancia, reclutamiento y estado reproductor) representaría una herramienta valiosa para intentar desarrollar alguna estrategia de prevención para evitar futuras pérdidas.

Objetivos y Estructura de la Tesis

OBJETIVOS

El objetivo principal de esta tesis doctoral es estudiar ciertos aspectos ecológicos y genéticos de la biología de cuatro ascidias invasoras en el Mediterráneo occidental: *Microcosmus squamiger*, *Styela plicata*, *Didemnum vexillum* y *Clavelina oblonga*. El conocimiento detallado de la genética y biología nos permitirá conocer mejor el papel de estas especies en las comunidades bentónicas donde se establecen, ya sean sobre sustrato natural o artificial, así como desarrollar una gestión preventiva eficaz. Concretamente los objetivos son los siguientes:

1. Estudiar los procesos post-frontera de *Microcosmus squamiger* analizando la variabilidad genética y la estructura genética dentro de poblaciones así como la conectividad interpoblacional, en relación al tipo de sustrato (natural o artificial);

2. Analizar el grado de mezcla que existe a nivel poblacional e individual en las poblaciones introducidas respecto a los dos acervos genéticos identificados previamente del área nativa (Australia) de *Microcosmus squamiger*;

3. Evaluar los efectos de la competencia espermática y larvaria (tanto conspecífica como heteroespecífica) en el éxito de la fertilización, en el asentamiento larvario y en la metamorfosis de las ascidias *Microcosmus squamiger* y *Styela plicata*;

4. Estudiar la interacción de las larvas de las ascidias *Microcosmus squamiger* y *Styela plicata* entre sí y con juveniles del mejillón mediterráneo *Mytilus galloprovincialis* en España (rango nativo del mejillón) y en Sudáfrica (donde el mejillón es introducido) así como también con el mejillón marrón *Perna perna*, nativo del sur de África, que también coexiste con las ascidias en puertos y marinas de Sudáfrica;

5. Analizar genéticamente las poblaciones de *Didemnum vexillum* del Delta del Ebro y de Venecia y compararlas con poblaciones previamente analizadas de la distribución mundial de la especie;

6. Determinar si *Clavelina oblonga* y *C. phlegraea* son en realidad la misma especie mediante estudios moleculares y morfológicos;

7. Realizar un seguimiento del ciclo de vida de las dos ascidias *Didemnum vexillum* y *Clavelina oblonga* y así determinar su crecimiento, abundancia y reproducción a lo largo del año.

ESTRUCTURA DE LA TESIS

Para llevar a cabo los objetivos anteriormente comentados, esta tesis se ha estructurado en cuatro capítulos, compuesto cada uno por una publicación:

Primer Capítulo: Mixed but not admixed: a spatial analysis of genetic variation of an invasive ascidian on natural and artificial substrates.

En este capítulo analizaremos la dispersión post-frontera a pequeña escala de poblaciones introducidas de la ascidia *Microcosmus squamiger* en la costa catalana, evaluando la variabilidad genética de la ascidia tanto en poblaciones establecidas en sustrato natural como en artificial, estudiando la estructura genética dentro y entre poblaciones y determinar si ésta varía según el tipo de sustrato. Además estudiaremos el grado de mezcla que existe a nivel poblacional e individual respecto a los dos acervos genéticos del área nativa de la especie (Australia). Se conoce la historia del proceso pre-frontera de *M. squamiger*, pero poco se sabe de la capacidad de dispersión de la ascidia que le ha permitido establecerse en sustrato natural una vez ha sido introducida en los puertos.

Segundo Capítulo: Early biotic interactions among introduced and native species reveal cryptic predation and shifts in larval behaviour.

Aquí exploramos las interacciones ontogenéticas tempranas de las ascidias *Microcosmus squamiger* y *Styela plicata* entre ellas (ya que ambas ascidias coexisten y comparten el espacio de asentamiento en los puertos y marinas donde se han introducido) y con otros invertebrados abundantes del bentos marino, también presentes en estructuras artificiales en puertos y marinas, los mejillones. Este conjunto de organismos (ascidias y mejillones) constituye un buen modelo para explorar las interacciones entre especies dominantes ya que son abundantes y gregarios, coexisten y pueden competir por el sustrato como recurso principal. Evaluaremos los efectos de la competencia espermática conspecífica y heteroespecífica en el éxito de la fertilización de las ascidias *M. squamiger* y *S. plicata*, así como el efecto de la presencia conspecífica y heterospecífica de larvas de las dos ascidias en el éxito y comportamiento en el asentamiento y en la metamorfosis. Además, estudiaremos la interacción entre las larvas de estas dos ascidias con juveniles del mejillón mediterráneo *Mytilus galloprovincialis* en España (rango nativo) y en Sudáfrica, donde el mejillón es introducido. Como se ha comentado anteriormente, *M. squamiger* ha sido capaz de colonizar el bentos natural fuera de puertos en la costa mediterránea, donde comparte nicho con *M. galloprovincialis*, por lo que las interacciones entre estos dos organismos son más que probables. Además, se ha usado el mejillón marrón *Perna perna*, nativo del sur de África, para completar el estudio, ya que también coexiste con las ascidias en puertos y marinas de Sudáfrica.

Tercer Capítulo: Ongoing expansión of *Didemnum vexillum* in the Mediterranean Sea: biological cycle and genetic structure of a worldwide invader.

En el Tercer Capítulo haremos un monitoreo de la evolución del recubrimiento de *Didemnum vexillum* en las cuerdas de ostrón en el Delta del Ebro para conocer su ciclo de crecimiento y abundancia, así como también describir su ciclo reproductivo. Además, obtendremos datos genéticos de la población del Delta del Ebro y de la de la láguna véneta, única cita previa de la especie en el Mediterráneo. A continuación compararemos la variabilidad genética de las poblaciones del Mediterráneo con poblaciones previamente analizadas de la distribución mundial de la especie. *D. vexillum* tiene una gran capacidad de colonización y causa problemas tanto económicos como ecológicos en varias partes del globo, por lo que es importante un conocimiento exhaustivo de su biología para poder desarrollar medidas preventivas en la zona.

Cuarto Capítulo: When invasion biology meets taxonomy: presence of *Clavelina oblonga* Herdman, 1880 in the Mediterranean Sea.

En el último capítulo de esta tesis utilizamos la morfología y la genética para desvelar la verdadera identidad de la especie de *Clavelina* que ahogó la cosecha de mejillones en 2011 en el Delta del Ebro, y determinar si *Clavelina oblonga* y *C. phlegraea* son en realidad la misma especie. Asimismo, también realizaremos un seguimiento de la evolución del recubrimiento de la ascidia en las cuerdas de mejillón, así como en cuerdas con placas de PVC, para conocer su ciclo de crecimiento y abundancia y, por último, estudiaremos su ciclo reproductor y los patrones de reclutamiento.

Después de los cuatro capítulos, la tesis comprende una Discusión y Conclusiones generales, así como un apartado con la Bibliografía citada en la Introducción y Discusión, ya que cada capítulo posee su propio listado de referencias. Finalmente, la tesis incorpora los apéndices que corresponden a cada capítulo. Además, se incluyen como apéndices dos artículos en los que el doctorando es coautor: Rius *et al.* 2012 y Pineda *et al.* 2012. Aunque estos artículos no están incluidos en la presente tesis, el trabajo de Rius *et al.* 2012 fue fundamental para la concepción y planificación del Primer Capítulo. Por otro lado, el trabajo de Pineda *et al.* 2012 es complementario al del Segundo Capítulo de esta tesis, ya que, mientras el Segundo Capítulo se centra en interacciones bióticas, Pineda *et al.* 2012 aborda el efecto de variables abióticas sobre el desarrollo temprano de *Styela plicata* y *Microcosmus squamiger*, Por todo ello pensamos que disponer de estos artículos en sendos apéndices puede facilitar la lectura y comprensión de la tesis.

Primer Capítulo:

Juntos pero no revueltos: análisis espacial de la variación genética de una ascidia invasiva en sustratos naturales y artificiales

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RESUMEN

Siguiendo la introducción a una nueva área (dispersión pre-frontera), los procesos post-frontera determinan el éxito en el establecimiento de las especies no indígenas. Sin embargo, se conoce poco sobre cómo estos procesos post-frontera dan forma a la composición genética de las especies no indígenas a escalas regionales. En este capítulo analizamos la variación genética de poblaciones introducidas a lo largo de costas con un alto impacto humano, para inferir las dinámicas demográficas y de parentesco en el estado post-frontera. Usamos la ascidia *Microcosmus squamiger* como modelo, una especie introducida mundialmente. Esta ascidia puede colonizar y crecer rápidamente en estructuras artificiales creadas por el hombre, afectando instalaciones como las de maricultura. No obstante, también puede establecerse sobre sustrato natural, alterando así la comunidad natural convirtiéndose en un problema ecológico. Se han genotipado 302 individuos de ocho poblaciones establecidas en sustratos naturales y artificiales en la costa catalana (noroeste del Mediterráneo), mediante seis loci microsatélites. Comparamos también los genotipos resultantes con los encontrados en la región nativa de la especie (Australia). Encontramos niveles altos de diversidad genética y riqueza alélica en todas las poblaciones, con un déficit general de heterocigotos. El análisis de autocorrelación mostró que no hay estructura genética dentro de poblaciones (a escala de decenas de metros); asimismo, no se encontró diferenciación significativa en las comparaciones por pares entre las poblaciones (decenas de

kilómetros) ni tampoco se encontró un patrón de aislamiento por distancia. Los resultados sugieren que *M. squamiger* tiene una gran capacidad natural de dispersión desde una zona de sustrato duro a otra sin presentar diferencias entre sustrato natural y artificial. Es de destacar que se detectaron dos grupos de individuos genéticamente diferenciados asociados con las dos áreas de origen ancestrales de las poblaciones introducidas mundialmente. El test de asignación individual mostró la coexistencia de individuos de estos dos acervos en todas las poblaciones pero con poco cruzamiento entre ellos, ya que la frecuencia de individuos mezclados fue sólo del 15%. Se desconoce el mecanismo responsable de que estos acervos genéticos se mantengan sin mezclarse, pero este hecho no parece comprometer a la colonización post-frontera de las poblaciones introducidas.

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ORIGINAL PAPER

Mixed but not admixed: a spatial analysis of genetic variation of an invasive ascidian on natural and artificial substrates

Víctor Ordóñez · Marta Pascual · Marc Rius · Xavier Turon

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Abstract Following the introduction to a new area (pre-border dispersal), post-border processes determine the success in the establishment of non-indigenous species (NIS). However, little is known on how these post-border processes shape the genetic composition of NIS at regional scales. Here, we analyse genetic variation in introduced populations along impacted coastlines to infer demographic and kinship dynamics at the post-border stage. We used as a model system the ascidian species *Microcosmus squamiger* that has been introduced worldwide. This species can colonize and grow fast on man-made artificial structures, impacting activities such as mariculture. However, it can also establish itself on natural substrates, thus altering natural communities and becoming an ecological problem. We genotyped 302 individuals from eight populations established on natural and artificial substrates in the north-western Mediterranean Sea, using six microsatellite loci. We then compared the resulting genotypes with those found within the native range of the species. We found high levels of genetic diversity and allelic richness in all populations, with an overall deficit of heterozygotes. Autocorrelation analyses showed that there was no within-population genetic structure (at a scale of tens of metres); likewise, no significant differentiation in pairwise comparisons between populations (tens of kilometres apart) and no isolation-by-distance pattern was found. The results suggest that *M. squamiger* has a natural capacity for high dispersal from one patch of hard substrate to another and no differences whatsoever could be substantiated between natural and artificial substrates. Interestingly, two groups of genetically differentiated individuals were detected that were associated with the two ancestral source areas of the worldwide expansion of the species. Individual assignment tests showed the coexistence of individuals of these two clusters in all populations but with little interbreeding among them as the frequency of admixed individuals was only 15 %. The mechanism responsible for maintaining these genetic pools unmixed is unknown, but it does not appear to compromise post-border colonization of introduced populations.

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Introduction

The artificial introduction of species to new areas, defined as pre-border (Forrest et al. 2009), or extra-range dispersal (Wilson et al. 2009) is mediated by several vectors (Carlton and Geller 1993; Ruiz et al. 1997; Wonham et al. 2001) and is an ongoing process that will continue in the foreseeable future despite prevention efforts (Bax et al. 2001; Hulme 2006). However, the establishment of a species translocated to a new area depends on the success of post-border processes, that is, those involving the establishment and spread after the initial inoculation (Forrest et al. 2009). Such processes determine whether the species will become invasive or not and open valuable opportunities for the management and prevention of invasive species (Wotton and Hewitt 2004; Forrest et al. 2009; Airoldi and Bulleri 2011). Survival, reproduction, dispersal and local environmental conditions are the main factors that determine the success of an introduced population during the post-border stage (Blackburn et al. 2011). A key process is the regional spread of the species that, contrary to what happens in extra-range dispersal, relies heavily on its natural dispersal abilities, as well as on human-mediated transport at local scales (Wasson et al. 2001; Branch and Steffani 2004).

A common feature of many successful invaders is that their colonization histories often involve multiple introductions from multiple sources (Sakai et al. 2001; Geller et al. 2010), which in the sea is often accomplished through repeated transport following the main routes of ship traffic or via aquaculture activities (Roman and Darling 2007). This often leads to a high genetic diversity of introduced populations through admixture of genetically differentiated sources (Geller et al. 2010). Furthermore, the interaction between different genetic pools (e.g. McIvor et al. 2001; Meusnier et al. 2002; Kelly et al. 2006; Pineda et al. 2011) from different origins and, potentially, different ecophysiological characteristics can significantly affect the success of introduced populations in a way that remains largely unexplored.

In heavily urbanized coastlines, dispersal is also favoured by the existence of marinas and harbours, with associated shipping activities. While primary introductions can depend on ship traffic between main ports, a network of marinas with boating activities can contribute to secondary dispersal (Lambert and Lambert 1998; Wasson et al. 2001; Lacoursière-Roussel et al. 2012). Natural dispersal between suitable habitats can lead to stepping stone models of dispersal, while boat-mediated dispersal can break down relationships between geographical distance and natural connectivity at local scales (Lacoursière-Roussel et al. 2012).

Artificial substrates can act as dispersal corridors for exotic rocky-bottom species (Bulleri and Airoldi 2005; Glasby et al. 2007; Vaselli et al. 2008; Bulleri and Chapman 2010; Airoldi and Bulleri 2011) and can represent a source of infiltration to natural benthos by non-indigenous species (Simkanin et al. 2012). These structures do not function as surrogates from natural substrates, as structure and dynamics of communities on artificial hard substrate are different from those in adjacent natural rocky bottoms (e.g. Pinn et al. 2005; Tyrrell and Byers 2007; Bulleri and Chapman 2010; Airoldi and Bulleri 2011). In general, species richness is lower in communities on artificial substrate than on well-established natural communities (e.g. Bacchiocchi and Airoldi 2003), and many ecological processes such as recruitment, competition or predation may differ between natural and artificial habitats (Bulleri and Chapman 2010; Dumont et al. 2011). Overall, natural communities are expected to be able to influence propagule settlement more than artificial assemblages and thus to be less prone to invasion (Stachowicz and Tilman 2005; Tyrrell and Byers 2007; Dumont et al. 2011, but see Osman and Whitlatch 2007). Therefore, in order to understand the patterns of colonization and connectivity with possible source areas (e.g. Fauvelot et al. 2009), it is essential to compare the genetic composition and diversity of introduced populations on natural and artificial substrates.

Among marine organisms, ascidians have become a worldwide problem as invader species (Lambert and Lambert 1998, 2003; Dijkstra et al. 2007; Lambert 2007; Locke 2009). Non-indigenous ascidians rapidly colonize artificial substrates in harbours such as pilings, docks, floating pontoons, boat hulls and buoys (Lambert 2002; Lambert and Lambert 2003). From these artificial substrates, some species can colonize adjacent natural environments (Rius et al. 2009; Simkanin et al. 2012). Ascidians expand their ranges naturally via their non-feeding larvae, which only allow a restricted dispersal. This group is therefore a good model to study the patterns of dispersal and connectivity of introduced species at small scales. Pre-border processes in non-indigenous ascidian species have been studied in a number of species with the aid of genetic tools, focusing on relatedness among colonized areas and on inferring the putative origin of colonizers (e.g. López-Legentil et al. 2006; Barros et al. 2009; Zhan et al. 2010; Goldstien et al. 2011; Lejeusne et al. 2011; Pineda et al. 2011; Rius et al. 2012). However, post-border dispersal processes are less studied in ascidians (e.g. *Styela clava*, Goldstien et al. 2010; *Ciona intestinalis*, Zhan et al. 2012; *Perophora japonica*, Pérez-Portela et al. 2012).

The solitary ascidian *Microcosmus squamiger* Michaelsen 1927 is native to Australia (Michaelsen 1927; Kott 1985; Rius et al. 2008a) and has been introduced in many parts of the world, such as the eastern Atlantic, western Mediterranean, California, South Africa, India and New Zealand (Rius et al. 2012 and references therein). *M. squamiger* has been introduced as a stowaway in ports and marinas via ballast water or ship fouling (Rius et al. 2008a) and has the capacity to colonize natural substrates (Turon et al. 2007) where it forms dense populations (Rius et al. 2009), and thus, this species represents a valuable model to study behaviour and dispersal capacities of invasive populations. Genetic data showed that its colonization process involved two source areas genetically differentiated (eastern and western Australia, Rius et al. 2012) which contributed to the genetic make-up of the introduced populations. Pre-border processes (global genetic patterns) in this species have been studied by Rius et al. (2008a, 2012), but there is no information on fine-scale genetic patterns and dispersal at local scales (i.e. at the post-border stage).

Microcosmus squamiger can entirely carpet artificial habitats (Turon et al. 2007; Rius et al. 2009). A plausible consequence of a fast colonization of coastal infrastructures would be a low genetic diversity of these populations, as found for other benthic invertebrates (Fauvelot et al. 2009), with high kinship values within the aggregations. On the other hand, high genetic diversity is also found in populations established on such artificial substrates (e.g. Rius et al. 2008a, Zhan et al. 2012). Natural habitats have less dense populations of *M. squamiger* (authors' pers. obs.), which may result in higher or lower genetic diversity as a function of the colonization process with drift, competition pressure and propagule dispersal playing an important role.

The study of genetic variability and population connectivity of invasive species can unravel post-border dispersal dynamics and is a necessary step to design management and intervention plans (Forrest et al. 2009). In this sense, the main goal of the present study was to assess post-border, fine-scale dispersal in artificial and natural substrates of introduced populations of *M. squamiger* by analysing genetic structure within and between populations. Our aims were (1) to study the genetic variability of both natural and artificial substrate populations, (2) to determine whether fine-scale population structure exists within populations and varies according to the type of substrate, (3) to assess inter-population connectivity related to substrate type and geographical distance and (4) to analyse the degree of admixture at the individual and population levels with respect to the two recognized genetic pools in the native area of the species.

Materials and methods

Sampling area

We sampled eight sites along a 50-km stretch of highly urbanized coast in the north-western Mediterranean Sea (Fig. 1). There were two big commercial ports north and south of the studied coastline, the port of Barcelona located 30 km north-eastwards and the Port of Tarragona 22 km south-westwards. These ports can act as entry points for the species in the area. This coast mainly consists of sandy areas interspersed with natural rocky outcrops and artificial breakwaters (spaced at most ca. 3 km). It provides therefore an adequate setting for testing stepping stone dispersal of the studied species, which does not settle on soft substrate. All sampled sites were located in exposed open coastal zones, that is, they are located outside confined areas such as marinas or embayments. Four of the sites were found on natural substrates: Garraf-natural (GAN), Sitges-natural (SIN), El Roc de Sant Gaietà (SG), Torredembarra (TB); and four on artificial substrates: Garraf-artificial (GAA), Sitges-artificial (SIA), Cubelles (CU) and

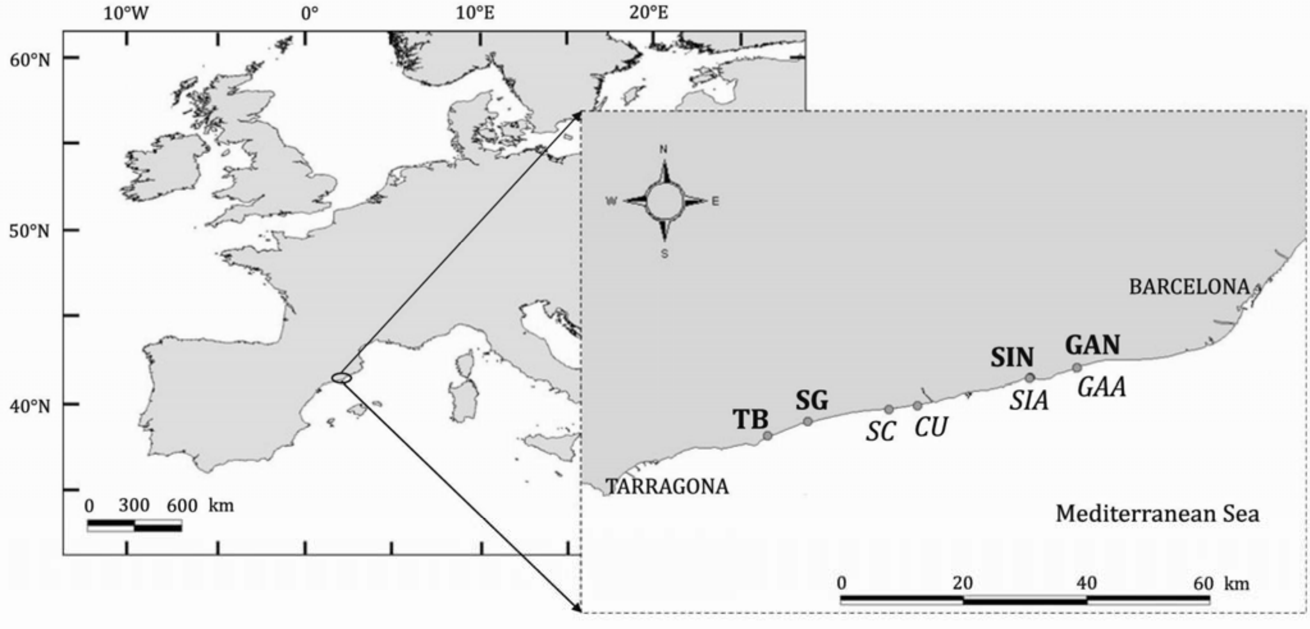

Fig. 1 Sampled localities between the ports of Barcelona and Tarragona (Spain, north-western Mediterranean). In bold, the localities sampled on natural substrate, and in *italics*, the localities sampled on artificial substrate. Population codes as in Table 1

Table 1 Genetic variability of natural and artificial substrate populations

	Locus						
	MS 6	MS 7	MS 10	MS 11	MS 12	MS 13	Mean
Populations on natural substrate							
Garraf (GAN)							
N	18	18	16	18	18	18	17.667
NA	2	2	5	6	5	4	4.000
AR	2.000	2.000	4.938	5.833	4.807	3.833	3.902
H_o	0.278	0.389	0.625	0.667	0.333	0.556	0.475
H_e	0.386	0.475	0.613	0.795	0.502	0.663	0.572
F_{IS}	0.286	0.185	−0.020	0.166	0.342	0.167	0.175
Sitges (SIN)							
N	59	59	59	59	59	59	59.000
NA	2	3 (1)	7	6	6	4	4.667
AR	1.997	2.254	4.853	5.776	5.174	3.694	3.958
H_o	0.271	0.576	0.712	0.441	0.254	0.627	0.480
H_e	0.261	0.458	0.621	0.792	0.666	0.646	0.574
F_{IS}	−0.040	**−0.262**	**−0.148**	**0.446**	**0.621**	0.030	**0.165**
El Roc de Sant Gaietà (SG)							
N	45	45	44	45	45	45	44.833
NA	3 (1)	2	7	7	7	5 (1)	5.167
AR	2.331	2.000	5.544	5.873	5.750	4.137	4.272
H_o	0.333	0.511	0.614	0.622	0.444	0.556	0.513
H_e	0.284	0.425	0.604	0.800	0.622	0.651	0.564
F_{IS}	**−0.174**	−0.206	−0.016	**0.224**	0.287	0.148	0.091
Torredembarra (TB)							
N	34	34	34	34	34	34	34.000
NA	3	2	7	6	8	4	5.000
AR	2.441	2.000	5.425	5.883	5.892	3.685	4.221
H_o	0.353	0.500	0.588	0.676	0.441	0.559	0.520
H_e	0.355	0.409	0.547	0.808	0.543	0.595	0.543
F_{IS}	0.006	−0.228	−0.076	0.165	0.191	0.062	0.043
Populations on artificial substrate							
Garraf (GAA)							
N	27	27	24	27	27	27	26.500
NA	2	2	8	7	5	4	4.667
AR	1.998	2.000	6.124	6.869	4.803	3.913	4.284
H_o	0.259	0.296	0.708	0.630	0.296	0.593	0.464
H_e	0.230	0.425	0.621	0.840	0.584	0.635	0.556
F_{IS}	**−0.130**	0.307	−0.143	**0.254**	**0.498**	0.068	**0.168**
Sitges (SIA)							
N	16	16	15	16	16	16	15.833
NA	2	2	3	6	3	4	3.333
AR	2.000	2.000	3.000	5.935	3.000	3.938	3.312
H_o	0.438	0.500	0.267	0.813	0.750	0.750	0.586
H_e	0.353	0.484	0.246	0.772	0.575	0.688	0.520
F_{IS}	**−0.250**	−0.034	−0.087	−0.054	−0.319	−0.094	−0.133
Cubelles (CU)							
N	56	57	57	57	56	57	56.667
NA	2	2	11	7 (1)	10 (1)	5 (1)	6.167

Table 1 continued

	Locus						
	MS 6	MS 7	MS 10	MS 11	MS 12	MS 13	Mean
AR	1.999	2.000	6.752	6.714	6.616	4.150	4.705
H_o	0.357	0.474	0.579	0.649	0.446	0.596	0.517
H_e	0.296	0.429	0.580	0.834	0.700	0.613	0.575
F_{IS}	**−0.209**	−0.104	0.001	**0.223**	**0.364**	0.028	**0.102**
Segur de Calafell (SC)							
N	46	46	42	46	45	46	45.167
NA	4 (1)	2	8	6	8	4	5.333
AR	2.650	2.000	6.415	5.896	6.075	3.698	4.456
H_o	0.304	0.326	0.548	0.587	0.378	0.717	0.477
H_e	0.297	0.379	0.618	0.818	0.527	0.656	0.549
F_{IS}	−0.024	0.141	0.116	**0.284**	**0.285**	−0.094	**0.133**

Number of amplified individuals (N); number of alleles (NA), private alleles (if any) are indicated inside parenthesis; allelic richness per locus and population (AR), based on a minimum amplified sample size (over all loci) of 15 diploid individuals; observed (H_o) and expected (H_e) heterozygosities; and inbreeding coefficient (F_{IS})

Significant F_{IS} values are in bold. Means over loci (or global value for F_{IS}) are also indicated

Segur de Calafell (SC) (Fig. 1, Table S1). Samples were collected in 2010 from natural rocky walls or groynes and breakwaters at depths between 1 and 4 m through snorkelling or SCUBA. Overall, we studied 302 individuals: 156 collected from natural substrate and 146 from artificial substrate (Table S1).

In order to investigate fine-scale (within-population) genetic structure, we collected samples along a fixed 90-m horizontal transect marked with a measuring tape in two of the natural substrate sites (Sitges and El Roc de Sant Gaietà) and two with artificial substrate (Cubelles and Segur de Calafell). Individuals were collected at 7 points along these transects at increasing distance from the initial point (Table S2). At each point, all individuals situated within a 20-cm radius were collected (resulting in sample sizes of 4–15 individuals per point). The design allowed for the comparison of relatedness among individuals located 0–90 m apart.

The remaining localities were sampled by collecting individuals haphazardly (i.e. picking individuals randomly but at distances larger than 0.5 m) across a total linear distance of ca. 100 m. These and the previous samples were used to test connectivity as related to substrate type and geographical distance. We obtained thus the two following datasets: one to assess genetic variability and differentiation among all populations and the other to assess fine-scale genetic structure in the four populations sampled following the transect line. Once in the laboratory, all individuals were dissected and a piece of muscular tissue preserved in absolute ethanol and stored at −80 °C.

DNA extraction and amplification

We used six microsatellite loci isolated from this species (Rius et al. 2008b): MS6, MS7, MS10, MS11, MS12 and MS13. These microsatellites do not show linkage disequilibrium so they can be considered independent loci (Rius et al. 2008b). The genomic DNA extraction was performed using the *Squishing Buffer* (SB) + *proteinase K* method, originally developed for *Drosophila* (Gloor et al. 1993).

The PCR amplification was performed with a final volume of 20 µl. MS7 and MS13 were amplified together at 57 °C annealing temperature, with 4 µl of buffer 5× (Green GoTaq, Promega), 2.5 µl of dNTPs (1 mM), 1.8 µl of $MgCl_2$ (25 mM), 8.9 µl of H_2O, 0.6 µl of DMSO, 0.2 µl of Taq corresponding to 1 unit (GoTaq, Promega), 0.25 µl of each primer (10 µM) and 1 µl of DNA. The other microsatellites were amplified separately using 4 µl of buffer 5× (Green GoTaq, Promega), 2.5 µl of dNTPs (1 mM), 3 µl of $MgCl_2$ (25 mM), 7.7 µl of H_2O, 0.6 µl of DMSO, 0.2 µl of Taq corresponding to 1 unit (GoTaq, Promega), 0.25 µl of each primer (10 µM) and 1 µl of DNA. The annealing temperature was 50 °C for MS10, 53 °C for MS6 and 57 °C for MS11 and MS12. The PCRs started with an initial denaturation at 94 °C for 5 min, followed by 35 cycles of a denaturation step at 94 °C for 1 min, an annealing step at the corresponding temperature for 30 s and an elongation step at 72 °C for 30 s, with a final extension at 72 °C for 5 min. The forward primer of each locus was marked with an appropriate fluorochrome (Rius et al. 2008b). We used the GeneMapper® software (version 3.7, Applied Biosystems, 2004) to assign allele sizes to each microsatellite and to genotype each individual.

We had a small number of failed amplifications for three of the loci. This was despite repeated attempts using different PCR conditions and new DNA extractions. The percentage of failed amplifications was low and varied among loci (0.3 % for MS6, 3.64 % for MS10, 0.66 % for MS12).

We also performed comparisons of the microsatellite dataset here obtained with the genotypes obtained by Rius et al. (2012) from two native localities (Manly and Bunbury). These samples were genotyped in the same machine but at different years than the present study, and the original chromatograms of these samples were manually checked for possible bias on allele calling between studies. No correction was necessary.

Analysis of genetic variability

The program Microsatellite toolkit version 3.1.1 (Park 2001) was used to transform the data files into the adequate formats for the different programmes used. In order to compare genetic diversity patterns among populations with different numbers of individuals, we calculated the allelic richness corrected per sample size with the program Fstat version 2.9.3.2 (Goudet 2002). We obtained the number of alleles and both the expected and observed heterozygosities for each locus and population using GeneClass version 2 (Piry et al. 2004). Deviations from Hardy–Weinberg equilibrium were tested with the inbreeding coefficient F_{IS}, and its significance assessed by 10,000 bootstrap replicates with the program Genetix version 4.05.2 (Belkhir et al. 2004). In order to compare genetic variability between populations in natural and artificial substrate, the permutation test implemented in Fstat was used.

Within-population spatial genetic structure

In order to assess fine-scale genetic structure, we used autocorrelation analysis, which allows an assessment of the scale at which discontinuities occur (Heywood 1991). We designed the spatial sampling across transects to evaluate potential genetic consequences of dispersal over fine spatial scales (Loiselle et al. 1995). Given all possible distances between the different sampling points (Table S2), we established 18 distance classes (in metres): 0.2, 2.5, 5, 7.5, 10, 15, 17.5, 20, 30, 35, 37.5, 40, 50, 70, 80, 85, 87.5 and 90. These distances designate the endpoints of each distance class. Distances were chosen to obtain the highest number of intervals but with enough data points at each interval to have at least 20 pairwise comparisons within each class (overall mean: 56.25 comparisons per distance class). The class 0.2 refers to individuals collected within each transect point and separated by less than 20 cm.

For individual comparisons, we computed two statistics: the kinship coefficient (Loiselle et al. 1995) and the $\hat{a}$ index defined by Rousset (2000). The kinship coefficient is a similarity measure that estimates the probability that two alleles of an autosomal locus are identical by descent (Ritland 1996). The advantage of this index is that it is not dependent on Hardy–Weinberg equilibrium conditions (Hardy and Vekemans 1999; Fenster et al. 2003). The $\hat{a}$ index is a distance measure analogous to the $F_{ST}/(1 - F_{ST})$ ratio (see Rousset 2000 for details). The 95 % confidence intervals of non-association at a given distance interval were assessed through 1,000 randomizations, permuting the position of the individuals along the transect, which effectively eliminated all spatial structure but preserved sample sizes and individual genotypes. We performed these analyses using the program SPAGeDi version 1.2 (Hardy and Vekemans 2002). In order to compare the kinship coefficient and the $\hat{a}$ index between natural and artificial substrates, SigmaStat version 3.11 (Systat Software, Inc.) was used to perform the nonparametric Mann–Whitney U test.

Genetic differentiation among populations

Recent studies have questioned the suitability of commonly used G_{ST}- and F_{ST}-like estimators to assess population differentiation, as they are highly dependent on the variability of the marker used, and new estimators have been proposed (Hedrick 2005; Jost 2008). Nevertheless, the issue is still in debate (Jost 2009, Whitlock 2011, Jakobsson et al. 2013), and it seems advisable for the time being to use both traditional and new estimators (Meirmans and Hedrick 2011). We have therefore assessed population genetic differentiation using conventional F_{ST} estimates and the new estimator D_{est} as in equation 12 of Jost (2008). The former was calculated with Genetix using the estimator of Weir and Cockerham (1984). The significance of the pairwise values was assessed with 1,000 permutations of data, after correcting for multiple comparisons using the Benjamini–Yekutieli method described in Narum (2006). For D_{est} estimates, we used the R package DEMEtics version 0.8.1 (Gerlach et al. 2010). We calculated a confidence interval around the obtained values by 1,000 bootstrap replicates. In this procedure, alleles are automatically randomized over populations when the compared populations are in Hardy–Weinberg equilibrium; otherwise, genotypes are randomized. The programme automatically adjusts confidence intervals for multiple comparisons. Significant differentiation was inferred when this confidence interval excluded zero. The program GenePop On The Web version 4.0.10 (Raymond and Rousset 1995) was used to test whether there is isolation by distance among the studied populations comparing the D_{est}

and F_{ST} pairwise matrices with the geographical distance matrix using a Mantel test. Analyses of molecular variance (AMOVA) grouping populations by substrate (natural and artificial) were performed using a F_{ST}-like statistic with the program Arlequin version 3.5.1 (Excoffier et al. 2005).

Admixture analysis

In order to assess whether there was any overall structure in our microsatellite dataset, we used the program STRUCTURE version 2.3 to find the most likely number of genetically differentiated clusters (K) (Pritchard et al. 2000; Hubisz et al. 2009). We used the admixture and loc prior model because it performs better than other models for detecting genetic structure even in the situations of low levels of genetic divergence or a limited number of loci (Hubisz et al. 2009). Twenty independent runs were performed for increasing values of K from 1–8 (number of populations) using 500,000 iterations and a burn-in period of 50,000. We ran CLUMPP version 1.1.2 (Jakobsson and Rosenberg 2007) to merge the results of the 20 runs for the most likely K, and DISTRUCT version 1.1 (Rosenberg 2004) was used to graphically display the results. Using the 20 runs, we plotted both the Inc$K$ statistic (Evanno et al. 2005) and the log probability of the data (LnP(D)) as a function of K and looked for the value that best captured the structure of the data. Additionally, we used a K-means clustering algorithm available in the R package *adegenet* 1.2–8 (Jombart 2008) to find groups of individuals that maximize between-group genetic variation (function *find.clusters*). We used the same K as obtained with STRUCTURE to compare the groups recovered by both methods.

In order to check for structure in our data with a different approach, we ran a factorial correspondence analysis (FCA) using Genetix. FCA provides a graphical representation of genetic distances among individuals without any a priori clustering and also evaluates the relative contribution of each allele to the ordination found.

We used discriminant analysis of principal components (DAPC, Jombart et al. 2010), to further analyse our data. DAPC is a recently developed technique that extracts information from genetic datasets (multivariate in nature) by first performing a principal component analysis (PCA) on pre-defined groups or populations and then using the PCA factors as variables for a discriminant analysis (DA). In order to assess the relationships of the groups defined with STRUCTURE with individuals from two populations, Bunbury (W Australia) and Manly (E Australia), representative of the two source areas of the worldwide expansion of the species (Rius et al. 2012), we ran DAPC (function *dapc* in the package *adegenet*) with our samples and the Australian populations. Variables were centred but not scaled, and 30 principal components of PCA were retained and input to DA. Additionally, an AMOVA grouping individuals as per genetic groups derived from STRUCTURE results were performed with Arlequin as explained above.

Results

Genetic variability of natural and artificial substrate populations

There were large differences in the genetic variability of the different loci, ranging from 3 to 13 alleles. Mean total allelic richness was similar between populations in natural and artificial substrates (natural substrate mean $\pm$ SE $=$ 24.530 $\pm$ 0.557; artificial substrate $=$ 25.136 $\pm$ 1.829). No significant differences were found with a permutation-based method between natural and artificial populations (P values for allelic richness $= 0.802$; $H_o = 0.769$; gene diversity $= 0.486$; $F_{IS} = 0.688$; 10,000 permutations). The inbreeding coefficient F_{IS} across loci (Table 1) was significant in one population from natural substrate (SIN) and three populations from artificial substrate (GAA, CU and SC). MS11 and MS12 loci featured the highest deviation from Hardy–Weinberg equilibrium (HWE), with significant positive F_{IS} values in five and four populations, respectively. Locus MS6 and MS10 had significant negative F_{IS} values in four and one population, respectively. Overall, there was a heterozygote deficiency in all populations (except for SIA, with negative but non-significant F_{IS} value), irrespective of the nature of the substrate (Table 1).

Within-locality spatial genetic structure

The kinship coefficient (Fig. 2a) and Rousset's $\hat{a}$ distance (Fig. 2b) for the different distance classes in the autocorrelation analyses showed a lack of pattern with increasing distance in all four populations, with values remaining mostly within the confidence interval of non-association (shaded areas in the figures). Only in a few cases, the actual values of the coefficients fell slightly outside this non-association interval, which we attributed to random outcomes rather than to any true biological structure. This lack of intra-population structure allowed us to use the sampled specimens in inter-population comparisons (see below).

The differences between natural and artificial substrates were not significant neither for the overall kinship coefficient (Mann–Whitney U test, $T = 15.5$; $P = 0.486$) nor for Rousset's $\hat{a}$ index ($T = 19$; $P = 0.886$).

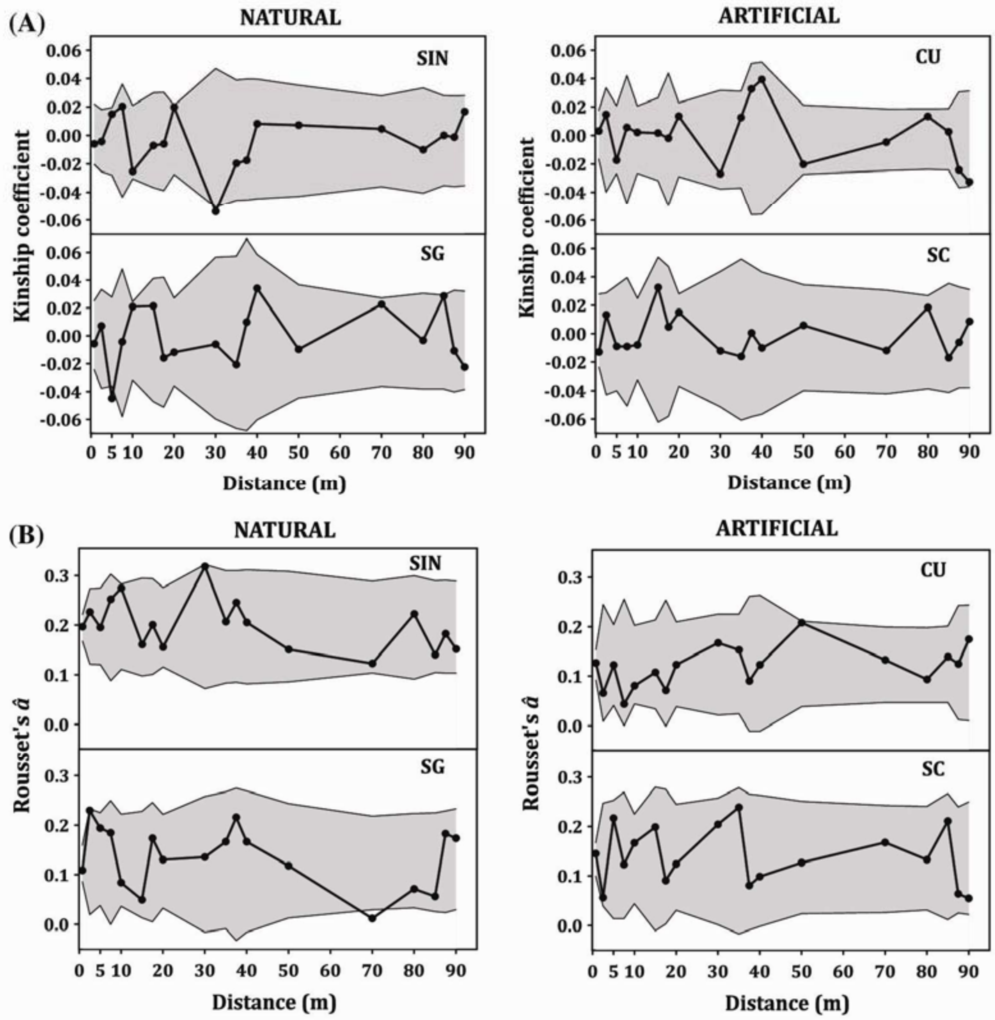

Fig. 2 Graphic representation of the **a** kinship coefficient and **b** Rousset's $\hat{a}$ index at each distance class in populations on natural substrate (Sitges and El Roc de Sant Gaietà) and artificial substrate (Cubelles and Segur de Calafell). The first distance class (0.2) corresponds to individuals collected in the different sampling points within a 20-cm radius. The shaded areas represent the 95 % confidence interval of non-association between genetic and spatial structures obtained by randomly permuting individuals across positions of the transects. Codes of localities as in Table 1

Between-population structure related to substrate type and geographical distance

Table 2 shows F_{ST} and D_{est} results. Both estimators' values were low in all cases. No pairwise F_{ST} comparison was significant after correction for multiple comparisons. Likewise, no pairwise D_{est} value was significantly different from 0, and neither was the global D_{est} value (0.0036). In addition, we computed the Pearson correlation between F_{ST} and D_{est} values to ensure that both estimators yielded comparable information, and the correlation was significant and very high ($r = 0.983$, $P < 0.001$). The Mantel test correlating the genetic and the geographical distances was not significant (using D_{est} matrix: $P = 0.977$; F_{ST} matrix: $P = 0.394$), so there was no indication of isolation by distance among these populations.

No significant variation attributable to differences between substrates (natural vs. artificial) was found in the AMOVA (per cent variation explained: 0.01 %, $P = 0.425$, Table 3), neither among populations within substrate. The highest percentage of variation was explained by differences within individuals (89.64 %, $P < 0.001$) and within populations (10.44 %, $P < 0.001$).

Admixture analysis

The most likely number of genetically differentiated clusters was two as assessed by the program STRUCTURE (Inc$K$ presented the highest value for $K = 2$, while the likelihood of the model presented the lowest standard deviation among the 20 replicates and a marked change in slope, which started to reach a plateau Fig. S1). In order to

Table 2 Genetic differentiation of the studied populations of *Microcosmus squamiger*

	GAN	SIN	SG	TB	GAA	SIA	CU	SC
GAN		−0.0079	−0.0113	−0.0134	*−0.0058*	*0.0092*	*−0.0009*	*−0.0074*
SIN	−0.0100		−0.0072	0.0029	*−0.0041*	*0.0202*	*0.0007*	*0.0025*
SG	−0.0170	−0.0102		−0.0056	*−0.0072*	*0.0141*	*−0.0038*	*−0.0059*
TB	−0.0198	0.0071	−0.0057		*−0.0018*	*0.0172*	*−0.0006*	*−0.0061*
GAA	*−0.0021*	*0.0013*	*−0.0052*	*−0.0006*		0.0252	−0.0059	−0.0077
SIA	*0.0081*	*0.0265*	*0.0150*	*0.0249*	0.0363		0.0214	0.0192
CU	*0.0023*	*0.0023*	*−0.0042*	*0.0028*	−0.0079	0.0354		0.0026
SC	*−0.0088*	*0.0055*	*−0.0066*	*−0.0074*	−0.0114	0.0254	0.0041	

D_{est}	Mean	±SE	F_{ST}	Mean	±SE
Nat–Nat	−0.0093	0.0039	Nat–Nat	−0.0071	0.0023
Nat–Art	0.0034	0.0031	Nat–Art	0.0013	0.0026
Art–Art	0.0137	0.0088	Art–Art	0.0091	0.0059

F_{ST} values are shown above the diagonal and D_{est} values below. Italicized values indicate pairwise comparisons between natural and artificial substrate. In addition, mean values of each estimator (±SE) are provided for comparison between populations on natural substrate (Nat–Nat), between natural and artificial substrate (Nat–Art) and between artificial substrate (Art–Art). Population codes as in Table 1

Table 3 AMOVA considering two groups of populations as per substrate type (natural and artificial), and considering the three genetic groups herein defined from the STRUCTURE results (A, B and AB)

Source of variation	*df*	Sum of squares	Variance components	Percentage of variation	*P* value
AMOVA by type of substrate					
Between substrates	1	1.736	<0.001	0.010	0.425
Among populations within substrates	6	10.360	−0.001	−0.090	0.613
Among individuals within populations	294	538.927	0.173	10.440	0.000
Within individuals	302	449	1.487	89.640	0.000
AMOVA by genetic groups					
Between groups	2	68.506	0.178	10.320	0.000
Among populations within groups	20	33.155	0.002	0.110	0.369
Among individuals within populations	279	449.362	0.062	3.580	0.019
Within individuals	302	449	1.487	85.990	0.000

test whether the two clusters defined were differentially influenced by some loci, we ran STRUCTURE analyses with five loci, leaving out one locus at a time. This procedure showed that loci MS11 and MS12 were determinant of the genetic structure found (results not shown).

The two clusters were found in all eight populations studied (Fig. 3). The program STRUCTURE also gave us the probability of each individual to belong to each of the two genetically differentiated clusters. Interestingly, most individuals (85.1 %) could be assigned to one of the two clusters with an assignment probability higher than 80 %. A total of 151 individuals were assigned to one of the clusters (henceforth group A) and 106 individuals to the other cluster (henceforth group B). Only 45 individuals (14.9 %) had probabilities of assignment lower than 80 % and were joined in a third group of individuals with mixed genotypes (henceforth referred to as group AB). The procedure *find.clusters* of the *adegenet* package with $K = 2$ classified the individuals into two groups fully coherent with those of STRUCTURE: 83 % of individuals of group A belonged to one of these groups, all individuals of group B belonged to the other and, as expected, the admixed individuals (AB) were classed almost equally (51:49 %) into each group.

Table 4 summarizes the genetic information of the three groups established. Group AB presented the highest total allelic richness (33.86). Only group A showed a significant (albeit low) F_{IS} value (0.097), which was due to the MS12 locus ($F_{IS} = 0.796$), and the overall F_{IS} values of the other two groups were close to zero and non-significant. Eighteen out of 45 alleles were exclusive of group A or B (irrespective of whether they were present in AB or not), but most of them were low-frequency alleles (<1 %) with the exception of loci MS11 and MS12, where four alleles

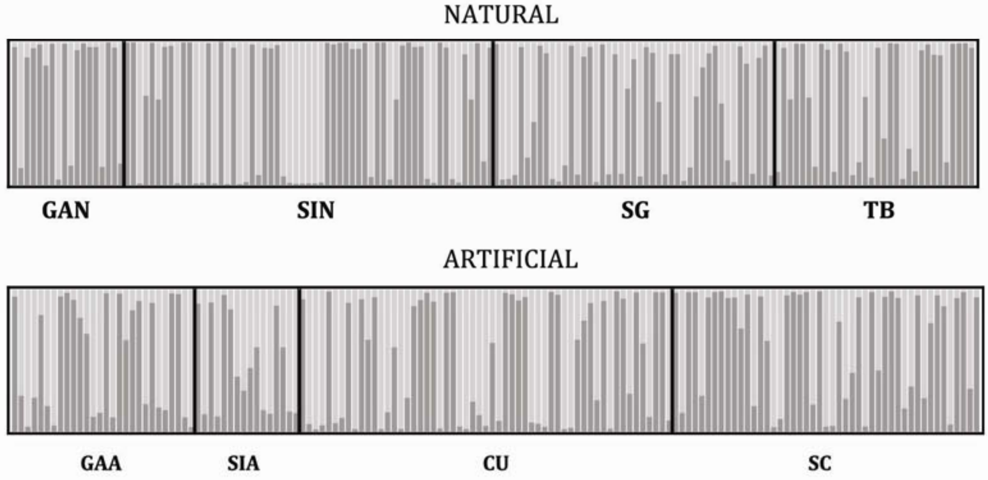

Fig. 3 Assignment of the 302 individuals to each of the two genetically differentiated clusters identified by STRUCTURE ($K = 2$). Each individual is represented by a *stacked bar*, where the *dark* and *light grey* segments represent the relative probability of assignment to each cluster. Populations (codes as in Table 1) are separated by *vertical black lines*. *Above* populations on natural substrate; *below* populations on artificial substrate

Table 4 Genetic variability of the three groups (A, B and AB) defined following STRUCTURE results

	Locus						
	MS 6	MS 7	MS 10	MS 11	MS 12	MS 13	Mean
A							
N	150	151	144	151	150	151	149.500
NA	4	3	10	6	3	6	5.333
AR	2.573	2.285	7.79	5.955	2.871	4.564	4.339
H_o	0.347	0.437	0.625	0.649	0.06	0.583	0.450
H_e	0.332	0.437	0.598	0.697	0.294	0.633	0.499
F_{IS}	−0.045	0.000	−0.046	0.069	**0.796**	0.079	**0.097**
B							
N	106	106	104	106	106	106	105.667
NA	3	2	11	7	7	4	5.667
AR	2.406	2	8.333	6.275	6.224	3.997	4.872
H_o	0.283	0.519	0.567	0.557	0.755	0.651	0.555
H_e	0.252	0.427	0.529	0.718	0.759	0.642	0.555
F_{IS}	**−0.123**	**−0.216**	−0.073	**0.226**	0.005	−0.014	−0.001
AB							
N	45	45	43	45	44	45	44.500
NA	3	2	10	7	8	4	5.667
AR	2.956	2	10	6.997	7.954	3.956	5.644
H_o	0.311	0.4	0.628	0.578	0.659	0.644	0.537
H_e	0.269	0.396	0.674	0.741	0.563	0.632	0.546
F_{IS}	**−0.159**	−0.011	0.069	**0.223**	**−0.174**	−0.019	0.017

Number of amplified individuals (N); number of alleles (NA); allelic richness per locus and population (AR) based on a minimum amplified sample size (over all loci) of 43 diploid individuals; observed (H_o) and expected (H_e) heterozygosities; and inbreeding coefficient (F_{IS})

Significant F_{IS} values are in bold. Means over loci (or global value for F_{IS}) are also indicated with frequencies 4–32 % were exclusive. Two alleles at low frequency (<4 %) were found only in AB individuals.

The first axis of the FCA accounted for most of the variance (82.9 %) and the second axis explained 17.0 %. Axis 1 separated individuals assigned to the group A in the positive values, individuals assigned to the group B in the negative values, and individuals assigned to the group AB in between, the latter with considerable spread over the second axis (Fig. 4). Examining the relative contribution of alleles to the different axes, it is apparent that alleles from loci MS11 and MS12, as found in the STRUCTURE analyses, were the main contributors to the inertia explained by the first axis, followed by MS6. Likewise, MS12, MS10 and MS11 were the main contributors to axis 2.

We performed a DA of principal components (DAPC) using the groups A, B and AB, together with individuals of two populations of Australia: Bunbury and Manly, representative of the two areas of origin of the worldwide introduced populations of *M. squamiger* (Rius et al. 2012) and genotyped for the same loci used in the present study. Along the first axis of the DAPC (explaining 57 % of the total variance), group A appears close to Bunbury and group B to Manly, while admixed individuals (group AB) appeared in between (Fig. 5). The second axis (26 % of variance) separates the native from the introduced populations. Additionally, we ran DAPC with only the Bunbury and Manly populations as groups and used the resulting discriminant function to assign individuals of groups A, B and AB to one of the two Australian populations using the function *predict.dapc*. A total of 78.14 % of individuals of group A were assigned to Bunbury, 77.35 % of group B were assigned to Manly and individuals of group AB were evenly assigned to Bunbury (55.55 %) or Manly (44.45 %).

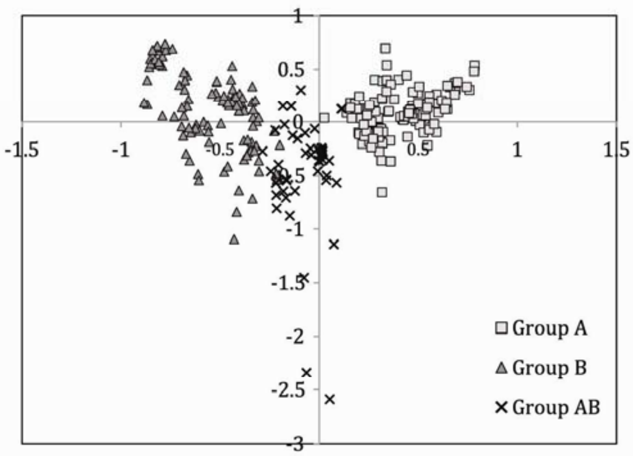

Fig. 4 Two-dimension plot of the factorial correspondence analysis (FCA) of the 302 individuals. The *three symbols* indicate in which group (*A*, *B*, and *AB*) each individual belongs

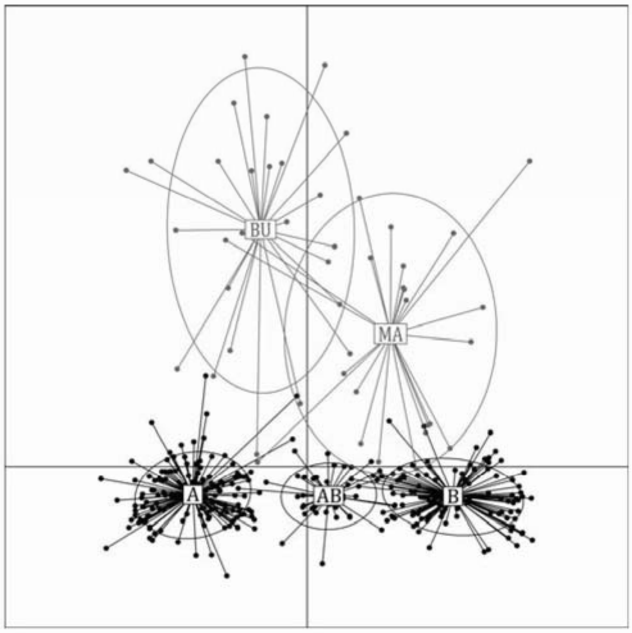

Fig. 5 Discriminant analysis of principal components (DAPC) using as groups the two Australian native populations: Bunbury (BU) and Manly (MA), and the three groups of individuals (*A*, *B* and *AB*) defined after the STRUCTURE results within the introduced populations studied herein

To ensure that the lack of inter-population genetic structure found in the previous analyses was not an artefact of the presence of individuals of different genetic pools mixed in all populations, we performed an AMOVA, grouping the individuals as per genetic group (A, B or AB) and examining the differentiation between populations within groups. A significant amount of the total variation (10.32 %, $P < 0.001$) was explained by differences among the three genetic groups. Reassuringly, after accounting for this group structure, the residual variance between populations was low (0.11 %) and not significant ($P = 0.369$) (Table 3), with most of the variability found within individuals (85.99 %, $P < 0.001$). Likewise, we repeated the autocorrelation analyses separately for groups A and B within each population and did not find any significant fine-scale genetic structure (results not shown). Furthermore, we calculated the F_{IS} values for each group separately in these four populations, since sample size was large and allowed performing the comparison. In all cases we observed that F_{IS} values for each group were lower than the population F_{IS} value and non-significant (Table S3).

Discussion

The present study revealed no genetic structure at any of the spatial scales analysed in populations of *M. squamiger* established on natural and artificial substrate, either within or between populations. No fine-scale (tens of metres) pattern was apparent, and no significant genetic differentiation was found among pairs of populations (tens of km). Likewise, in none of the studied parameters did the populations on natural and artificial substrate differ.

Our expectation of different genetic diversity and genetic relatedness in populations of *M. squamiger* on natural substrates was not borne out, as allelic richness, gene diversity and genetic relatedness (kinship coefficient) were not different when the populations on artificial substrate were compared with the ones on natural rocky reefs. Likewise, grouping localities according to substrate type did not explain any significant amount of genetic variability in our samples (AMOVA), indicating that they likely represent a collective source of propagules to ensure the post-border dispersal success of this species.

Two differentiated genetic pools were identified with STRUCTURE. These results were coherent with the FCA ordination and with groups found using a *K*-means clustering algorithm based on a different approach (i.e. maximizing inter-group differentiation, Jombart et al. 2010). The separation of the two groups was due mostly to two of the loci studied (MS11 and MS12). This most likely reflects differences in allele frequencies between the two ancestral sources due to drift, although we cannot rule out that this outcome is due to differential selection in the ancestral area on some genes linked to those two loci.

An unexpected pattern that appeared from the admixture analyses is that most individuals could be unambiguously assigned to one or another genetic group encountered, with only 15 % of individuals showing ambiguous membership probabilities attributable to admixture between the two genetic pools. The general low frequency of private alleles of one group or the other may explain why admixed individuals do not have a particularly higher degree of heterozygotes than either of the groups. Moreover, even if not admixed at the individual level, the two genetic pools detected appeared mixed at the population level and lack of spatial structure was also obtained when each identified genetic group was analysed separately. Remarkably, the groups seemed to correspond to an ancient split of the genetic make-up of the species, as they could be related to the two Australian populations of *M. squamiger* representative of the two native areas that were reported as the source of the worldwide introduction of this species (Rius et al. 2012).

Many studies have addressed the potential relationship between spatial genetic structure at several scales and dispersal capabilities of larvae in benthic invertebrates (reviewed in Grosberg and Cunningham 2001; Hellberg et al. 2002; Shanks et al. 2003; Palumbi 2004). For example, fine-scale (within-population) genetic structure can appear in species that are brooders and have extremely short-lived larvae such as sponges or cnidarians (e.g. Calderón et al. 2007; Blanquer et al. 2009; Ledoux et al. 2010; Mokhtar-Jamaï et al. 2011). All larval types of ascidians are short-lived and lecithotrophic, and solitary ascidians are commonly oviparous (Svane and Young 1989). In *M. squamiger*, time from fertilization to hatching is 11–12 h in the laboratory at 20 °C for most embryos (Rius et al. 2010), although lower temperatures increase this time considerably (M.R. unpublished data). Once hatched, most settlement occurs during the first eight hours in laboratory conditions (Rius et al. 2010). Nonetheless, this potential lifespan may not correlate with natural dispersal if retention mechanisms occur, and several such mechanisms have been described for solitary ascidians including, among others, negative buoyancy, stickiness of eggs and retention of eggs and larvae in mucus strings or bio-foam (Svane and Havenhand 1993; Petersen and Svane 1995; Marshall 2002; Castilla et al. 2007). In other solitary ascidians, extremely localized dispersal has been reported (e.g. *C. intestinalis* Petersen and Svane 1995; Howes et al. 2007) and autocorrelation analyses have also substantiated the existence of genetic structure at a scale of a few metres (*Styela plicata*, David et al. 2010; *S. clava,* Dupont et al. 2009). In *M. squamiger,* no evidence of fine-scale structure exists in the range of distances analysed in the present study (up to ca. 100 m), and the pattern is the same in the populations studied on natural and artificial substrates. Therefore, no local retention mechanisms of embryos or larvae seem to be acting.

We have found a lack of genetic differentiation among our populations, indicating that enough larvae travel between our study localities separated by tens of kilometres so as to prevent the drifting apart from allele frequencies. This may reflect the natural active dispersal capabilities of this species but, even if we have chosen only open-shore sites, marinas are present all along the shore studied, and *M. squamiger* is present on them. Therefore, recreational boating is likely to have contributed to the movement of the species, which can then spill out of the confined environment of marinas and colonize nearby open-shore substrates. We cannot assess at present the relative importance of natural and man-mediated dispersal at the scale studied. Both have probably an important role in generating a pattern of high gene flow over natural and artificial substrates in the area, which are representative of the spacing of available hard substrate that is common in the highly urbanized western Mediterranean Sea (Airoldi and Beck 2007; Airoldi and Bulleri 2011).

The genetic exchange between populations is likely to be high and involve enough number of individuals as to avoid secondary bottlenecks in post-border dispersal. In our case, it is remarkable that in such a short stretch of coastline, 45 alleles were found in the six microsatellite loci, representing 71.4 % of the total allelic richness found worldwide (63 alleles, Rius et al. 2012). This is in accordance with the source of the worldwide introduction of *M. squamiger* being traced to a single admixture event involving two genetically differentiated ancestral regions—the western and eastern coasts of Australia (Rius et al. 2012). However, in that work, true admixture (interbreeding of the genetic pools) was not to be distinguished from the coexistence of the two unmixed groups in introduced populations. Our findings indicate that the introduced populations studied have individuals attributable to one or the other putative ancestral genetic pools and that interbreeding among them is scarce, since only few individuals are actually admixed. The maintenance of this genetic structure is surprising, especially since historical records and genetic analyses show that the Mediterranean Sea was probably the first area of introduction of the species (Monniot 1981; Rius et al. 2012) more than 50 years ago, an ample time frame for the genetic pools to interbreed. Given the presence of two genetic clusters in each population, the overall deficit of heterozygotes detected and the significant F_{IS} values found in the four studied populations could be explained by a Wahlund effect. The fact that F_{IS} values of the individuals belonging to group A or B are smaller (and not significant) than the overall values of the populations (Table S3) reinforces the idea of a Wahlund effect. The alternative explanation for positive F_{IS} values, an artefact due to null alleles, seems unlikely since only MS10 had an appreciable percentage of failed amplifications (3.6 %) and this locus had more negative than positive F_{IS} values. Only one population (SIA) presented a non-significant negative F_{IS} value. However, it is also the population with the smallest sample size and thus stochasticity in the collection could influence this outcome.

We cannot tell at present which are the causes that prevent the interbreeding of the two genetic pools. Among other explanations, gamete recognition mechanisms or temporal separation of the breeding periods could be acting at the pre-zygotic stage. Previous studies of the life cycle of *M. squamiger* within our study area have shown a long reproductive period, from late spring to early autumn (Rius et al. 2009), which can accommodate differential reproductive timings. Reproductive post-zygotic isolation mechanisms reducing fitness of the admixed individuals are also a possibility. An alternative explanation would be that one of the groups (A or B) has recently arrived into the zone as a secondary introduction and little admixture has been realized since. Although this possibility cannot be discounted without a proper temporal sampling, it seems unlikely given that the Mediterranean was the first area colonized by a pool that already comprised a mixture of the two native sources (Rius et al. 2012). Furthermore, that study (whose sampling was done in 2006) included one population (Cubelles) in common with the present work. A reanalysis of that population (done with STRUCTURE with $K = 2$) showed that the two genetic groups (A and B) here found were already present in 2006. Similarly, only a few admixed individuals (16.7 % AB) were found in the 2006 collection and this proportion did not vary in relation to the 2010 samples of Cubelles used in the present work (17.5 % AB individuals) ($\chi^2 = 0.009$, $df = 1$, $P = 0.924$), so the low amount of admixture seems stable over time. Another potential explanation for the presence of two genetic pools, cryptic speciation, can be discarded using the sequences of COI for the Cubelles population sampled in 2006 available in Rius et al. (2008a). The sequences of the majority (70 %) of individuals were included in the most abundant haplogroup in the introduced range (Rius et al. 2008a), irrespective of whether the individuals belonged to groups A, B or AB.

Whatever the proximate cause, the maintenance over time of differentiated genetic pools can have a strong effect on the introduction process itself. It has been recognized that multiple introductions, if originating from different sources, contribute to increase genetic diversity and, perhaps more importantly, to the generation of novel allelic combinations that can foster the adaptive potential of invasive populations (Geller et al. 2010). In another introduced solitary ascidian, *S. plicata*, two ancestral genetic groups were also recognized but the populations had in general an excess of admixed genotypes with respect to expectation (Pineda et al. 2011), so that an interbreeding of the two genetic pools seemed to be favoured in this case. Although not demonstrated empirically, admixture has been suggested to increase the ability of invasive species to adapt and to thrive in new environments in terrestrial, freshwater and marine systems (e.g. McIvor et al. 2001; Kelly et al. 2006; Blum et al. 2007; Facon et al. 2008; Kolbe et al. 2008; Lombaert et al. 2011). In *M. squamiger*, however, admixture seems to be limited by unknown factors, without having compromised the colonizing potential of the species. In any case, the existence of two distinct pools that may differ in ecophysiological characteristics (e.g. reproductive timing and adaptive plasticity) is undoubtedly to be kept in mind when studying the introduction process and the invasive potential of *M. squamiger*.

Taken together, our results indicate that the expansion of *M. squamiger* is highly likely across different hard substrate types available for colonization. Mitigation measures are thus difficult to undertake at the post-border stage in shores with enough patches of suitable substrate. Efforts would be better invested in preventing the arrival of this species at the pre-border stage, a consideration that seems obvious for species with wide dispersal abilities and that, considering our results, applies also to organisms with short-lived larval stages.

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Segundo.Capítulo:

Interacciones biológicas tempranas entre especies bentónicas introducidas y nativas nos revelan depredación críptica y cambios en el comportamiento larvario

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Interacciones biológicas tempranas entre especies bentónicas introducidas y nativas nos revelan depredación críptica y cambios en el comportamiento larvario

RESUMEN

Las introducciones recurrentes de especies no indígenas generan interacciones nuevas que varían con las condiciones locales y la composición de la comunidad receptora. La mayoría de estudios examinan las relaciones de los recién llegados con las especies nativas, pero las interacciones entre las especies introducidas pueden también afectar cambios en la comunidad. Como los estados ontogenéticos tempranos son particularmente vulnerables a las interacciones biológicas, en este capítulo exploramos las interacciones directas e indirectas en diferentes estados tempranos del ciclo de vida de invertebrados marinos dominantes por el espacio: las ascidias introducidas *Microcosmus squamiger y Styela plicata*, y mejillones tanto nativos como introducidos. Para completar el estudio, se realizó el mismo experimento en dos regiones distantes, una en el hemisferio norte (España) y la otra en el hemisferio sur (Sudáfrica). No se encontró interferencia espermática entre las ascidias, ni tampoco efectos interespecíficos en el asentamiento o en el éxito de metamorfosis. Sin embargo, las larvas de las dos especies de ascidias reaccionaron a la presencia de las otras cambiando de un asentamiento agregado a uno aleatorio. Los juveniles de mejillones consumieron gran número de larvas de ascidia, aunque en presencia de mejillones las larvas que presentaban una mayor tasa de asentamiento. Las especies de mejillón en Sudáfrica (el nativo *Perna perna* y el introducido *Mytilus galloprovincialis*) consumieron más larvas de ascidia que los mejillones en

España (nativo *M. galloprovincialis*), con una tendencia de las larvas de ascidia a evitar asentarse cerca de los mejillones en España. Concluimos que el consumo por parte de los mejillones afecta el establecimiento de las ascidias, pero la magnitud de este efecto depende del contexto. Estos resultados enfatizan la importancia de la composición de la comunidad receptora en determinar su susceptibilidad a la invasión. No importa que las especies que comprenden esta comunidad sean nativas o introducidas, sino lo que importa es el tipo de especies que son.

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Early biotic interactions among introduced and native benthic species reveal cryptic predation and shifts in larval behaviour

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ABSTRACT: Recurrent introductions of non-indigenous species generate novel interactions that vary with local conditions and the composition of the receiving community. Most studies examine relationships of newcomers with native species, but interactions among introduced species could also affect community shifts. As early ontogenetic stages are particularly vulnerable to biotic interactions, we explored direct and indirect interactions across early life-history stages in space-dominating marine invertebrates. We used introduced ascidians and both native and introduced mussels. To increase generality, we ran our experiments in 2 distant locations, one in the northern and one in the southern hemisphere (Mediterranean and South Africa). We found no sperm interference between the ascidians, nor were there interspecific effects on settlement or metamorphosis success. However, larvae of the ascidian species reacted to each other by shifting from aggregated to random settlement. Juvenile mussels consumed large numbers of ascidian larvae, though larvae that avoided mussel predation showed higher settlement success. Mussel species in the southern hemisphere locality (native *Perna perna* and introduced *Mytilus galloprovincialis*) consumed more ascidian larvae than mussels in the northern locality (native *M. galloprovincialis*), with a tendency for ascidian larvae to avoid settling close to mussels in the northern locality. We conclude that larval consumption by mussels affects the establishment of ascidians, but that the magnitude of this effect is context dependant. These results emphasize the importance of the composition of the receiving community in determining its susceptibility to invasion. Whether the species comprising this community are native or introduced is, however, less important than what manner of species they are.

KEY WORDS: Competition · Settlement · Larviphagy · Invasive species · *Microcosmus squamiger* · *Styela plicata* · *Mytilus galloprovincialis* · *Perna perna* · Ascidians · Mussels

INTRODUCTION

The introduction of species as a result of human activities is a major concern in the maintenance of biodiversity in marine systems (Carlton & Geller 1993, Ruiz et al. 1997, Harris & Tyrrell 2001). Accordingly, the study of biological introductions often focuses on their negative effects on native species (e.g. Caro et al. 2011) and there has been the suggestion that interspecific facilitation among invasive species can result

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in invasion meltdown (Simberloff & Von Holle 1999, Grosholz 2005). Although there is still no clear evidence of the widespread nature of this effect (Simberloff 2006), the intensity of recurrent introduction events often leads to an increase in community heterogeneity through the coexistence of diverse non-indigenous species that interact both among themselves and with the resident community (Byrnes & Stachowicz 2009, Dijkstra & Harris 2009). Indeed control of newcomers to a particular community can be exerted by already established introduced species rather than by native species. For example, habitats dominated by introduced species may be more resistant to further invasions than native communities (Osman & Whitlatch 2007). It is important, therefore, to understand interactions among dominant introduced species, which in marine systems often compete aggressively for space. This is a largely unexplored field, although the notion of complementarity (temporal, trophic or otherwise) among introduced species has been put forward as one factor that can modulate community function and dynamics (Byrnes & Stachowicz 2009). Nevertheless, few studies have analyzed the potential effects of biotic interactions among non-indigenous species colonizing a new area (e.g. Simberloff 2006, Rius et al. 2011), and our study aimed to contribute towards filling this gap.

Early ontogenetic stages of marine organisms (e.g. fertilization, larval settlement and survival) are particularly vulnerable to competitive interactions, which can strongly influence species fitness, and are key to the establishment of marine organisms (Grosberg 1981, Bingham & Walters 1989, Osman & Whitlatch 1995a,b, Lambert 2000, Porri et al. 2008). Despite this being a well-established idea, studies of interactions between introduced and resident species usually focus on adult interactions (e.g. Byers 2000, Grosholz et al. 2000, Nyström et al. 2001, Decottignies et al. 2007) or how adults affect new recruits (e.g. Osman & Whitlatch 1995a,b, Lohrer & Whitlatch 2002), with less attention to species interactions during early life-history stages (e.g. Rius et al. 2009a).

There is a range of potential direct and indirect competitive effects during the early life-history stages of marine benthic invertebrates. For closely related broadcast spawning species that share spawning periods, interference competition could happen at the fertilization stage owing to interspecific sperm competition (Lambert 2000). Once embryonic development is completed, some marine invertebrate larvae select their point of settlement taking into account future risks such as the presence of predators (Johnson & Strathmann 1989, Stoner 1994) or dominant competitors (Grosberg 1981). Hence, the behaviour of larvae during settlement is a crucial determinant of successful recruitment (Young & Chia 1984, Young & Cameron 1989, Stoner 1994). Predation of larvae and juveniles can also play an important role in determining recruitment and population connectivity (Bingham & Walters 1989, Young & Cameron 1989, André et al. 1993) and may control the establishment of newcomers (Osman & Whitlatch 1998). Such biotic interactions can occur not only between introduced and resident species, but also among non-indigenous species in the same habitats.

The aim of the present work was to explore early ontogenetic interactions among introduced species using dominant introduced and native species. Ascidians and mussels are major ecosystem engineer species along the world's coastlines and have been widely introduced via human-mediated transport (e.g. Lambert 2007, McQuaid & Arenas 2009). They often colonize artificial substrata in harbours such as pilings, docks, floating pontoons, boat hulls and buoys (e.g. Bax et al. 2002). As these species are generally space dominating, coexistence on such substrata is likely to lead to either exploitation or interference competition. Co-occurring ascidians and mussels constitute a good study system for potential interactions as they are abundant and gregarious, coexist and can compete for substratum as a primary resource. Specifically, we wanted to (1) assess the effects of competition of conspecific and heterospecific sperm on fertilization success of ascidian species; (2) assess the effect of the presence of conspecific and heterospecific ascidian larvae on settlement success, settlement behaviour and metamorphosis; (3) assess the interaction between ascidian larvae and mussel juveniles; and (4) compare the outcome of this interaction in 2 locations, one in the northern and one in the southern hemisphere, allowing us to compare the effects of the same mussel species where it is native and where it is introduced. Our rationale was to evaluate interactions among benthic species that may take place when these dominant space competitors co-occur, and how propagules react to the presence of the same heterospecifics in different geographic regions.

MATERIALS AND METHODS

Study species and regions

Both *Microcosmus squamiger* Michaelsen, 1927 and *Styela plicata* (Lesueur, 1823) are solitary ascidians that have been widely introduced to harbours and marinas of warm and temperate oceans (Pineda et al. 2011, Rius et al. 2012). *S. plicata* is probably native to the NW Pacific Ocean (Pineda et al. 2011 and references therein), although stochastic and recurrent introductions over a long period of time make its source difficult to ascertain (Pineda et al. 2011). *M. squamiger* is native to Australia (Rius et al. 2012 and references therein). Both species are highly tolerant of abiotic stressors, such as pollution and changes in temperature and salinity, particularly *S. plicata* (Naranjo et al. 1996, Pineda et al. 2012). Although *S. plicata* has a wider distribution around the world than *M. squamiger*, both species often coexist at high densities. In addition, *M. squamiger* has a noticeable capacity for regional dispersal, which allows it to spread once it has reached a new area (Ordóñez et al. 2013). This species colonizes natural substrata in the western Mediterranean (Rius et al. 2009b, Ordóñez et al. 2013), where it coexists with the native mussel *Mytilus galloprovincialis* (Lamarck, 1819) (authors' pers. obs.).

The Mediterranean mussel *Mytilus galloprovincialis* is a key space occupier in shallow sublittoral and intertidal parts of the Mediterranean Sea (Bacchiocchi & Airoldi 2003, Rius & Zabala 2008), but is also found in many regions around the world (Apte et al. 2000). This mussel has become an invasive species in many temperate zones of the world (Branch & Steffani 2004), including the south coast of South Africa (McQuaid & Phillips 2000) where the mussel *Perna perna* (Linnaeus, 1758) is native (Siddall 1980). Both mussels co-occur in natural intertidal beds and can be found together with the ascidians *Styela plicata* and *Microcosmus squamiger* in harbours and marinas of the region (authors' pers. obs.).

Gamete collection and sperm interactions experiment

Microcosmus squamiger and *Styela plicata* were collected from ropes and floating pontoons in the SW Indian Ocean at the marina of Port Elizabeth, South Africa (33° 58′ 01.38″ S, 25° 38′ 02.47″ E) (Fig. 1A) during the austral spring of 2010. Both species are reproductively active during this time of the year elsewhere (Yamaguchi 1975, Rius et al. 2009b, Pineda et al. 2013). Samples were stored in seawater and transported (2 h) to the laboratory in insulated containers. Once in the lab, ascidians were kept in aerated tanks at 19 to 20°C. We used constant light conditions to prevent light-induced spawning (West & Lambert 1976).

Individuals of both ascidian species (see details below) were dissected to separate the gonads as described in Marshall et al. (2000). Ascidians are hermaphroditic (Lambert 2005), thus separation of eggs from sperm was necessary. Gonads from each individual were smashed separately in Petri dishes and filtered using an upper filter of 500 µm mesh and a lower filter of 100 µm mesh. The upper filter retained the tissue remains and the lower retained the eggs. Sperm passed with water through both filters and was collected in a beaker. Eggs retained in the 100 µm filter were rinsed with seawater and collected in a separate beaker. Thus, we obtained the sperm and eggs from the same individual separately. Filtered seawater was used at every step. This protocol was repeated for all experiments.

A total of 20 individuals of each species were used to perform this experiment. To prevent possible self-fertilization, the sperm of 10 individuals were pooled and used to fertilize the eggs of the 10 remaining individuals. Sperm concentration of the sperm homogenate was calculated using a Neubauer hemocytometer as spermatozoids ml^{-1}. For each species, 1 ml of egg homogenate (~300 eggs ml^{-1}) was added to

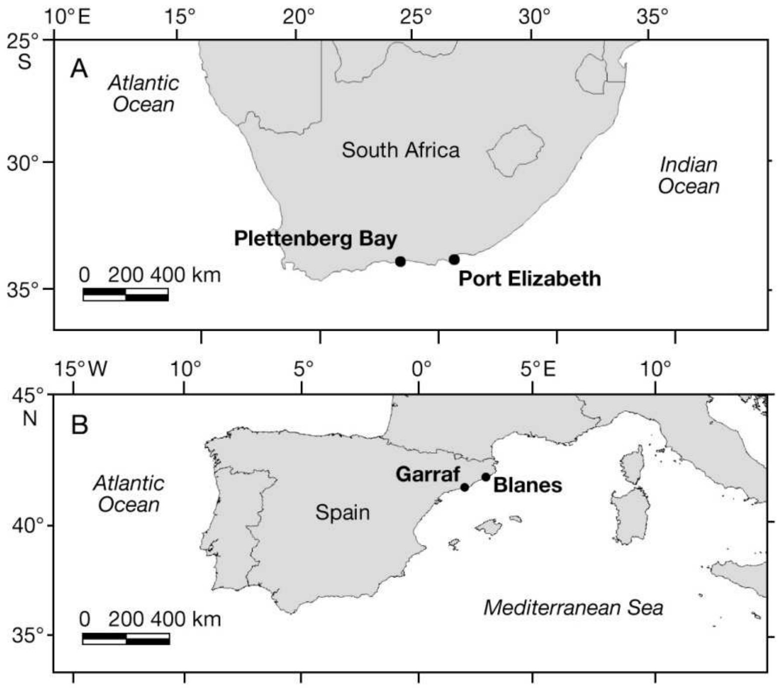

Fig. 1. Sampling sites in the 2 regions. (A) Southern hemisphere—south coast of South Africa (SW Indian Ocean). (B) Northern hemisphere—NE coast of Spain (NW Mediterranean Sea)

Table 1. Sperm concentrations (spermatozoids ml^{-1}) of each ascidian species (*Microcosmus squamiger* or *Styela plicata*) used for the fertilization of conspecific eggs: eggs fertilized in the presence of conspecific sperm only (C1 and C2); treatments using mixed sperm of both species at different concentrations (M1, M2 and M3)

	Non-mixed treatments		Mixed treatments		
	C1	C2	M1	M2	M3
Conspecific sperm	1×10^7	2×10^7	1×10^7	0.5×10^7	1.5×10^7
Heterospecific sperm	–	–	1×10^7	1.5×10^7	0.5×10^7

a Petri dish (65 mm in diameter) with 15 ml of filtered seawater. Then, the appropriate volume of sperm was added to the Petri dish to obtain the desired final concentration for each treatment (see details in Table 1). C1 and C2 were Petri dishes with eggs exposed to 2 different conspecific sperm concentrations to test for intraspecific interactions. M1, M2 and M3 were 'mixed' Petri dishes with eggs of each ascidian exposed to sperm of *M. squamiger* and *S. plicata* together in different relative proportions. For each treatment and species, 10 replicates (i.e. 10 Petri dishes) were run. Petri dishes were stored at 19 to 20°C under constant light. After 14 h, when embryonic development was already completed, each Petri dish was examined under a binocular microscope to obtain a measure of fertilization success as follows:

$$\% \text{ fertilization} = [\text{embryos} / (\text{embryos} + \text{unfertilized eggs})] \times 100$$

Larval interactions

Individuals of both ascidian species were sampled at the marina of Port Elizabeth, South Africa (Fig. 1A) as indicated above. As hundreds of larvae were required, 30 individuals of each species were dissected to obtain the gametes for cross-fertilization. Reciprocal crosses were carried out using eggs of half of the individuals of each species and sperm from the remaining individuals. Gametes were kept together for fertilization for 50 min, and eggs were filtered afterwards with a 100 µm mesh filter to wash off the sperm. Eggs were collected in a 1 l beaker with filtered seawater, oxygenated by an aquarium pump and stored at 19 to 20°C. This was done separately for each species. After ca. 14 h the larvae started hatching and were carefully pipetted into 65 mm Petri dishes with 15 ml of filtered seawater. 'Low density' (LD) and 'high density' (HD) treatments were single species treatments with 20 and 40 larvae, respectively, to test for intraspecific interactions. 'Mixed' was a treatment in which 20 larvae of *Microcosmus squamiger* were mixed with 20 larvae of *Styela plicata* (added at the same time) to assess interspecific interactions using LD and HD treatments as controls for comparison. For each treatment, 11 replicates (i.e. Petri dishes) were performed (a total of 880 larvae for each species). Petri dishes had been previously submerged in seawater for 24 h to create a biofilm, which favours the settlement of ascidian larvae (Keough & Raimondi 1995, Wieczorek & Todd 1997). After 48 h, all larvae were either settled or dead, and their numbers were counted under a microscope. After 72 h the number of completely metamorphosed settlers (hereafter 'post-metamorphs') was also counted. Settler positions were marked on the Petri dish lid using a felt-tip pen. Results were recorded as percentages as follows:

$$\% \text{ settlement} = \frac{\text{settlers}}{\text{larvae added}} \times 100$$

$$\% \text{ metamorphosis} = \frac{\text{post-metamorphs}}{\text{settlers}} \times 100$$

Pictures of each marked dish lid were taken using a digital camera (Canon PowerShot G11) and the distances between settlers on each dish (see 'Image treatment and data analyses') were analysed by the Laboratory of Image Analysis and Treatment of the CCiTUB (Scientific and Technological Centre of the University of Barcelona, see details below) using the proprietary program IMAT.

Ascidian–mussel interactions

Fertilizations were carried out to obtain larvae from both ascidian species as above. To obtain juvenile mussels, we collected adults directly from natural intertidal populations at low tide and carefully removed the juveniles found among byssus threads of adults under a binocular microscope.

In the southern hemisphere (SW Indian Ocean), *Microcosmus squamiger* and *Styela plicata* were collected at the Port Elizabeth marina, South Africa (Fig. 1A) as above. The 2 species of mussels, *Perna perna* and *Mytilus galloprovincialis*, were sampled at the same time as the ascidians from the shore of Port Elizabeth (33° 58′ 47.27″ S, 25° 39′ 27.61″ E) and the shore of Plettenberg Bay, South Africa (34° 03′ 35.44″ S, 23° 22′ 48.75″ E), respectively (Fig. 1A). Both species of mussels were stored in seawater and transported to the laboratory in insulated containers (~2 and 6 h journeys, respectively). In the lab, mussels were kept in tanks with water aeration at 19 to 20°C, while juveniles were removed from adult byssus threads. Juvenile mussels were kept in aerated containers at the same temperature and with seawater from the place where they had been collected until they were used for the experiments.

The ascidian–mussel interactions study was repeated in the northern hemisphere (NW Mediterranean Sea). Adults of both *Microcosmus squamiger* and *Mytilus galloprovincialis* were sampled by snorkelling at the outer edge of the breakwater of the marina in Garraf, Spain (41° 14′ 08.50″ N, 1° 54′ 08.63″ E), while *Styela plicata* was collected at the marina in Blanes, Spain (41° 40′ 30.57″ N, 2° 47′ 58.43″ E) by pulling up ropes (Fig. 1B). All of the collections in Spain were carried out during early summer 2011. Both ascidians and mussels were stored in seawater, transported to the lab (~1 h) and processed as in the previous experiment.

In both geographic regions, we randomly selected 25 juvenile mussels of 3 to 9 mm shell length, and placed them haphazardly in a 65 mm Petri dish with 25 ml of filtered seawater. Mussels released byssal threads for adhesion soon after their deployment. Subsequently, 40 ascidian larvae were carefully released in the Petri dish using a micropipette. Dishes with 40 ascidian larvae alone were used as controls simultaneously to the mixed dish experiments. All Petri dishes had been previously submerged in seawater for 24 h as above. For Spain, the different treatments and numbers of replicates were: mixed *Microcosmus squamiger*–*Mytilus galloprovincialis*, n = 12; mixed *Styela plicata*–*M. galloprovincialis*, n = 10; controls, n = 10 for each ascidian species. For South Africa, the treatments and number of replicates were: mixed *M. squamiger*–*M. galloprovincialis*, n = 6; mixed *S. plicata*–*M. galloprovincialis*, n = 6; mixed *M. squamiger*–*Perna perna*, n = 8; mixed *S. plicata*–*P. perna*, n = 8; controls, n = 14 for each ascidian species.

To calculate mortality due to mussel consumption (see 'Results'), the number of settlers and the number of dead and unattached larvae was recorded after 48 h, and a consumption percentage was calculated as:

$$\% \text{ consumption} = [(\text{added larvae} - \text{settlers} - \text{dead larvae}) / \text{added larvae}] \times 100$$

All non-consumed larvae were either settled or dead after 48 h in all treatments. Mussels were carefully scrutinized to look for settled or hidden larvae, and none were found. Settlement percentage was also recorded among the non-consumed larvae as:

$$\% \text{ settlement} = [\text{settlers} / (\text{settlers} + \text{dead larvae})] \times 100$$

Mussels and ascidian settlers of all dishes were also outlined on the lids with a felt-tip pen to take pictures, which were treated with the program IMAT as above.

Image treatment and data analyses

Every picture (taken in the larval interactions and ascidian–mussel interactions experiments) was manually checked to ensure that the program IMAT correctly identified the marks of settlers and/or mussels. We chose to measure the shortest distance between a given settler and the closest conspecific and/or heterospecific individual. These were distances between ascidians for the larval interaction experiments. For mussel–ascidian interaction experiments, distances were measured between ascidian settlers, between mussels, and between ascidians and mussels. For the distance between ascidians and mussels, we recorded the distance between the settler and the nearest edge of the closest mussel or mussel aggregate. The distribution of nearest-neighbour distances is a good descriptor of spatial patterns (Clark & Evans 1954), as they tend to be smaller than expected (under random settlement) for aggregated settlement, and larger when there is settler avoidance (leading to regular distribution).

To generate the expected nearest-neighbour distances between ascidians and/or mussels in the different replicates, a simulation program was written ad hoc in Turbo Pascal. The program generates a virtual arena of the same circular area as the Petri dish and places ascidian settlers and/or mussels into this arena at random to match the actual numbers in every experimental replicate. To match the experimental setting, for the larval interaction experiment, 'settlers' assigned to the 2 species were added alternately to the virtual arena, whereas for the mussel–ascidian interaction experiment, 'mussels' were allowed to settle first and 'ascidian settlers' were added afterwards. The virtual ascidian settlers were given an area equivalent to the mean area recorded for the actual settlers (as variation in this parameter was low). Virtual mussel settlers were given exactly the same area as observed in the mussels of the corresponding replicate (as mussel areas changed considerably because they often grouped in clumps). The program avoided placing a new virtual settler on top of existing ones or mussels. To compensate for potential edge effects

(Sinclair 1985), virtual mussels or ascidians were allowed to settle at the margins of the virtual Petri dish even if the whole area of the settler did not fit inside it. The same distance parameter recorded for the actual replicate was calculated from the simulated Petri dish, and the procedure was repeated 1000 times to generate a mean distance value under random settlement. The actual values recorded were transformed to percentage deviation (positive or negative) from the generated mean values.

Mean and SE of the percentage deviation for each experiment were obtained by averaging all replicates, and significant departures from randomness were recorded whenever the confidence interval of the mean did not include zero. Significant positive deviations (i.e. nearest-neighbour distances larger than expected) indicate avoidance behaviour, and significant negative deviations indicate aggregative behaviour.

ANOVA was performed to assess the effects of the different treatments. The variables analysed (fertilization, settlement, metamorphosis and consumption) did not comply in general with the assumptions of parametric analyses, as assessed by the Kolmogorov-Smirnov test (normality) and Levene median test (homogeneity of variances) even after trying several transformations, including some recommended for percentage data, such as arcsine or logit (Warton & Hui 2011). We finally adopted the rank-transformation method (Conover & Iman 1981, Potvin & Roff 1993), in which data are transformed to ranks and parametric models are then fitted to the ranked data (Quinn & Keough 2002). Rank-transformed data in all cases met the assumptions of parametric tests. Post-hoc tests were made using the Student-Newman-Keuls (SNK) test. Significant interactions were interpreted by examining plots of effects and performing SNK comparisons for levels of one factor at each level of the other factor using the common error mean square (Quinn & Keough 2002). The programs Statistica v. 8 (StatSoft) and SigmaStat v. 3.11 (Systat Software) were used to run the analyses.

RESULTS

Sperm interactions

Overall, fertilization success was low for all experiments even though they were done during the reproductive season. There were no significant differences in the fertilization success among treatments, nor was there a significant Species × Treatment interaction (ANOVA, Table 2). There was a significant effect of species due to higher success in *Styela plicata* (mean % ± SE, 3.51 ± 0.20) than in *Microcosmus squamiger* (2.62 ± 0.12). Thus, at the concentrations tested here, there was no effect of increasing conspecific sperm concentration (C1 vs. C2), or of the presence of different proportions of heterospecific sperm (M1 to M3) (Fig. 2).

Table 2. ANOVA results of the ascidian sperm interactions experiment. Treatment levels as in Table 1

	df	SS	MS	F	p
Fertilization success					
Species	1	10197.79	10197.79	14.98	<0.001
Treatment	4	1966.59	491.65	0.72	0.579
Species × Treatment	4	6553.26	1638.32	2.41	0.055
Residual	88	59909.09	680.78		

Larval interactions

No significant differences were found for the variables analysed (% settlement, % metamorphosis) as a result of intraspecific competition (2 densities, HD and LD) or interspecific competition (mixed treatment compared to HD) (Fig. 3, Table 3), nor was there a significant Treatment × Species interaction. Overall, there was a marked species effect, reflecting the fact that *Styela plicata* had better success than

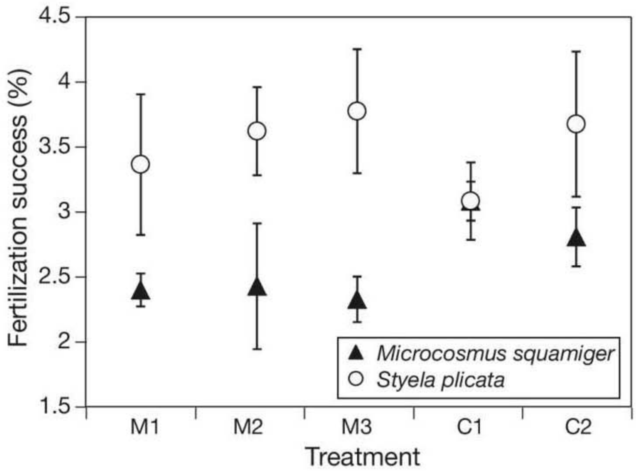

Fig. 2. *Microcosmus squamiger* and *Styela plicata*. Mean (±SE) fertilization success (%) of both species' eggs in each experimental condition. C1 and C2: eggs fertilized in the presence of conspecific sperm only; M1, M2 and M3: treatments using mixed sperm of both species at different concentrations

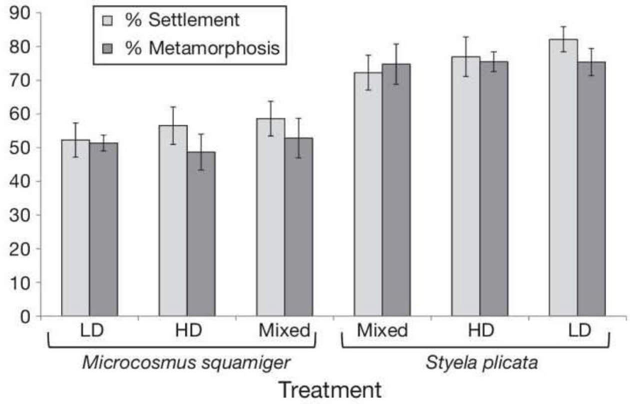

Fig. 3. *Microcosmus squamiger* and *Styela plicata*. Mean (±SE) % settlement and % metamorphosis in each treatment for both ascidian species. LD (low density): 20 larvae; HD (high density): 40 larvae; mixed: 20 larvae of each species. The % settlement is relative to the number of larvae added, while the % metamorphosis is relative to the number of settled larvae; differences from 100% reflect larval and settler mortality, respectively

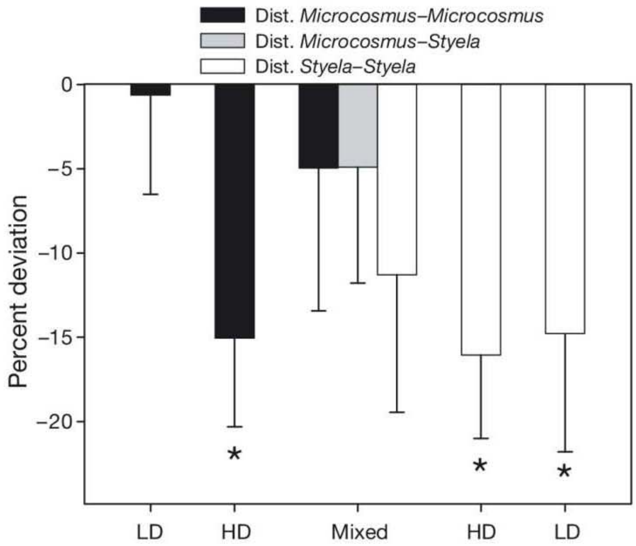

Fig. 4. Mean (±SE) percent deviations [(observed – expected)/expected × 100] of the shortest distances between ascidian settlers in the larval interaction experiment. Expected values obtained through simulation. Asterisks: significant departures from zero (i.e. non-random distribution)

Microcosmus squamiger in terms of % settlement of larvae (mean ± SE, 77.13 ± 2.89 vs. 55.82 ± 2.97, respectively) and % metamorphosis of settlers (mean ± SE, 75.23 ± 2.50 vs. 50.98 ± 2.68, respectively).

The distribution of settlers in the experimental dishes, as assessed by nearest-neighbour distances, showed that *Microcosmus squamiger* settled at random at low densities, but significantly aggregated at high densities, while *Styela plicata* had an aggregated settlement distribution at both densities (Fig. 4). When the 2 species were together (mixed treatment), the behaviour of the larvae changed. Although there was a tendency towards aggregation both for intra- and interspecies comparisons (negative deviations), there was considerable variability and the mean values were not significantly different from what would be expected if settlement was random.

Table 3. ANOVA results of the ascidian larval interactions experiment

	df	SS	MS	*F*	p
Settlement					
Species	1	6740.74	6740.74	24.66	<0.001
Treatment	2	41.20	20.60	0.08	0.927
Species × Treatment	2	700.46	350.23	1.28	0.285
Residual	60	16403.09	273.38		
Metamorphosis					
Species	1	10792.97	10792.97	50.37	<0.001
Treatment	2	57.02	28.51	0.13	0.876
Species × Treatment	2	214.59	107.29	0.50	0.609
Residual	60	12857.41	214.29		

Ascidian–mussel interactions

Some of the 25 initial mussels attached separately to the Petri dish using their byssal threads, but most showed gregarious behaviour and tended to group in clumps. *Perna perna* had a higher tendency to form clumps and, as a result, formed fewer aggregates with higher numbers of individuals in each aggregate. On average, *P. perna* formed 7.06 ± 0.40 (mean ± SE) aggregates per Petri dish, while *Mytilus galloprovincialis* formed 8.54 ± 0.30 aggregates, the difference being significant (*t*-test: $t = 2.82$; df = 97; p = 0.006).

There was strong larval consumption by mussels in all trials (Fig. 5). When we compared consumption by *Mytilus galloprovincialis* between South Africa and Spain, the factor region was significant (Table 4A), with consumption being less marked in Spain than in South Africa. The overall consumption percentage, pooling both ascidians in the mussel's native Mediterranean, was smaller than in its introduced range of South Africa (mean % ± SE, 73.75 ± 1.52 and 91.25 ± 1.52, respectively). A significant species effect (Table 4A) reflected the higher consumption of *Styela plicata* larvae (Fig. 5). The differences were coherent across geographical regions (non-significant interaction term).

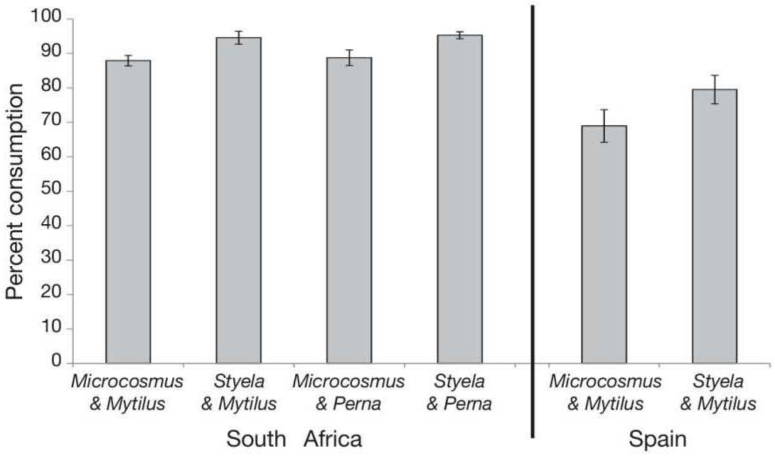

Fig. 5. Mean (±SE) consumption of ascidian larvae by mussels in South Africa and Spain in mixed treatments (ANOVA results in Table 4)

In South Africa, the 2 mussel species consumed larvae similarly (non-significant species effect), but the response of the 2 ascidians was different (Table 4B). The overall consumption percentage (both mussel species pooled) was lower for *Microcosmus squamiger* (mean % ± SE, 88.40 ± 0.01) than for *Styela plicata* (95.00 ± 0.01). The interaction between ascidian species and mussel species was non-significant (Table 4B).

Non-consumed larvae showed significantly higher settlement success in the presence of mussels in both geographic regions and for both mussel species (Fig. 6). The comparison between Spain and South Africa showed significant effects of the main factors: region (higher settlement in the Mediterranean), ascidian species (higher settlement in *Styela plicata*) and treatment (higher settlement of surviving larvae in the presence of mussels). However, the Ascidian × Treatment interaction was significant, as was the 3-way interaction (Table 5A), which could hinder interpretation of the main effects. Comparisons at fixed levels of region showed that the Ascidian × Treatment interaction was significant in South Africa but not in Spain, and examination of plots of effects and post-hoc SNK tests indicated that this significant interaction was due to a more marked response for *S. plicata* than *Microcosmus squamiger* to the presence of mussels in South Africa. The interactions were due, therefore, to a difference in the magnitude, not in the direction of the response (an overall increase in settlement success of both ascidian species when in the presence of mussels, with the effect being stronger for *S. plicata*).

In the comparison of settlement percentage between the controls and the 2 mussel species, *Mytilus galloprovincialis* and *Perna perna,* in South Africa, the factors ascidian species and treatment were highly significant (Table 5B), while no significant interaction was found. Settlement success was higher for *Styela plicata* than *Microcosmus squamiger*, and a SNK test on treatment effects revealed no differences associated to the 2 mussel species, with both mussel treatments featuring significantly higher settlement than the controls (Fig. 6).

For *Microcosmus squamiger,* the spatial distribution of the ascidians and mussels in the experimental plates showed that larvae significantly avoided settling close to mussels or mussel clumps in Spain (Fig. 7), while the pattern of distribution of settlers relative to mussels was not different from random in South Africa for either mussel species. The ascidian settlers themselves tended to adopt a regular distribution when mussels were present (significant only in South Africa). The mussels (individuals or clumps) showed in all cases a significant tendency towards avoiding other clumps.

In the case of *Styela plicata* (Fig. 7), again an avoidance reaction was detected in the presence of native *Mytilus galloprovincialis* in Spain, while in South Africa the distribution of settlers was not different from random with respect to mussels. In this case, the ascidian settlers showed no significant spatial pattern, with mean positive deviations of ex-

Table 4. ANOVA results for consumption percentage of the ascidian–mussel interactions experiment. (A) Testing differences in the effect of *Mytilus galloprovincialis* on both ascidian species (Ascidian factor, *Microcosmus squamiger* and *Styela plicata*) in the 2 different geographical regions (Region factor, South Africa and Spain). (B) Testing differences in the effect of *Perna perna* and *M. galloprovincialis* (Mussel factor) on both ascidians (Ascidian) in South Africa

	df	SS	MS	*F*	p
(A) Comparison between regions					
Ascidian	1	358.86	358.86	6.04	0.020
Region	1	1043.25	1043.25	17.55	<0.001
Ascidian × Region	1	18.01	18.01	0.3	0.586
Residual	30	1783.35	59.44		
(B) Comparison between mussel species					
Ascidian	1	613.44	613.44	13.37	0.001
Mussel	1	42.15	42.15	0.92	0.347
Ascidian × Mussel	1	0.29	0.29	0.01	0.936
Residual	24	1100.77	45.86		

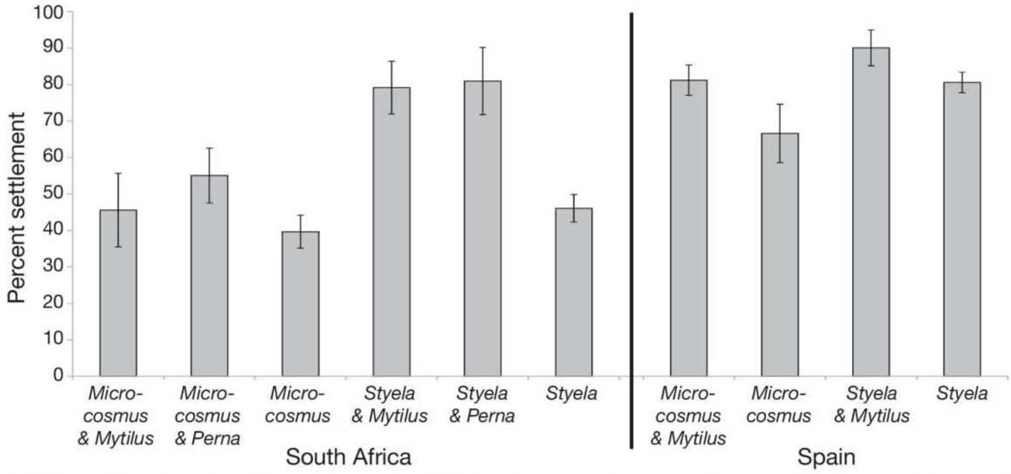

Fig. 6. Mean (±SE) ascidian larval settlement success (%) for the experiment testing ascidian–mussel interactions in mixed and control Petri dishes. Note that values were obtained by considering only non-consumed larvae (ANOVA results in Table 5)

pected distances in Spain, and negative in South Africa, albeit with large standard errors. The mussels and mussel aggregates showed the same significant pattern of avoidance as before.

DISCUSSION

We found interactions ranging from subtle to very strong, including both direct and indirect effects. The studied ascidian species interacted in quite mild ways. There was a strong difference in fertilization success between the 2 species, but no effect of either conspecific sperm concentration or the presence of heterospecific sperm. The same was true for larval interactions; settlement success differed between species with no effects of the presence of heterospecific larvae on settlement success, though this did alter spatial patterns of settlement. On the other hand, the effects of mussels on ascidians were much more dramatic. Cryptic predation of ascidian larvae by juvenile mussels was observed, with similar high levels of larviphagy by the 2 studied mussel species. However, when we compared consumption by *Mytilus galloprovincialis*, we found greater values within its introduced range than in its home region. Thus the presence of mussels stimulated 2 indirect responses in ascidians: (1) larval settlement was higher and (2) larvae avoided settling near their predators. Taken together, the results indicate differ-

Table 5. ANOVA results for settlement percentage of the ascidian–mussel interactions experiment. (A) Testing differences in the effect of *Mytilus galloprovincialis* on both ascidian species in a 3-way ANOVA: Ascidian factor, *Microcosmus squamiger* and *Styela plicata*; Region factor, South Africa and Spain; Treatment factor, control and mixed (with M. *galloprovincialis* only). (B) Testing differences in the effect of both mussel species on both ascidians in South Africa in a 2-way ANOVA: Ascidian factor, as above; Treatment factor: control (without mussels), mixed with *M. galloprovincialis*, mixed with *Perna perna*

	df	SS	MS	*F*	p
(A) Comparison between regions					
Ascidian	1	1294.88	1294.88	7.03	0.010
Region	1	5946.21	5946.21	32.29	<0.001
Treatment	1	2054.88	2054.88	11.16	0.001
Ascidian × Treatment	1	778.75	778.75	4.23	0.044
Ascidian × Region	1	0.24	0.24	0.00	0.971
Treatment × Region	1	24.24	24.24	0.13	0.718
Ascidian × Treatment × Region	1	854.79	854.79	4.64	0.036
Residual	56	10313.25	184.16		
(B) Comparison between mussel species					
Ascidian	1	7515.59	7515.59	19.65	<0.001
Treatment	2	12114.59	6057.30	15.83	<0.001
Ascidian × Treatment	2	587.37	293.69	0.77	0.468
Residual	78	29839.36	383.56		

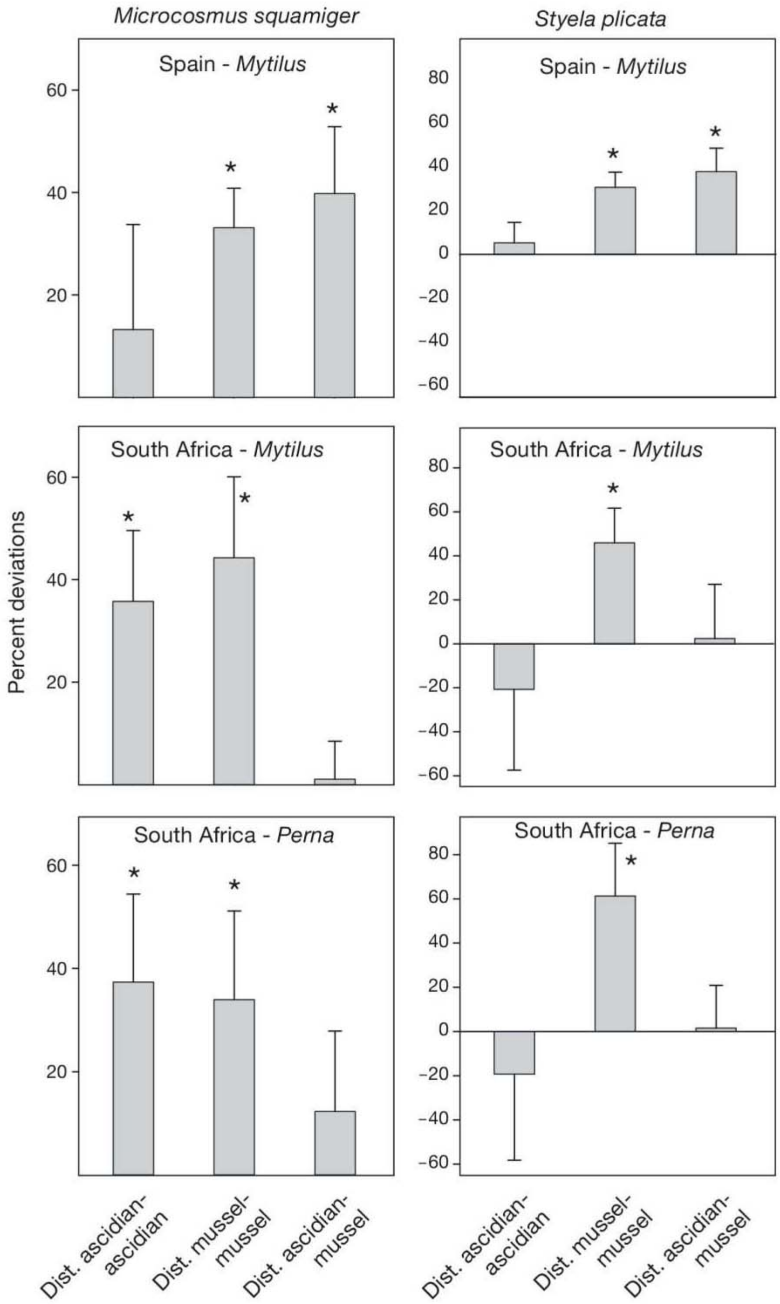

Fig. 7. Mean (±SE) percent deviations [(observed − expected)/expected × 100] of the shortest distances between ascidian settlers, between mussel juveniles (or aggregates), and between ascidians and mussels for the mixed ascidian–mussel experimental plates (in Spain and in South Africa). Expected values obtained through simulation. Asterisks: significant departures from zero (i.e. non-random distribution)

In our experiments, *Styela plicata* had higher success in fertilization, settlement and metamorphosis than *Microcosmus squamiger*, and the only interspecies interaction found at the early-life history stages tested concerned spatial patterns at settlement. There was no effect of heterospecific sperm on the fertilization process, nor any density-dependent effect of homologous sperm at the concentrations assayed. In ascidians, besides intraspecific sperm competition (Yund 1998), interspecies sperm competition can occur (Lambert 2000). Even though fertilization is species-specific in most ascidians, in solitary species heterologous sperm can trigger mechanisms that block polyspermy, hindering subsequent fertilization by homologous sperm (Lambert 2001). Follicle cells enveloping the eggs are involved in the fertilization process and in the establishment of this primary block to polyspermy in response to conspecific and heterospecific sperm (Lambert 2009). This process did not seem to act in the present work. In previous experiments with the same species used here, Rius et al. (2009a) first exposed eggs of *M. squamiger* in its native range (Australia) to the sperm of introduced *S. plicata*, then the eggs were washed, and finally exposed to conspecific sperm. That study found no effects of the heterologous sperm on the fertilization process. In our study, we exposed oocytes of each ascidian species to conspecific and heterospecific sperm at the same time and at different concentrations with similar results. The species studied here are space dominant and sympatric; they are both free spawners and largely overlap in the timing of their reproduction (Yamaguchi 1975, Rius et al. 2009b, Pineda et al. 2013). Thus, it seems likely that, in nature, oocytes of the 2 species are often exposed to sperm of both species, especially as ascidian sperm is able to fertilize eggs 12 h or more after dilution (Bolton & Havenhand 1996). In this sense, lack of interaction with heterologous sperm would be beneficial for these invaders. However, even though we found no direct effect of one species on the other, the sperm environment plays an important role in gamete phenotype and quality in at least one of the studied species (*S. plicata*; Crean & Marshall 2008), so we cannot exclude the possibility of more subtle intraspecific and interspecific effects that could not be detected with our approach.

ent interactions among introduced and native species at early life-history stages, which can combine to produce both direct and indirect effects that can fundamentally influence invasion success and community assembly.

There is evidence that the resident community can influence the larvae of marine invertebrates (e.g. Grosberg 1981, Johnson & Strathmann 1989, Osman et al. 1989, Bingham & Young 1991, Stoner 1994) including adult ascidians (Svane & Young 1989), which suggests important interactions affecting the settlement process. We found no evidence of species interference when looking at the effect of the larvae of one species on the larvae of the second species in terms of settlement or metamorphosis success. The only effect observed was on the spatial arrangement of the settlers. Larvae of both species settled gregariously (*Microcosmus squamiger* only so at the highest concentration tested), and showed more random settlement in the presence of larvae of the other species. In contrast to our results, Rius et al. (2009a) found that the settlement success of *M. squamiger* in its native Australian range was negatively affected by previously settled recruits of the introduced *Styela plicata* and vice versa. We expected some kind of interference effect as both species form dense aggregations in nature on artificial human-made substrata, so that competition for space is likely to be strong between them. We hypothesized that such competition would be detectable already at the larval stage, but in our experimental setup no effect was found when larvae of both ascidian species were placed together. Interference effects, therefore, seem to occur when a larva faces already established settlers rather than larvae of the other species. Although larval encounters are likely in the studied environments, finding already settled competitors may be a more common situation, and indeed recruitment timing can play an important role in introduction success (Stachowicz et al. 2002).

The effects of marine predators on the success of biological introductions have been extensively studied (e.g. Osman & Whitlatch 1998, Rilov et al. 2002, Epelbaum et al. 2009, Dumont et al. 2011). Such studies often concentrate on the role of small, vagile predators preying on new recruits. However, suspension-feeding organisms processing large volumes of water can potentially exert considerable predation pressure on free planktonic larvae (Porri et al. 2008), thus preventing successful recruitment (Bingham & Walters 1989, Young & Cameron 1989). Mussels are highly efficient filter feeders (Norén et al. 1999, Porri et al. 2008) and, although traditionally thought to be basically consumers of small phytoplankton, there is compelling evidence that they can also efficiently ingest mesozooplankton species (e.g. Davenport et al. 2000, Lehane & Davenport 2002, 2006) and thus have an omnivorous diet (Maloy et al. 2012). *Mytilus* species have been suggested to be major determinants of mesozooplankton abundance through grazing (Lehane & Davenport 2006). In a study focusing on cannibalism, the 2 mussel species studied in South Africa ingested mussel larvae and settlers of up to 400 µm in length (Porri et al. 2008). Our ascidian larvae measured around 500 µm total length (Pineda et al. 2012). This fits nicely in the range of zooplankton sizes found in the stomach contents of mussels, which is generally about 400 to 600 µm (Lehane & Davenport 2002), but can reach several millimetres (Davenport et al. 2000, Lehane & Davenport 2006). These studies are biased towards detection of prey with hard parts (molluscs, crustaceans), and the rarity of soft-bodied prey may be an artefact. Indeed, ascidian larvae are the right size and, lacking appendages or cilia, are unable to avoid being trapped in the mucus on the filtering apparatus of mussels.

In our experiments, we found a marked effect of consumption by mussels of ascidian larvae with 70 to >90% of total larvae being consumed. This was despite the small size of the juvenile mussels. Consumption by adult mussels would presumably be dramatically higher. Previous experimental studies on mussel consumption of zooplankton showed consumption even at the smallest size classes tested (1.5 to 2.0 cm; Lehane & Davenport 2002). According to our results, the ability to capture larvae is already established at earlier stages of mussel development (sizes smaller than 1 cm). We can safely conclude that the effect of a well-developed mussel bed on larvae swimming by would be far stronger. Bivalves are able to select food before ingestion and reject unwanted particles as pseudofaeces (e.g. Foster-Smith 1975, Kiorboe & Mohlenberg 1981, Bougrier et al. 1997) within a few hours after ingestion (Foster-Smith 1975). We did not find production of pseudofaeces by our juvenile mussels after 48 h of larval addition, coherent with reports of zooplankton in the stomachs rather than the pseudofaeces of mussels (Davenport et al. 2000). Moreover, mussels and mussel aggregates were scrutinized for hidden larvae or settlers and none were found, so the only explanation for the disappearance of larvae is that mussel juveniles ingested them. Among non-consumed larvae, a general response was a higher settlement success, which could be due to an acceleration of the settlement process as an adaptative response to minimize risks of being captured by mussels.

The effects of *Mytilus galloprovincialis* on ascidian larvae were stronger in the introduced range of the mussel (South Africa), where consumption was higher. The increase in settlement response of ascidians to the presence of *M. galloprovincialis* was stronger where the mussel was native than in its introduced range, which could explain in part the lower consumption. Changes in consumption rates in *M. galloprovincialis* could be due to an adaptation to the invasion of new areas (Lee 2002), but other explanations can be put forward as well. *M. galloprovincialis* comprises several genetic groups (Sanjuan et al. 1997, Daguin & Borsa 2000). In particular, the South African introduced populations are related to the European Atlantic stock, while the Mediterranean populations belong to a different genetic clade (Daguin & Borsa 2000). Thus, we have used *M. galloprovincialis* individuals from 2 genetically differentiated sources, which could explain the different behaviours observed. Alternatively, and considering that the consumption by the native *Perna perna* in South Africa was of the same level as that by *M. galloprovincialis* there, environmental factors could be driving an increased filtering activity in South Africa. Temperature changes the metabolic activity of mussels (Anestis et al. 2007). The mean annual surface water temperature for the past 30 yr in the 2 regions where we performed the experiments are significantly different (Mann-Whitney *U*-test, T = 200.00; $p = 0.004$) with a mean temperature (±SE) of 17.14 ± 0.90°C in the Mediterranean Sea (www.meteoestartit.cat), and 20.86 ± 0.36°C off the south coast of South Africa (M. Rius unpubl. data). Thus, although the laboratory temperature was the same in both experiments, adaptation to water temperature in each area could play a role in determining filtration activity of the mussels used in the present study. Assessing the ultimate causes of the observed differences in ascidian larval consumption would require further studies beyond the scope of the present work.

When comparing predatory effects between mussel species in South Africa, no differences in consumption or settlement percentages were found between native and introduced mussels. In all cases, *Styela plicata* experienced significantly greater losses through consumption by mussels than *Microcosmus squamiger*. Accordingly, *S. plicata* also had a stronger response in terms of settlement, which was higher than in *M. squamiger* in the presence of mussels. The greater change in settlement for *S. plicata* explains the significant interactions found in these analyses.

Both *Styela plicata* and *Microcosmus squamiger* avoided settling close to *Mytilus galloprovincialis* in its native area (Mediterranean Sea), as shown by ascidian–mussel distances being larger than expected. In South Africa, however, the pattern of ascidian settlement was not different from random for either mussel species tested. The settlement of *M. squamiger* changed from aggregated (as seen in the settlement experiment) to a regular pattern in the presence of mussels (particularly in South Africa), while that of *S. plicata* changed from aggregated to random. Overall, then, there was an increase in the spacing among ascidian settlers in the presence of mussels, allowing the testing of as many different places as possible in an environment where competition for space with mussels will be predictably high. However, laboratory experiments are simplified systems and thus caution is needed when interpreting these results.

In summary, we found weak competitive interactions between the studied ascidian species and strong predatory effects of juvenile mussels on ascidian larvae. This dramatic reduction of the larval pool of ascidians suggests that propagule pressure must be intense to allow ascidians to colonize space in habitats where mussel beds are well developed, such as the fouling communities or rocky shores. We found evidence that ascidian larval behaviour shifted in the presence of predators, by increasing settlement success and avoidance of mussels. A general lack of interaction between gametes or larvae of 2 dominant introduced ascidians was found, with only slight changes in spatial distribution of settlers. As both ascidian species can form dense populations that monopolize space (Rius et al. 2009b, Pineda et al. 2013), our results suggest that interference competition occurs at later, post-metamorphic stages. We showed that co-occurring introduced species can interact intensely at early ontogenetic stages, especially in terms of predatory interference among broadcast spawners. Our results reinforce the importance of the composition of the target community in determining the susceptibility to invasion success. Whether the species comprising the target community are native or introduced seems to be less important than what manner of species they are.

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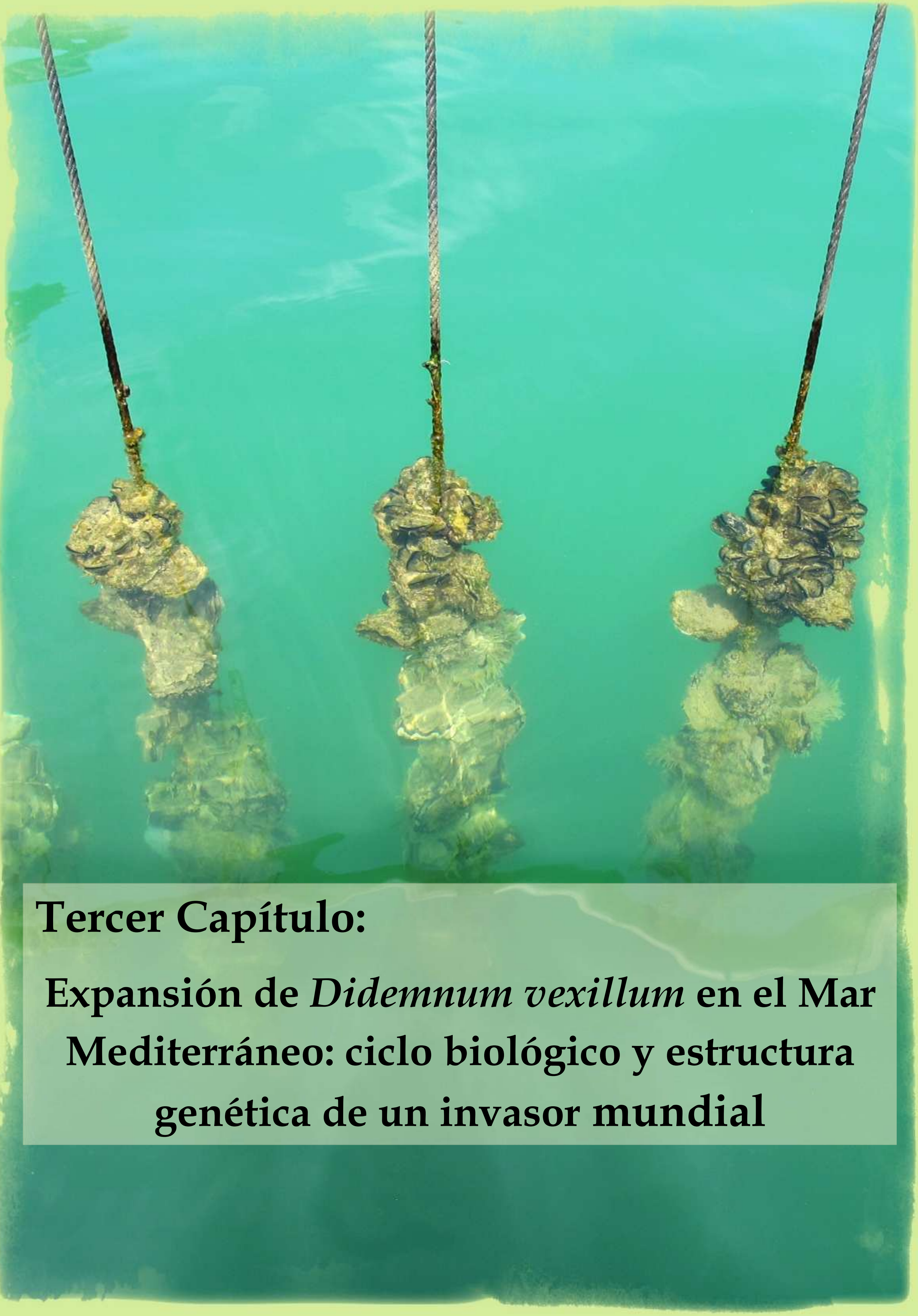

Tercer Capítulo:

Expansión de *Didemnum vexillum* en el Mar Mediterráneo: ciclo biológico y estructura genética de un invasor mundial

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RESUMEN

Las ascidias no indígenas son una especial preocupación para la industria de acuacultura y, paradójicamente, el stock de bivalvos representa una importante vía para transportar estas ascidias por todo el mundo. En 2012 una ascidia no indígena se encontró cubriendo las cuerdas de ostrones en el Delta del Ebro (Mediterráneo occidental). Hemos identificado la ascidia genética y morfológicamente como *Didemnum vexillum* Kott, 2002. Este descubrimiento nos indica que la especie está expandiendo su distribución por el Mediterráneo, ya que se ha encontrado recientemente en la cuenca este (Venecia, mar Adriático). En este capítulo estudiamos el ciclo de vida (crecimiento y reproducción) de la ascidia en el Delta del Ebro. La especie presenta un ciclo marcadamente estacional, con una regresión en los meses más cálidos reapareciendo durante el invierno. En primavera, *D. vexillum* alcanza su máxima abundancia seguido por un pico reproductor justo antes de la regresión. También analizamos la estructura genética de la población del Delta del Ebro y de la establecida en la Laguna de Venecia, mediante secuencias del *COI*, para comparar con las poblaciones mundiales de la ascidia. La baja diversidad genética de nuestras muestras (tres haplotipos) fue coherente con lo observado en las poblaciones introducidas mundialmente. Esta variabilidad genética baja en las poblaciones introducidas impide el esclarecimiento de la vía de introducción en el mar Mediterráneo, pero es muy probable que la ascidia fuera introducida con el stock de cultivos de bivalvos de la costa atlántica francesa. Finalmente, la gran actividad de embarcaciones en el Delta del Ebro

hace que el transporte mediado por el hombre sea muy probable en la zona, amenazando los fondos pesqueros próximos, por lo que se necesita especial cuidado para evitar la continua expansión de esta plaga.

Ongoing expansion of *Didemnum vexillum* in the Mediterranean Sea: biological cycle and genetic structure of a worldwide invader

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ABSTRACT

Non-indigenous ascidians are of special concern to aquaculture industry and, paradoxically, the stock of bivalve culture represents an important way to translocate these species worldwide. In 2012 a non-indigenous ascidian was found covering the oyster crops in the Ebro Delta (Western Mediterranean). We have identified the ascidian genetically and morphologically as *Didemnum vexillum* Kott, 2002. This finding indicates that the species is currently expanding its distribution in the Mediterranean Sea, as it has been recently found in the eastern Basin (Venice, Adriatic Sea). We assessed the life cycle (growth rates and reproduction) of the ascidian at the studied site. The species has a marked seasonal cycle, with regression in the warmest months and reappearance during the winter. In spring *D. vexillum* reaches its maximum abundance, followed by a peak in reproduction just before regression. We analysed the genetic structure of the population of the Ebro Delta and the one established in the Lagoon of Venice using *COI* sequence data for comparison with the worldwide populations of the ascidian. The low genetic diversity in our samples (3 haplotypes) was coherent with what is observed in the introduced populations worldwide. This low genetic variability in introduced populations precluded the elucidation of the pathway of the introduction in the Mediterranean, but it is likely that the ascidian was introduced with oyster stock from bivalve cultures in the Atlantic French coasts. Finally, the high boating activity in the Ebro Delta makes further human-mediated transport of the species highly likely, threatening nearby fishing grounds, thus caution is needed to avoid the continuous expansion of this ascidian pest.

Key words: ascidian, life cycle, maturity index, growth rate, bivalve culture, aquaculture, *COI*, introduced species

INTRODUCTION

Biological invasions are a highly relevant environmental issue, with a large number of introductions of many taxa worldwide over the last century (e.g. Carlton & Geller 1993, Lowe *et al.* 2000, Wonham & Carlton 2005, Strayer 2010). These invasions have both ecological and economic implications (Grosholz 2002, Clavero & García-Berthou 2005, Didham *et al.* 2005, Pimentel *et al.* 2005), and the costs of eradicating invasive organisms is usually very high, if at all possible (Williams *et al.* 2010). Environmental features of the new range of distribution, such as climate conditions, are crucial for the success of the establishment of these nonindigenous species (e.g. Ashton *et al.* 2007, Bomford *et al.* 2009, Bomford *et al.* 2010). Once established, knowledge of biological features (f.i., responses to environmental conditions, natural capacity for dispersal) and life cycle of these species are crucial to better understand their invasive potential, as well as to develop efficient management tools (eradication and preventive programs).

In marine ecosystems, the intense shipping traffic around the world, whether short or long-distance travels, has facilitated the introduction of nonindigenous species of many taxa via ballast waters or as a biofouling on ship hulls (Carlton & Geller 1993, Ruiz *et al.* 1997, Lambert 2002), although other vectors such as those associated with industries or management practices have also been involved (Ruiz *et al.* 1997). Aquaculture, particularly, has become a leading vector of aquatic invasive species worldwide (Naylor *et al.* 2001, Minchin 2007). Among marine introduced species, ascidians have become one of the most important groups, with many nonindigenous species spread around the world (Lambert 2007, Locke & Hanson 2011). Harbours, marinas, aquaculture facilities and other man-made structures are often the habitats where ascidians are first introduced and proliferate (Lambert & Lambert 2003, Lambert 2007, Tyrrell & Byers 2007, Locke *et al.* 2009). Shellfish aquaculture industry is commonly affected by introduced ascidians worldwide (Gittenberger 2009, Arens *et al.* 2011), causing economic losses (Coutts & Forrest 2007, Daigle & Herbinger 2009, Adams *et al.* 2011). Nonindigenous ascidians overgrow bivalves, adding weight, restricting water exchange and nutrients, and decreasing bivalve productivity (LeBlanc *et al.* 2002, Gittenberger 2009, Daigle & Herbinger 2009).

A non-native didemnid species was found in spring 2012 covering oyster crops in aquaculture facilities in the Ebro Delta, Spain (North-western Mediterranean Sea). The morphological traits of the colonial ascidian matched those of *Didemnum vexillum*, Kott 2002. This ascidian was previously recorded in the Eastern Mediterranean Sea by Tagliapietra *et al.* (2012) at the Lagoon of Venice (Adriatic Sea), although the species was said to be already present in the Lagoon since at least 2007. *D. vexillum* is one of the most important pest species among marine invertebrates due to its high invasiveness, dispersion capabilities and its high capacity to alter benthic communities (see Lambert 2009 and references therein). It is native to the Northwest Pacific Ocean (Lambert 2009, Stefaniak *et al.* 2012), and has become a very successful invader in temperate regions worldwide, including New Zealand, both coasts of North America, the Netherlands, the Atlantic coast of France, Great Britain, Ireland, the west coast of the Iberian Peninsula and the Adriatic Sea (Lambert 2009, El Nagar *et al.* 2010, Tagliapietra *et al.* 2012, Stefaniak *et al.* 2012 and references therein). Multiple vectors of introduction of *D. vexillum* have been identified (Lambert 2009), including the hull-fouling of recreational (slow-moving) vessels (Herbog *et al.* 2009) or aquaculture activities, as epifauna on cultured oysters (Dijkstra *et al.* 2007) or in mussel seed stock (Denny 2008). The ascidian represents an issue of special concern to aquaculture industry, as the species overgrows commercial shellfish smothering them (Coutts & Forrest 2007, Bullard *et al.* 2007a, Valentine *et al.* 2007a). Moreover *D. vexillum* is not confined to enclosed areas on man-made artificial structures or to aquaculture facilities, but also occurs in exposed outer coasts on hard-bottom natural substrates, where it severely affects important fishing grounds (Bullard *et al.* 2007a, Valentine *et al.* 2007b).

We report here the presence of *Didemnum vexillum* for the first time in the Western Mediterranean. This points to a worrisome ongoing expansion of *D. vexillum* in this sea. Our purpose was to characterize morphologically and genetically the population found on the oyster cultures in the Ebro Delta, as well as to establish genetic similarities with other populations of the species worldwide. Moreover, we studied the life cycle of the species in the Ebro Delta, by documenting its abundance, growth rates and reproduction. Knowledge of the biology of introduced species in the newly colonized areas might be decisive, allowing stakeholders to evaluate and take the appropriate management actions to mitigate their damaging effects in the aquaculture industry.

MATERIAL & METHODS

Study site

Didemnum vexillum was first observed in May 2012 at the Fangar bay, at the northern side of the Ebro Delta (Spain, West Mediterranean Sea) (Fig. 1) growing as epifauna on oyster ropes. The species apparently competes for the surface of the oyster's shell with other organisms, clearly overgrowing algae, polychaetes, the solitary ascidian *Styela plicata* or the colonial ascidian *Clavelina lepadiformis*, and even occasionally *Mytilus galloprovincialis* growing on the oyster ropes (Fig.2).

The Fangar bay has nine km^2 of extension with a muddy bottom up to 4.2 m depth and houses aquaculture facilities with 77 bivalve beds. Each bivalve bed consists of a rectangular structure of wooden beams arranged in a grid, sustained by cement columns, where the bivalve ropes hang. In a single bivalve bed there can be up to 5000 ropes. Both the oyster *Crassostrea gigas* and the mussel *Mytilus galloprovincialis* are grown in the bay, but the former is the species most commonly cultured in this bay.

Monitoring of abundance and growth cycle

From May 2012 to July 2013 we monitored five ropes with *Crassostrea gigas* granted by the owner and placed along oyster beds in the middle of the Fangar bay. These ropes had been there since the previous autumn. The ropes were monthly surveyed with the exception of September 2012 and February 2013 when the monitoring could not be done due to logistic problems. In order to obtain an estimate of the covering of *Didemnum vexillum*, pictures of each rope were taken with a digital camera (Canon PowerShot G11, 10MP). Ropes were photographed including a ruler to scale every picture. In order to measure the monthly area covered by the ascidian, the perimeter of each colony or fragment was manually marked in every picture with Photoshop CS4. Pictures were subsequently analysed by the Laboratory of Image Analysis and

Treatment of the CCiTUB (Scientific and Technological Centre of the University of Barcelona), using the proprietary program IMAT. The total area of didemnid on every rope (in cm^2) was divided by the total length of the rope (in m), thus obtaining a relative estimate of abundance (cm^2/m).

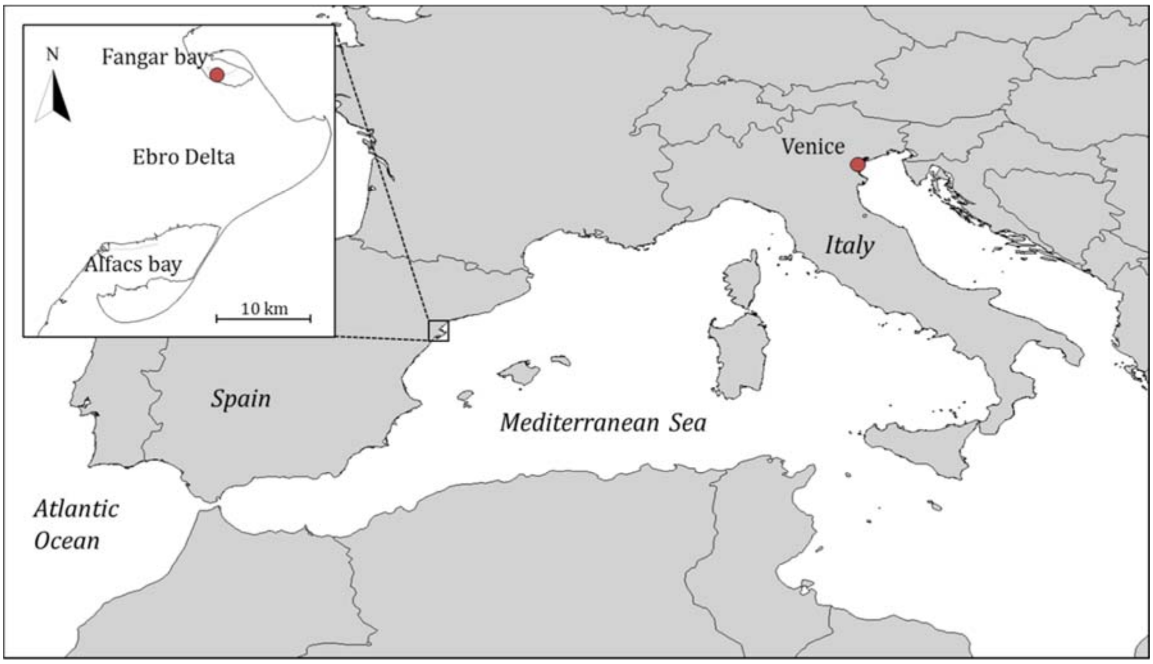

Figure 1. Sampling and monitoring site of *Didemnum vexillum* in the Fangar bay (Ebro Delta, Spain, western Mediterranean Sea), and sampling site in the Lagoon of Venice (Italy, eastern Mediterranean Sea).

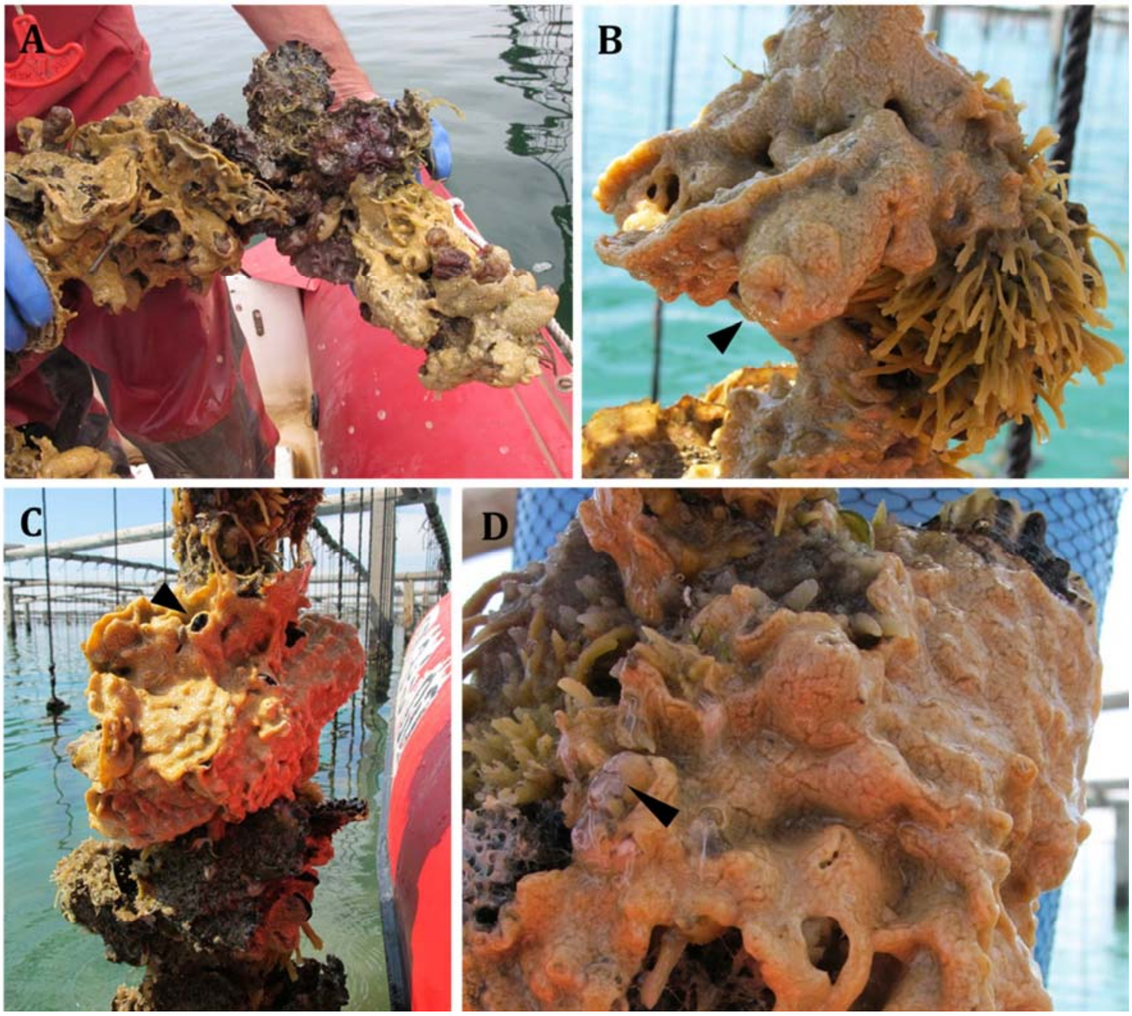

Figure 2. Colonies of *Didemnum vexillum* in oyster ropes at the Fangar bay (Ebro Delta, Spain) A) overgrowing oysters and its epifauna; B) completely covering the oysters and the solitary ascidian *Styela plicata* (black arrow); C) overgrowing oysters and *Mytilus galloprovincialis* (black arrow); D) the ascidian overgrowing *Clavelina lepadiformis* (black arrow) on the oyster ropes.

Reproductive cycle

To study the reproductive cycle we collected on a monthly basis fragments of five colonies of the ascidian during the monitoring from June 2012 to August 2013. Samples were stored *in situ* in formaldehyde 10%. Colonies were dissected under a binocular microscope. Ten zooids were randomly selected per colony in order to determine their reproductive status. As most colonial ascidians, this didemnid is hermaphrodite and protandric, so each zooid was categorized as follows: (1) immature; (2) presence of testes; (3) presence of testes and oocytes; (4) presence of oocytes alone; (5) presence of embryos; and (6) presence of larvae. We calculated a maturity index per month (MI) as in López-Legentil *et al.* (2005), averaging the category number of the ten zooids per colony and calculating the mean of the five colonies studied.

Statistical analysis

Cross-correlation analyses were used to study relationships of the abundance cycle and the maturity index with the environmental parameters: temperature (ºC), salinity (psu), levels of oxygen (%) and chlorophyll *a* (μgL^{-1}) in the water column. In cross-correlation analysis, two time series are compared using the Pearson correlation coefficient with an increasing lag of one series with respect to the other. Correlation at time lag 0 is the usual Pearson correlation. Correlations at negative lags relate values in the first series with previous values in the second series. Correlations at positive lags analyse relationships of the values in the first series with future values in the second series. Physical data and chlorophyll *a* values were obtained from the weekly monitoring of the Bay performed by the Institute of Agriculture and Food Research and Technology (IRTA) of Sant Carles de la Ràpita.

In order to correct for missing data (September 2012 and February 2013), the values of these months were calculated averaging the value of the previous and the posterior months. In the case of the MI, we assigned stage 1 (immature) to the months when colonies were absent in order to avoid missing data and to be able to perform the cross-correlation analysis. The package Systat v. 12.02 was used for these analyses.

Genetic analyses

Sequences were obtained of colonies from the Ebro Delta and from the Venice Lagoon. The latter area was reported as the first occurrence of *Didemnum vexillum* in the Mediterranean Sea (Tagliapietra *et al.* 2012) but no genetic data was available for this location. Fragments of a total of 29 colonies spaced at least two metres apart were collected from ropes with *Crassostrea gigas* in the Fangar bay (Ebro Delta). A total of 28 samples of *D. vexillum* from artificial structures in the Arsenale and Lio Grando marinas inside the Lagoon of Venice were also collected as above. Samples were fixed in 96% ethanol in the field and stored at -20ºC in the laboratory. For DNA extraction, we dissected the colonies and pooled ca. 30 thoracic regions of zooids or 30 larvae (when available) from each colony, and total DNA extraction was made using QIAamp® DNA Mini Kit (QIAGEN).

We used the primers Tun_forward, 5′-TCGACTAATCATAAAGATATTAG-3′ and Tun_reverse2, 5′AACTTGTATTTAAATTACGATC-3′ described in Stefaniak *et al.* (2009), for the amplification of a fragment of the mitochondrial gene *Cytocrome C Oxidase I* (*COI*).

PCR amplifications were carried out in a total volume of 20 µl with 14.7 µl H_2O, 2 µl 5x buffer (GoTaq, Promega), 1 µl $MgCl_2$ (25mM), 0.5 µl dNTP's (1 mM), 0.4 µl (10 µM) of each primer, 1U *Taq* Polymerase (GoTaq, Promega) and 1 µl of DNA. The PCRs started with an initial denaturation at 94 ºC for 5 min, followed by 35 cycles of a denaturation step at 94 ºC for 1 min, an annealing step at 50ºC for 1 min and an elongation step at 72 ºC for 1 min 30 s, with a final extension at 72 ºC for 7 min. The amplified DNA was purified with Exo-SAP and both strands were cycle sequenced by Macrogen Inc. with the EZ-seq V2.0 service. For each individual the forward and reverse sequences were visually inspected, edited and aligned with BioEdit Sequence Alignment Editor (Hall 1999) using the ClustalW Multiple alignment option to obtain a 586 bp sequence length after trimming.

To match previously identified haplotypes, the sequences were aligned with known haplotypes of *Didemnum vexillum* available from GenBank (Stefaniak *et al.* 2009, 2012).

Using the data on haplotype frequencies per population, we calculated the population genetic divergence using the estimator D_{est} (Jost 2008) with SPADE (Chao & Shen 2009). To visualize the resulting pattern of pairwise dissimilarities among worldwide populations of *Didemnum vexillum*, we constructed a Multidimensional Scaling plot (MDS) using Systat v. 12.02..

RESULTS

Morphological observation

The colonies grew as encrusting sheets, of yellow-orange color (Fig. 2). When they occupy all the available substrate they start forming lobes and digitations. The spicules in the tunic are not densely distributed. The zooids are arranged in systems, separated by darker meandering zones with less dense spiculation. The spicules (Fig. 3A) are mostly between 20 and 30 µm in diameter, with relatively short and pointed rays, about 8-9 in optical section.

The zooid morphology is typical of the genus. The thorax has a wide atrial aperture without languet, thoracic lateral organs posteriorly situated and a retractor muscle originating in the oesophageal neck region. When mature, the zooids have a spherical testicle surrounded by a spermduct with 8-9 coils and one developing oocyte.

Larvae (Fig. 3B) are incubated in the basal tunic of the colonies. They are about 500 µm long (in the coiled stage), and feature 3 adhesive papillae and 6 pairs of elongated ectodermal ampullae (Fig 3C).

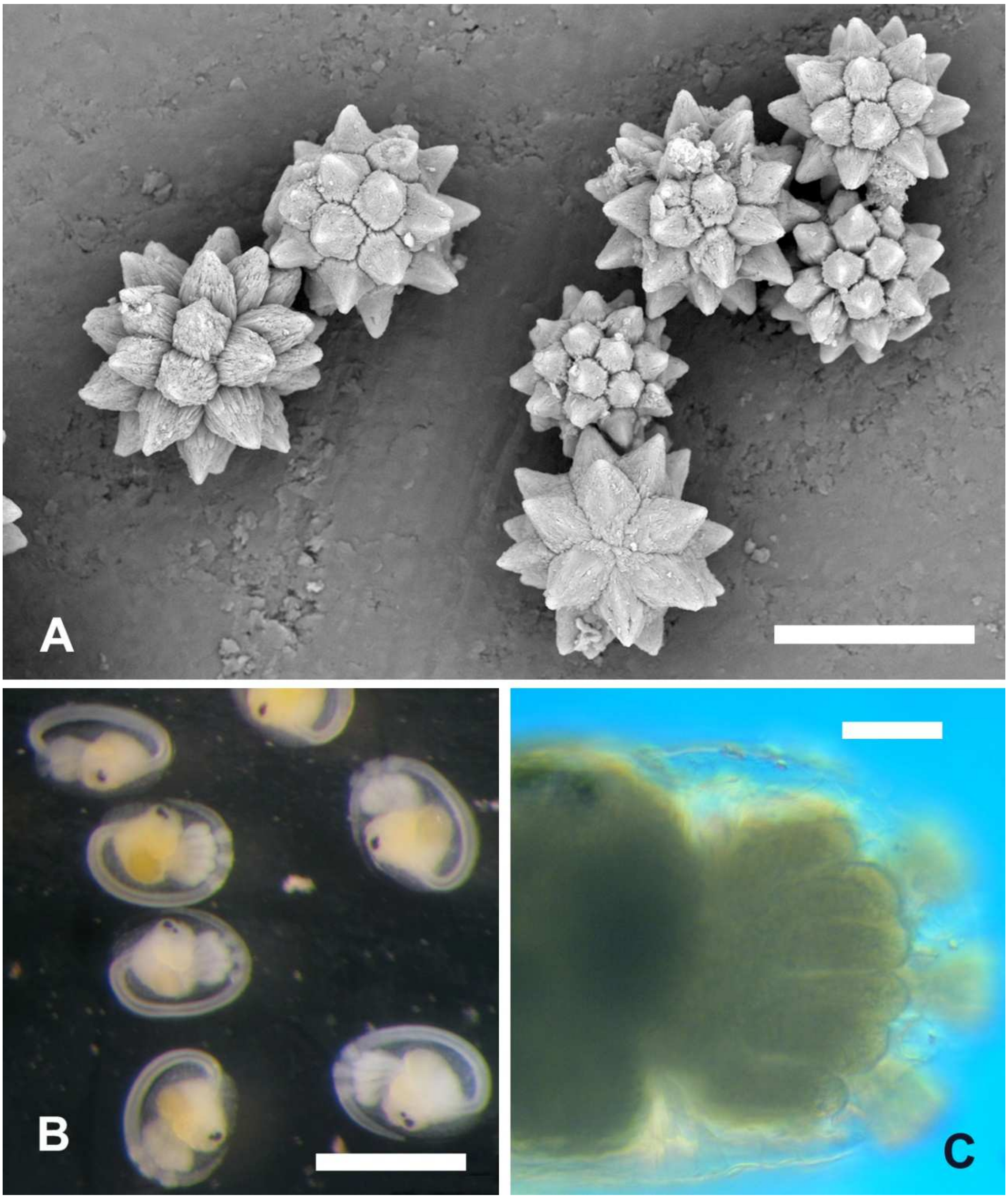

Figure 3. A) Image of the spicules in the tunic of colonies of *Didemnum vexillum* from the Ebro Delta. B) view of unhatched larvae obtained from the basal tunic. C) close-up of the anterior part of a larva, showing ectodermal ampullae. Scale bars: A, 25 µm; B, 500 µm; C, 50 µm

Growth cycle

The growth cycle showed a marked seasonal fluctuation in the coverage of *Didemnum vexillum* in the Fangar bay with a maximum coverage in spring (Fig. 4). There was a marked increase in the area occupied by the ascidian in 2013 in relation to 2012. From May 2012 to August 2012 only one of the five ropes surveyed was colonized by *D. vexillum*. During summer, the colonies entered in regression and disappeared completely in October 2012. Unfortunately, we do not have data from September 2012, and we cannot tell exactly when *D. vexillum* completely disappeared from the ropes, but it is likely that the ascidian was already missing in that month given the very low cover observed in August 2012. In November 2012 small colonies of *D. vexillum* began to grow, this time on all five ropes, with a mean coverage of 3.33 cm^2/m. The ascidian grew all along winter, reaching its maximum abundance in spring (May 2013), occupying over 400 cm^2/m in average. The colonies of *D. vexillum* started again to regress and by July 2013 their coverage was as low as 67.52 cm^2/m.

The time course of environmental variables (temperature, chlorophyll *a*, salinity, and levels of O_2) over the study period is shown in (Fig 5). The relationship of these variables with ascidian abundance was evaluated by cross-correlation analysis (Fig. 6). The results showed a significant negative effect of the temperature in the 2-4 previous months (lag of -2, -3, and -4) in the coverage of the current month. Likewise, levels of chlorophyll *a* of the current and up to three previous months were also negatively significant correlated with the covering of *Didemnum vexillum*. Salinity showed only a negative significant correlation of the fifth previous month (lag -5), which does not seem to have a biological meaning and may be due to a random outcome. There was no significant correlation with the levels of O_2 at any time lag.

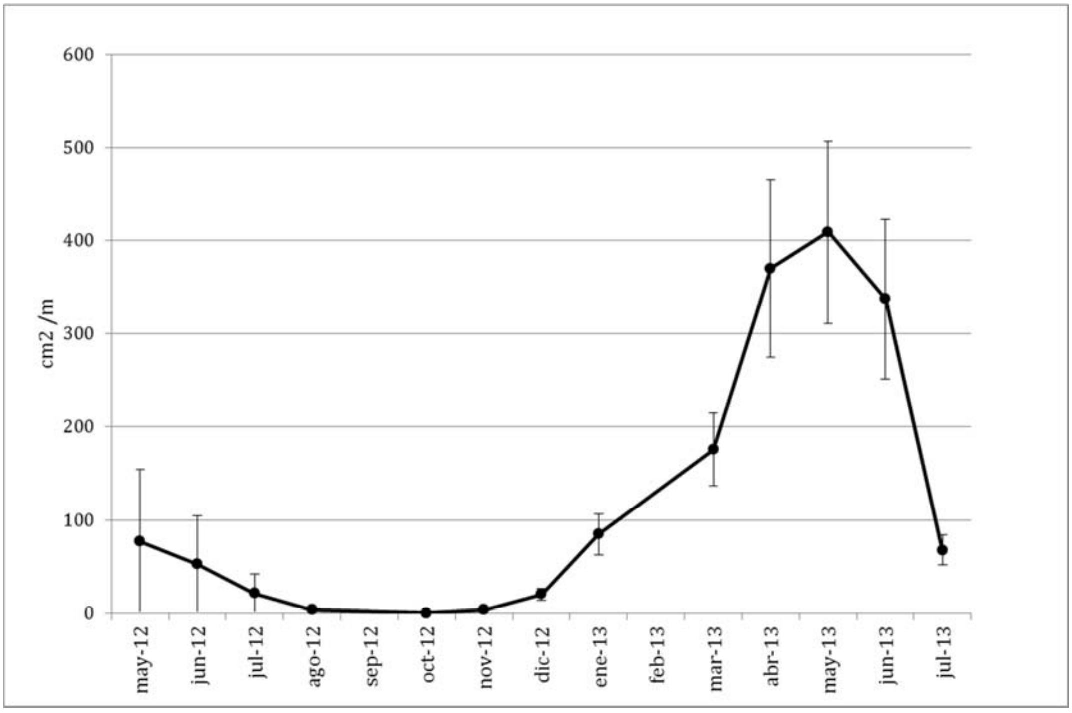

Figure 4. Growth cycle of *Didemnum vexillum* from May 2012 to July 2013. Monthly values represent the mean coverage (cm²/m ± SE) of the five ropes surveyed. In October 2012 the coverage was zero since no colonies were present on the ropes.

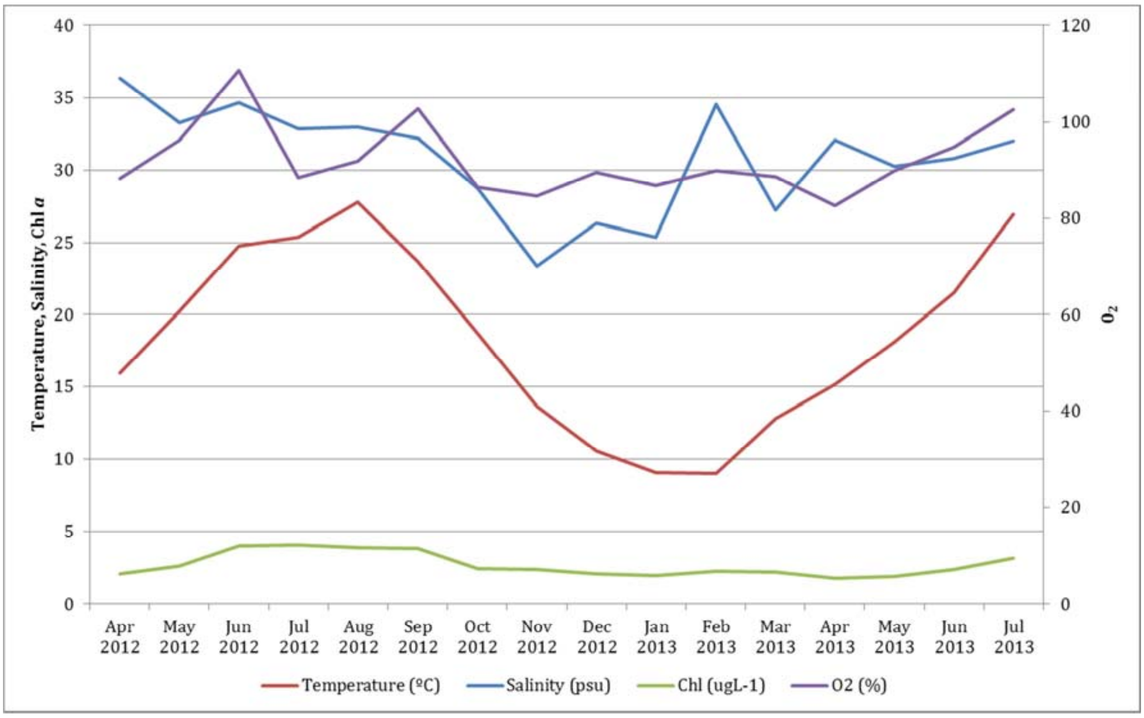

Figure 5. Environmental parameters (temperature, salinity, levels of O_2), and levels of chlorophyll *a* (Chl *a*) in the Fangar bay (Ebro Delta, Spain) during the monitoring period.

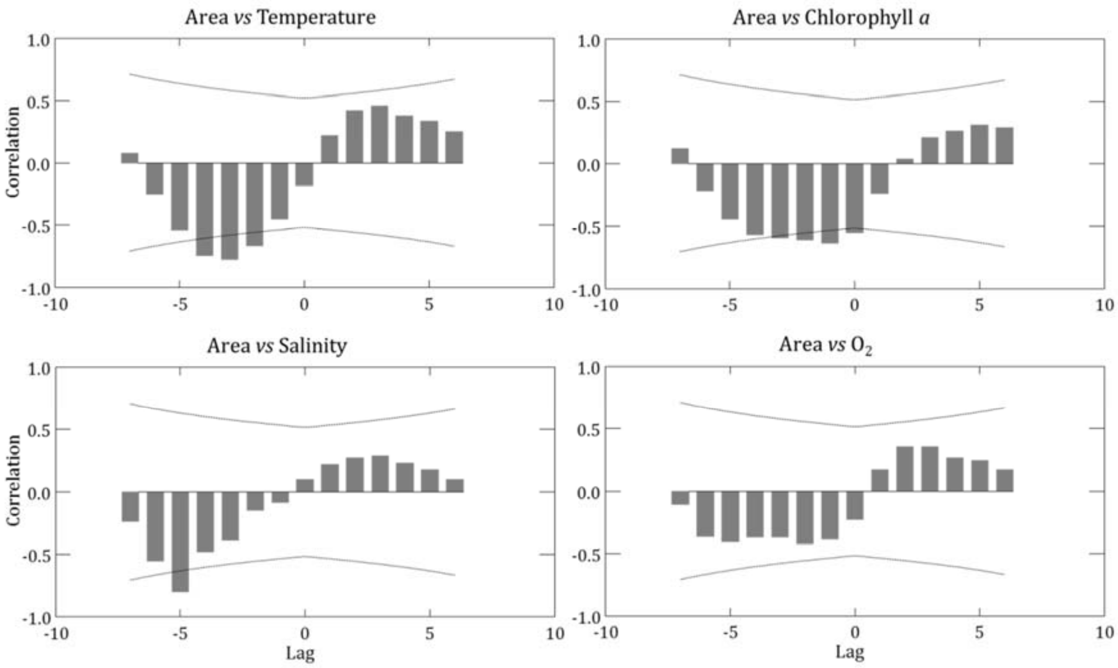

Figure 6. Cross-correlation analyses between the mean monthly coverage (cm²/m) of *Didemnum vexillum* and temperature (ºC), chlorophyll *a* (µgL^{-1}), salinity (psu), and levels of O_2 (%) in the water column. Data series were lagged with respect to one another and the Pearson correlation coefficient computed for each time lag. The curved lines represent the threshold for significant (p = 0.05) correlation values. Time lags are in months.

Reproductive cycle

As in the growth cycle, there was a strong seasonal fluctuation in the reproductive status of *Didemnum vexillum* zooids (Fig. 7). Larvae were present only in late spring and early summer. There were still some larvae in incubation in August, when colonies are regressing and almost all zooids had released their larvae becoming immature. Immature zooids were dominant in the colonies from August 2012 to January 2013. Unfortunately we do not have data for February 2013 due to logistic problems, but we can observe that in March 2013, most of the zooids featured already developed gonads. The Maturity Index (MI) peaked in June and July 2012, and decreased markedly in August 2012. In autumn 2012 and early winter 2013, the ascidian remained immature, but in spring 2013 the MI increased reaching again its maximum levels in early summer 2013. As in the case of cover, there were significant correlations between MI and temperature and chlorophyll *a* levels (Fig. 8). For temperature the MI was positively correlated with current temperatures, but more strongly so with the temperatures of the two forthcoming months (time lags +1 and +2). For chlorophyll *a*, the correlation was significant with future values of this variable (time lag +2). Thus, it seems that the cycle of reproduction is somewhat advanced with respect to temperature and chlorophyll cycles, and may be triggered by a different factor. No significant correlation was found between MI and salinity or levels of O_2 in the water column.

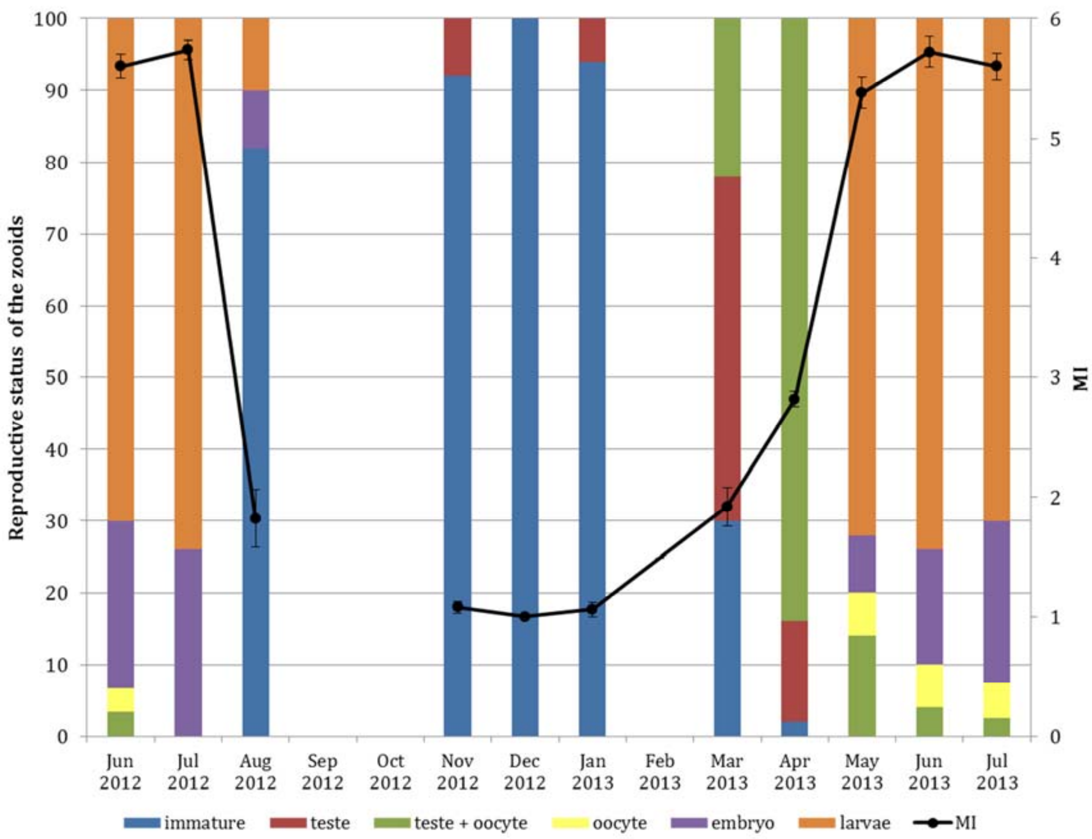

Figure 7. Reproductive status of the zooids of *Didemnum vexillum* from the Fangar bay (Ebro Delta) over the monitored period. Columns indicate the percentage of zooids belonging to each stage. The solid line represents the mean maturity index (MI) of the colonies (bars are standard errors). No colonies were present in October 2012.

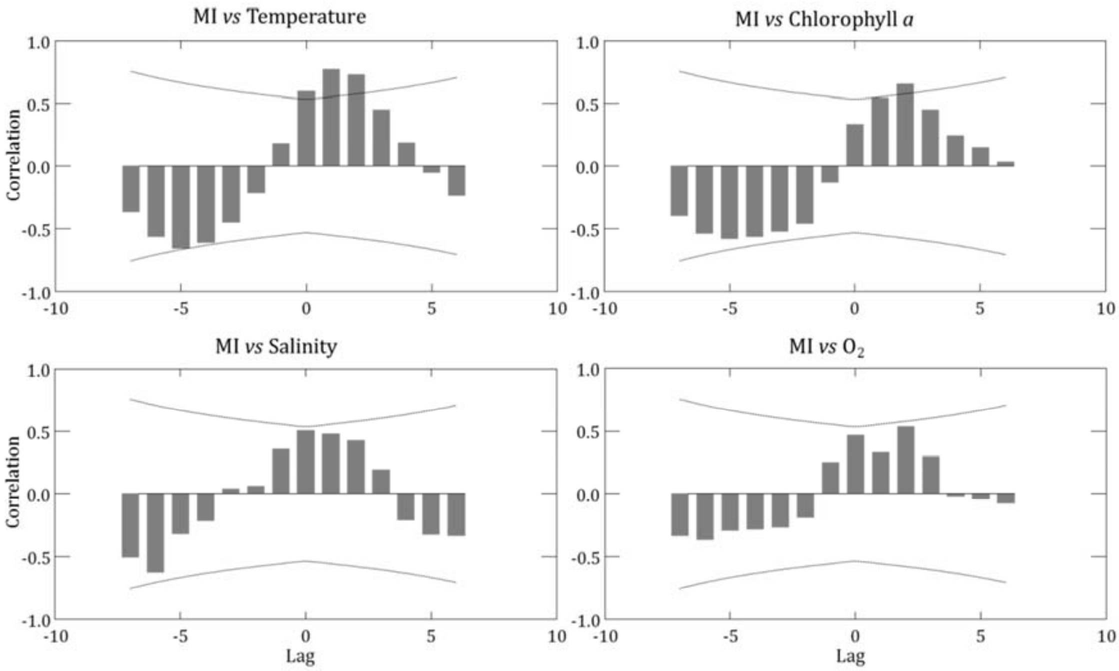

Figure 8. Cross-correlation analyses, correlating the Maturity Index (MI) of *Didemnum vexillum* with temperature (ºC), chlorophyll *a* (μgL^{-1}), salinity (psu), and levels of O_2 (%) in the water column. The curved lines represent the threshold for significant (p = 0.05) correlation values. Time lags are in months.

Genetic analyses

Our *COI* sequences from the Fangar bay, Ebro Delta (Spain) and from the Lagoon of Venice (Italy) matched those of *Didemnum vexillum* from Stefaniak *et al.* (2009). Three haplotypes (haplotype codes as in Stefaniak *et al.*, 2009) were found in the Fangar bay [Haplotype (frequency) = Hp 2 (0.07); Hp 3 (0.79) and Hp 5 (0.14)], and only two in the Lagoon of Venice [Haplotype (frequency) = Hp 3 (0.89) and Hp 5 (0.11)].

In order to assess the putative origin of our samples, we compiled the population genetic information for the *COI* gene from all sites of *Didemnum vexillum* worldwide where at least seven individuals had been sequenced (Stefaniak *et al.* 2009, 2012). We selected 26 populations including our two localities (see Appendix). According to the MDS representation of the genetic divergence matrix, our two populations were clearly grouped with the NE America and New Zealand populations (Fig. 9), although some NW European populations were also situated near Ebro Delta and Venice as well (Fig. 9).

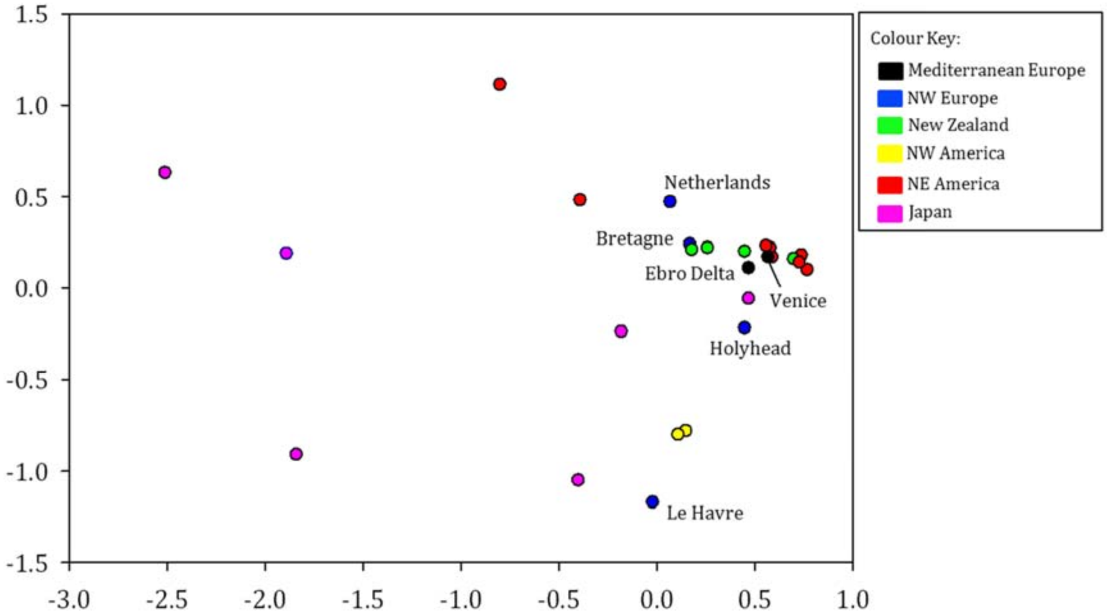

Figure 9. Multidimensional Scaling (MDS) of 26 worldwide populations of *Didemnum vexillum*: our two Mediterranean populations (Ebro Delta and Venice) and 24 populations selected from Stefaniak *et al*. (2009, 2012). Only codes of European populations are indicated to avoid cluttering. Populations are colour coded by region. Stress of the final configuration: 0.06.

DISCUSSION

Both morphological and genetic data confirmed that the colonial ascidian that grows in the Fangar bay is *Didemnum vexillum* Kott, 2002. This species overgrows other epifaunal organisms of the oysters and completely covers the bivalves. According to farmers in the Ebro Delta, *D. vexillum* creates an unwanted foreign covering that diminishes the visual appeal of the oysters, and it is time-consuming to clean oysters before commercialization. Although at its present degree of development the ascidian apparently does not smother the bivalves, it has been reported elsewhere that covering by *D. vexillum* slows down oyster growth (Restrepo *et al.* 2013).

Didemnum vexillum in our study site has a markedly seasonal life cycle, growing actively during the cold season, reaching its highest coverage in late spring and regressing at the end of summer, while reproduction peaked right at the end of the active period. There are studies describing features of the time course of *D. vexillum* over the year (e.g. Gittenberger 2007, Valentine *et al.* 2007a, Tagliapietra *et al.* 2012), and some studies also analysed the recruitment (e.g. Valentine *et al.* 2009, Fletcher *et al.* 2013), but in general there is a lack of detailed information about the life history of the species.

Didemnum vexillum is a temperate species, which in the Mediterranean is in its lower range of latitudes, although it has recently been found in more tropical waters (Restrepo *et al.* 2013). Many colonial ascidians show seasonal life cycles in temperate waters, and temperature appears to be the main driver of them (e.g. Turon 1988, De Caralt *et al.* 2002, López-Legentil *et al.* 2005, Pérez-Portela *et al.* 2007, López-Legentil *et al.* 2013). *D. vexillum* appears to possess a very plastic life cycle that can be adapted to different temperature regimes. Thus, in temperate waters with cold winters the species has reduced growth or colony regression in winter (e.g. Gittenberger 2007, Valentine *et al.* 2007a, Lindeyer & Gittenberger 2011, Fletcher *et al.* 2013). In zones with extremes of both summer and winter temperatures, such as the Venice Lagoon in the Adriatic Sea, the species seems to pass through two periods of arrested growth during these seasons, when water temperatures can be as low as 0ºC or as high as >30ºC (Tagliapietra *et al.* 2012). In our case, the range of temperatures found in the Fangar bay during our study was between 7 and 28 ºC. Summer is here clearly the unfavourable season and thus the species aestivates, likely reduced to unconspicuous resistance forms that regenerate colonies as temperatures decrease in late autumn. Based on population observations in the Netherlands and the USA, it has been suggested that 4°C may be a critical lower temperature that limits the spread or growth of *D. vexillum* colonies (Daniel & Therriault 2007). Laboratory experiments have shown that *D. vexillum* grows better approximately at the range of 16-20ºC (McCarthy *et al.* 2007). In our case, the maximum coverage reached by the ascidian was in May 2013, when the mean temperature of the water was around 18ºC. There are other instances of colonial ascidians that hibernate in the Atlantic and aestivate in the Mediterranean (e.g, Mukai 1977, De Caralt *et al.* 2002). Summer is in general the unfavourable season in the Mediterranean for many invertebrates (Coma *et al.* 2000, Coma & Ribes 2003), and colonial ascidians in particular (e.g. Turon 1988, Turon & Becerro 1992, López-Legentil *et al.* 2013).

Even if the Fangar bay is a shallow embayment with freshwater inputs, the levels of salinity did not fluctuate widely during the study period, remaining over 30 ‰ for most of the year, and the same can be said for the oxygen levels. It is not surprising, therefore, that no meaningful correlation with these parameters could be found in our study. For chlorophyll *a* content, on the other hand, an apparently negative relationship of growth with the chlorophyll *a* values of the previous months was found, which seems paradoxical. However, we attribute this outcome to the seasonal pattern in the abundance of the phytoplankton, highly correlated with the temperature of the water, rather than to an intrinsic negative influence of the levels of chlorophyll *a* in the abundance of the species.

The populations analysed showed a low genetic diversity (three haplotypes in the Ebro Delta and two in Venice Lagoon). However, this is true of most introduced populations of *Didemnum vexillum* worldwide. In fact, only four haplotypes had been previously found in European waters, compared to 22 in the putatively native region in NW Pacific (Stefaniak *et al.* 2012). Of the three haplotypes found, haplotype 3 was present in all introduced areas, while the other haplotypes had been found previously in Europe and NW America (haplotype 2) and in Europe, NE America and New Zealand (haplotype 5). Ascertaining the source of the Mediterranean populations is not straightforward. Although genetic markers are a very useful tool to track the process of colonization of introduced species (e.g. Lejeusne *et al.* 2011, Rius *et al.* 2012), the low variability found for the *COI* gene in all introduced populations hinders any conclusion. The two Mediterranean populations appear in the MDS near New Zealand and NE America *D. vexillum* populations. This is surprising at least for the Ebro Delta, since according to fishermen, the oysters came from the Atlantic coast of France and it seems highly likely that the ascidian was introduced as epifauna on the oyster stock. The lack of resolving power of the marker and the small number of sequenced individuals (Stefaniak *et al.* 2009, 2012) available for some regions, such as France (we could only include two French populations in our analyses), can explain this discrepancy. It is necessary to perform a more complete sampling of the worldwide populations of *D. vexillum* and to use polymorphic genetic markers such as microsatellites (Abbott *et al.* 2011) in order to unravel the invasion history of this colonial ascidian around the world, and in the Mediterranean in particular.

Our results suggest that *Didemnum vexillum* is already widely distributed in the Mediterranean, as it has been found in both the eastern and western basins. In the Ebro Delta, according to local farmers, the species has been present for at least 10 years, although never reached the abundance levels observed in the last three years. The species seems to be increasing in abundance, as we detected a much higher coverage in 2013 than in 2012. Also, some colonies have been seen in 2012 (authors pers. obs.) in the southern bay of the Ebro Delta (Alfacs bay), another site with aquaculture (mussels and oysters) facilities, which suggests that the species is colonizing nearby localities. It could be that populations of the ascidian in the Fangar bay are within an initial phase of the invasion. It is known that some nonindigenous species do not proliferate massively soon after their introduction, but have a lag-phase of slow increase before a blooming growth of the population, becoming widely invasive (Crooks 2005, Suarez & Tsutsui 2008).

The finding of *Didemnum vexillum* in increasing abundances in an area of important aquaculture production and with nearby fishing grounds should raise concern, as the species is capable not only of causing damage to bivalve farms, but also to proliferate in deeper waters covering trawling bottoms (Bullard *et al.*. 2007a, Valentine *et al.* 2007b). While the summer regression and the scarcity of hard substrate in the Ebro Delta may have contributed to limit so far its expansion, the species has biotic features that can boost its proliferation, such as its capacity to reproduce not only sexually but also asexually by fragmentation (McCarthy *et al.* 2007, Bullard *et al.* 2007b, Valentine *et al.* 2007a, Morris & Carman 2012). Fragmentation can also act as dispersion vector, as fragments can be dispersed by currents, waves and tides, or travel via ballast waters (Bullard *et al.* 2007a,b) and so be able to reattach on a new hard substrate. On the other hand, *D. vexillum* has low attachment strength (Murray *et al.* 2012) that seems to prevent its attachment on fast-moving vessels, thus hindering its dispersion via this kind of vectors (Herbog *et al.* 2009). However, in the Ebro Delta bays, slow-moving vessels are the most commonly used for sailing throughout the aquaculture facilities, thus representing a potential factor of future man-mediated dispersion of the ascidian all along the Ebro Delta and adjacent coasts. Eradication measures could be deployed to clean infected stock in order to avoid further introductions via aquaculture transfers, but the methods assayed are time-consuming and not free of environmental concerns (Denny 2008).

We conclude that we are probably in the verge of a potentially important ecological and economic issue. The biological knowledge gained here may assist to counteract the impact and spread of the species. For instance, seeding new oyster stock during the months when larvae are present in the water column (May-July) can be avoided. In addition, harvesting can be concentrated during the period of regression of the colonies to avoid cumbersome cleaning of shells. In spite of this, close monitoring of the Ebro Delta aquaculture facilities and nearby coasts is necessary in the forthcoming years, and stringent control measures should be implemented in the short term if the species proliferation continues.

Acknowledgements

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Cuarto Capítulo:

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RESUMEN

El estudio de especies invasoras es a menudo confundido por cuestiones taxonómicas y por lo tanto estas especies pueden no ser reconocidas como introducidas en nuevas áreas colonizadas. *Clavelina oblonga* Herdman, 1880 es una especie abundante en la costa sureste de los Estados Unidos y del mar Caribe. Fue introducida en el este del Atlántico y Brasil hace algunas décadas. En el mar Mediterráneo se describió una especie similar como *C. phlegraea* Salfi, 1929, que ha sido referenciada en el sur de Italia y Córcega en ambientes confinados. En los últimos años una especie de *Clavelina* proliferó en las instalaciones de acuacultura del Delta del Ebro (noroeste Mediterráneo), una zona con una industria activa de bivalvos, donde la ascidia ahogó la cosecha de mejillón causando gran preocupación. En este capítulo aportamos la identidad morfológica y genética de esta especie con la atlántica *C. oblonga* y con la mediterránea *C. phlegraea*, la cual es por lo tanto un sinónimo de la primera. Por tanto, *C. oblonga* fue introducida en el Mediterráneo hace más de 80 años. Además, estudiamos sus ciclos de vida y de reproducción así como sus patrones de asentamiento. El ciclo de la especie fue marcadamente estacional, con la mayor abundancia y los estados reproductivos más avanzados coincidiendo con los meses más cálidos. Evitando plantar los mejillones y ostrones durante finales de verano y principios de otoño se puede reducir fácilmente el daño causado por esta especie.

When invasion biology meets taxonomy: presence of *Clavelina oblonga* Herdman, 1880 in the Mediterranean Sea

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ABSTRACT

The study of invasive species is often confounded by taxonomic issues and thus these species can fail to be recognized as introduced in the newly colonized areas. *Clavelina oblonga* Herdman, 1880 is an abundant species in the South East coast of the United States and the Caribbean Sea. It was introduced into E Atlantic and Brazil decades ago. In the Mediterranean Sea a similar species had been described as *C. phlegraea* Salfi, 1929 and reported from S Italy and Corsica from confined environments. In the last few years a *Clavelina* species has proliferated in the embayments of the Ebro Delta (NW Mediterranean), a zone of active bivalve culture industry, where their outbursts have smothered mussel spat causing great concern. We report here the morphological and genetic identity of this species with the Atlantic *C. oblonga* and the Mediterranean *C. phlegraea*, which is therefore a synonym of the former. Thus, *C. oblonga* was introduced in the Mediterranean for over 80 years now. Further, we studied its growth and reproductive cycles as well as its settlement patterns. The cycles of the species were markedly seasonal, with the highest abundance and advanced reproductive stages of the species matching the warmest months. Avoidance of mussel and oyster seeding during late summer and early autumn can easily reduce the damage caused by this species.

Key words: *Clavelina phlegraea*, ascidian, life cycle, maturity index, recruitment, bivalve culture, aquaculture, *COI*, introduced species

INTRODUCTION

The introduction of alien species by human-mediated vectors is a well-recognized problem worldwide, involving both terrestrial and aquatic ecosystems (Lowe *et al.*. 2000, Wonham & Carlton 2005, Strayer 2010). It is well known that exotic organisms can modify native communities, including their species composition, even causing extinctions of native biota (Grosholz 2002, Orensanz *et al.* 2002, Clavero & García-Berthou 2005, Didham *et al.* 2005). Management and eradication of these non-indigenous species can be economically expensive and is often ineffective (Pimentel *et al.* 2000, Williams *et al.* 2011), and prevention may be the best solution in order to avoid unwanted introductions and their consequences (Myers *et al.* 2000, Strayer 2010, Williams *et al.* 2010). Nonetheless, there are evidences that introductions of non-indigenous species are increasing (Simberloff 2004, Gittenberger 2009).

In marine ecosystems, biological invasions are closely associated with the intense shipping around the world, introducing alien species of many taxa mostly via ballast waters or hull fouling (Carlton & Geller 1993, Lambert 2002, Gollasch 2006). Moreover, aquaculture activities have also become a leading vector of introductions for aquatic exotic species (Naylor *et al.* 2001, Minchin 2007). Associated to this industry, new introductions can be intentional (target species for economic purposes) or unintentional (epibiota related with commercial stock) (Gollasch 2006). Among marine introductions, ascidians are a prominent instance, featuring important species translocated around the world (Lambert 2007, Locke & Hanson 2011). Harbours, marinas, aquaculture facilities and other man-made structures are often the habitats where ascidians are introduced and proliferate (Lambert & Lambert 2003, Lambert 2007, Tyrrell & Byers 2007, Locke *et al.* 2009). Although many introduced ascidians are confined to these artificial habitats that act as gateways, and thus are of little ecological concern, they can be of high economic impact if they reduce aquaculture yield.

Correct identification of species is the crucial initial step for any biologically meaningful study, including a correct management (Geller *et al.* 1997). However, the study of invasive species is often confounded by taxonomic issues and thus these species can fail to be recognized as introduced in the newly colonized areas, resulting in the so-called pseudoindigenous species (Carlton 2009). Upon closer scrutiny, "endemic" species, particularly in highly urbanized areas, may well correspond to species that have been named elsewhere. The long list of synonymies of some cosmopolitan species, such as the ascidians *Botryllus schlosseri*, *Styela plicata*, or *Ciona intestinalis* (Kott 1985) bears testimony to how often taxonomy has been unable to cope with a global-scale perspective.

This indeed continues to happen, in part due to declining taxonomic expertise and to the taxonomic problems posed by groups with few diagnostic characters. In these cases, apart from conventional morphological tools for species' recognition, genetic techniques have proven unvaluable for a correct identification of alien newcomers, as for example in cryptic invasions (Geller *et al.* 1997, McGlashan *et al.* 2008, Turon *et al.* 2003). On the other hand, shellfish aquaculture is a growing industry around the world, and also in the Mediterranean Sea (Basurco & Lovatelli 2003, Galil 2009, Theodorou *et al.* 2011). Shellfish aquaculture industry is commonly affected by introduced ascidians worldwide (Bourque *et al.* 2007, Gittenberger 2009, Arens *et al.* 2011a), causing economic losses (Coutts & Forrest 2007, Daigle & Herbinger 2009). Nonindigenous ascidians overgrow bivalves adding weight and restricting water exchange and nutrients, decreasing bivalve productivity (Gittenberger 2009, LeBlanc *et al.* 2002, Daigle & Herbinger 2009).

In late summer 2011, a colonial ascidian completely covered mussel spat in bivalve cultures in the Ebro Delta, Spain (Northwestern Mediterranean Sea). The ascidian smothered the bivalves, causing the loss of almost all the juvenile mussels, while oyster cultures were also affected, although to a lesser extent. Two species matched the morphological characters of this colonial ascidian (hereafter *Clavelina* sp.). One was *Clavelina oblonga* Herdman, 1880, an ascidian that seems native of the Northeastern American tropical waters and the Caribbean Sea, and it has been introduced in Brazil, Azores Islands, Cape Vert and Senegal (Rocha *et al.* 2012 and references therein). *C. oblonga* has not been recorded in the Mediterranean Sea. Another similar species is *Clavelina phlegraea* Salfi, 1929, described in Lago Fusaro (SW Italy). This colonial ascidian is considered to be native to the Mediterranean Sea and has been reported from lagoons in Corsica (Monniot *et al.* 1986), and Italy: Naples (Salfi 1929), Rome (Brunetti 1987), and Taranto (Mastrototaro *et al.* 2008).

In the present work we aimed at determining the taxonomic status of the potential pest species *Clavelina* sp. found in NW Mediterranean as well as that of the Mediterranean records of *C. phlegraea*, by combining morphological and molecular methods. Likewise, as knowledge of the biology of introduced species is crucial for their management (Myers *et al.* 2010), we also aimed at studying the life cycle of *Clavelina* sp. by documenting abundance, reproduction, and recruitment in the affected bivalve cultures. Our final goal was to produce meaningful advice for minimizing losses due to ascidian overgrowth of bivalves.

MATERIAL & METHODS

Study site and sampling

This study was carried out in Alfacs bay, at the southern side of the Ebro Delta (Spain, West Mediterranean Sea) (Fig. 1). This bay is 55 km^2 in surface area and up to eight m in depth, with a muddy bottom. The bay houses aquaculture facilities, with ca. 90 bivalve beds. Each bivalve bed consists of a rectangular structure of wooden beams arranged in a grid, sustained by cement columns. From the wooden beams hang ropes with the bivalves. They grow the mussel *Mytilus galloprovincialis* and the oyster *Crassostrea gigas*, but the former is the most common culture in the bay. In a single bivalve bed there can be up to 5000 ropes.

In summer 2011 the ropes of mussels and oysters appeared heavily fouled with a clavelinid ascidian (*Clavelina* sp.) whose colonies formed balls up to 15 cm in diameter (Fig. 1). Samples were obtained for morphological and genetic identification during 2011-2012. The northern Bay of the Ebro Delta, El Fangar, was also examined and the species was found there, albeit in less abundance than in Els Alfacs Bay.

For morphological comparison we examined colonies of *Clavelina oblonga* previously collected by XT from Bocas del Toro, Panama, and material from Faial Island (Azores) from a previous study (Turon *et al.* 2003). For morphological and genetic analyses we also used specimens of *Clavelina phlegraea* from the Mediterranean. Formalin-preserved samples from the Urbino Lagoon (Corsica) logged in the Museum National d'Histoire Naturelle (Paris) were examined. Further samples could be obtained from the Mar Piccolo of Taranto (Fig. 1). Careful search of the type locality in Fusaro Lagoon (Naples, Italy) in July 2013 by one of us (XT) could not detect any colony. Previous attempts by collaborators did not find any specimen, either.

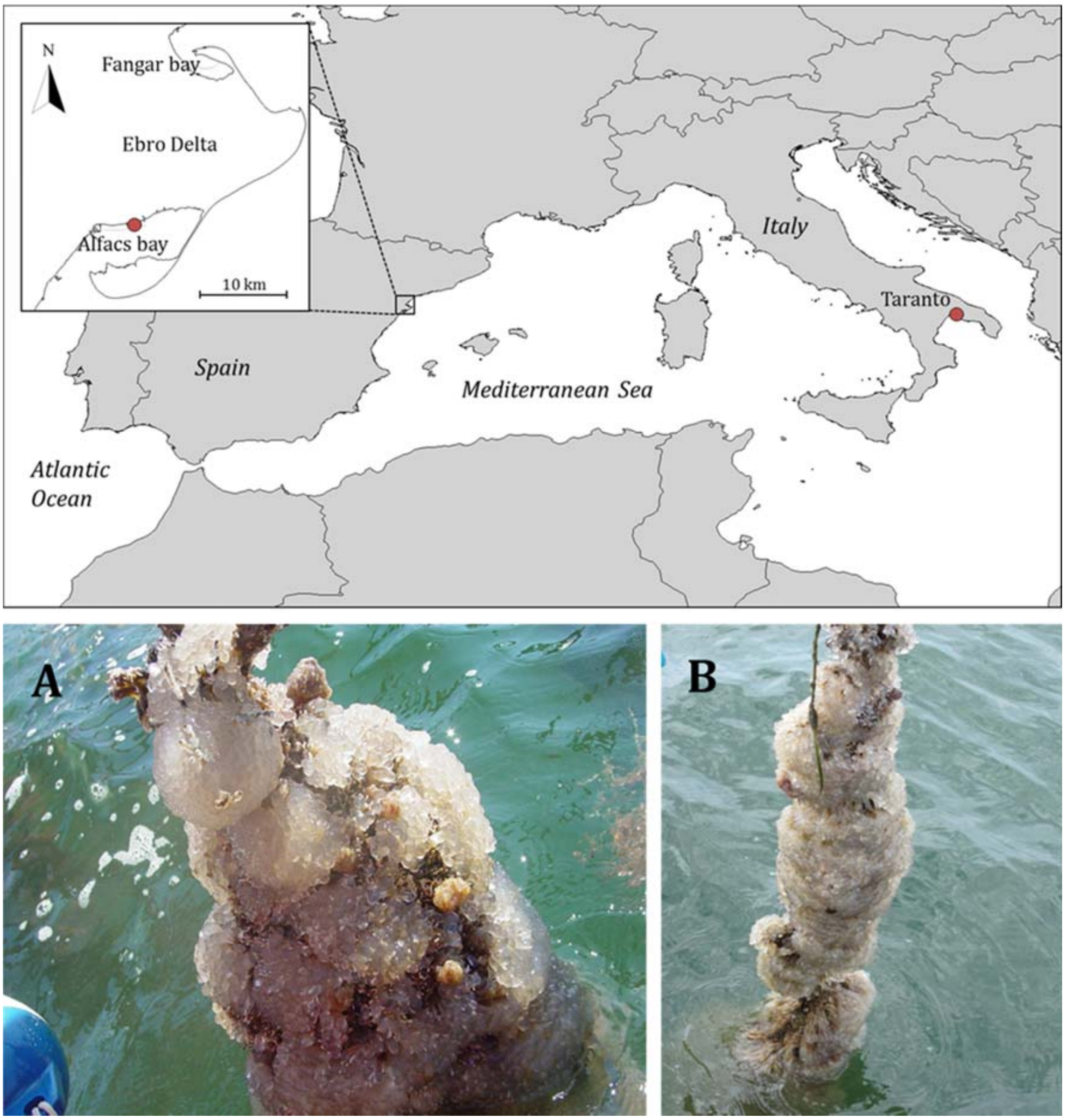

Figure 1. Sampling and monitoring site of *Clavelina* sp. in the Alfacs bay (Ebro Delta, Spain, western Mediterranean Sea), and sampling site of *C. phlegraea* in Taranto (Italy, eastern Mediterranean Sea). *Clavelina* sp. overgrowing mussel crops (A) (September 2011) and oyster crops (B) (September 2012) in the Alfacs bay (Ebro Delta, Spain).

Genetic analysis

Colonies of *Clavelina* sp from mussel crops in Alfacs bay (Ebro Delta, Spain) (n=27) and colonies of *C. phlegraea* from Taranto (Italy) (n=9) were fixed in ethanol and stored in the laboratory at -20ºC. For DNA isolation, one zooid was dissected from each colony in order to obtain its branchial sac, and its DNA was extracted using QIAamp® DNA Mini Kit (QIAGEN) and resuspended in 200 µl distilled water.

We used the universal primers HCO2198 and LCO1490 (Folmer *et al.* 1994) for the amplification of a fragment of the mitochondrial gene *Cytocrome C Oxidase I* (COI). PCR amplifications were carried out in a total volume of 20 µl with 14.7 µl H_2O, 2 µl 5x buffer (GoTaq, Promega), 1 µl $MgCl_2$ (25mM), 0.5 µl dNTP's (1 mM), 0.4 µl (10 µM) of each primer, 1U *Taq* Polymerase (GoTaq, Promega) and 1 µl of DNA. The PCRs started with an initial denaturation at 94 ºC for 5 min, followed by 35 cycles of a denaturation step at 94 ºC for 1 min, an annealing step at 50ºC for 1 min and an elongation step at 72 ºC for 1 min 30 s, with a final extension at 72 ºC for 7 min. The amplified DNA was purified with Exo-SAP and both strands were sequenced in Macrogen Inc. with the EZ-seq V2.0 service. Forward and reverse sequences were edited, aligned and confirmed visually with BioEdit Sequence Alignment Editor (Hall 1999) using the ClustalW Multiple alignment option. Final sequence length after trimming was 658 bp. Likewise, sequences of all our individuals were aligned with haplotypes of *C. oblonga* from populations worldwide available in GenBank from Rocha *et al.* (2012).

Monitoring of abundance and growth cycle

Two approaches were used in order to have an estimate of the abundance of *Clavelina* sp. over the year. First we monitored five mussel (*Mytilus galloprovincialis*) ropes granted by the owner in mussel beds in the middle of the bay. The ropes were placed in November 2011 and were ca. 2-3 m long. We monitored them monthly from December 2011 to March 2013 when they were removed by the owners, with the exception of November 2012 and February 2013 due to logistic problems. We took pictures of the ropes with a digital camera (Canon PowerShot G11, 10MP). Ropes were photographed together with a ruler used to scale every picture. The perimeter of each colony of the ascidian was manually outlined in every picture with Photoshop CS4 in order to measure the cover area. Pictures were treated by the Laboratory of Image Analysis and Treatment of the CCiTUB (Scientific and Technological Centre of the University of Barcelona), using the proprietary program IMAT. The total area of colonies on every rope (in cm^2) was divided by the total length of the rope (in m), thus obtaining a relative estimate of abundance (cm^2/m) every month. Second, we deployed plates for the study of the abundance cycle. In a mussel bed located in the centre of the bay we placed three ropes, each with three PVC plates (19.5 x 19.5 x 0.3 cm) at three depths: 20 cm, 1m, and 2m, in order to assess the pattern of abundance and distribution in depth of *Clavelina* sp. These ropes were placed in December 2011 and monitored monthly until July 2013 (again with the exception of November 2012 and February 2013). Pictures of every PVC plate (both sides) were taken and processed as above, except for the fact that the total cover (cm^2) of ascidian colonies in each plate was divided by the area of the plate (760.5 cm^2).

Reproductive cycle and recruitment

In order to study the reproductive cycle of *Clavelina* sp. in the Alfacs bay, we collected monthly five colonies from June 2012 to August 2013, with the exception of February 2013 when no collection could be done due to logistic problems. Samples were stored *in situ* in formaldehyde 10%. Colonies were dissected under a binocular microscope. Ten zooids were randomly selected per colony in order to determine their reproductive status. As most colonial ascidians, this clavelinid is hermaphrodite, protandric, and brooder. This allowed us to categorize each zooid as follows: (1) immature; (2) presence of testes; (3) presence of ovary; and (4) presence of brooded larvae. Note that stages 2, 3, and 4 are not mutually exclusive, and we assigned each zooid to the highest stage observed. We calculated a maturity index per month (MI) as in López-Legentil *et al.* (2005), averaging the category number of the ten zooids per colony and calculating the mean of the five colonies studied.

In order to assess the recruitment pattern of *Clavelina* sp., we placed in the same mussel where the ropes with permanent plates were located three further ropes with PVC plates (19.5 x 19.5 x 0.3 cm) at three depths (20 cm, 1m, and 2m), as in the previous experiment. We replaced the plates monthly and counted the number of colonies established on them as an estimate of recruitment of the species at every month and depth.

Statistical analyses

Cross-correlation analyses were used to study relationships of the abundance cycle and the maturity index with the environmental parameters: temperature (ºC), chlorophyll *a* (μgL^{-1}), salinity (psu), and levels of O_2 (%) in the water column. In cross-correlation analysis, two time series are compared using the Pearson correlation coefficient with an increasing lag of one series with respect to the other. Correlation at time lag 0 is the usual Pearson correlation. Correlations at negative lags relate values in the first series with previous values in the second series. Correlations at positive lags analyse relationships of the values in the first series with future values in the second series. Physical data and chlorophyll *a* values were obtained from the weekly monitoring of the Bay performed by the Institute of Agriculture and Food Research and Technology (IRTA) of the nearby locality of S. Carles de la Ràpita.

In order to correct for missing data for growth cycle (November 2012 and February 2013), the values of these months were calculated as the average of the previous and the posterior months. In the case on the MI, the months in which the colonies were absent (regressed) were assigned stage 1 (immature) in order to be able to perform the cross-correlation analysis.

One way ANOVA was used to assess differences in coverage and in recruitment intensity with depth in the months with the highest values for these parameters (September 2012 for coverage, and October 2012 for recruitment, see Results), after checking that the assumptions of normality and homoscedasticity were met. The analyses were carried out using SigmaStat v 3.1 and Systat v 12.02.

RESULTS

Morphological observation

The colonies of *Clavelina* sp. collected in the Ebro Delta formed circular masses of up to 15 cm in diameter and 10 cm in height. The masses are made of thick, anastomosed digitations of tunic coalescing towards the base. Each digitation bears apically from one to eight zooids. The tunic is soft apically and more consistent basally, mostly transparent with whitish flecks scattered. On the thick proximal tunic there are numerous fine stolons ending in budding chambers marked with white pigment.

The zooids measure up to 25 mm and have some white pigment in the branchial sac and stomach (Fig 2). They have ca. 20 simple tentacles of various orders. The neural gland aperture is a vertical oval. There are ca. 20 rows of stigmata (with 50-60 stigmata per half row in well-developed zooids). The digestive system comprises a descending oesophagus and a subterminal squared stomach with marked ridges, followed by a mid-intestine and an ascending rectum. The gonads lie to the left of the intestinal loop (considering the stomach dorsal). They comprise numerous ovoid and small male follicles, with a mass of oocytes in the middle of the testes. Up to 100 larvae can be incubated in the posterior part of the peribranchial cavity, on the right side. The final part of the oviduct forms a dilated pouch filled with embryos that protrudes postero-basally from the thorax. The fully formed larvae measured up to 0.8 mm. As is typical of the genus *Clavelina* they have well developed ocellus and otolith in the sensory vesicle, and bear an anterior process with three simple adhesive papillae arranged in a triangle connected by a ventral peduncle to the trunk of the larva.

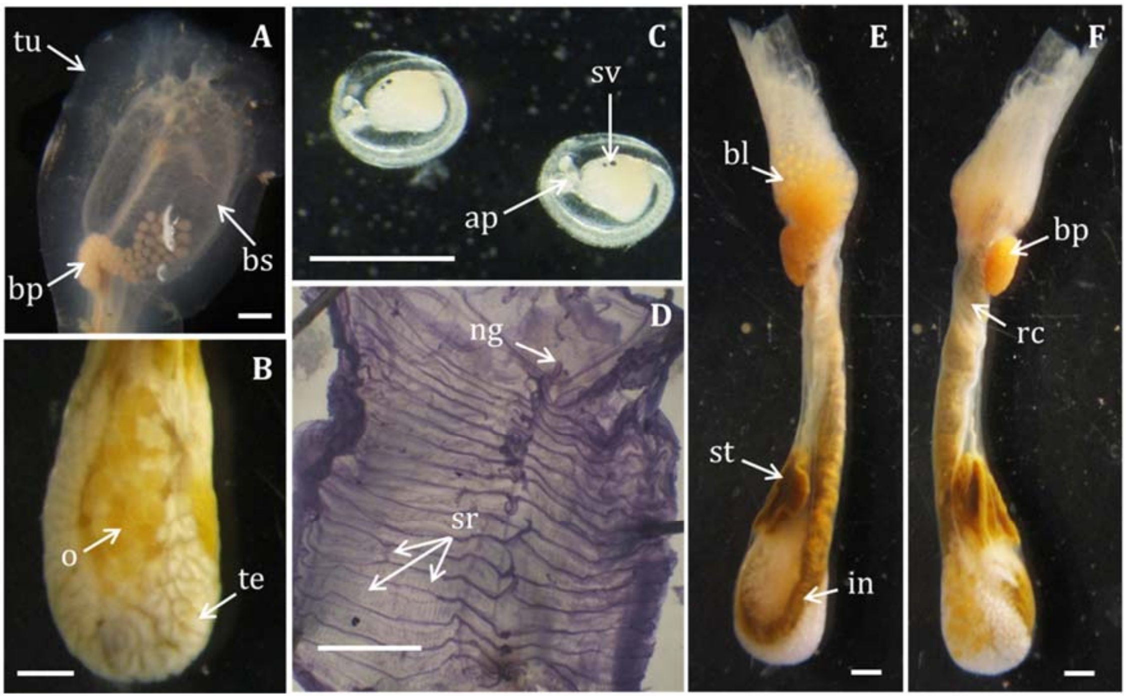

Figure 2. Morphological features of the *Clavelina* species found in the Ebro Delta. A) distal part of a zooid; B) gonads in basal part; C) larvae; D) dissected branchial sac; E) and F) full view of a *Clavelina* zooid. Abbreviations: ap (adhesive papillae); bl (brooded larvae); bp (brooding pounch); bs (branchial sac); in (intestine); ng (aperture of neural gland); o (oocytes); rc (rectum); sr (stigmata rows); st (stomach); sv (sensory vesicle); te (testes); tu (tunic). Scale bars: A, B, D, E, F, 1mm; C, 0.5 mm.

The morphological characters of the other colonies examined (*Clavelina oblonga* from Panama and Azores, *Clavelina phlegraea* from Taranto and Corsica) were similar to each other and to those observed in *Clavelina* sp. from the Ebro Delta.

Genetic analysis

All of our samples from the Ebro Delta and from Taranto presented the same COI sequence, which corresponds to Haplotype 3 of *Clavelina oblonga* as defined in Rocha *et al.* (2012).

Growth cycle

Growth cycle showed a marked seasonal fluctuation in the coverage of *Clavelina* sp. in Alfacs bay (Fig. 3). No colonies were found in the five ropes of mussels deployed in November 2011 before July 2012 (24.49 cm^2/m ± 2.56, mean ± SE). The ascidian reached its maximum coverage in September 2012 (706.26 cm^2/m ± 328.07), and then entered in regression to almost disappear in January 2013 (0.41 cm^2/m ± 0.25). In March 2013 they had completely disappeared; unfortunately we do not have data from February 2013, so we cannot tell exactly when *Clavelina* sp. completely disappeared from the ropes, but it seems likely that the ascidian was already missing in that month. Mussel ropes were removed by fishermen and thus the monitoring of these five ropes came to an end in March 2013.

Results of the monitoring of the three ropes with permanent PVC plates at three depths are also shown in Fig. 3 and is fully coherent with the pattern observed in the mussel ropes. The month with highest cover percentage was again September 2012, in which the ascidian almost completely occupied the plates at 1m (93.36 % ± 6.64, mean ± SE) and 2m (98.51 % ± 1.49) while plates at 20 cm presented the lowest coverage (45.03 % ± 1.54), likely due to the abundance of the solitary ascidian *Styela plicata* on the shallower plates. This difference in covering among depths was significant (ANOVA $F = 53.69$, $p < 0.001$), due to the higher cover in the plates at 1 and 2 m with respect to those at 20 cm (post-hoc Student-Newman-Keuls tests, $p < 0.001$).

In October 2012 the ascidian showed a drastic regression diminishing its coverage under 10% at the three depths. In January 2013, the area occupied by *Clavelina* sp. hardly reached 1% at any depth. The colonies completely regressed afterwards, and no colony was found in March 2013. It was not until April 2013 that the ascidian reappeared, although with less than 1% cover. These colonies most probably grew from stolons that resisted winter conditions, since at that time we found very few colonies around the facilities and they were all immature, and no recruitment was detected (see below). In July 2013, *Clavelina* sp. grew again mostly at 2m, reaching 10.27 % of covering (Fig. 3). Shallower plates were at that time completely covered by recruits of *Mytilus galloprovincialis*.

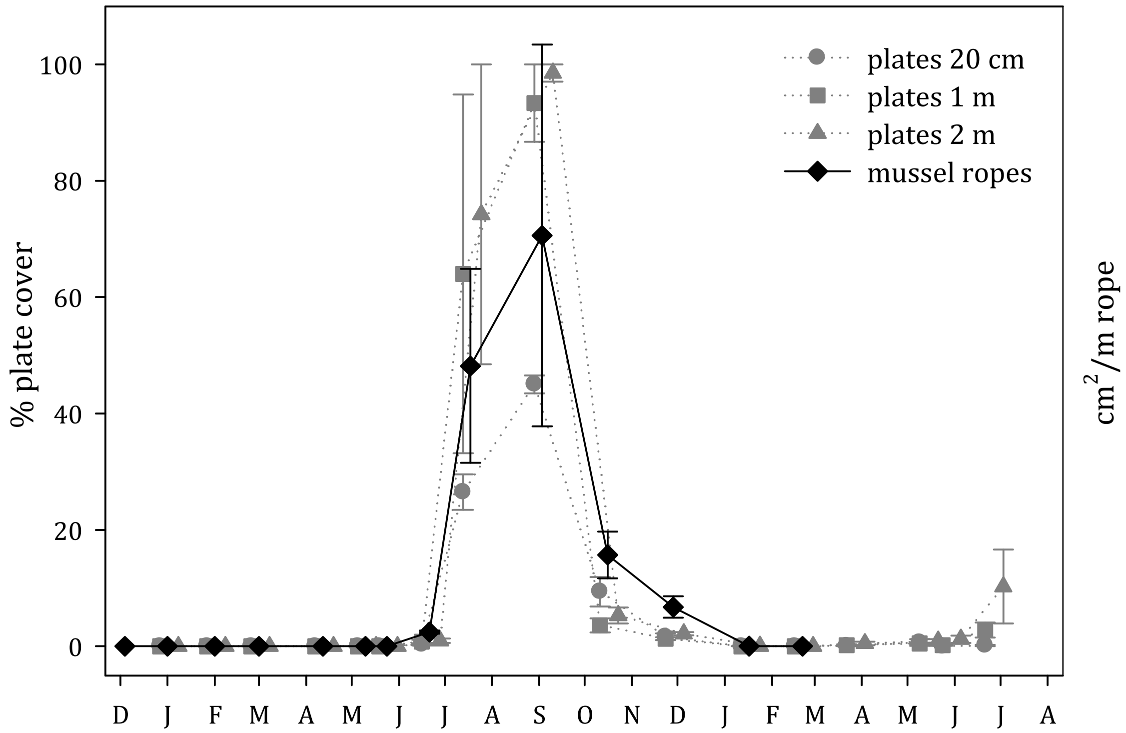

Figure 3. Growth cycle of *Clavelina* sp. in Alfacs bay, Ebro Delta (Spain). Values are mean percentage of coverage of the three ropes with PVC plates at each depth (grey dotted lines), and mean of monthly coverage (in cm^2 of ascidian per m of rope) on the five mussel ropes (black line and diamonds). Bars are standard errors.

The time course of environmental variables (temperature, salinity, levels of O_2 and levels of chlorophyll *a*) over the study period is shown in (Fig 4). The relationship of these variables with ascidian abundance was evaluated by cross-correlation analysis (Fig. 5). We chose to use the mean coverage of the ropes with the PVC plates since it correlated tightly with the mean coverage of the mussel ropes (Spearman rank correlation $R = 0.972$, $p < 0.001$), and it allowed us to perform the analyses over a longer period of time (from January 2012 to July 2013). Cross-correlation analysis showed a positive significant correlation of coverage with the temperature of the current and the two previous months (Lag of 0, -1, -2, Fig. 5). Chlorophyll *a* showed a significant correlation with the coverage of the current month, which is attributable to the peak of chlorophyll *a* in September 2012 (Fig. 4) coinciding with the blooming of *Clavelina* sp. A negative significant correlation was found between coverage and oxygen levels of the previous month (Lag of -1, Fig. 5). Likewise, coverage showed a significantly negative correlation with salinity of future months (time-lag +4). We could not assign any biological meaning to these correlations with oxygen levels or salinity, and they are interpreted as random outcomes of our data. This view is supported by the corresponding correlation plots not showing the wave-like pattern typical of correlated time-series (f.i., compare with the pattern for temperature).

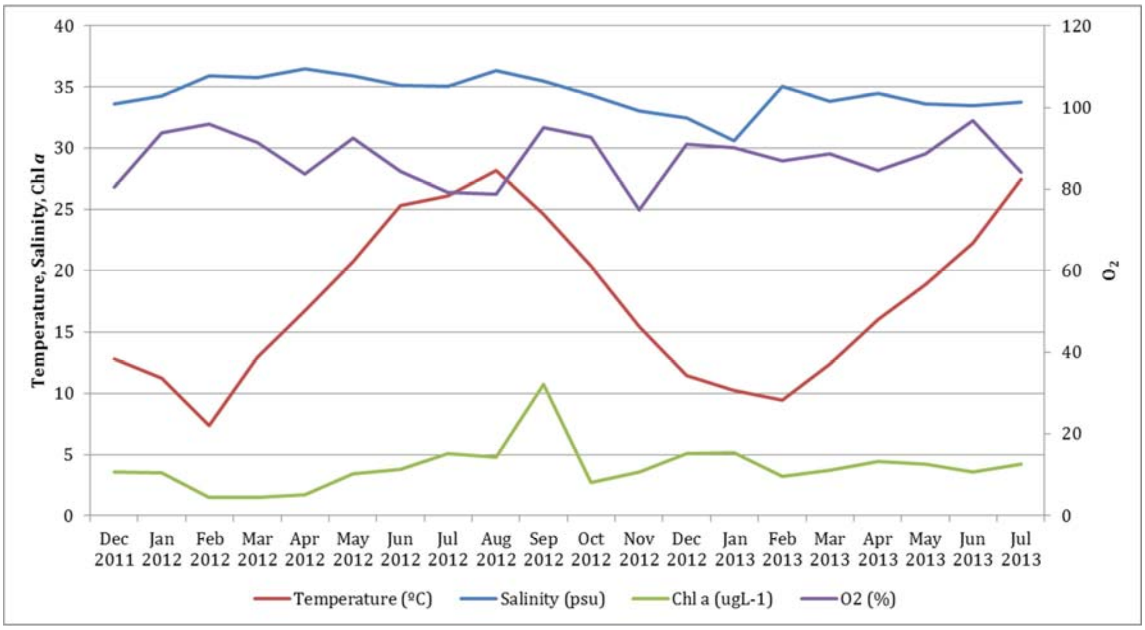

Figure 4. Environmental parameters (temperature, salinity, levels of O_2), and levels of chlorophyll *a* (Chl *a*) in Alfacs bay (Ebro Delta, Spain) during the monitoring period.

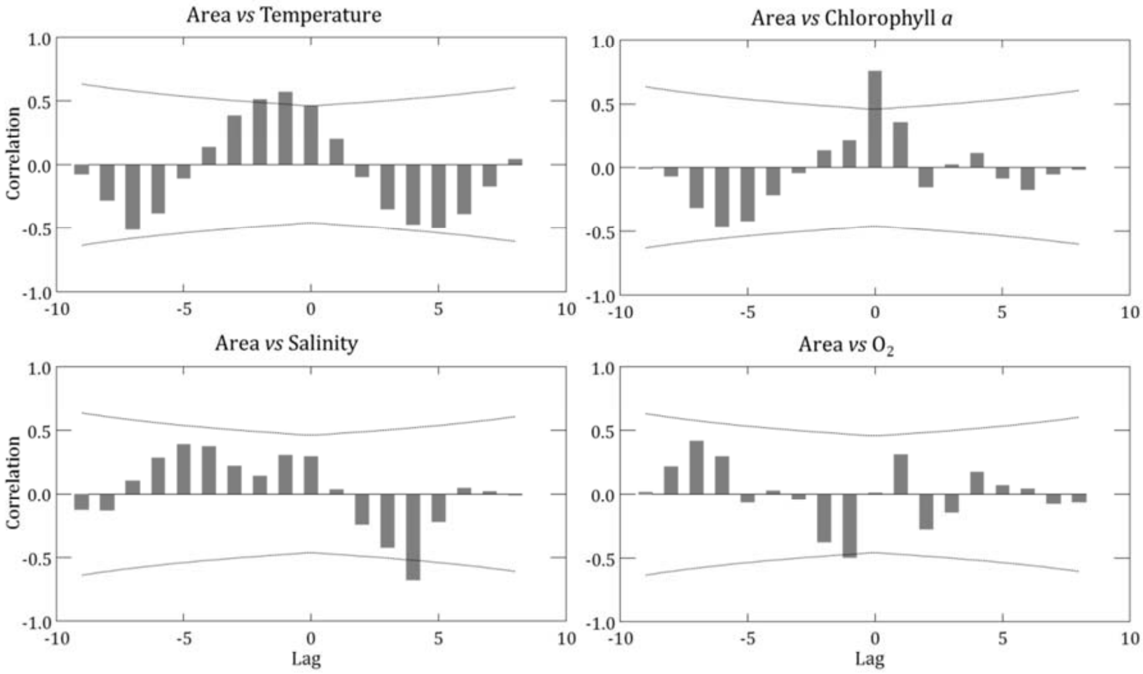

Figure 5. Cross-correlation analyses relating the mean monthly coverage (in percent) of *Clavelina* sp. with temperature (ºC), chlorophyll *a* (μgL^{-1}), salinity (psu), and levels of O_2 (%) in the water column. The curved lines represent the threshold for significant (p = 0.05) correlation values. Time lags are in months.

Reproductive cycle and recruitment

There was a strong seasonal cycle in the reproductive status of *Clavelina oblonga*. Brooding larvae were observed during summertime and early autumn, being August and September 2012 the months with highest percentage of zooids brooding larvae (Fig. 6). From December 2012 to April 2013, colonies remained completely immature or where absent altogether as in March 2013.

Maturity Index (MI) increased during summertime in 2012 reaching its highest value in September 2012 (Fig. 6) and later on diminished until colonies were completely immature by December 2012. In March 2013 there were no colonies in the site, indicating regression. In April 2013 we found only a few small immature colonies. MI increased again in spring 2013, with brooding colonies found again in July 2013.

The pattern of temperature seems to match the one of MI. In fact, cross-correlation analysis showed a significant positive correlation of the MI with the temperature of the current month and up to two months earlier (Fig.7). Moreover, MI had a significant positive correlation with salinity of the current month and the previous one (Fig. 7). Levels of O_2 and chlorophyll *a* had no correlation with MI.

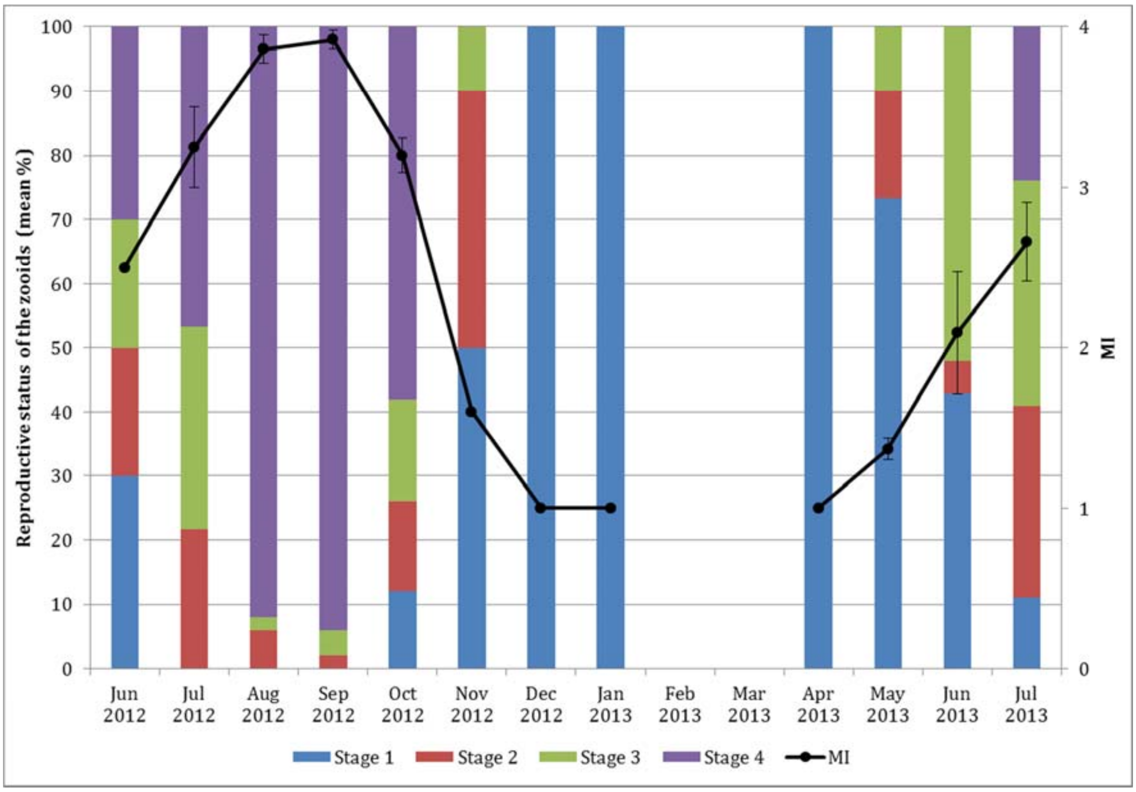

Figure 6. Reproductive status and Maturity Index (MI) of the *Clavelina* sp. zooids from Alfacs bay (Ebro Delta, Spain) over the monitoring period. Columns indicate the percentage of zooids belonging to each stage or category as explained in the text. No data were available for February 2013. In March 2013 there were no colonies in the site. Bars in MI are standard errors.

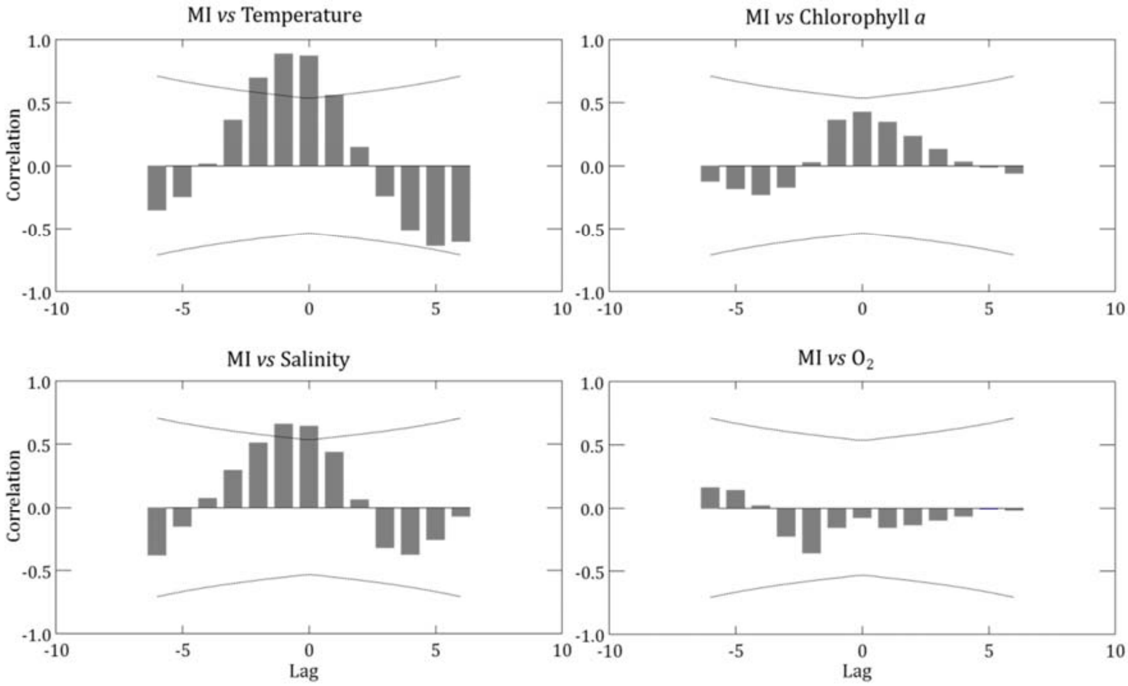

Figure 7. Cross-correlation analyses correlating the mean monthly MI of *Clavelina* sp. with temperature (ºC), chlorophyll *a* (μgL^{-1}), salinity (psu), and levels of O_2 (%) in the water column. The curved lines represent the threshold for significant ($p = 0.05$) correlation values. Time lags are in months.

Concerning recruitment, it was relatively low during summertime 2012, but increased considerably in October, with more than 100 recruits per plate at 20 cm (Fig. 8). Interestingly, recruitment was higher in the shallowest plates, a pattern opposite that of plate cover values (Fig. 3). The differences in recruitment were significant between all depths in the month with highest recruitment (October 2012) (ANOVA, $F = 21.466$, $p = 0.002$, followed by Student-Newman-Keuls tests, data log-transformed to meet normality). In December 2012 only three recruits could be found in all plates. No recruitment was observed afterwards, until July 2013, when only one recruit was found in one plate at 2m depth.

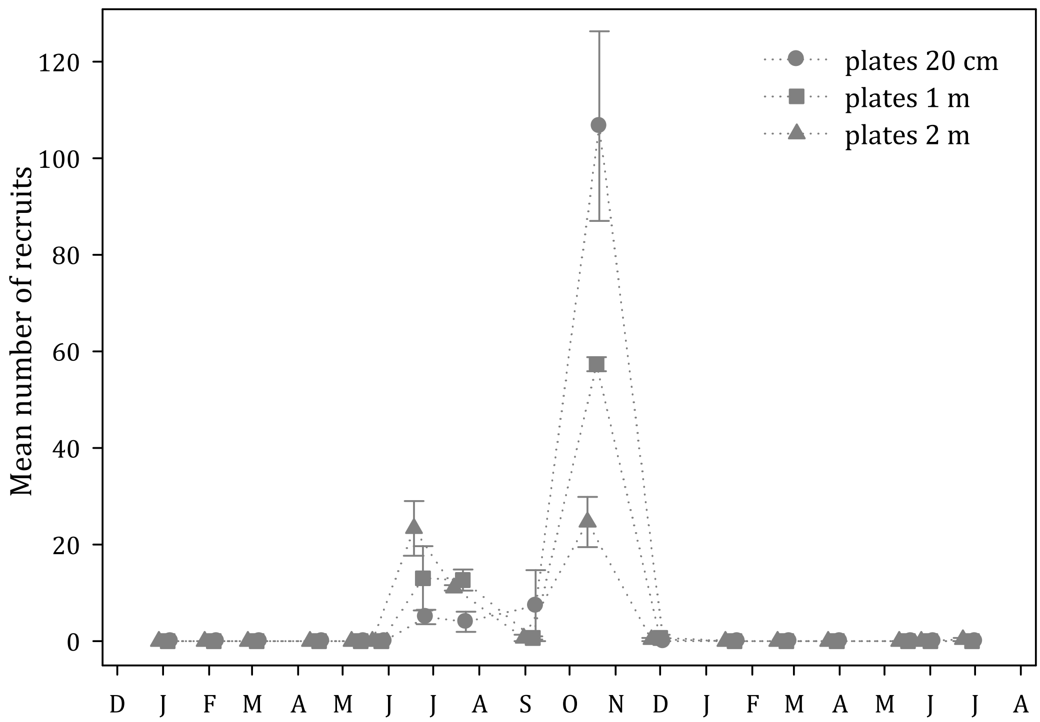

Figure 8. Recruitment of *Clavelina* sp. on the monthly PVC plates at the three depths. Bars are standard errors.

DISCUSSION

Morphological analyses showed that our *Clavelina* sp. matched well previous descriptions of *C. oblonga* (e.g. Van Name 1945) and *C. phlegraea* (e.g. Brunetti 1987), as well as the morphology of examined material from Panama and Azores (*C. oblonga*), and from two of the four locations where *C. phlegraea* has ever been reported (Mar Piccolo of Taranto and Urbino Lagoon). Furthermore, our genetic analyses indicated that the *Clavelina* sp. from the Ebro Delta and *C. phlegrea* from Taranto have the same *COI* haplotype, which is the only one found in populations of *C. oblonga* in its introduced range (Rocha *et al.* 2012). The conclusion is, therefore, that *C. oblonga* and *C. phlegraea* are the same species, the former name having precedence. Thus, *Clavelina* sp. that bloomed in recent years in bivalve cultures in the Ebro Delta constitutes a new report of *C. oblonga* in the Mediterranean Sea.

It is unfortunate that no material from the type locality of *Clavelina phlegraea* (Fusaro Lagoon, SW Italy) could be found, in spite of repeated attempts and a thorough survey in July 2013. This is hardly surprising, though, given the harsh history of the Fusaro Lagoon in the last century, with increased concentration of pollutants in recent decades and, particularly, intense dredging of the bottom that was performed in the 1980s (De Pippo *et al.* 2004). We have to conclude that this species does not exist anymore in the phlegraean fields that originated its name. Even so, the account by Salfi (1929) allows us to safely conclude that this species is the same found in other Mediterranean lagoons and here shown to be conspecific with *C. oblonga*.

Clavelina oblonga, described from Bermuda, is considered native of tropical western Atlantic (from S Carolina to Panama), from where it spread to the South (S Brazil) and to the East (Açores, Cape Verde, Senegal) (Rocha *et al.* 2012). These introductions were relatively old, as it was detected in Brazil in 1925 (Rocha *et al.* 2012); in Cape Vert it was mentioned by Hartmeyer (1912), in the African Coast by Pérès (1951), and in Açores by Monniot (1974). It has not been previously reported in the Mediterranean, but it was there in fact before at least 1929, although it was known under a different name, providing yet another instance of a pseudoindigenous species (Carlton 2009). Its presence exclusively in lagoons with mariculture activities should have raised suspicions earlier on.

The genetic composition of the populations of *Clavelina oblonga* is coherent with this picture of introduction history. In spite of an overall low diversity, four *COI* haplotypes could be found in Caribbean waters, while only one (Haplotype 3) was present in the putatively introduced regions of S Brazil and Açores (Rocha *et al.* 2012). This same haplotype was the only one found in the Mediterranean. Explaining why one haplotype has become widespread while the others seem confined to the native area can have diverse interpretations (Rocha *et al.* 2012) and is beyond the scope of the present paper; further studies with nuclear markers can contribute to elucidate this point.

According to local farmers, *C. oblonga* was already present in the Ebro Delta some 3-4 years before its blooming in 2011, while in subsequent years we have observed high abundances in summertime. It was likely introduced from Italy (one common source of mussel spat in the Ebro Delta cultures). Again according to local farmers, the species poses a threat to mussel cultures (*Mytilus galloprovincialis*), which can be totally smothered, and slows growth of oysters (*Crassostrea gigas*). A bloom of *C. oblonga* in the northern side of the Ebro Delta, the Fangar bay (also with important aquaculture facilities) was observed in 2012, one year after the proliferation in Alfacs bay. Thus there is much concern about its proliferation and a demand for prevention measures, where biological knowledge should play a crucial role.

Clavelina oblonga in the Ebro Delta showed a markedly seasonal life cycle, where peak abundance and reproduction coincided with the warmest months. There was a significant positive correlation of temperature with coverage and MI. Brooding of larvae occurred mostly at the end of summer, followed by their mass release in early autumn, with a peak of recruitment in the plates in October. Water temperature has been shown to be crucial in the growth cycle of colonial ascidians (e.g. Turon 1988, De Caralt *et al.* 2002, López-Legentil *et al.* 2005, Pérez-Portela *et al.* 2007, López-Legentil *et al.* 2013). In our case, temperature seems to determine the growth period of *C. oblonga*, being the optimal for the species around 25ºC. Nevertheless, the peak of chlorophyll *a* in the Bay is also coincident with the warmest months, so increased food ability can also contribute to the peak observed in growth and reproduction. Further evidence for a role of temperature comes from the abnormally cold spring 2013; there was a delay in the rise of temperature in early summer 2013 (up to four degree less in June 2013 compared to June 2012), which was reflected by a delay in the maturity of the zooids (Fig. 6). In addition, this cold spring 2013 was also reflected in the recruitment pattern of the ascidian. In monthly plates we found up to 20 colonies per plate in July 2012, but only one colony was found in July 2013.

In the Mediterranean, *Clavelina oblonga* sits at the northern extreme of its distributional range. In Alfacs Bay water temperature ranges from less than 10ºC in winter to about 28ºC in summer. The winter conditions are likely too harsh for this tropical species, as it is known that cold waters affect negatively this ascidian (Rocha *et al.* 2009). This is reflected by the disappearance of the colonies during winter. Regression during unfavourable periods is common in clavelinids (De Caralt *et al.* 2002), and resting buds ensure the quick population recovery when conditions are re-established. In fact, *C. oblonga* grew at amazing rates after overwintering, totally covering the experimental plates in less than two months. Among the few studies providing data for this species, Rocha (1991) found also an increased abundance in the warmest months in Brazilian waters. In the Mar Piccolo de Taranto, Mastrototaro *et al.* (2008) also found the highest abundances of *C. oblonga* (referred to as *C. phlegraea*) in summer but, surprisingly, the species does not seem to overwinter in that area and is abundant all year round, despite winter temperatures fall below 10 ºC. More detailed comparisons of environmental parameters in Alfacs Bay and Mar Piccolo is needed to understand this difference in life-cycles.

There was segregation in the distribution in depth of the ascidian, being more abundant at one and two metres of depth (permanent plates), while recruitment, on the contrary, was higher near the surface (monthly plates). No doubt competition with other species that also grew on the plates near the surface (such as *Styela plicata* and *Mytilus galloprovincialis*) could explain this contrasting pattern. *Clavelina oblonga* can become a dominant competitor for space, but it apparently needs an initial period of growth and favourable conditions (Rocha 1991). Further experimental studies can clarify the role of biotic and abiotic interactions in the distribution of *C. oblonga*.

In this study we have shown how taxonomic problems can confound studies of invasion biology, and unmasked an old Mediterranean introduction that is cause of concern in bivalve cultures. We also provided information about the life cycle of *Clavelina oblonga*, and showed that, as a species with tropical affinities, its populations bloom in summertime. Knowledge is a powerful weapon, and learning about the biology of introduced species is crucial in order to achieve a successful management. Several methods have been suggested for eradication or control of invasive ascidians in mussel aquaculture, including the use of pressurized seawater (Arens *et al.* 2011a, b), acetic acid (Page *et al.* 2011), or bleach (Denny 2008), but success is not guaranteed and application is problematic and labour-intensive. Some fishermen of Alfacs Bay kept mussel crops outside the water, exposing them to the sun, for one day. This method was highly effective in order to eliminate ascidian colonies attached to the mussel crop, but it represented an extra effort, adding economic costs to the fishermen.

Instead of these methods, a better tuning of the mariculture activities with the biological cycle of the ascidian can greatly reduce its negative impact. The bloom of 2011 was caused by an attempt by the fishermen at obtaining an extra cohort of marketable mussels, thus they re-seeded mussels in July, which became totally covered when mass recruitment of *Clavelina oblonga* started in September. Farming in July and waiting until autumn for placing new spat has been the traditional technique used by local farmers and seems the best suited to avoid damage due to the summer proliferation of *C. oblonga*. Overall, our work illustrates the importance and usefulness of a correct identification of introduced species and of a basic knowledge of their biology for correct management decisions mitigating their impact.

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Discusión general

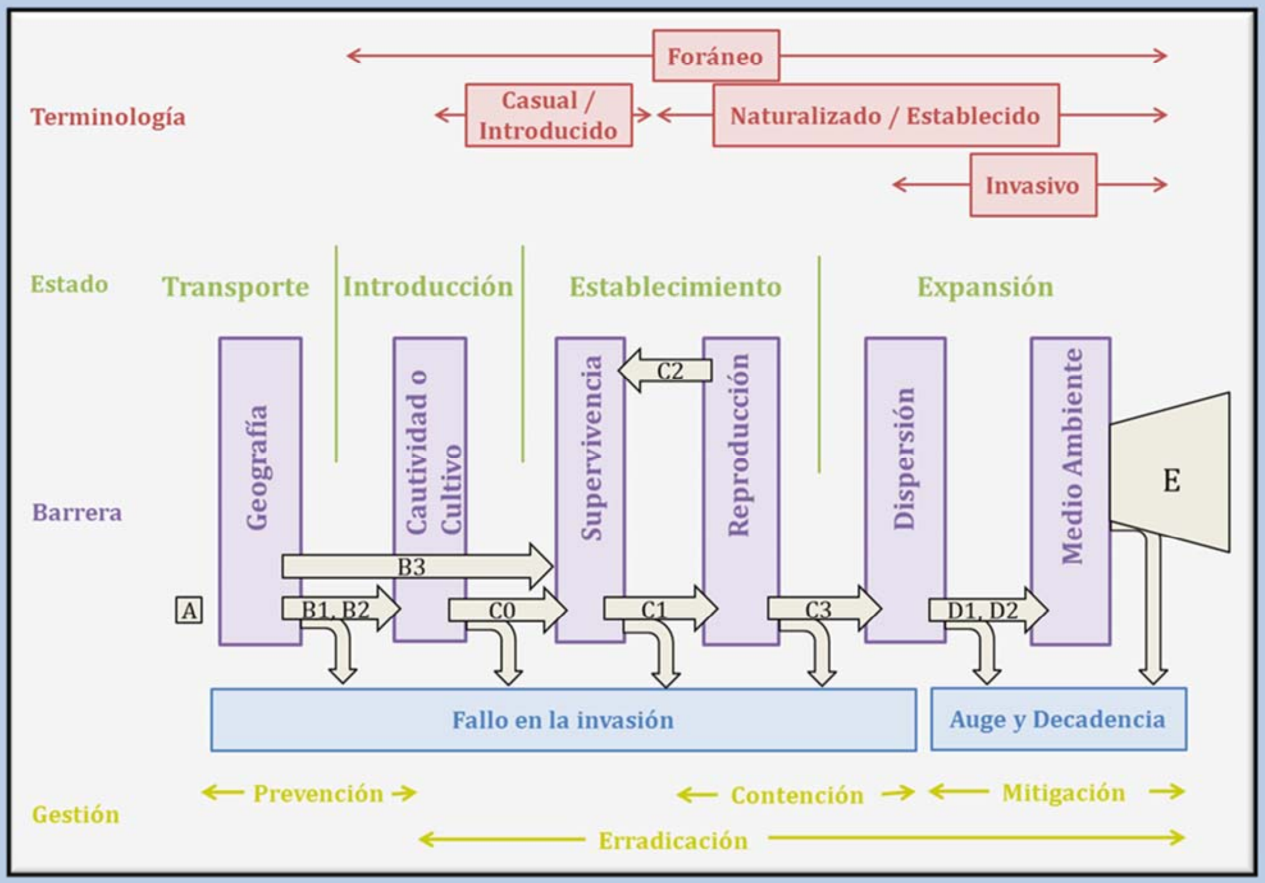
DISCUSIÓN GENERAL

¿Introducidas o invasoras?

Cuando nos referimos a una especie alóctona (no indígena o exótica) entendemos una especie que se ha introducido intencionadamente o no fuera de su rango, más allá de su potencial de dispersión natural (Richardson *et al.* 2000, Galil 2009, Zenetos *et al.* 2010, Blackburn *et al.* 2011). Después de introducida, esta especie puede ser capaz de adaptarse, sobrevivir y reproducirse en el lugar donde fue inoculada (Blackburn *et al.* 2011), entonces se considera que está establecida o naturalizada (Richardson et la 2000, Blackburn *et al.* 2011). Por otra parte, los criterios para considerar una especie como invasiva o no son objeto de cierto debate. Para algunos autores, una especie invasora (Panel 5) es una especie naturalizada que ha sido capaz de dispersarse y establecerse fuera del rango de introducción (Richardson *et al.* 2000, Kolar & Lodge 2001, Colautti & MacIsaac 2004, Blackburn *et al.* 2011). En cambio, otras veces se considera una especie invasora como sinónimo simplemente de especie no indígena (Goodwin *et al.* 1999, Radford & Cousens 2000), o una especie no indígena con una amplia distribución que presenta efectos adversos en el hábitat invadido (Davis & Thompson 2000, Mack *et al.* 2000), incluyendo problemas ecológicos, económicos y de salud para el hombre (Zenetos *et al.* 2010). En esta tesis hemos considerado las cuatro especies estudiadas como invasoras. Aunque *Microcosmus squamiger* es la única que hemos encontrado en sustrato natural fuera de puertos y marinas en la zona estudiada, *Didemnum vexillum* y *Clavelina oblonga* también se han encontrado establecidos en sustratos naturales en otros sitios del globo (Bullard *et al.* 2007a, Rocha *et al.* 2009, respectivamente), y también aunque más raramente *Styela plicata* (Pineda 2012). Además, resulta indiscutible la gran capacidad de colonización, competencia y supervivencia que presentan estas cuatro ascidias. Por otro lado, las especies marinas no indígenas (ya no sólo las ascidias) establecidas en puertos, marinas o instalaciones de acuacultura, aun estando confinadas en estos sustratos artificiales también forman toda una comunidad, en la que unas especies interaccionan con las otras, incluso con especies nativas. Algunas de estas especies (como por ejemplo las ascidias) se vuelven dominantes en estas comunidades "artificiales". Por tanto, la capacidad de ocupación de toda esta serie de sustratos sería suficiente para considerar a estas especies no indígenas como invasoras. Finalmente, hay que considerar que, aún confinadas en ambientes concretos, estas especies dominantes pueden tener un gran impacto económico por su capacidad para recubrir estructuras sumergidas y por sus efectos adversos en actividades de acuacultura.

Panel 5. ¿Introducidas o invasoras?

Propuesta de esquema para una terminología estándar en las invasiones biológicas (Modificado a partir de Blackburn *et al.* (2011))



A No transportado más allá del rango nativo

B1 Individuos transportados más allá del rango nativo, en cautividad o cuarentena (provisionados de condiciones óptimas pero con medidas explícitas de contención en un lugar determinado)

B2 Individuos transportados más allá del rango nativo, en cultivo (provisionados de condiciones óptimas pero con medidas explícitas de prevención de la dispersión)

B3 Individuos transportados más allá del rango nativo, directamente liberados en el nuevo ambiente

C0 Individuos liberados en zonas salvajes (fuera de cautividad o cultivo) una vez introducidos, pero incapaces de sobrevivir por un período significativo

C1 Individuos que sobreviven en zonas salvajes (fuera de cautividad o cultivo) una vez introducidos, pero no llegan a reproducirse

C2 Individuos que sobreviven en zonas salvajes una vez introducidos, se reproducen, pero la población no es autosuficiente

C3 Individuos que sobreviven en zonas salvajes una vez introducidos, se reproducen y la población es autosuficiente

D1 Población autosuficiente en zonas salvajes, con individuos sobreviviendo en distancias significativas del punto inicial de introducción

D2 Población autosuficiente en zonas salvajes, con individuos sobreviviendo y reproduciéndose en distancias significativas del punto inicial de introducción

E Especie totalmente invasiva, con individuos dispersándose, sobreviviendo y reproduciéndose en múltiples lugares por todo un territorio/hábitat más o menos extenso

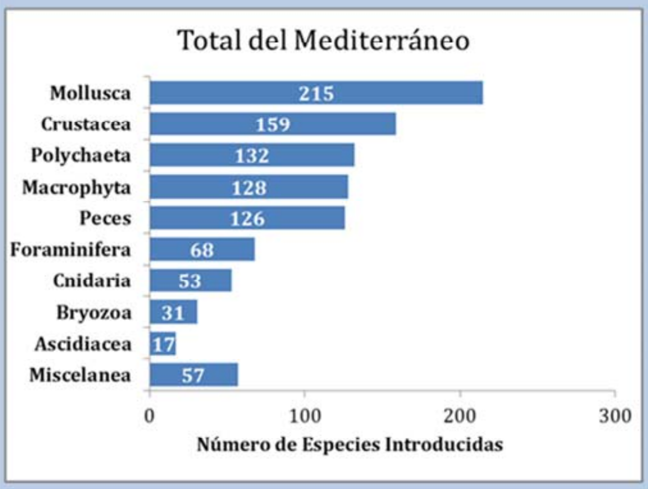
Las ascidias no indígenas en el Mar Mediterráneo

Las ascidias representan una pequeñísima proporción del total de especies no indígenas en el Mar Mediterráneo. Encontramos un total de 17 ascidias de las casi 1000 especies exóticas descritas en censos publicados en la bibliografía (Galil 2009, Izquierdo-Muñóz *et al.* 2009, Zenetos *et al.* 2012), lo que representa un modesto 1,7% (Panel 6). En cambio, es indiscutible la gran importancia ecológica y/o económica que tienen las ascidias no indígenas, tanto en infraestructuras hechas por el hombre o cultivos de acuacultura, como en comunidades naturales y, tal y como hemos comentado anteriormente, no sólo en el Mediterráneo, sino mundialmente.

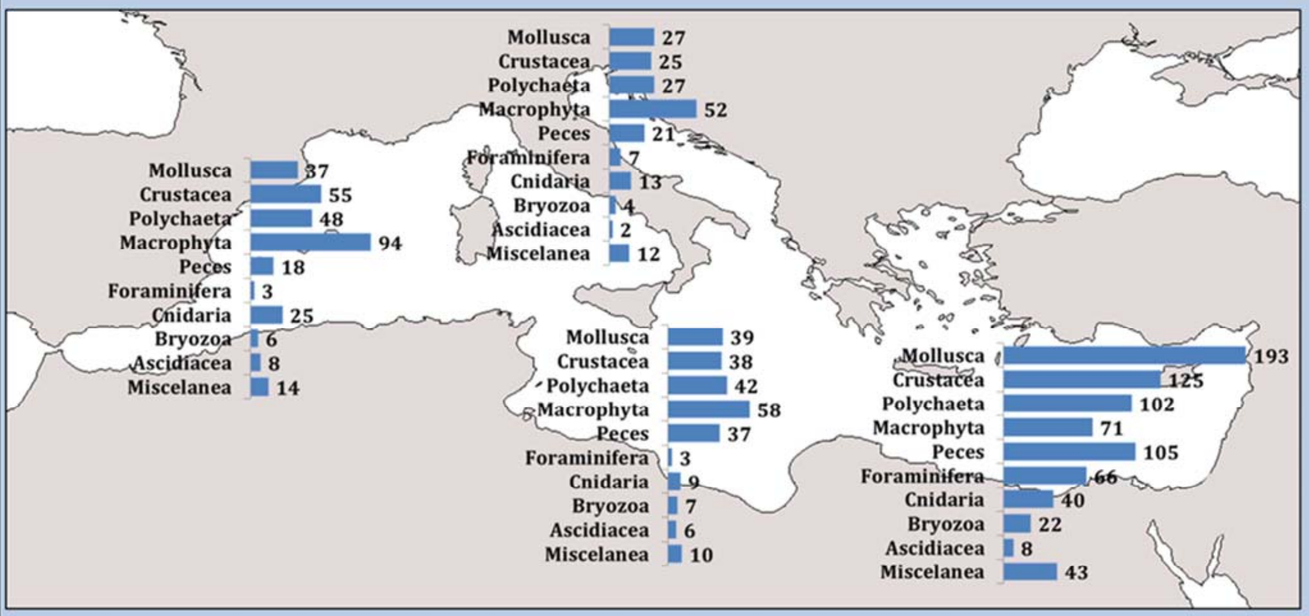
De las cuatro especies que estudiamos en esta tesis, sólo dos aparecen en los censos: *Microcosmus squamiger* y *Didemnum vexillum*. Resulta sorprendente que *Styela plicata* no haya sido incluida aún, mientras que *Clavelina oblonga* habría pasado inadvertida debido a su errónea identificación como *C. phlegraea*, considerada nativa de la región.

La mayoría de ascidias no indígenas en el Mar Mediterráneo han estado introducidas a través del recubrimiento de los barcos (Zenetos *et al.* 2012). Asimismo, *Microcosmus squamiger* y *Styela plicata* habrían estado introducidas por esta vía (Rius *et al.* 2008, Pineda *et al.* 2011), mientras que el vector de introducción para *Didemnum vexillum* y *Clavelina oblonga* seguramente sería el stock comercial de bivalvos.

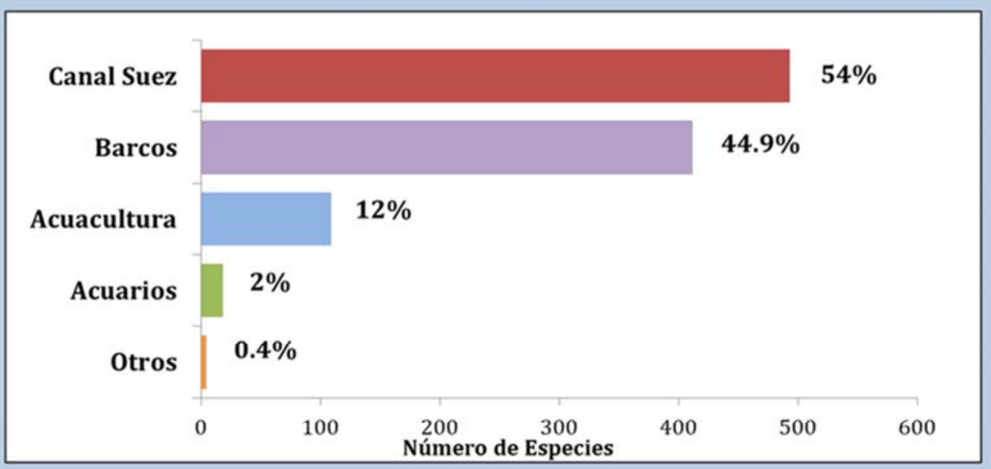
Panel 6. Invasiones biológicas en el Mar Mediterráneo (II)



De las casi 1000 especies no indígenas censadas en el Mar Mediterráneo, menos del dos por ciento son ascidias (izq.). En el mapa se muestra el número de especies no indígenas en cada subregión del Mar Mediterráneo (OMED: Mediterráneo Oeste; CMED: Mediterráneo Central; ADRI: Mar Adriático; EMED: Mediterráneo Este). Algunas especies pueden encontrarse en más de una subregión (Modificado a partir de Zenetos *et al.* (2012)).



Vías de introducción de especies no indígenas en el Mar Mediterráneo. En el caso de las ascidias, su larva lecitotrófica tiene un período pelágico más bien corto (de minutos a horas), por lo que el principal vector de introducción a grandes distancias es sobretodo mediante el recubrimiento de los barcos. La acuacultura sería también un vector importante de introducción de estos tunicados (Modificado a partir de Zenetos *et al.* (2012)).



Estructura genética de Microcosmus squamiger

Pensábamos encontrar diferente diversidad genética y niveles de consanguinidad en poblaciones de *Microcosmus squamiger* establecidas en diferentes sustratos, pero no fue así ya que los valores fueron los mismos al comparar poblaciones establecidas en sustrato artificial y natural. Estos resultados sugieren que los individuos colonizando los dos tipos de sustratos representarían un mismo conjunto de propágulos que permiten asegurar el éxito en la dispersión post-frontera de la especie.

Por otro lado, no hemos encontrado ninguna estructura genética espacial a pequeña escala (autocorrelación a decenas de metros). En otros organismos marinos incubadores, con larvas de vida muy corta, se ha descrito la existencia de estructura genética a este nivel, como por ejemplo en esponjas o cnidarios (Calderón *et al.* 2007, Blanquer *et al.* 2009, Ledoux *et al.* 2010, Mokhtar-Jamaï *et al.* 2011). Las larvas de todas las ascidias son de vida corta y lecitotróficas, pero las formas solitarias suelen ser ovíparas (Svane & Young 1989), por lo que el periodo embrionario se produce también en la columna de agua. En *Microcosmus squamiger* pueden pasar en el laboratorio hasta 20 horas, desde que se fertilizan los huevos, se desarrolla la larva y ésta se asienta (Rius *et al.* 2010), aparentemente tiempo suficiente para asegurar la dispersión del propágulo. Si embargo, algunas ascidias solitarias presentan mecanismos de retención de los propágulos, como por ejemplo flotabilidad negativa, viscosidad de los huevos, o retención de huevos y larvas en cordones de mucus o bio-espuma (Svane & Havenhand 1993, Petersen & Svane 1995, Marshall 2002, Castilla *et al.* 2007). En otras ascidias solitarias se ha registrado una dispersión extremadamente localizada (*Ciona intestinalis*, Petersen & Svane 1995, Howes *et al.* 2007) y los análisis de autocorrelación han corroborado la existencia de estructura genética a escala de pocos metros (*Styela plicata*, David *et al.* 2010, *S. clava*, Dupont *et al.* 2009). En cambio, *M. squamiger* no presenta evidencias de ningún tipo de estructura genética en el rango estudiado (hasta 100 metros), siendo patrón idéntico para poblaciones en sustrato natural y artificial. En esta especie no parece actuar ningún mecanismo de retención de embriones y la dispersión natural de la ascidia tendría un alcance suficiente para asegurar una eficiente dispersión post-frontera.

Para nuestra sorpresa, encontramos dos acervos genéticos en las poblaciones de *Microcosmus squamiger* en la costa catalana (resultados de STRUCTURE y FCA). La mayoría de los individuos podían asignarse a uno u otro acervo genético, con sólo un 15% de individuos que presentaban probabilidades ambiguas de asignación a cualquiera de los dos grupos. Sin embargo, aunque no mezclados a nivel de individuo, los dos acervos genéticos sí se han detectado mezclados a nivel poblacional. Estos grupos genéticos parecen corresponder a la separación de la composición genética de la especie en su área original, ya que pueden ser relacionados con las dos poblaciones australianas de *M. squamiger* representativas de las dos áreas nativas que han sido descritas como fuente de las poblaciones introducidas de *M. squamiger* (Rius *et al.* 2012). Por otro lado, el mantenimiento de esta estructura genética interna (dos acervos genéticos distribuidos por todas las poblaciones, con pocos individuos mixtos) es sorprendente, especialmente porque tanto los registros históricos como los datos genéticos muestran que el Mar Mediterráneo fue probablemente la primera área de introducción de la especie (Monniot 1981, Rius *et al.* 2012) hace más de 50 años, un amplio marco de tiempo para que los acervos genéticos se mezclen. No podemos asegurar cuales son las causas que previenen el cruce entre estos dos grupos. Entre otras explicaciones, los mecanismos de reconocimiento gamético o la separación temporal de los períodos reproductores pueden estar actuando en el aislamiento pre-zigótico. Estudios previos del ciclo de vida de *M. squamiger* en nuestra área de estudio han mostrado un período reproductivo largo, desde finales de primavera hasta principios del otoño (Rius *et al.* 2009a), el cual puede alojar diferentes tiempos de reproducción de los dos grupos. No obstante, fertilizaciones *in vitro* realizadas en julio y agosto del 2012 han mostrado que, al menos en condiciones de laboratorio, oocitos de individuos pertenecientes a uno de los grupos genéticos pueden ser fertilizados por esperma de individuos del otro grupo genético, y obtener así larvas (resultados no publicados) por lo que podemos descartar una incompatibilidad gamética. Otra posibilidad es que haya mecanismos reproductivos de aislamiento post-zigóticos que estén reduciendo la eficacia biológica de los individuos mezclados. Una explicación alternativa sería que uno de los dos grupos haya llegado recientemente a la zona en una introducción secundaria y desde entonces se haya dado poca mezcla. Sin embargo, aunque no se puede descartar esta posibilidad sin un muestreo temporal apropiado, esto es poco probable dado que el Mediterráneo fue la primera área colonizada por un acervo que ya comprendía una mezcla de las dos fuentes nativas (Rius *et al.* 2012).

Sea cual sea la causa, el mantenimiento a lo largo del tiempo de los acervos genéticos diferenciados puede tener un fuerte efecto en el proceso de introducción. Está reconocido que múltiples introducciones, si son originarias de diferentes fuentes, contribuyen a incrementar la diversidad genética y la generación de nuevas combinaciones alélicas que pueden fomentar el potencial adaptativo de una población invasora (Geller *et al.* 2010). Aunque no demostrado empíricamente, se ha sugerido que la mezcla genética incrementa la habilidad de invasión de especies para adaptarse y prosperar en nuevos ambientes en sistemas terrestres, marinos y de agua dulce (McIvor *et al.* 2001, Kelly *et al.* 2006, Blum *et al.* 2007, Facon *et al.* 2008, Kolbe *et al.* 2008, Lombaert *et al.* 2011). Sin embargo, en *Microcosmus squamiger* la mezcla parece estar limitada por factores desconocidos, sin comprometer el potencial colonizador de la especie.

En conjunto, nuestros resultados indican que la expansión de *Microcosmus squamiger* es muy probable por todos los diferentes sustratos duros disponibles tanto en ambientes naturales como artificiales. En el estado post-frontera resultaría muy difícil de llevar a cabo medidas de control mínimamente eficientes, más aún en costas con bastante sustrato duro disponible. En consecuencia, es mejor centrarse en desarrollar medidas de prevención de nuevas entradas de la ascidia en el estado pre-frontera, algo que de hacerse las cosas bien, se aplicaría para otros organismos introducidos por la misma vía de introducción, no solo con capacidades altas de dispersión sino también incluyendo organismos con dispersión restringida.

Interacciones biológicas tempranas

Las ascidias y los mejillones son de las mayores especies ingenieras en los ecosistemas a lo largo de las costas de todo el mundo, y se han introducido ampliamente a través del transporte humano (Lambert 2007, McQuaid & Arenas 2009). Suelen colonizar sustratos artificiales en puertos, tales como muelles, columnas, plataformas flotantes, casco de los barcos y boyas (Bax *et al.* 2002). Ya que estas especies son dominantes en el espacio, la coexistencia en tales sustratos probablemente lleva tanto a la competencia entre ellas por explotación de recursos como a la competencia directa por interferencia.

En nuestros experimentos, *Styela plicata* tuvo más éxito en la fertilización, asentamiento y metamorfosis que *Microcosmus squamiger*, y la única interacción interespecífica encontrada fue en el patrón espacial de asentamiento. No hubo efecto del esperma heterospecífico en el proceso de la fertilización, ni tampoco hubo efecto dependiente de la densidad del esperma homólogo. En ascidias, aparte de la competencia espermática intraespecífica (Yund 1998), también se da la competencia espermática entre especies (Lambert 2000). Aunque la fertilización es especie- específica en la mayoría de ascidias, se ha visto que el esperma heterólogo puede desencadenar los mecanismos que bloquean la polispermia, dificultando la fertilización por el esperma homólogo (Lambert 2001, 2009b). Este proceso no parece actuar en nuestro caso. Las dos especies de ascidias son dominantes en el espacio y simpátricas, ambas son ovíparas y se solapan ampliamente en su período reproductor (Yamaguchi 1975, Rius *et al.* 2009a, Pineda *et al.* 2013). Consecuentemente, parece probable que, en la naturaleza, los oocitos de ambas especies estarán expuestos al esperma de ambas especies, sobretodo porque el esperma de ascidias es capaz de fertilizar huevos 12 horas o más después de la dilución (Bolton & Havenhand 1996). En este sentido, la falta de interacciones con el esperma heterólogo sería beneficioso para estos invasores.

Las larvas de los invertebrados marinos pueden estar influenciadas por la comunidad residente (Grosberg 1981, Johnson & Strathmann 1989, Osman *et al.* 1989, Bingham & Young 1991, Stoner 1994) incluso por adultos de ascidias (Svane & Young 1989), lo que sugiere importantes interacciones que afectan el proceso de asentamiento. Nosotros no encontramos evidencia de interferencia entre las larvas de *Microcosmus squamiger* y *Styela plicata* en términos de asentamiento y metamorfosis. El único efecto fue en el ordenamiento espacial de los asentados: las larvas de ambas especies se asentaban de manera gregaria, pero lo hacían de manera más aleatoria en presencia de las larvas de la otra especie. Esperábamos algún tipo de interferencia ya que ambas especies forman densos agregados en la naturaleza en sustratos artificiales hechos por el hombre, así que era probable que la competencia por el espacio fuese fuerte entre ellas. Nuestra hipótesis era que si hubiera tal competencia sería detectable en el estado larvario. En nuestro experimento no hubo interferencia entre larvas de *M. squamiger* y *S. plicata* sugiriendo que los efectos de interferencia ocurrirían cuando las larvas se encuentran con asentados ya establecidos más que con las larvas de la otra especie (Rius *et al.* 2009b).

Por otro lado, en las placas de PVC permanentes usadas para el experimento del Capítulo Cuatro con *Clavelina oblonga*, vimos que en septiembre del 2012, en las placas más superficiales, *S. plicata* era capaz de ocupar gran parte del espacio impidiendo que *C. oblonga* se estableciera en la placa entera, tal y como hizo en las placas de mayor profundidad (1m y 2m) ese mismo mes. Sin embargo, un mes antes, en agosto del 2012, *Styela plicata* ocupaba muy poco espacio en esas mismas placas (resultados no mostrados), en forma de pequeños reclutas aparentemente repartidos por toda la placa. Además, como hemos visto en los resultados del Capítulo Cuarto, el reclutamiento de *C. oblonga* fue mayor en las placas de superficie durante el pico de reclutamiento. Esto nos estaría mostrando la intensa competencia por el espacio que hay entre ambas especies una vez los individuos ya están establecidos y crecen en su fase adulta. Por otro lado, nos estaría mostrando la gran tasa de crecimiento que presenta *S. plicata*, que en un mes incrementó alarmantemente su abundancia, característica que la dotaría de un gran potencial competidor contra otros invertebrados marinos, no solo con *Microcosmus squamiger* o *C. oblonga*. De todos modos, se tendrían que realizar más estudios para averiguar si es la competencia entre estas dos especies (tanto a nivel larvario como entre reclutas o adultos) u otros factores los que determinan que *C. oblonga* sea menos abundante en las placas de superficie, así como determinar por qué *S. plicata* no aparecía dominante (o estaba incluso ausente) en las placas de profundidad de 1m y 2m del mismo mes.

El efecto de los depredadores marinos en el éxito de las introducciones biológicas se ha estudiado extensivamente (Osman & Whitlatch 1998, Rilov *et al.* 2002, Epelbaum *et al.* 2009, Dumont *et al.* 2011), pero estos estudios se centran en pequeños depredadores vágiles que pueden consumir nuevos reclutas. Sin embargo, los organismos filtradores que procesan un gran volumen de agua pueden potencialmente ejercer una fuerte presión de depredación sobre las larvas planctónicas de vida libre (Porri *et al.* 2008), previniendo así un reclutamiento exitoso (Bingham & Walters 1989, Young & Cameron 1989). Los mejillones son unos eficientes filtradores de fitoplancton, y se ha visto que también pueden ingerir especies del mesozooplancton (Davenport *et al.* 2000, Lehane & Davenport 2002, 2006) teniendo así una dieta omnívora (Maloy *et al.* 2012). Las larvas de *Microcosmus squamiger* y *Styela plicata* miden alrededor de 500 µm de longitud (Pineda *et al.* 2012a), dentro del rango de medidas del zooplancton encontrado en contenidos estomacales de mejillones, el cual es generalmente alrededor de 400-600 µm (Lehane & Davenport 2002) aunque puede alcanzar varios milímetros (Davenport *et al.* 2000, Lehane & Davenport 2006). En nuestros experimentos de competencia con mejillones, encontramos un efecto marcado del consumo de larvas de las dos ascidias (desde un 70% hasta más del 90%) por parte de los juveniles de las dos especies de bivalvos de manera que el consumo por parte de los adultos sería presumiblemente mayor. El consumo de zooplancton por parte de mejillones se ha demostrado en clases de tamaño pequeñas (1,5-2 cm, Lehane & Davenport 2002). Según nuestros resultados, la habilidad de los mejillones para capturar larvas de ascidias ya está establecida en estadios incluso más tempranos del desarrollo (tamaños más pequeños que 1 cm). Además, entre las larvas no consumidas, una respuesta general fue una mayor tasa de asentamiento, la cual puede explicarse por una aceleración en el proceso de asentamiento como respuesta adaptativa para minimizar el riesgo de ser capturado por los mejillones.

Por otro lado, *Mytilus galloprovincialis* presentó una mayor depredación sobre las larvas de las ascidias en Sudáfrica, donde la especie está introducida. Un mayor asentamiento de las ascidias en presencia de *M. galloprovincialis* donde el mejillón es nativo (España) además de evitar asentarse cerca de éste, podría explicar en parte el menor consumo. Los cambios en la tasa de filtración del mejillón podrían ser debidos a que cada región (España y Sudáfrica) tienen poblaciones de *M. galloprovincialis* pertenecientes a diferentes acervos genéticos (Daguin & Borsa 2000, Sanjuan *et al.* 1997), que podría explicar su diferente comportamiento. Alternativamente, y considerando que el consumo por parte de *Perna perna* en Sudáfrica no difiere significativamente del de *M. galloprovinvialis* en la región, podemos pensar que factores ambientales pueden estar determinando un incremento de actividad filtradora en Sudáfrica. Se ha visto que la temperatura puede cambiar la actividad metabólica de los mejillones (Anestis *et al.* 2007), y la temperatura media anual de la costa de Sudáfrica es significativamente mayor, unos tres grados, que la del Mar Mediterráneo. Por lo tanto, la adaptación a la temperatura del agua en cada región puede estar determinando la actividad filtradora de los mejillones usados en este estudio. No obstante, se necesitaría profundizar más en el asunto con otros diseños experimentales para averiguar la verdadera causa de este resultado.

Expansión de Didemnum vexillum en el Mar Mediterráneo

Hemos confirmado morfológica y genéticamente que el didémnido que crece en el stock de bivalvos en la bahía del Fangar (Delta del Ebro) es *Didemnum vexillum* Kott, 2002. La ascidia crea una capa sobre el ostrón cultivado que resulta trabajosa de limpiar antes de la comercialización del bivalvo aunque parece ser que no los ahoga. *D. vexillum* muestra un ciclo de vida marcadamente estacional en la bahía del Fangar, creciendo activamente durante el invierno, alcanzando su máxima cobertura a finales de primavera y entrando en regresión en verano. Las colonias alcanzan su máximo estado reproductivo justo antes de entrar en regresión.

Didemnum vexillum es una especie templaada, que se encuentra en su rango más bajo de latitudes en el Mediterraneo, aunque se ha encontrado recientemente en aguas más tropicales (Restrepo *et al.* 2013). Muchas ascidias coloniales muestran ciclos estacionales en aguas templadas y la temperatura aparece como factor responsable principal (Turon 1988, De Caralt *et al.* 2002, López-Legentil *et al.* 2005, Pérez-Portela *et al.* 2007, López-Legentil *et al.* 2013). *D. vexillum* muestra generalmente un ciclo de vida plástico que se puede adaptar a diferentes regímenes de temperaturas. Así, en aguas templadas con inviernos fríos la especie reduce su crecimiento o presenta regresión en invierno (Gittenberger 2007, Lindeyer & Gittenberger 2011, Valentine *et al.* 2007a, Fletcher *et al.* 2013). En zonas con temperaturas extremas tanto en verano como en invierno, como la Laguna de Venecia en el Mar Adriático, la especie parece pasar por dos períodos sin crecimiento durante estas estaciones, cuando la temperatura del agua puede ser desde 0ºC a >30ºC (Tagliapietra *et al.* 2012). En nuestro caso, la temperatura anual del agua en la bahía oscila entre los 7 y los 28ºC. El verano es claramente la estación desfavorable y la especie estiva, probablemente reducida a formas de resistencias poco visibles que generan colonias una vez la temperatura disminuye a finales de otoño. Se ha sugerido que 4ºC es la temperatura inferior crítica que limita la dispersión y el crecimiento de las colonias de poblaciones de *D. vexillum* (Daniel & Therriault

2007), mientras que experimentos en el laboratorio sugieren que la temperatura óptima de crecimiento de la ascidia son entre los 16 y 20ºC (McCarthy *et al.* 2007). En nuestro caso, el máximo de cobertura de la ascidia fue en mayo 2013, cuando la temperatura media era sobre los 18ºC. El verano es en general la estación desfavorable en el Mediterráneo para muchos invertebrados (Coma *et al.* 2000, Coma & Ribes 2003), particularmente para ascidias coloniales (Turon 1988, Turon & Becerro 1992, López-Legentil *et al.* 2013).

Las dos poblaciones analizadas muestran una diversidad genética baja (tres haplotipos en el Delta del Ebro y dos en la Laguna de Venecia). Sin embargo, esto también pasa en la mayoría de poblaciones introducidas mundialmente de *Didemnum vexillum*. De hecho, solo cuatro haplotipos han sido encontrados previamente en las poblaciones europeas, comparado con los 22 encontrados en la región de origen del noroeste del Pacífico (Stefaniak *et al.* 2012). De los tres haplotipos encontrados, solo el haplotipo 3 se encuentra en todas las zonas introducidas, mientras que los otros dos han sido encontrados en Europa y noroeste de América (haplotipo 2) y en Europa, noreste de América y Nueva Zelanda (haplotipo 5). Determinar la fuente de las poblaciones mediterráneas no resulta sencillo. Aunque los marcadores genéticos son buenas herramientas para el estudio de los procesos de colonización de especies introducidas (Lejeusne *et al.* 2011, Pineda *et al.* 2011, Rius *et al.* 2012), la baja variabilidad encontrada para el *COI* en todas las poblaciones introducidas dificulta esta tarea. Nuestras dos poblaciones aparecen en el MDS cerca de las poblaciones de NE América y de Nueva Zelanda. Esto es sorprendente, al menos para la población del Delta del Ebro ya que, según los trabajadores de la zona, el stock de ostrón proviene de la costa atlántica francesa, y es muy probable que *D. vexillum* haya venido junto con el stock comercial como epibionte.

Los resultados obtenidos en relación a la similitud genética entre poblaciones indicaría que las poblaciones Mediterráneas provienen de otras regiones (Nueva Zelanda y NE América), aunque este resultado podría estar afectado por el pequeño número de individuos disponibles para algunas regiones en trabajos previos (Stefaniak *et al.* 2009, 2012), como por ejemplo Francia. Para llevar a cabo la comparación entre diferentes poblaciones seleccionamos poblaciones con al menos siete individuos, como sería el caso de las dos poblaciones francesas usadas en los análisis (Bretaña y Le Havre) mientras que no usamos otras poblaciones francesas debido al pequeño tamaño de la muestra (Stefaniak *et al.* 2009, 2012). De esta manera, un error de muestreo debido tanto al pequeño número de individuos usados así como al no usar otras posibles poblaciones de origen, pueden estar determinando este resultado inesperado. En cualquier caso, sería necesario realizar un muestreo intensivo de las poblaciones mundiales de *Didemnum vexillum* para revelar la historia de las invasiones de la ascidia, usando quizás otros marcadores genéticos polimórficos como los microsatélites (Abbott *et al.* 2011), ya que han mostrado ser muy útiles para inferir las rutas de invasión y las similitudes poblacionales de especies invasoras por todo el mundo (Pascual *et al.* 2007, Rius *et al.* 2012).

Nuestros resultados sugieren que *Didemnum vexillum* se encuentra ya ampliamente distribuido en el Mediterráneo, ya que se ha encontrado en las cuencas este y oeste. En el Delta del Ebro, según los trabajadores locales, la especie está presente en la zona desde hace al menos 10 años, aunque nunca ha alcanzado la abundancia que ha tenido en los últimos tres años. La especie parece incrementar su abundancia, ya que hemos detectado un aumento considerable de cobertura en 2013 respecto 2012. Además, se han visto algunas colonias en la parte sur del Delta del Ebro, en la bahía de Alfacs, que alberga también instalaciones de acuacultura (mejillones y ostrones), lo que sugiere que la especie está colonizando localidades cercanas. Podría ser que las poblaciones de *D. vexillum* en la bahía del Fangar estén en una fase inicial de invasión. Se sabe de algunas especies no indígenas que no proliferan masivamente justo después de su introducción, sino que presentan una fase de latencia de crecimiento lento antes de presentar un crecimiento explosivo, volviéndose ampliamente invasivo (Crooks 2005, Suarez & Tsutsui 2008).

El descubrimiento de *Didemnum vexillum* en crecientes abundancias en un área con una importante producción de acuacultura y con zonas de pesca cercanas debería ser motivo de preocupación, ya que la especie es capaz no sólo de causar daños en el cultivo de bivalvos, sino que también prolifera en aguas más profundas cubriendo fondos de pesca de arrastre (Bullard *et al.* 2007a, Valentine *et al.* 2007b). Mientras que la regresión en verano y la falta de sustrato duro en el Delta del Ebro pueden haber contribuido a limitar su expansión, la especie cuenta con características bióticas que pueden impulsar su proliferación, como por ejemplo, su alta capacidad de reproducirse no sólo sexualmente, sino también asexualmente mediante la fragmentación (McCarthy *et al.* 2007; Bullard *et al.* 2007b; Valentine *et al.* 2007a; Morris & Carman 2012). La fragmentación puede ser incluso un método de dispersión, ya que los fragmentos pueden dispersarse mediante corrientes, olas y mareas, o viajar mediante aguas de lastre (Bullard *et al.* 2007a, b) y ser capaces de adherirse de nuevo al sustrato duro. Por otro lado, la ascidia presenta una fuerza de adhesión baja (Murray *et al.* 2012) que parece prevenir su adhesión en embarcaciones de navegación rápida, previniendo así su dispersión a través de este tipo de vectores (Herbog *et al.* 2009). Sin embargo, en la bahía del Fangar, las embarcaciones de navegación lenta son el tipo más usado para navegar por las instalaciones de acuacultura, representando así un factor potencial para futuras dispersiones mediadas por el hombre en el Delta del Ebro y sus regiones adyacentes. Por tanto, se deberían emprender medidas de erradicación además de limpiar el stock comercial de bivalvos y así evitar introducciones recurrentes a través de estos medios.

Llegamos a la conclusión de que es probable que estemos en el borde de un problema potencialmente importante, tanto ecológico como económico. El conocimiento biológico obtenido aquí puede ayudar a contrarrestar el impacto y la propagación de la especie. Por ejemplo, se podría evitar la siembra de nuevo stock de ostrones durante los meses cuando las larvas están presentes en la columna de agua (mayo-julio). Además, la cosecha se puede limitar durante el período de la regresión de las colonias para evitar la limpieza engorrosa de las conchas. A pesar de ello, sería necesaria una estrecha vigilancia de las instalaciones de acuacultura del Delta del Ebro durante los próximos años, así como de las costas cercanas, y deberían aplicarse medidas de control estrictas a corto plazo si la proliferación de la especie continúa.

Presencia de Clavelina oblonga Herdman, 1880 en el Mar Mediterráneo

En el cuarto capítulo hemos visto que la descripción morfológica de *Clavelina* sp. en el Delta del Ebro es la misma que las descripciones previas tanto de *Clavelina oblonga* (atlántica) como de *C. phlegraea* (italiana). Asimismo, los resultados genéticos nos muestran un único haplotipo para el gen *COI* en las muestras de *Clavelina* sp. del Delta del Ebro así como de *C. phlegraea* de Taranto (Italia). Por lo tanto, *C. oblonga* y *C. phlegraea* son en realidad la misma especie, siendo la segunda sinónimo de la primera. Por lo tanto *Clavelina* sp. en el Delta del Ebro sería una nueva referencia de *C. oblonga* en el Mar Mediterráneo, habiendo sido introducida desde hace al menos 80 años atrás. Desafortunadamente, no hemos podido encontrar ejemplares de *C. phlegraea* en el Lago Fusaro, la localidad tipo en Italia donde fue descrita la especie por Salfi en 1929, debido probablemente a la alta contaminación del lago así como el intenso drenaje del fondo que se llevó a cabo en los 1980s (De Pippo *et al.* 2004).

Se han descrito cuatro haplotipos para el gen *COI* en la región nativa del Caribe de *Clavelina oblonga*, aunque solo uno (haplotipo 3) se encuentra en las regiones presumiblemente introducidas de la especie, incluyendo Brasil y Azores (Rocha *et al.* 2012) y, a raíz de nuestros resultados, ahora también en el Mediterráneo.

Clavelina oblonga muestra un ciclo de vida marcademente estacional en el Delta del Ebro, alcanzando su máxima abundancia y el estado reproductor más avanzado en los meses más calurosos. El ciclo está caracterizado también por la regresión de las colonias durante los meses más fríos hasta desaparecer en invierno, para volver a regenerarse en primavera. La temperatura está positivamente correlacionada con el recubrimiento y con el Índice de Madurez (IM). Al final del verano se da la incubación de las larvas y éstas se liberan a principios de otoño, reflejándose en el pico de reclutamiento en octubre. La temperatura del agua es crucial para el ciclo de crecimiento de las ascidias (Turon 1988, De Caralt *et al.* 2002, López-Legentil *et al.* 2005, Pérez-Portela *et al.* 2007, López-Legentil *et al.* 2013). En nuestro caso, la temperatura parece determinar el ciclo de vida de *C. oblonga*, y la temperatura óptima para el crecimiento de la ascidia sería sobre los 25ºC. Además, la mayor disponibilidad de fitoplancton en los meses de máximo crecimiento favorecería al crecimiento y reproducción de la especie. Más evidencia de la influencia de la temperatura en el ciclo de vida de *C. oblonga* la tenemos en la primavera del 2013 que fue más fría de lo normal, con lo que a principios de verano del 2013 la temperatura media del agua fue de hasta cuatro grados menos que en 2012. Esto re reflejó en un retraso en el IM y el reclutamiento en julio del 2013 respecto de julio del 2012.

En el Delta del Ebro la ascidia se encuentra en el extremo norte de su rango de distribución, con temperaturas que varían desde menos de 10ºC en invierno hasta los 28ºC en verano. Las bajas temperaturas afectan negativamente a *Clavelina oblonga* (Rocha *et al.* 2009). Esto se refleja en la desaparición de las colonias durante el invierno. La regresión durante los períodos desfavorables es común en clavelínidos (De Caralt *et al.* 2002) y las yemas de reposo aseguran una recuperación poblacional rápida cuando vuelven las condiciones favorables. De hecho, después de la hibernación y con condiciones favorables, *C. oblonga* es capaz de crecer a un ritmo espectacular, llegando a cubrir las placas de PVC por completo en menos de dos meses. Rocha (1991) también encontró un incremento en la abundancia de la especie para los meses de verano en aguas brasileñas. Por otro lado, Mastrototaro el at (2008) describen la máxima abundancia de la ascidia en el Mar Piccolo de Taranto (refiriéndose a ella como *C. phlegraea*) en verano pero sin presentar hibernación en los meses más fríos. No obstante, hay pocos estudios realizados sobre esta especie, y se necesitarían más trabajos enfocados a la relación del ciclo de vida de la ascidia en las diferentes zonas con los parámetros ambientales, para así acabar de comprender estas diferencias.

Hubo una segregación en profundidad en la distribución de la ascidia, siendo más abundante en uno y dos metros bajo el agua (placas permanentes), mientras que el reclutamiento fue mayor cerca de la superficie (placas mensuales). Lo más probable es que la competencia con otras especies pueden explicar esta diferencia. Tanto *Styela plicata* como *Mytilus galloprovincialis* crecieron de manera abundante en las placas cerca de superficie, ocupando el espacio y dificultando el crecimiento a *Clavelina oblonga*. Se ha visto que *C. oblonga* puede llegar a ser dominante en la competencia por el espacio, aunque necesitaría un período inicial de crecimiento y condiciones favorables (Rocha 1991). En cualquier caso, serían necesarios más estudios experimentales para clarificar la relación de la ascidia tanto con factores bióticos como abióticos en su distribución.

Según los trabajadores locales, *Clavelina oblonga* estaba presente en el Delta unos 3-4 años antes del bloom del 2011. Es probable que la ascidia fuera introducida con la semilla del mejillón proveniente de Italia. A parte de ahogar la cosecha de mejillón del verano del 2011, *C. oblonga* también cubrió las cuerdas de ostrón (*Crassostrea gigas*) sin llegar a ahogarlos pero provocándoles un menor crecimiento. La siembra del verano 2011 fue un intento de los propietarios para tener una cohorte extra de mejillón comercializable para diciembre, lo que acabó con el total recubrimiento de las cuerdas a finales de verano. Algunos propietarios intentaron salvar lo que quedaba de su cosecha exponiendo las cuerdas al aire libre durante 24h, lo que resultó bastante efectivo ya que la ascidia moría mientras que el mejillón resistía bien la desecación. Según nuestros resultados, evitando plantar los mejillones hasta principios de otoño y recogiendo la cosecha a principios de verano, se reducirían futuras pérdidas ocasionadas por la especie.

La prevención es la mejor herramienta para evitar y reducir los daños causados por las especies invasoras, y todo comienza por una correcta identificación temprana de éstas, seguido de un conocimiento profundo de su biología para desarrollar estrategias que permitan mitigar los efectos nocivos de estas y reducir el impacto económico y ecológico de las mismas.

La ciencia y la gestión

En muchas ocasiones la ciencia tiene el compromiso de obtener un beneficio para el hombre. En cambio, la irresponsabilidad del hombre ha transportado miles de especies de una parte a la otra del globo como si fueran fichas en un tablero, provocando alteraciones en las comunidades nativas a veces con grandes repercusiones ecológicas y económicas. Estamos tirándonos piedras en nuestro propio tejado. Es nuestra responsabilidad hacernos cargo de la conservación de estas comunidades alteradas por la introducción de biota exótica. El conocimiento es un arma poderosa con la que podemos intentar reducir los daños causados por las introducciones de especies y para ello, es imprescindible conocer de dónde vienen, cómo vinieron, sus características y su biología.

La genética está resultando ser un aliado imprescindible para desvelar los procesos y la historia de la introducción de las especies, así como para averiguar su procedencia. De esta manera, combinando la genética con la taxonomía y los estudios tradicionales observacionales y experimentales, podemos construir una lista con los atributos más destacados a tener en cuenta para la gestión de las especies invasoras. Así sabemos que para controlar la expansión de *Microcosmus squamiger* hemos de evitar su transporte primario mediante embarcaciones, ya que una vez introducido en un nuevo puerto o marina, es muy probable que por sus propios medios sea capaz de colonizar el sustrato rocoso natural adyacente. Sabemos que *Styela plicata* tiene un gran potencial colonizador y, aunque en la costa catalana no la hayamos encontrado en sustrato natural, no podemos predecir si en un futuro será capaz de hacerlo provocando daños a la comunidad autóctona. Sabemos que, aunque *Didemnum vexillum* parece no afectar demasiado (de momento) a los cultivos de ostrón en el Delta del Ebro, su dispersión mediante las embarcaciones es muy probable, lo que llevaría a una expansión peligrosa de esta ascidia con un gran potencial invasor. Sabemos que, con algo tan sencillo como evitar sembrar una nueva cosecha de mejillón en la época de máximo crecimiento y reproducción de *Clavelina oblonga*, se pueden evitar grandes pérdidas económicas. Además, sabiendo la vía de introducción de estas ascidias, se podrían evitar futuras introducciones simplemente limpiando el stock comercial de bivalvos (se han descrito medidas efectivas para ello como ya hemos comentado a lo largo de la tesis), o limpiando el casco de las embarcaciones de toda la comunidad que crece sobre ellos. Algunos se echarían atrás en realizar estas medidas de prevención por el gasto o quizás las molestias que representan, pero como ya se ha demostrado, la prevención es siempre la mejor opción, es más efectiva y más barata.

Hay gente que me pregunta "¿de qué sirve eso que estás estudiando?" Si no hay un beneficio comercial detrás de la investigación, la creencia popular es que no sirve de nada. Nada más lejos. Es necesario un cambio de mentalidad para que se empiece a apreciar el ecosistema que nos rodea y la importancia en su mantenimiento, para así poder conservar la maravillosa diversidad con la que tenemos el privilegio de convivir.

Conclusiones

CONCLUSIONES

1. *Microcosmus squamiger* tiene una gran capacidad de dispersión post-frontera. La variabilidad genética es alta, sin haber diferencias entre poblaciones establecidas en sustrato natural respecto las de sustrato artificial. Además, no hay estructura genética espacial ni dentro ni entre poblaciones, independientemente del tipo de sustrato.

2. Hay dos acervos genéticos en las poblaciones de *Microcosmus squamiger* asociados a las dos zonas australianas, originarias de las introducciones mundiales de la especie. Estos acervos están mezclados a nivel poblacional pero no a nivel de individuo, existiendo sólo un pequeño porcentaje (15%) de individuos mezclados. El mecanismo que previene la mezcla de estos acervos a nivel de individuo se desconoce, aunque no parece comprometer la capacidad invasora de la especie.

3. No hay interferencias a nivel espermático entre *Microcosmus squamiger* y *Styela plicata*, ni a nivel conspecífico ni entre especies. Tampoco encontramos efectos ni en el éxito de asentamiento ni en el de metamorfosis entre las dos especies. No obstante, las larvas de las ascidias reaccionan cambiando el patrón espacial de asentamiento de agregado a al azar con la presencia de las larvas de la otra especie. Esta falta de interacción en los estadios tempranos del ciclo de vida sería beneficioso para dos especies que coexisten y comparten el espacio en los puertos y marinas de todo el mundo donde han sido introducidas.

4. Las larvas de *Microcosmus squamiger* y *Styela plicata* son consumidas por los juveniles de los mejillones *Mytilus galloprovincialis* y *Perna perna*. El consumo larvario es mayor en Sudáfrica que en España y, a su vez, las larvas no consumidas en esta última localidad evitan asentarse cerca de sus consumidores. Por lo tanto, el consumo de larvas por parte de una comunidad adulta de mejillones estaría afectando notablemente al establecimiento de las ascidias en la comunidad bentónica. Además, no es tan importante que las

especies sean nativas o no, sino que lo principal a tener en cuenta es el tipo de especie que son.

5. La presencia de la ascidia invasora *Didemnum vexillum* en el Mediterráneo occidental nos indica que la especie está expandiendo su rango de distribución en este mar. Además, la diversidad genética de las poblaciones de *D vexillum* en el Mediterráneo es baja (sólo tres haplotipos), en concordancia con la escasa diversidad de las poblaciones introducidas de la distribución mundial de la especie previamente analizadas. Esto junto al escaso muestreo en algunas zonas dificulta determinar la historia de la introducción de *D. vexillum* en la región.

6. *Didemnum vexillum* presenta una marcada estacionalidad en su ciclo de vida, caracterizado por una regresión en los meses más calurosos del año debido a las altas temperaturas. El período reproductor de la ascidia es durante el verano. A pesar de cubrir por completo los ostrones, parece ser que no compromete la supervivencia de éstos, y la regresión durante el verano parece evitar mayores problemas de la invasión de *D. vexillum*. No obstante, la dispersión de la ascidia por parte del hombre es un factor de riesgo en la futura proliferación de *D. vexillum*.

7. Las especies *Clavelina oblonga* Herdman, 1880 y *C. phlegraea* Salfi, 1929 son en realidad la misma especie, siendo la segunda sinónima de la primera, lo que significaría la introducción de *C. oblonga* en el Mediterráneo desde hace al menos 80 años.

8. El ciclo de vida de *Clavelina oblonga* es marcadamente estacional, caracterizado por un crecimiento explosivo a finales de verano y por una regresión en los meses más fríos del año debido a las bajas temperaturas. La especie se encuentra en su pico reproductor también a finales de verano, mientras que el reclutamiento se realiza sobre todo a principios de otoño. Evitando plantar nueva semilla de mejillón hasta principios de otoño y recogiendo el cultivo a principios de verano del siguiente año, se reduciría

considerablemente el daño económico que pudiera causar la especie en la industria de acuacultura de la zona.

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Informe dels directors sobre la coautoria i factor d'impacte de les publicacions derivades de la tesi presentada per Víctor Ordóñez Sánchez

Val a dir que CAP INFORMACIÓ d'aquestes publicacions ha estat ni serà utilitzada en altres Tesis Doctorals.

Publicació 1: Mixed but not admixed: a spatial analysis of genetic variation of an invasive ascidian on natural and artificial substrates

V. Ordóñez, M. Pascual, M. Rius and X. Turon

Marine Biology 160: 1645-1660 (2013)

Tasques del doctorand:

En aquest article el doctorand ha contribuït obtenint les mostres analitzades, ha fet les amplificacions de tots els loci microsatèl·lits per a totes les poblacions. Conjuntament amb els altres autors ha fet les anàlisis estadístiques pertinents i ha redactat la primera versió del manuscrit

Marine Biology té un índex d'impacte de 2.468 (SCI 2012) i es troba al primer quartil (lloc 18 de 100 revistes) a l'àrea de "MARINE & FRESHWATER BIOLOGY".

Publicació 2: Early biotic interactions among introduced and native benthic species reveal cryptic predation and shifts in larval behavior

V. Ordóñez, M. Rius, C.D. McQuaid, M.C. Pineda, M. Pascual and X. Turon

Marine Ecology Progress Series 488: 65–79 (2013)

Tasques del doctorand:

En aquest article el doctorand ha contribuït obtenint les mostres analitzades, ha fet els assajos en el laboratori per a totes les zones amb l'ajuda en alguns cassos d'alguns autors. Conjuntament amb els altres autors ha fet les anàlisis estadístiques pertinents i ha redactat la primera versió del manuscrit.

Marine Ecology Progress Series té un índex d'impacte de 2.546 (SCI 2012) i es troba al primer quartil (lloc 16 de 100 revistes) a l'àrea de "MARINE & FRESHWATER BIOLOGY".

Publicació 3: Ongoing expansion of *Didemnum vexillum* in the Mediterranean Sea: biological cycle and genetic structure of a worldwide invader

Ordóñez V, Pascual M, Fernández-Tejedor M, Pineda MC, Tagliapietra D and Turon X

Article en revisió final pels altres autors i pendent d'enviar.

Tasques del doctorand:

En aquest article el doctorand ha contribuït realitzant el seguiment de la població invasora del Delta de l'Ebre i obtenint totes les mostres analitzades d'aquesta zona. Ha realitzat l'anàlisi de les imatges així com l'estat reproductor estacional. Ha realitzat l'anàlisi genètic de les mostres d'aquesta zona i d'Itàlia. Conjuntament amb els altres autors ha fet les anàlisis estadístiques pertinents i ha redactat la primera versió del manuscrit.

Publicació 4: When invasion biology meets taxonomy: presence of *Clavelina oblonga* Herdman, 1880 in the Mediterranean Sea

V. Ordóñez, M. Pascual, M Fernández-Tejedor and X. Turon

Article en revisió final pels altres autors i pendent d'enviar.

Tasques del doctorand:

En aquest article el doctorand ha realitzat el seguiment mensual de la població invasora del Delta de l'Ebre i obtingut totes les mostres analitzades d'aquesta zona. Ha realitzat l'anàlisi de les imatges així com l'estat reproductor estacional. Ha realitzat l'anàlisi genètic de les mostres d'aquesta zona i d'Itàlia. Conjuntament amb els altres autors ha fet les anàlisis estadístiques pertinents i ha redactat la primera versió del manuscrit.

Barcelona, 12 de setembre de 2013

Els Directors de la Tesi:

Marta Pascual Berniola | Francesc Xavier Turon Barrera

Apéndices

APÉNDICES PRIMER CAPÍTULO

Table S1. Populations studied, with code names and coordinates (Latitude and Longitude); type of substrate (S): natural (Nat) or artificial (Art); number of individuals sampled from each population (N).

Population	S	Code	Latitude	Longitude	N
Garraf	Nat	GAN	41°15'8.85"N	1°54'9.15"E	18
Sitges	Nat	SIN	41°14'11.16"N	1°49'45.64"E	59
El Roc de Sant Gaietà	Nat	SG	41°10'6.97"N	1°28'55.13"E	45
Torredembarra	Nat	TB	41° 8'46.19"N	1°25'9.09"E	34
Garraf	Art	GAA	41°15'8.19"N	1°54'8.37"E	27
Sitges	Art	SIA	41°14'8.99"N	1°49'39.39"E	16
Cubelles	Art	CU	41°11'37.93"N	1°39'12.08"E	57
Segur de Calafell	Art	SC	41°11'16.95"N	1°36'30.04"E	46

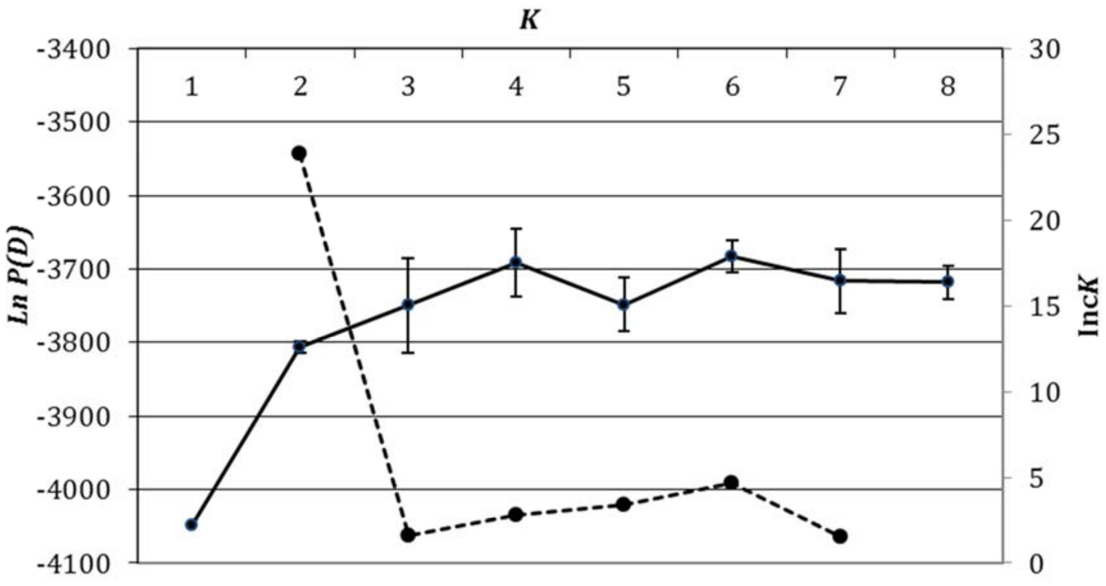
Table S2. Point distances and number of individual sampled in the transects laid out for the autocorrelation study in natural (Sitges and El Roc de Sant Gaietà) and artificial substrate populations (Cubelles and Segur de Calafell). In each transect point all individuals were collected within a 20 cm radius.

	NATURAL		ARTIFICIAL	
Distance	Sitges	El Roc de Sant Gaietà	Cubelles	Segur de Calafell
0m	9	6	4	6
2.5m	9	5	4	6
5m	9	6	9	5
10m	7	6	9	7
20m	9	7	9	6
40m	6	4	7	6
90m	10	11	15	10
Total	59	45	57	46

Table S3. F_{IS} values of the four populations used in the autocorrelation analysis. The values were calculated separately for individuals only belonging to group A, only belonging to group B and over all individuals, in each of the four populations. Significant F_{IS} values are in **bold.** Population codes as in Table 1.

	A	B	All
SIN	0.115	0.047	**0.165**
SG	0.063	-0.048	0.091
CU	0.085	0.048	**0.102**
SC	0.086	0.023	**0.133**

Figure S1. Change in likelihood of the model (*Ln P(D)* ± SD) (continuous line) and Inc*K* (dashed line) statistic for increasing numbers of differentiated genetic clusters (*K*).



APÉNDICES TERCER CAPÍTULO

Appendix. Number of individuals of each haplotype for the worldwide populations of *Didemnum vexillum* (Stefaniak *et al.* 2009, 2012) and our two populations (in bold).

	Haplotypes																							
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**	**18**	**19**	**20**	**21**	**22**	**23**	**Total**
Asamushi (Japan)		5	1			4	2	2										3	1					18
Boothbay (NE America)	1			6	1																			8
Bretagne (NW Europe)			4		3																			7
Bushy Rock (NE America)			9																					9
Butler Cove (NE America)			9		1																			10
Ebro Delta (Med. Europe)		2	23		4																			29
Holyhead Marina (NW Europe)		3	7																					10
Izu Peninsula (Japan)											3	2		3	1									9
Jamestown (NE America)			6	1																				7
Le Havre (NW Europe)		6	2																					8
Lemmens (NW America)		4	4			5																		13
Lowes Cove (NE America)	2		2	2	3																			9
Marlborough (Nez Zealand)			29		15																			44
Millstone (NE America)			7																					7
Misaki (Japan)									8	2	3			1						3	2			19
Nelson (Nez Zealand)			6		4																			10
The Netherlands (NW Europe)			8	6	1																			15
Picton (Nez Zealand)			10	1	2																			13
Sandwich (NE America)			10	2																				12
Shizugawa (Japan)		4	6		6	2										2								20
Sugashima (Japan)	1		10										1				4							16
Usujiri (Japan)								4														1	2	7
Venice (Med. Europe)			25		3																			28
Whangamata Habour (Nez Zealand)			14																					14
Winchester Bay (NW America)		4	3			3																		10
Woods Hole (NE America)			11																					11

PUBLICACIONES ANEXAS

A continuación se adjuntan las publicaicones de Rius *et al.* (2012) y Pineda *et al.* (2012) como comentamos anteriormente en la Estructura de la Tesis:

Rius M, Turon X, **Ordóñez V**, Pascual M (2012) Tracking invasion histories in the sea: facing complex scenarios using multilocus data. *PLoS ONE* 7: e35815. doi:10.1371/journal.pone.0035815

Pineda MC, McQuaid CD, Turon X, López-Legentil S, **Ordóñez V**, Rius M (2012a) Tough adults, frail babies: an analysis of stress sensitivity to abiotic factors across early life-history stages of widely introduced marine invertebrates. *PLoS ONE* 7(10): e46672. doi:10.1371/journal.pone.0046672

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PLoS one

Tracking Invasion Histories in the Sea: Facing Complex Scenarios Using Multilocus Data

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Abstract

In recent years, new analytical tools have allowed researchers to extract historical information contained in molecular data, which has fundamentally transformed our understanding of processes ruling biological invasions. However, the use of these new analytical tools has been largely restricted to studies of terrestrial organisms despite the growing recognition that the sea contains ecosystems that are amongst the most heavily affected by biological invasions, and that marine invasion histories are often remarkably complex. Here, we studied the routes of invasion and colonisation histories of an invasive marine invertebrate *Microcosmus squamiger* (Ascidiacea) using microsatellite loci, mitochondrial DNA sequence data and 11 worldwide populations. Discriminant analysis of principal components, clustering methods and approximate Bayesian computation (ABC) methods showed that the most likely source of the introduced populations was a single admixture event that involved populations from two genetically differentiated ancestral regions - the western and eastern coasts of Australia. The ABC analyses revealed that colonisation of the introduced range of *M. squamiger* consisted of a series of non-independent introductions along the coastlines of Africa, North America and Europe. Furthermore, we inferred that the sequence of colonisation across continents was in line with historical taxonomic records - first the Mediterranean Sea and South Africa from an unsampled ancestral population, followed by sequential introductions in California and, more recently, the NE Atlantic Ocean. We revealed the most likely invasion history for world populations of *M. squamiger*, which is broadly characterized by the presence of multiple ancestral sources and non-independent introductions within the introduced range. The results presented here illustrate the complexity of marine invasion routes and identify a cause-effect relationship between human-mediated transport and the success of widespread marine non-indigenous species, which benefit from stepping-stone invasions and admixture processes involving different sources for the spread and expansion of their range.

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Introduction

Selective forces and demographic processes have shaped community composition and biogeographic patterns of the world's ecosystems over millions of years of evolution [1]. Genetic tools have enabled researchers to infer the evolutionary history and to understand the biogeography of many taxa in order to reveal how these processes have occurred [2–4]. However, the recent increase in large-scale environmental impacts of anthropogenic origin [5] has extensively modified evolutionary trajectories of populations across species ranges [6]. Biological invasions are a direct consequence of such broad human-driven habitat alteration, constituting a crucial factor shaping biodiversity and biogeographic patterns worldwide [7]. The recent increase in research on biological invasions has stimulated debates regarding the evolutionary importance of such invasions [8,9] and the key aspects of the invasion process such as its long-term consequences and predictability [10–12].

Genetic studies have been recognized as crucial to uncover the pathways of the introduction of non-indigenous species (NIS), colonisation histories, and the origin of these introductions [13,14]. However, genetic studies of NIS have certain methodological, and especially analytical, limitations [15,16]. For instance, assignment tests and dendrograms might fail to provide accurate assignments of introduced populations, especially when studies analyse populations that have been under severe genetic drift during and after introduction, or that have multiple or unsampled sources [17]. New analytical tools, such as the approximate Bayesian computation (ABC) methods [18–21], have recently been utilized in studies of biological invasions to overcome some of these limitations and reconstruct demographic history using genetic data [22–26].

Most research studies which have successfully implemented ABC methods have focussed on terrestrial ecosystems, analysing species in their native environment [27,28] and assessing historical processes within their introduced range (e.g. [22,23,26,29]). In marine ecosystems only a few studies have looked at long-term evolutionary scenarios using ABC methods (e.g. [30,31]), while no study to date has used this approach to investigate marine NIS. The use of ABC methods in studies of marine NIS is particularly relevant because the sea contains ecosystems that are amongst the most heavily affected by biological invasions worldwide [32]. Furthermore, there is a growing recognition that marine biological invasions are often extremely complex due to the high prevalence of multiple sources and non-independent introductions [15,33].

The majority of the species responsible for marine invasions are from lower trophic levels [34]. Among these, sessile filter feeder invertebrates are recognized as one of the most important groups [35,36]. Most of these organisms have a planktonic larval stage with limited dispersal capabilities [37–39]. However, larvae can be caught in ballast pumps and survive in transit to other harbours, or adults attached to floating structures such as drift algae or loose debris can be pumped in or gravitated into the ship (through large openings where the water simply flows into the vessel) [40]. In addition, adults can also be transported as fouling on the hulls and sea chests of ships and recreational vessels [41–43], and can release their offspring in the locations where these ships stop.

Here, we studied a sessile marine invertebrate, *Microcosmus squamiger* (Tunicata, Ascidiacea), to infer the colonisation histories and routes of invasion of its world populations. We obtained a dataset comprising microsatellite and mitochondrial DNA (mtDNA) data from populations located within the vast distributional range of *M. squamiger*, and analysed them using a variety of analytical tools, including the ABC methods. We specifically compared different scenarios to understand: (1) whether the introductions were independent or not, (2) the origin of the colonisers, and (3) the sequence of the different introduction events worldwide.

Methods

Studied species

The solitary ascidian *Microcosmus squamiger* is native to Australia [33,44,45] but is now well established on most continents. This species was first recorded outside its introduced range in Bizerte (Tunisia) in the early 1960s [46], but has since been recorded all over the western Mediterranean Sea and adjacent Atlantic Ocean region [47,48]. Subsequently, this species was detected in California in 1986 [49], on the NE Atlantic coast in 1994 [50,51], in South Africa in 2000 [52], in New Zealand in 2003 [53], in India in 2006 [54], and recently in Japan (T. Nishikawa, pers. comm.). However, the first report of *M. squamiger* outside its native range might in fact have been from along the southern African coast. Millar [55,56] reportedly found the congeneric *Microcosmus exasperatus* in surveys conducted between 1950 and 1956 along this coastline. However, considering that: 1) Both *M. squamiger* and *M. exasperatus* are widespread species that can easily be confused [47], 2) Millar's descriptions [55,56] were oversimplified and not sufficiently detailed to distinguish between the two species, and 3) *M. exasperatus* has never again been reported along the coast of South Africa [52,57]; it is highly likely that Millar [55,56] misidentified the samples and that they were in fact *M. squamiger* (see also [52]). Consequently, we consider that the first records of *M. squamiger* as a NIS could have been in South Africa in the 50 s and in the Mediterranean Sea in the 60 s, and not in 1983 as stated elsewhere [58]. Within the localities of its introduced range, *M. squamiger* is abundantly found on both natural and artificial substrata [59–61]. It can generally be found in or close to large shipping harbours or marinas [47,61–63], and has occasionally been found in open coastal habitats where *M. squamiger* can be highly invasive and colonise all available hard substrata forming dense aggregates [47,59]. Ascidians are sessile organisms that have very poor dispersal capabilities, restricted to their short-lived lecithotrophic larvae [37,38]. Consequently, the natural active spread of *M. squamiger* is restricted to an extremely limited swimming period [64]. Thus, transoceanic relocation of this species is through shipping [33,47], which makes tracking the movement of NIS very challenging [65,66].

Sample collection

We sampled *Microcosmus squamiger* specimens from 11 sites covering most of the introduced and native ranges of the species (see Table 1 and Fig. 1). No specific permits were required for the described field sites. We collected 24–28 specimens per sampling site through SCUBA diving or by pulling up harbour ropes, ensuring in all cases a distance of a few meters among the different individuals when collected. We dissected the specimens *in situ* and a piece of muscular tissue from the mantle was immediately preserved in absolute ethanol. Once in the laboratory we replaced the ethanol with new absolute ethanol and stored the samples at -80°C until DNA extraction.

DNA extraction, PCR amplification and genotyping

We extracted DNA from each individual using the REALPURE extraction kit (Durviz, València, Spain). We amplified by PCR six polymorphic loci (MS6, MS7, MS10, MS11, MS12 and MS13) that had been isolated for this species [67]. The PCR conditions used were based on 20 µL total reaction volume, with 0.5 µL of each primer (10 µM), 2.5 µL dNTPs (10 mM), 4 µL 5× buffer, 1.8–3 µL $MgCl_2$ (25 mM), 9.5 - 8.3 µL H_2O, 1 U Taq polymerase (Promega) and 1 µL DNA. An initial denaturation at 94°C for 5 min was followed by 30 cycles consisting of a denaturation step at 94°C for 1 min, an annealing step at 53 to 57°C (see [67] for details) for 30 sec and an extension step at 72°C for 30 sec, and a final extension at 72°C for 5 min. The forward primer of each locus was labelled with different fluorescent dyes [67]. We estimated allele sizes based on the standard Rox (70–500 bp, Bioventures) using a capillary sequencer 3730 DNA Analyzer (Applied Biosystems) and the software GeneMapper™ (v. 3.5, Applied Biosystems) from the Serveis Científico-Tècnics of the Universitat de Barcelona. In addition to the microsatellite genotypes, we obtained mtDNA sequences of the same individuals from a previous study [33].

Analysis of genetic diversity and population structure using microsatellite data

We used the GenAlex programme v. 6.1 [68] to calculate allele frequencies found in each population and microsatellite locus and to run a Mantel test to compare pairwise-population matrices, and to convert our data file to required formats for other programmes. We calculated linkage disequilibrium between pairs of loci in each population using Genepop v. 4.0 [69], while we used Genetix v. 4.05.2 [70] to test deviations from Hardy-Weinberg equilibrium using the inbreeding coefficient (F_{IS}) and estimated its significance (10000 permutations).

We assessed pairwise population differentiation using the microsatellite dataset and the *D* estimator [71] computed with the programme DEMEtics v. 0.8–3 [72]. The significance of the pairwise comparisons was evaluated using the built-in randomization procedure of DEMEtics. The use of *D* has been advocated to overcome some of the shortcomings of conventional statistics such as F_{ST} or G_{ST}, as it performs better when the goal is to estimate genetic differentiation [72,73]. Nonetheless, and follow-

Table 1. Collection sites of *Microcosmus squamiger* including geographical regions, population abbreviations (Code), type of habitat (O: outside harbour, I: inside harbour) and number of individuals analysed.

	Geographical region	Country	Sites	Code	Latitude/Longitude	Habitat	Sample size	He	H_O	Na	Np	AR	F_{IS}	*h*
Native populations														
	Australasia	Australia	Bunbury	BU	33°19′13″S/115°39′39″E	I	24	0.558	0.514	5.17	1	4.935	0.081	0.692
	Australasia	Australia	Albany	AL	35°01′56″S/117°53′25″E	O	24	0.611	0.512	5.17	4	4.965	**0.165**	0.712
	Australasia	Australia	Manly	MA	27°27′10″S/153°11′22″E	O	24	0.535	0.410	5.50	8	5.146	**0.238**	0.867
Introduced populations														
	North America	Mexico	Bahía Falsa	BF	37°56′18″N/122°25′36″W	O	24	0.550	0.431	4.67	1	4.504	**0.220**	0.825
	Southern Africa	South Africa	Port Elizabeth	PE	33°57′60″S/25°38′06″E	I	24	0.531	0.438	4.17	1	4.029	**0.179**	0.596
	NE Atlantic Ocean	Spain	Santander	SA	43°27′45″N/3°47′22″W	I	28	0.622	0.548	5.17	0	4.900	0.121	0.641
	NE Atlantic Ocean	Portugal	Cascais	CAS	38°41′34″N/9°25′03″W	I	24	0.529	0.354	4.50	0	4.366	**0.335**	0.841
	NE Atlantic Ocean	Spain	Cádiz	CAD	36°31′51″N/6°17′03″W	I	24	0.515	0.394	4.00	0	3.811	**0.239**	0.674
	Mediterranean Sea	Spain	Ceuta	CE	35°53′43″N/5°18′44″W	I	24	0.546	0.511	4.83	4	4.691	0.065	0.587
	Mediterranean Sea	Spain	Cubelles	CU	41°11′37″N/1°39′17″E	O	24	0.545	0.445	4.50	2	4.449	**0.187**	0.853
	Mediterranean Sea	Spain	Barcelona	BA	41°20′33″N/2°09′41″E	I	24	0.509	0.444	4.50	1	4.311	0.129	0.875

Diversity estimates based on microsatellites are as follows: He - mean expected heterozygosity (Nei's gene diversity), H_O - mean observed heterozygosity, Na - mean number of alleles per locus, Np - number of private alleles, AR - mean Allelic Richness, F_{IS} - inbreeding coefficient with significant values in **bold**, and. *h* - haplotype diversity in COI (data from [33]).

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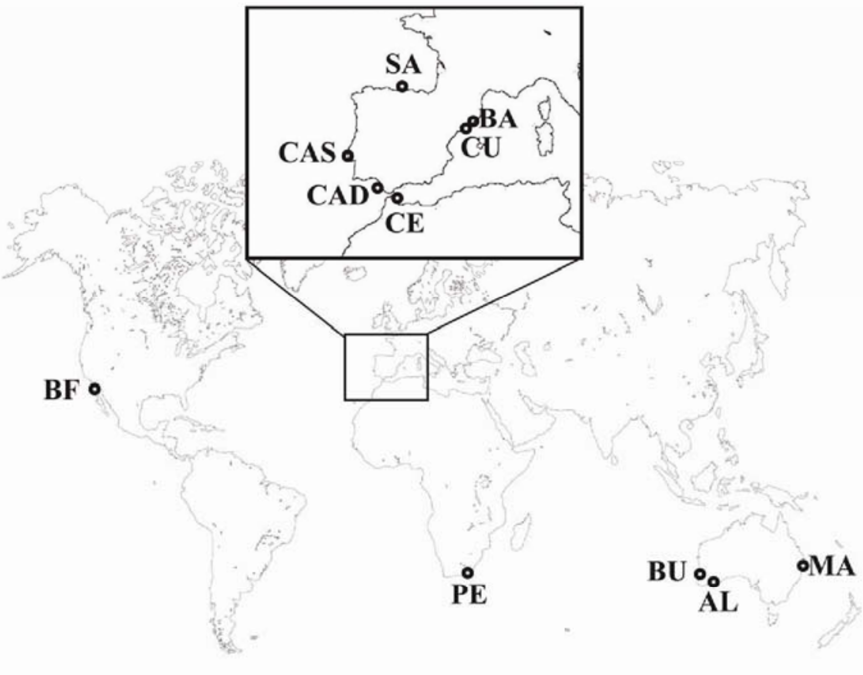

Figure 1. Map of the sampled sites of *Microcosmus squamiger*. The Atlanto-Mediterranean region has been enlarged. Collection sites are abbreviated as in Table 1.

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ing recent advice [73], we also estimated population divergence using the conventional F_{ST} estimates for comparison, while a randomization test was used to test the existence, or lack thereof, of significant genetic differentiation for each population pair across all loci. These analyses were done using the Arlequin v. 3.5 programme [74]. For both measures of population differentiation we corrected pairwise *P*-values using the sequential Bonferroni method [75].

Identification of clusters of genetically related individuals and spatial ordination of between-group structures

In order to have a visual assessment of between-population differentiation, we performed a discriminant analysis of principal components (DAPC) [76]. This technique extracts information from genetic datasets (multivariate in nature) by first performing a principal component analysis (PCA) on pre-defined groups or populations, and then using the PCA factors as variables for a discriminant analysis (DA), which seeks to maximize the inter-group component of variation. The previous PCA step ensures that the variables input to DA are uncorrelated [76].

We performed DAPC using the *adegenet* package for R [77]. A file including microsatellite and mtDNA data was used and DAPC was performed (function dapc) using pre-defined groups corresponding to populations or groups of populations (see Results). Variables were centred but not scaled. In all analyses 50 principal components of PCA were retained as input to DA. The procedure also provides estimates of the probability with which the DA recovers the true group membership of the individuals.

The programme STRUCTURE v. 2.3 was used to detect the number (K) of genetically homogeneous populations in our microsatellite dataset [78]. We used the Admixture and loc prior model because it performs better than other models for detecting genetic structure even in situations of low levels of genetic divergence or a limited number of loci [79]. Following the recommendations of Evanno et al. [80], we calculated an ad hoc statistic IncK based on the rate of change in the log probability of data between successive K-values, since it provides a good estimator to accurately detect the number of population groups. For each dataset we quantified, using 20 runs, the mean and standard deviation of the likelihood of each K. We tested a range of K values depending on the number of populations included in the analysis: 1 to 13 (when all populations were included), 1 to 5 (for native populations) and 1 to 10 (for introduced populations). CLUMPP v.1.1.2 [81] was used to merge the results across the 20 runs for the best selected K while DISTRUCT v.1.1 [82] was used to visualize the results.

Unravelling the routes of invasions and colonisation histories using ABC methods

In order to obtain relevant and detailed information of the routes of invasions and colonisation histories of *M. squamiger*, we designed a series of sets of evolutionary scenarios and analysed them with ABC methods using the DIYABC v. 1.0.4.41 programme [83]. Given that mtDNA could be affected by adaptive selection [84], we first ran all analyses using microsatellites and subsequently used a combined dataset of microsatellites and mtDNA. Preliminary simulations indicated an absence of genetic bottlenecks when comparing introduced and ancestral populations, and no founder effects in introduced populations (see Results). Thus, we did not consider the presence of bottlenecks for the different sets of competing scenarios. In some scenarios we incorporated the presence of an unsampled population, which acted as an invasive bridgehead population (see [26]), from which other introductions originated.

In order to define each set of scenarios we used prior distributions of demographic parameters (Table S1) based on information regarding the biological and invasion traits of the studied species. To estimate the time of events (in number of generations) for this species, we considered that *M. squamiger* has two overlapping generations per year based on what is know about its life cycle [59]. To estimate the effective population size of each population, we considered information on past geological processes of the Australian region and marine vectors of invasive species. During the late Pleistocene, periglacial deposits formed as a result of cold-climate processes in Australia [85], which most-likely prevented *M. squamiger* from surviving in this area. At the beginning of the Holocene, the global climate became warmer [86] and Australia acquired a climate similar to that found today, allowing species such as *M. squamiger* to thrive in this area. Consequently, we considered that the populations included in this study could have originated ca. 10000 years BP (Table S1). Regarding the introduced populations, we assumed that all *M. squamiger* introductions occurred after the first European sailors visited the Australian shores around 400 years BP [87] (Table S1). Regarding extra-range colonisations, taxonomic records indicate that *M. squamiger* has been outside Australia for at least 60 years, and thus we assumed that introductions within the introduced range only occurred over the last 100 years. Finally, we assumed that the effective population size was the same for all populations and used a uniform distribution bounded between 10–100000 individuals (Table S1).

The first set of scenarios aimed to infer whether the colonisations within the introduced range were independent or not. For this we first divided the introduced populations into four groups according to major geographical regions as defined in Table 1. Three scenarios were compared: two that considered independent colonisations from Australia to the different regions of the introduced range, and one that incorporated an unsampled population from where all introduced populations originated as a result of a non-independent colonisation (see Fig. S1A). In order to establish the sequence of colonisation for the two independent scenarios, we used the taxonomic records of each region as a guideline, and because the Mediterranean Sea and South Africa were colonised around the same time, we interchanged the order of appearance of these regions in the two independent scenarios.

The second set of scenarios focussed on the origin of the introduced populations. For this, we first considered each of the native populations as separate entities, and then followed the outcome of the analysis of the previous set of scenarios (see Results) to group the introduced populations together. Regarding the ancestral populations, both the DAPC and STRUCTURE (see below) showed that Bunbury and Manly were the closest populations genetically to the introduced cluster, while Albany remained separated. Accordingly, preliminary simulations revealed that the exclusion of the population of Albany from the analyses did not affect the final outcome. We thus excluded this population from subsequent analyses. In the first scenario we considered that the introduced populations originated from Manly, as suggested by mtDNA data [33]. The second scenario considered that the colonisers originated from Bunbury. Finally, a third scenario contemplated an admixture event that involved these two Australian populations (Fig. S1B).

The third set of scenarios aimed to assess the sequence of worldwide colonisations across regions. We first grouped the populations according to geographic regions as in Table 1 and assumed that all introduced populations originated from an unsampled ancestral population (see Results). As stated above, the faunistic records indicate that the first introduction of *M. squamiger* was in South Africa or the Mediterranean Sea, followed by California and the NE Atlantic. However, because recurrent colonisations might have obscured the reconstruction of the introduction history of this species, we did not limit our analyses to what the faunistic records indicated but included several other geographical sequences (see Fig. S1C). This approach allowed us to simultaneously examine the independence of the different introductions from an ancestral bridgehead population and the colonisation sequence that these introductions followed.

For all sets of scenarios we used 10^6 simulated data per scenario to build a reference table. To compute posterior probabilities of the competing scenarios, we used the 1% of the simulated datasets closest to the observed data (using Euclidean distances between each simulated and observed dataset) to estimate the relative posterior probability (with 95% confidence intervals) of each scenario with a logistic regression [19]. For each set of scenarios, the most likely scenario was the one with the highest posterior probability value and non-overlapping 95% confidence intervals, using both a dataset with only microsatellite loci and one that combined microsatellites and mtDNA.

We assessed the sensitivity of different priors that included different effective population sizes and mutation models for the combined dataset of microsatellites and mtDNA. For this, we performed simulations using the first set of scenarios, which focussed on whether or not the colonisations within the introduced range were independent. Different prior sets were used to test the robustness of demographic estimates as follows: Prior set 1) standard priors as described in Table S1; Prior set 2) uniform distributions of effective population sizes bound between 10 and 10^6 diploid individuals for N, Nau and Nu; Prior set 3) stepwise mutation model for microsatellite loci; and Prior set 4) no insertion-deletion mutation rate in microsatellites flanking regions.

Results

Genetic diversity and differentiation of *M. squamiger* populations

A total of 268 individuals were genotyped using six microsatellite loci. The number of alleles per locus ranged from 4 at the MS7 locus to 18 for the MS10 locus (Table S2). No linkage disequilibrium was observed between loci pairs after correction for multiple tests by false discovery rate [88] and thus all loci were considered independent. The mean expected heterozygosities were higher than the observed ones in all populations and the global F_{IS} values were significant in all cases except for Bunbury (Australia) and three sites in Spain (Santander, Ceuta and Barcelona) (Table 1). The significant homozygote excess was mainly due to loci MS11 and MS12 in the remaining introduced populations (Table S3). The population that showed the highest number of private alleles was Manly, Australia (Table 1). On average, native populations showed higher numbers of private alleles than the introduced ones (mean ± S.E., 4.33±2.03 and 1.12±0.48, respectively) but the differences were not significant (*t*-test, $t=-1.54$, $P=0.25$). The mean allelic richness was significantly higher in native than in introduced populations (mean ± S.E., 5.01±0.07 and 4.38±0.12, respectively; *t*-test, $t=-4.55$, $P<0.01$). However, when we analysed the numbers of mtDNA COI haplotypes [33], no significant differences among native and introduced populations were found (mean ± S.E., 10±1 and 7.87±0.85 respectively; *t*-test, $t=-1.62$, $P=0.16$). Both native and introduced populations showed similar microsatellite heterozygosity (mean ± S.E., 0.568±0.02 and 0.54±0.01, respectively; *t*-test, $t=-0.96$, $P=0.40$) and mtDNA haplotype diversity (mean ± S.E., 0.757±0.05 and 0.736±0.04, respectively; *t*-test, $t=-0.29$, $P=0.78$). When we compared the observed and expected gene diversity of the microsatellite alleles found in each population, the programme BOTTLENECK [89] did not detect bottlenecks for the populations of the introduced range (Table S4). We used IAM and TPM mutation models given the allele size distribution found in the *M. squamiger* microsatellite dataset.

The mean *D* in pairwise comparisons was smaller among introduced (0.005±0.006, mean ± SE) than among native (0.227±0.058) populations, and the same was found for the F_{ST} estimates (0.047±0.006 and 0.105±0.027, respectively). After correcting for multiple tests, 38 out of 55 *D* values indicated significant genetic differentiation (Table 2), while 17 comparisons were not significant, of which 16 involved introduced populations. Pairwise F_{ST} values yielded very similar results (Table 2), although in this case 22 comparisons were not significant, again involving mostly (20 pairs) introduced populations. Both estimators of population-pairwise differentiation were strongly correlated as shown by the Mantel test ($r=0.922$, $P<0.001$).

Spatial ordination of the studied populations

The DAPC showed that the 50 principal components of the retained PCA explained 95.3% of the total variance. The scatterplot of the first two components of the DA (Fig. 2A) showed that the native populations were set apart from the introduced ones, which formed a tight cluster with no discernible structure. The first axis separated the two Australian sites of Albany and Bunbury populations from the rest, while the second axis set apart the other Australian population (Manly). No clear overlap of the inertia ellipses existed between the introduced and the native populations, although Bunbury and Manly appeared closer to the introduced group than Albany. When we considered individuals belonging to four groups (three for each of the Australian populations and a single group for the introduced ones), a high proportion (92.2%) of individuals were correctly assigned to their original group using the classification functions obtained in the DA. We then repeated the analysis using only the introduced populations to infer subtle patterns that could have been obscured by the analysis of the entire dataset. A sum of 98.5% of the total variance was then explained by the 50 retained principal components of the PCA. The populations appeared mixed in the space delimited by the first two axes of the DA (Fig. 2B). The first axis showed the South Africa population at one extreme and Santander (Spain) at the other. The second axis mainly separated Bahía Falsa (California) from the rest. The percentage of individuals correctly assigned to their population of origin was relatively low (67.3%), which was in accordance with the low population differentiation observed.

When we ran the analysis using the programme STRUCTURE and included all populations, lncK presented the highest value for $K=2$ (Fig. S2A), suggesting the presence of two genetically differentiated units. The graphical representation of all populations using $K=2$ (Fig. 3A) revealed that the Australian population of Albany had a higher percentage of individuals assigned to one of the two groups, while the introduced populations included the majority of individuals belonging to the other group with a probability of assignment higher than 80%. The individuals belonging to the other two Australian populations (Manly and Bunbury) were mostly assigned to the same group of the introduced populations although the probability of assignment was much lower (Fig. 3A). When we repeated the analysis with only the native populations, lncK was the highest for $K=2$ (Fig. S2B) and the graphical output grouped individuals from the

Table 2. Measures of genetic differentiation based on the microsatellite loci in pairwise comparisons of the studied populations of *Microcosmus squamiger*.

	BU	AL	MA	BF	PE	SA	CAS	CAD	CE	CU	BA
BU		**0.1303**	**0.2209**	**0.0648**	**0.1054**	**0.1504**	0.0532	**0.1227**	**0.1164**	**0.1327**	**0.1040**
AL	**0.0519**		**0.3304**	**0.2497**	**0.2834**	**0.3468**	**0.2622**	**0.3017**	**0.2850**	**0.3399**	**0.2843**
MA	**0.1261**	**0.1374**		**0.1507**	**0.1227**	**0.2346**	**0.1321**	**0.1853**	**0.1174**	**0.1661**	**0.1341**
BF	0.0324	**0.0860**	**0.0941**		0.0323	**0.0707**	0.0404	**0.0752**	0.0182	0.0401	0.0342
PE	**0.0581**	**0.1081**	**0.0899**	0.0188		**0.1199**	0.0215	**0.0538**	0.0344	**0.0557**	0.0338
SA	**0.1087**	**0.1351**	**0.1370**	**0.0624**	**0.0864**		**0.1090**	**0.1089**	**0.0732**	0.0341	**0.0780**
CAS	0.0258	**0.1067**	**0.0945**	0.0227	0.0128	**0.0766**		0.0061	**0.0569**	0.0323	0.0189
CAD	**0.0731**	**0.1229**	**0.1294**	**0.0482**	0.0285	**0.0686**	0.0040		**0.0926**	**0.0467**	0.0433
CE	**0.0606**	**0.1072**	**0.0747**	0.0134	0.0217	**0.0455**	0.0342	**0.0575**		0.0198	0.0017
CU	**0.0823**	**0.1416**	**0.1171**	0.0307	0.0416	0.0131	0.0247	0.0278	0.0163		−0.0024
BA	**0.0531**	**0.1144**	**0.0948**	0.0212	0.0202	**0.0448**	0.0121	0.0280	−0.0001	−0.0009	

D values are shown above the diagonal and F_{ST} values below the diagonal. In **bold** are significant comparisons after sequential Bonferroni correction. Population abbreviation names as in Table 1.

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western (Bunbury) and southwestern (Albany) Australian populations which appeared highly differentiated from Manly, the eastern population (Fig. 3B). In the analysis including only introduced populations, the highest IncK was observed for K = 4 (Fig. S2C). The resulting assignment detected mixed origin for all individuals with varying contributions from the four clusters (Fig. 3C). However, four groups were observed: the first included individuals from South Africa and California (Port Elizabeth and Bahía Falsa, respectively), the second consisted of a single population (Santander), which was the most differentiated of the four groups (Table 2), the third group included the other Atlantic populations (Cascais and Cádiz), while the fourth group consisted of all Mediterranean populations (Barcelona, Cubelles and Ceuta).

Revealing the colonisation histories and invasion routes of *M. squamiger* using ABC methods

We found a good fit between the scenario-posterior combination and the pseudo-observed data for all sets of scenarios, as shown by both the PCAs and the comparisons between the observed and simulated summary statistics (i.e. model-check option) as implemented in the DIYABC programme (data not shown). The results from the first set of scenarios convincingly showed that the most likely scenario was the one that included non-independent colonisations across *M. squamiger*'s introduced range (Table 3, Fig. 4A). When testing the reputed origin of the colonisers (i.e. the second set of scenarios), the results favoured the admixed scenario when the analysis was run only with microsatellites (Table 3, Fig. 4B). However, when the analysis was run with microsatellite and mtDNA data together, the most likely scenario was the one that assumed Bunbury as the single origin of the introduced populations (Table 3). To resolve these conflicting results, we ran an analysis with a dataset that only included mtDNA and we obtained contradictory results between the direct and logistic regression estimates of the posterior probabilities - the direct estimate showed that the most likely scenario was the one that considered Manly (eastern Australia) as the only origin of colonisers ($P = 0.926$, C.I = 0.696, 1.000), while the logistic regression showed that the most supported scenario was the one that considers Bunbury (western Australia) as the origin ($P = 0.978$, C.I. = 0.867, 1.000). Thus, the outcome of the direct estimate was in accordance with a previous study [33] but the logistic regression supported a different outcome. Considering this lack of consistency of the posterior probability, we considered that the DIYABC programme could be selecting incorrect scenarios when divergent population histories revealed by mtDNA and nuclear DNA were mixed, possibly due to selection acting upon mtDNA. Therefore, we considered the results obtained with unlinked microsatellite loci as the most plausible.

Regarding the third set of scenarios (i.e. sequence of worldwide introductions), no scenario could be conclusively selected because the confidence intervals of the scenarios with the highest posterior probabilities overlapped with the intervals from other scenarios (Table 3). However, when considering the microsatellite data alone, the confidence intervals of the scenario with the highest probability (scenario 5, Fig. 4C) marginally overlapped with those of scenarios 3 and 4 (Table 3). Scenario 5 represented a sequential colonisation of the introduced range from an unsampled population that was in accordance with the historical taxonomic records. Conversely, the highest probability for the dataset including both microsatellites and mtDNA was scenario 3 (Table 3), although its confidence intervals overlapped with those of scenario 4. For the same reasons stated above we considered the results obtained with unlinked microsatellite loci as the most reliable.

The demographic parameters obtained for the most supported scenario (i.e. the scenario with the highest posterior probability) of each set of scenarios are shown in Table S5. A high effective population size was detected in both native and introduced populations, although this was slightly larger in the former. The colonisation times among the sites of the introduced range differed by approximately 100 years. When we tested, for the first set of scenarios, the robustness of our inferences on demographic parameters using different priors, the posterior probability indicated that under all prior sets the most supported scenario was Scenario 3 (Table S6, Fig. S1A). Regarding the inferences on demographic parameters, the estimated values of colonization times varied slightly among prior sets, while the differences of effective population sizes were not as negligible (Table S6). Nevertheless, we found that in all cases the confidence intervals for the posterior probabilities were the smallest for the standard prior (prior set 1), which was used to test the different sets of scenarios.

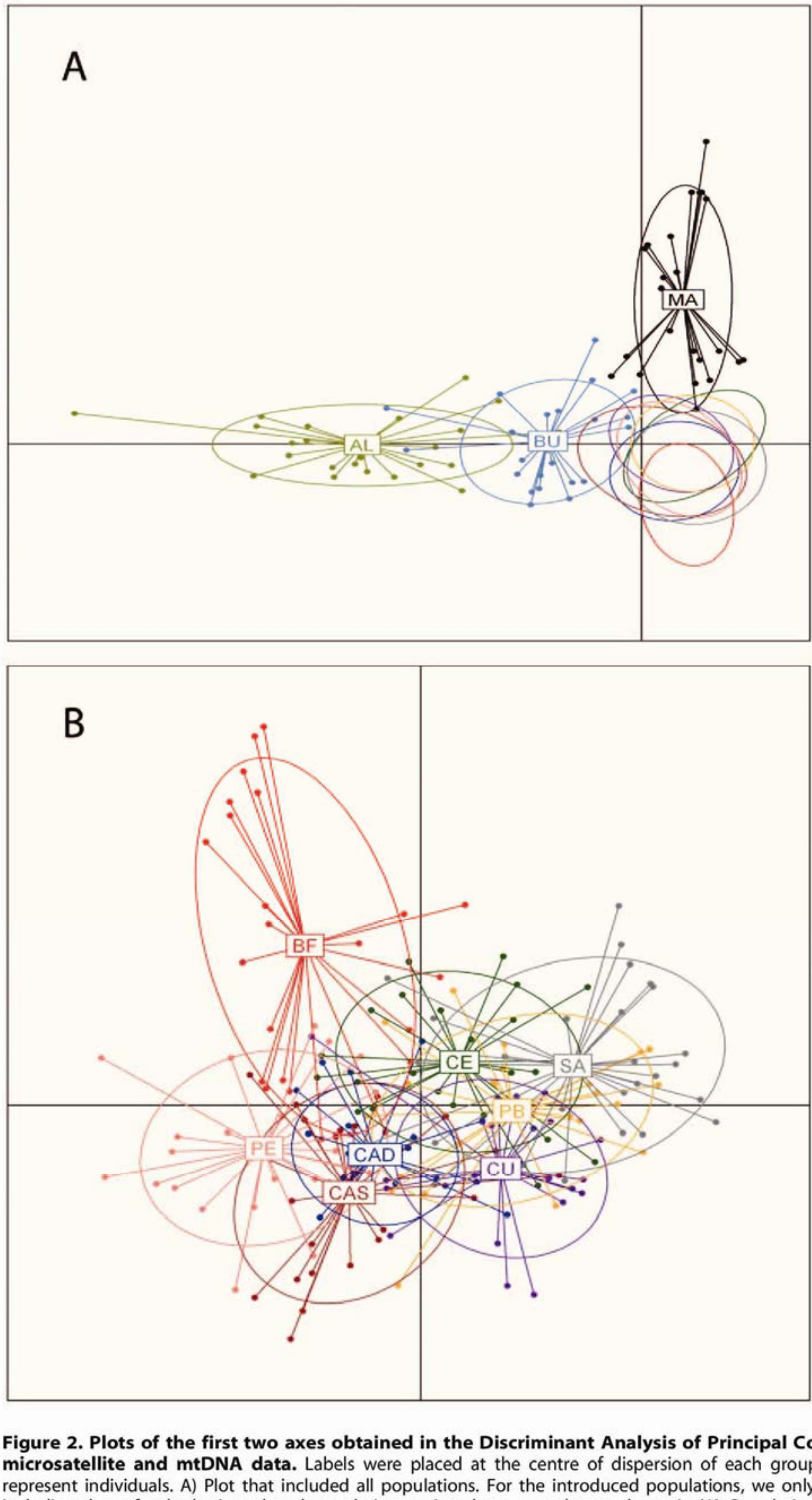

Figure 2. Plots of the first two axes obtained in the Discriminant Analysis of Principal Components using a combined dataset of microsatellite and mtDNA data. Labels were placed at the centre of dispersion of each group, further delineated by inertia ellipses. Dots represent individuals. A) Plot that included all populations. For the introduced populations, we only included ellipses to avoid cluttering, B) Plot including data of only the introduced populations, using the same colour codes as in (A). Population names abbreviated as in Table 1.
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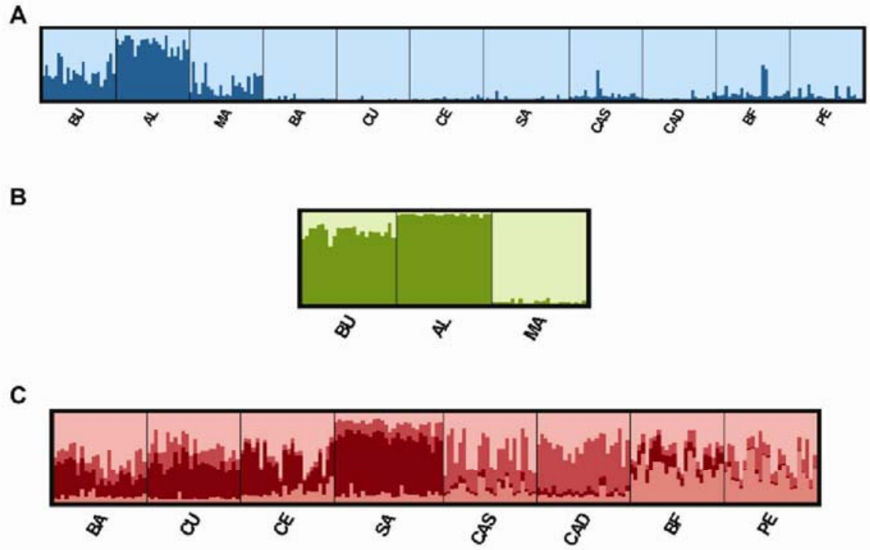

Figure 3. Population structure in native and introduced populations of *Microcosmus squamiger* with the most likely number of populations (K) inferred with the STRUCTURE programme. A) Including all populations, B) Native populations, and C) Introduced populations. Population abbreviations as in Table 1.
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Discussion

Genetic diversity and differentiation

The results of the present study are consistent with a scenario of high population connectivity among introduced populations and absence of genetic bottleneck. The analysis of microsatellite data of *M. squamiger* showed that the introduced populations have elevated levels of genetic diversity, similar to those found in native populations. The role of genetic diversity during the colonisation process has been at the heart of recent debates in invasion biology research [15,90–93]. This is because the relative importance of the trade-off between loses of genetic diversity due to bottleneck processes during colonisation, and high genetic diversity as a result of multiple origins or introduction events, remains to be fully understood. The results found for *M. squamiger* indicated that the spread of introduced populations has not been limited by reductions of genetic diversity both in terms of nuclear (this study) and mtDNA [33]. However, we found higher allelic richness and a higher number of private alleles in native populations compared to the introduced ones, as previously seen for mtDNA [33], although these differences were not large. This indicates that despite an absence of bottlenecks in the introduced populations, part of the genetic make-up of the species was lost during the colonisation process and only retained in the native area.

Strong genetic differentiation was detected among Australian populations using microsatellite loci, with a major genetic break between the population found on the eastern coast and the two western populations. This genetic differentiation was observed by pairwise comparisons, either using D or F_{ST} values, as well as with STRUCTURE and DAPC. Moreover, this genetic barrier was also found using F_{ST} statistics on mtDNA data from the same populations [33]. This genetic break could be due to isolation by distance with low connectivity between these two coastal regions or as a result of selection due to local adaptation [94]. However, caution is necessary to interpret these findings, as we only had data from three Australian populations and thus we had a limited representation of the genetic diversity in that region. Further studies with an increased sampling size within the native range of *M. squamiger* are needed to ensure a fine-scale analysis of this pattern.

The introduced populations had smaller values of differentiation (both estimated as D and as F_{ST}) than those found when comparing native populations. Only 43% (D) and 29% (F_{ST}) of the pairwise comparisons among introduced populations showed significant differentiation in terms of allelic frequencies. A higher genetic similarity among introduced populations than among native ones was also shown by the clustering of these populations in the DAPC and STRUCTURE. This similarity among introduced populations was also reflected by the presence of some loci of low frequency alleles that were only shared among introduced populations (see Table S2). Finally, ABC methods supported non-independent colonisation in distant introduced populations, suggesting recurrent human-mediated transportation among basins, as observed in other invasive species [15,26,90,95]. When we tested for isolation by distance by correlating genetic and geographic distances between all population pairs using a Mantel test (data not shown), we found marginally significant correlations among genetic divergence and geographical distance. When this analysis was restricted to the introduced populations, we found a non-significant relationship. The lack of correlation between genetic and geographic distance among introduced populations strongly points to the role of anthropogenic dispersal as a key factor shaping the genetic composition of these introduced populations. Therefore, transoceanic ship transport has enabled the colonisation of localities that are separated by long distances.

Tracking the origin of introduced populations

In the present study, the Australian sites of Bunbury and Manly comprised the native populations that appeared closer to the introduced populations in the DAPC and STRUCTURE. In line with this, the ABC analyses supported an admixture of both Manly and Bunbury as a source of the introduced populations, when the analysis was run with microsatellite loci only. However, when both mtDNA and microsatellite markers were combined the most likely scenario that emerged was the one considering Bunbury as the origin of colonisers. This contradictory result might be indicative

Table 3. Posterior probabilities and 95% confidence interval of the competing scenarios of each set of scenarios using approximate Bayesian computation methods.

Set of Scenarios	Scenario	Dataset	Posterior Prob	Confidence Interval
Independent vs non-independent colonisations				
AUS→PE; AUS→MED; AUS→BF; AUS→ATL	1	MICROS	0.0000	[0.0000,0.0000]
AUS→MED; AUS→PE; AUS→BF; AUS→ATL	2	MICROS	0.0000	[0.0000,0.0000]
AUS→U→Merge four introduced regions	**3**	**MICROS**	**1.0000**	**[1.0000,1.0000]**
AUS→PE; AUS→MED; AUS→BF; AUS→ATL	1	MICROS+mtDNA	0.0000	[0.0000,0.0000]
AUS→MED; AUS→PE; AUS→BF; AUS→ATL	2	MICROS+mtDNA	0.0000	[0.0000,0.0000]
AUS→U→Merge four introduced regions	**3**	**MICROS+mtDNA**	**1.0000**	**[1.0000,1.0000]**
Origin of colonising populations				
BU→MA; MA→Pooled introduced populations	1	MICROS	0,0004	[0.0002,0.0005]
BU→MA; BU→Pooled introduced populations	2	MICROS	0.3448	[0.2892,0.4003]
BU→MA; BU+MA→Pooled introduced populations	**3**	**MICROS**	**0.6549**	**[0.5994,0.7104]**
BU→MA; MA→Pooled introduced populations	1	MICROS+mtDNA	0.0048	[0.0000,0.0104]
BU→MA; BU→Pooled introduced populations	**2**	**MICROS+mtDNA**	**0.8241**	**[0.6654,0.9829]**
BU→MA; BU+MA→Pooled introduced populations	3	MICROS+mtDNA	0.1710	[0.0166,0.3254]
Sequence of worldwide introduction				
AUSc→U; U→MED; U→PE; U→BF; U→ATL	1	MICROS	0.0079	[0.0058,0.0099]
AUSc→U; U→PE; U→MED; U→BF; U→ATL	2	MICROS	0.0305	[0.0232,0.0378]
AUSc→U; U→MED; U→PE; U→BF; MED→ATL	3	MICROS	0.1980	[0.1660,0.2300]
AUSc→U; U→PE; U→MED; U→BF; MED→ATL	4	MICROS	0.1971	[0.1629,0.2313]
AUSc→U; U→MED; MED→PE; PE→BF; MED→ATL	**5**	**MICROS**	**0.2710**	**[0.2292,0.3128]**
AUSc→U; U→PE; PE→MED; PE→BF; MED→ATL	6	MICROS	0.1666	[0.1368,0.1965]
AUSc→U; U→PE; PE→ATL; ATL→MED; PE→BF	7	MICROS	0.1015	[0.0804,0.1227]
AUSc→U; U→BF; BF→PE; PE→ATL; ATL→MED	8	MICROS	0.0274	[0.0207,0.0340]
AUSc→U; U→MED; U→PE; U→BF; U→ATL	1	MICROS+mtDNA	0.0122	[0.0071,0.0173]
AUSc→U; U→PE; U→MED; U→BF; U→ATL	2	MICROS+mtDNA	0.0300	[0.0186,0.0415]
AUSc→U; U→MED; U→PE; U→BF; MED→ATL	**3**	**MICROS+mtDNA**	**0.2889**	**[0.2198,0.3579]**
AUSc→U; U→PE; U→MED; U→BF; MED→ATL	4	MICROS+mtDNA	0.2210	[0.1613,0.2806]
AUSc→U; U→MED; MED→PE; PE→BF; MED→ATL	5	MICROS+mtDNA	0.1664	[0.1200,0.2129]
AUSc→U; U→PE; PE→MED; PE→BF; MED→ATL	6	MICROS+mtDNA	0.1243	[0.0881,0.1605]
AUSc→U; U→PE; PE→ATL; ATL→MED; PE→BF	7	MICROS+mtDNA	0.1273	[0.0891,0.1656]
AUSc→U; U→BF; BF→PE; PE→ATL; ATL→MED	8	MICROS+mtDNA	0.0298	[0.0199,0.0398]

The dataset used (microsatellite loci - MICROS or both MICROS and mtDNA) is indicated. The scenarios with the highest probability are shown in **bold**. Abbreviations of single and clustered populations are as in Figure 4. 'U' indicates the unsampled population. Scenario numbers are as in Figure S2.
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of different evolutionary processes affecting nuclear and mitochondrial markers [96]. Mitochondrial DNA acts as a single unit due to the lack of recombination, thus selection in one mtDNA locus might leave a footprint in all remaining mitochondrial loci by hitchhiking. In line with this, it has been shown that this widely used marker is affected by selection in animals, which questions its usefulness in evolutionary studies [84]. However, we must also consider the possibility that putative source populations remain unsampled.

The results of our study suggest that the widespread distribution of *M. squamiger* has stemmed from the combined introductions of individuals from the east and west coasts of Australia - two highly differentiated areas in terms of genetic composition - through an unsampled bridgehead population. This process might have granted genetic variability and adaptation potential, which could subsequently have promoted the invasion fitness of this species (e.g. [97,98]). Multiple introductions have been attributed as the driver that enables the establishment of several invasive terrestrial and aquatic species (e.g. [58,99]) and recurrent human-mediated introductions might promote their range expansion (e.g. [100]). It is remarkable that most of the regions where *M. squamiger* has been introduced are coastal areas with a Mediterranean climate, where this species exhibits phenological cycles that peak in summer [59]. Temperature therefore seems to be an important factor influenc-

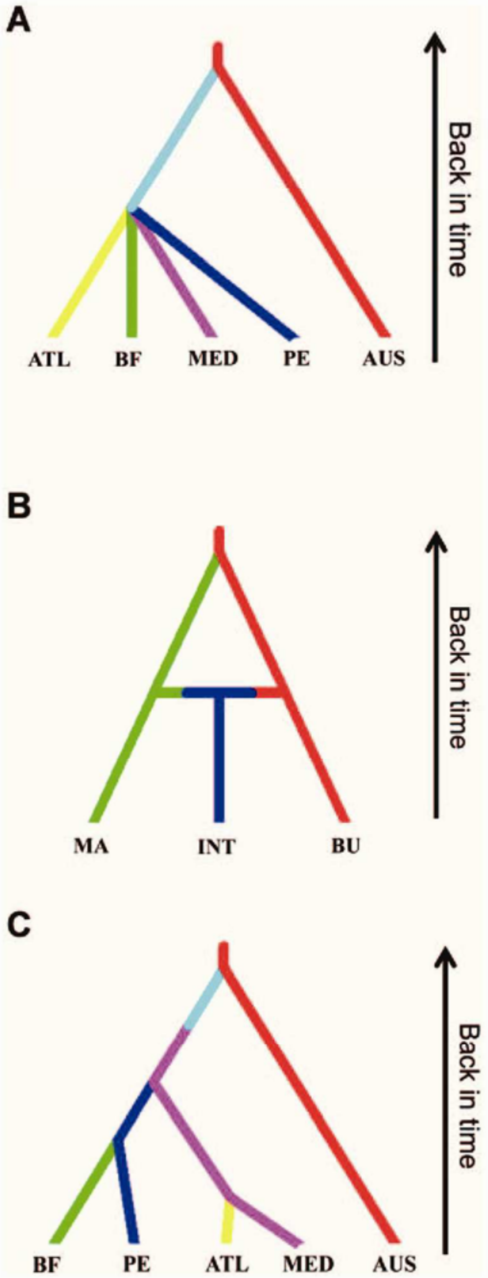

Figure 4. Most-likely scenarios of each set of scenarios using approximate Bayesian computation methods on microsatellite data of *Microcosmus squamiger*: A) Independent vs non-independent colonisations; B) Origin of colonising populations, indicating the admixture process between the two ancestral populations; C) Sequence of worldwide introductions. The Y-axis indicates the time of events (not to scale). Abbreviations of single and clustered populations are as follows: Bahía Falsa (BF), Bunbury (BU), Manly (MA), Port Elizabeth (PE), Introduced (INT), Australian (AUS), NE Atlantic (ATL) and Mediterranean Sea (MED). The unsampled population in A) and C) is indicated by a faint blue colour.
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ing this species' welfare within the introduced areas. It might also represent a relevant factor influencing the distribution of this species worldwide, by limiting the range expansion of *M. squamiger* in other non-Mediterranean climatic regions, especially those at high latitudes.

Tracking the sequence of introduction

The ABC analyses based on microsatellite data revealed a sequence that matched with the temporal series from the historical taxonomic records. This result was surprising considering the extensive artificial transportation of exotic ascidians in recent times (e.g. [35,49]), which have been responsible of homogenizing the genetic composition among regions, as seen in other widespread species [101,102].

We found a strong link between the populations of the NE Atlantic Ocean and the Mediterranean Sea, while California and South Africa appeared as genetically separated regions. The close genetic relationship between the NE Atlantic Ocean populations and those from the Mediterranean Sea could be a matter of geographical proximity that enhanced connectivity both through commercial and recreational shipping [42,103] within the region. The Atlanto-Mediterranean transition offers a unique geographic location for the study of marine biological invasions as it has a high concentration of harbours and marinas in the area, and shipping has been intense for many centuries. Genetic studies analysing the similarity between populations of benthic organisms of this region indicate that the Strait of Gibraltar represents an important barrier for dispersal (see [104–106]), although human mediated transport may easily overcome this obstacle [107,108]. In the case of *M. squamiger*, Rius et al. [33] indicated that the Strait of Gibraltar did not seem to act as an important barrier for this species. The results of the present study, however, do not seem to support this hypothesis, as three groups based on STRUCTURE were detected. The first group comprised the Mediterranean populations (Ceuta, Barcelona, Cubelles), which had a similar genetic composition and a consequent lack of significant differences in allele frequencies. The high genetic similarity between these populations could be due to a higher rate of human transportation within the same basin. The second group included two of the Atlantic populations, namely Cascais and Cadiz, and their similarity could be as a result of shipping. The last group comprised the Atlantic population of Santander, which appeared highly differentiated from the rest of the populations. This population also appeared in a peripheral position in the DAPC. Given that this introduced population also had higher allelic richness than the others in the area, we hypothesize that this population might have been established before the other NE Atlantic populations by a larger number of individuals or that recurrent introductions might have shaped its genetic composition. If Santander was excluded, the genetic differentiation (*D* values) in inter-basin (i.e. on both sides of the Strait of Gibraltar) comparisons were low (0.048 ± 0.010, mean $\pm$ SE), but much higher than those in intra-basin comparisons (0.006 ± 0.005), which indicates that the Strait of Gibraltar (and associated intense shipping) plays a relevant role in structuring these populations.

In the present study we revealed important characteristics of the introduction process of a widespread marine species. Firstly, we obtained consistent evidence of the non-independent nature of the world colonisations. Subsequently, we found that an admixture event between populations from the east and west Australian coasts was most likely responsible for shaping the genetic composition of the introduced populations of the species. In addition, this admixture event resulted on an unsampled population that served as a stepping-stone between native and introduced populations. Finally, we found that the sequence for the introduced range followed the historical taxonomic records, with the first colonisations occurring in the Mediterranean Sea and South Africa, followed by California and thereafter in the NE Atlantic.

We showed that the combined use of multilocus data obtained from samples of both native and introduced populations with new analytical methods allows for discrimination among complex evolutionary scenarios, and is a powerful approach to understand invasion histories of widespread marine organisms. The translocation of coastal marine species from one distant region to another is increasing [57,65,109,110], enhancing admixture processes and invasive bridgehead effects, which ultimately facilitate marine introduced species to spread and thrive within their introduced range. Such processes enhance NIS population connectivity and constitute an increased threat to native communities.

Supporting Information

Figure S1 Set of scenarios used to infer the colonisation histories of *Microcosmus squamiger* using approximate Bayesian computation analyses: A) Independent vs non-independent colonisations, B) Origin of colonising populations, C) Sequence of worldwide introductions. The Y-axis indicates the prior time of events (not to scale) as in Table S1. Abbreviations for populations and groups of populations are as in Figure 4. The unsampled population in A) and C) is indicated by a faint blue colour, and the temporal parameters are as in Table S1.
(DOC)

Figure S2 Plot of lncK as described in Evanno et al. [80] as a function of the number of clusters (K) across the 20 runs: (A) for the whole dataset, (B) for the native populations and (C) for the introduced populations.
(DOC)

Table S1 Prior distribution parameters describing the set of scenarios investigated for *Microcosmus squamiger*. The time priors were constrained ($t1<t2<t3<t4<t5<t6$) and comprised of split or admixed events. Nau: effective population size of the ancestral populations, Nu: effective population size of the unsampled bridgehead population, N: effective population size of each cluster of introduced populations, SNI: single nucleotide indel. All populations had the same prior regarding their effective population size.
(DOC)

Table S2 List of allele frequencies found in each population of *Microcosmus squamiger*. Population abbreviations as in Table 1.
(DOC)

Table S3 Inbreeding coefficient (F_{IS}) and its significance (significant results in bold) for each locus in each population of *Microcosmus squamiger*. Total number of alleles (N_A) per locus are indicated.
(DOC)

Table S4 Probability of Wilcoxon signed rank test to detect bottlenecks for the introduced populations of *Microcosmus squamiger*. Mutation model abbreviations are as follows: IAM - Infinite alleles model and TPM - Two-phase model.
(DOC)

Table S5 Demographic parameters obtained for the most supported scenario in each set of scenarios using microsatellite markers (see Table 3). Demographic parameter abbreviations as in Table S1. The establishment of the unsampled population (t5), colonisation of the Mediterranean Sea (t4), Southern Africa (t3), North America (t2) and NE Atlantic (t1) regions, are indicated.
(DOC)

Table S6 Robustness of prior choice in discriminating among introduction scenarios (Table 3, Independent *vs* non-independent colonisations) and inferences of demographical parameters. All values correspond to the highest probability scenario in each prior set (i.e. Scenario 3).
(DOC)

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Author Contributions

Conceived and designed the experiments: MR. Analyzed the data: MR VO XT MP. Contributed reagents/materials/analysis tools: MR VO XT MP. Wrote the paper: MR VO XT MP. Generated the data: MR VO.

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Tough Adults, Frail Babies: An Analysis of Stress Sensitivity across Early Life-History Stages of Widely Introduced Marine Invertebrates

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Abstract

All ontogenetic stages of a life cycle are exposed to environmental conditions so that population persistence depends on the performance of both adults and offspring. Most studies analysing the influence of abiotic conditions on species performance have focussed on adults, while studies covering early life-history stages remain rare. We investigated the responses of early stages of two widely introduced ascidians, *Styela plicata* and *Microcosmus squamiger*, to different abiotic conditions. Stressors mimicked conditions in the habitats where both species can be found in their distributional ranges and responses were related to the selection potential of their populations by analysing their genetic diversity. Four developmental stages (egg fertilisation, larval development, settlement, metamorphosis) were studied after exposure to high temperature (30°C), low salinities (26 and 22‰) and high copper concentrations (25, 50 and 100 µg/L). Although most stressors effectively led to failure of complete development (fertilisation through metamorphosis), fertilisation and larval development were the most sensitive stages. All the studied stressors affected the development of both species, though responses differed with stage and stressor. *S. plicata* was overall more resistant to copper, and some stages of *M. squamiger* to low salinities. No relationship was found between parental genetic composition and responses to stressors. We conclude that successful development can be prevented at several life-history stages, and therefore, it is essential to consider multiple stages when assessing species' abilities to tolerate stress. Moreover, we found that early development of these species cannot be completed under conditions prevailing where adults live. These populations must therefore recruit from elsewhere or reproduce during temporal windows of more benign conditions. Alternatively, novel strategies or behaviours that increase overall reproductive success might be responsible for ensuring population survival.

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Introduction

Abiotic factors such as temperature, salinity and habitat characteristics have long been considered primary factors affecting survival, fitness and distribution of marine organisms [1]. More recently, anthropogenic changes to the environment have yielded new agents of selection, with resistance to pollution being one of the most important [2,3]. Thus, the persistence of human-mediated stressors in the environment nowadays contribute to shaping the distribution of marine organisms, excluding some (e.g. [4]) and facilitating the establishment of others (e.g. [5]). Moreover, a species' long-term performance is modulated by abiotic factors across multiple life-history stages, including adulthood [6–9], and embryonic and larval development (e.g. [10–12]). Among these, embryogenesis, settlement and metamorphosis are critical life-history phases for many organisms (e.g. [13,14]), especially when exposed to anthropogenic stressors [15–17]. For sessile marine organisms, where adults are unable to escape unfavourable abiotic conditions, the importance of successful early stages is even more striking as it determines the viability of local adult populations [18–20]. This in turn can have community-level consequences as many sessile species act as ecosystem engineers, *sensu* [21], providing habitat for multiple associated organisms while excluding competitors for space.

The arrival and establishment of non-indigenous species (NIS) via man-mediated transport is a major factor altering communities worldwide (e.g. [22,23]). Shipping facilities such as harbours and marinas often act as entrance gates for NIS [24–28], and thus newcomers have to be able to cope with the stressful conditions (e.g. pollution, disturbance) that characterize these altered habitats. Establishment of NIS in such environments depends on physical and biological conditions being suitable not only for adults [29–31] but also for juvenile stages (e.g. [32]).

Genetic diversity is an important factor influencing the establishment of NIS [33–36] and it is generally assumed that the richer the genetic composition of a species' population, the wider its potential ability to adapt to stressful environmental situations [35,37]. The heritability of traits under selection depends on stress-response variation within a population, and the potential for rapid evolution in new environments [17,38]. For NIS, the latter can be problematic as introduced species often experience genetic bottlenecks that can reduce the genetic diversity needed for selection [35,39,40]. The study of genetic variability of introduced populations is essential to understanding NIS tolerance of environmental stresses and their potential to spread. To date, however, few studies have considered how different levels of parental genetic diversity in NIS influence offspring responses to multiple stressors.

Genotype-environment interactions are generally considered when differences in response between genotypes are not consistent from one environment to another, and have been investigated to assess, for instance, phenotypic stability [41] or genotypic responses to lethal and non-lethal stresses [42]. Most studies on genotype-environment interaction have analysed the influence of abiotic conditions during adulthood (e.g. [43]), while studies covering different, presumably more sensitive, early life-history stages remain rare. In line with this, genetic markers can be used to characterize different populations and to relate differences in biological response to genetic diversity and differentiation between and within populations.

Here we investigated the performance across multiple life-history stages of two widely introduced marine invertebrate species in locations where both species coexist. The solitary ascidians *Styela plicata* (Lesueur, 1823) and *Microcosmus squamiger* (Michaelsen, 1927) are sessile organisms that have been introduced worldwide [44,45] and that often inhabit places with highly variable abiotic conditions [7,46–48]. The success of introductions of *S. plicata* to new habitats has been linked to its high tolerance of polluted waters and changes in temperature and salinity [7,10,49], while *M. squamiger* is known to be resistant to low salinities as adults [46]. In addition, previous genetic studies of these widespread species based on a fragment of the mitochondrial gene Cytochrome Oxidase subunit I (*COI*) have revealed the existence of two highly divergent and widely distributed haplogroups for each species [44,45]. No information is available, however, on the functional significance of this intraspecific genetic structure in terms of responses to stress. In this study, we targeted several early life-history stages (fertilization, larval development, settlement, and metamorphosis) and we genetically characterized the progenitors. We tested species performance under thermal and salinity stress, and with several concentrations of a heavy metal (Cu). We hypothesised that *S. plicata* and *M. squamiger* offspring would develop well under realistic environmental conditions found in sheltered habitats where adults occur, although different haplogroups might respond to stress differently.

Methods

Field sites and general methods

Adult individuals of *Styela plicata* and *Microcosmus squamiger* were collected during the austral spring of 2010 (October and November) when both species are known to reproduce [50–52]. Two sites along the South African coast, approximately 160 km apart, were sampled: Port Elizabeth (33°57′44″S, 25°38′8″E) and Knysna (34°02′32″S, 23°02′40″E). The selection of the sampling sites was based on the co-occurrence and abundance of the studied species and the availability of genetic data from previous studies [44,45]. The sites feature slightly different characteristics in terms of abiotic parameters and ship traffic. Port Elizabeth harbour is an industrial port, with temperature oscillating around 16–18°C in winter and 22–22°C in summer, although there can be rapid fluctuations of up to about 10°C (range c. 14–24°C) due to periodic influx of warm water from the Agulhas Current and cooler upwelled water from nearby Cape Recife [53]. There is little freshwater input into the bay as a whole and salinities are consistently close to oceanic norms of 35.2‰ [54]. Copper concentrations in this harbour are between 0.5 μg/L and 11.3 μg/L [55]. In contrast, Knysna lagoon supports only recreational boating and can have a partially estuarine regime, with alternation of freshwater influx and tidal input. Salinities in the lagoon are generally similar to the open ocean [56], although freshwater conditions can reign in the area during unusually wet periods, displacing non-euryhaline organisms (M.R., *pers. obs.*). Temperatures generally oscillate around 15°C in winter and 22–24°C in summer. Copper levels in the waters of Knysna Lagoon range between <0.1 to 4.7 μg/L [57,58].

After collection, individuals were transported to the laboratory (located less than 6 h away) in insulated containers containing water from the collection site. Since seawater from harbours usually contains high concentrations of pollutants [55,57,59,60], we collected seawater from nearby clean sites far from any urban or industrial influence (33°58′47″S, 25°39′29″E for Port Elizabeth, 34°03′42″S, 23°22′38″E for Knysna). Animal storage and all laboratory experiments were conducted using this seawater, which we previously filtered using a vacuum filtration unit with 10 μm pore filters. Individuals were kept in the laboratory at constant temperature (20°C) and water aeration, for a minimum of 12 h and a maximum of four days for acclimatization. During the storage period in aquaria, temperature and salinity were monitored and modified to maintain the desired conditions. We used constant artificial illumination to prevent light-induced spawning [61]. All the samples were obtained according to current South African regulations. This species are not protected by any law and all sampling was conducted outside protected areas.

Experimental trials

We chose an array of abiotic factors (temperature, salinity and pollution) that are known to influence survival of marine invertebrates [10,46], and analysed four early life-history processes: fertilisation, development of the larvae, settlement and metamorphosis. Temperatures were set to either 20°C (control) or 30°C (treatment) in a Constant Environment (CE) room. Seawater temperature of 30°C represents the higher values occasionally reached in summer within the distributional area of both species [62]. Distilled water was added to seawater to achieve reduced salinity values (26‰ and 22‰) similar to those that are known to affect ascidian development and survival and can be found in estuaries [48,63]. For the pollution treatments, we used copper because it is known to be one of the most toxic heavy metals for marine invertebrates [64], especially during early life-history stages [65–67]. We added liquid copper (Spectrosol® ref.14139 1000 ppm copper standard solution) to filtered seawater to attain the desired concentrations: 25 μg/L, mean concentration in a polluted harbour [2]; 50 μg/L, common in highly polluted harbours or near boats recently painted with antifouling paint [68] and 100 μg/L, an extreme copper concentration often used in this type of studies [69].

Gamete extraction, fertilisation and experimentation

Gametes were extracted by dissecting the ripe gonads as described in Svane and Young [70] and Marshall et al. [71]. A mix of eggs and sperm was poured through a 100-µm filter with seawater into a small beaker to retain the eggs in the filter and gather the sperm and seawater in the beaker. For each fertilisation attempt (see Table 1 for details), around 10 individuals were dissected: 5 individuals for eggs and 5 for sperm (both species are simultaneous hermaphrodites). The oocytes obtained from the 5 female donors (around 12 to 18 ml per individual, ~500 eggs ml^{-1}) were subsequently pooled together, and the same was done with the sperm obtained from the 5 male donors (~10^7 sperm ml^{-1}).

For the fertilization and larval development assays, 6 ml of the oocyte suspension, 12 ml of the corresponding treatment solution (filtered seawater for the temperature treatment, other treatments adjusted to obtain the desired final concentrations after mixing with gametes), and 2 ml of concentrated sperm mix were added to a 65 mm Petri dish. The cultures were then immediately taken to the appropriate CE room for fertilisation. After 1 hour, the eggs were washed with the treatment solution to remove excess sperm using a 100-µm filter and then distributed among five Petri dishes (~100–500 eggs per dish) containing 12 ml of the treatment solution at the appropriate concentrations, and closed with a lid to avoid evaporation during the experimental period. This first set of cultures was used to assess fertilisation and development rates.

To obtain enough larvae to conduct the settlement and metamorphosis assays, new individuals were obtained from each species (Table 1) and fertilized in an aerated beaker containing 500 ml filtered seawater and maintained in a CE room at 20°C to maximize development rates [72]. Post-hatching experiments consisted of 40 larvae carefully pipetted out and placed in a Petri dish with 12 ml of the corresponding treatment solution (5 replicates per treatment and location). Petri dishes were previously submerged in seawater for 24 h to develop a biofilm in order to facilitate larval settlement [73,74]. All Petri dishes were then placed in CE rooms (30°C for the temperature treatment and 20°C for the rest of experimental conditions) and kept for 4 days.

Data collection and analyses

For both species, most of the larvae hatched within 14 hours of fertilisation at 20°C. Numbers of viable larvae, larvae with deformities (or immature larvae), undeveloped embryos and unfertilized eggs (Fig. 1) were then recorded using a stereomicroscope. Likewise, the numbers of settled, completely metamorphosed and unattached larvae were assessed every 24 h over 4 days (96 h) in the settlement and metamorphosis assays. The fertilisation rate (FR), development rate (DR), settlement rate (SR) and metamorphosis rate (MR) were calculated as follows:

$$FR = \frac{(\text{viable larvae} + \text{larvae with deformities} + \text{undeveloped embryos})}{(\text{total initial number of eggs})} * 100$$

$$DR = \frac{(\text{viable larvae})}{(\text{viable larvae} + \text{larvae with deformities} + \text{undeveloped embryos})} * 100$$

$$SR = \frac{(\text{settled individuals after 96 h} + \text{metamorphosed individuals 96 h})}{(\text{total number of initial larvae})} * 100$$

$$MR = \frac{(\text{metamorphosed individuals after 96 h})}{(\text{settled individuals 96h} + \text{metamorphosed individuals 96 h})} * 100$$

We analyzed two types of variables, the proportion of success at each developmental stage (i.e. fertilization rate, development rate, settlement rate, and metamorphosis rate) for controls and treatments, and the relative success ratios (RS) obtained by dividing the value of each rate by the mean of the corresponding controls. The former was used to assess treatment effects against the controls. For site effects, as differences between sampled sites often occurred even in the controls, the RS were an appropriate assessment of the effect of interest (i.e., whether development was impaired differentially in one site with respect to the other, after eliminating the effect of differences in controls).

For both types of variables, we performed separately two-way analyses of variance (ANOVA) per species with site and treatment as fixed factors. We used a logit transformation of the FR, DR, SR and MR data as it is known to stabilize the variances of proportional data better than other commonly used methods [75]. Our transformed data had homogeneity of variances in all datasets, although normality was only accomplished in a few cases. Nonetheless, we performed the ANOVA tests as they are robust to departures from normality when variances are homogeneous [76]. For the relative success rates (RS), the data complied in all cases with the homoscedasticity assumption, although they weren't normally distributed in some cases. As several transformations tried did not improve this, we proceeded with the raw data in the analyses.

For the proportion data, used to assess treatment effects, if the interaction between factors was significant, post-hoc analyses of treatments were performed at each site against the control with Dunnett's test. If the interaction was not significant, post-hoc tests on treatment levels were done combining both sites. For the RS variables, used to determine site differences, when interaction was significant, site effects were assessed within each level of treatment (using a post-hoc Student-Newman-Keuls test). If interaction was not significant, no test was necessary as site had only two levels. In

Table 1. Artificial fertilisation runs for each species and sampled site.

Species	Population	Fertil. Date	N.Indiv.	Parameters studied
S. plicata	Port Elizabeth	8th October	10	Settlement & Metamorphosis
		16th October	10	Fertilization & Larval Development
	Knysna	24th October	10	All parameters
M. squamiger	Port Elizabeth	8th October	9	Settlement & Metamorphosis
		5th November	6	Fertilization & Larval Development
	Knysna	24th October	10	All parameters

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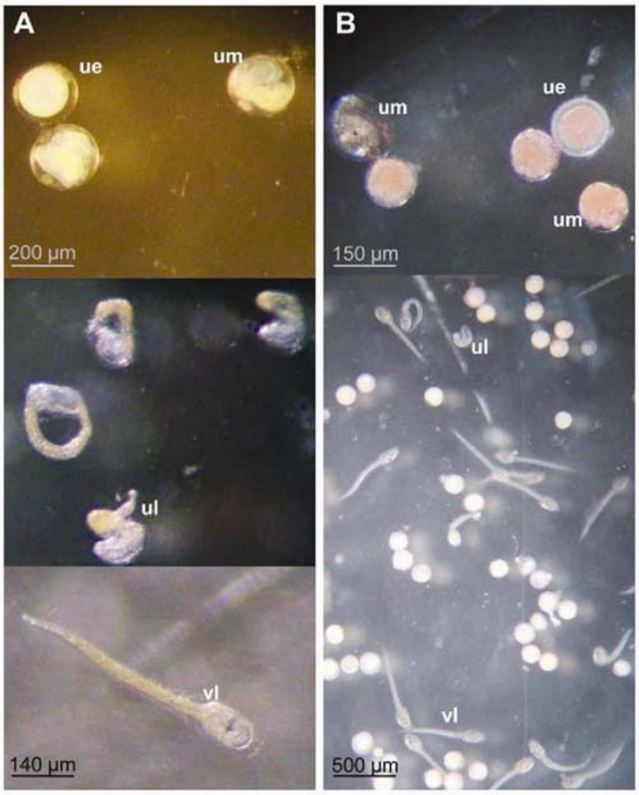

Figure 1. Picture of eggs and larvae under stereomicroscope. a) *S. plicata* and b) *M. squamiger* (ue: unfertilized egg; um: undeveloped embryo; ul: unviable larvae; vl: viable larvae).
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all post-hoc analyses, the residual mean square obtained from the original two-way ANOVAs was used to calculate the standard errors of the means for the post-hoc comparisons [76,77]. Statistical analyses were performed using the software STATISTICA v. 6.1 (©StatSoft, Inc. 1984–2004).

In order to obtain an overall estimate of success (from egg fertilisation to post-metamorphic formation), we also calculated the cumulative % success of the different stages, for each of the treatments. For this purpose, each of the different rates (FR, DR, SR, MR) was multiplied by the mean of the previous stage.

Screening of parental genotypes

A piece of muscular tissue from the mantle or the siphon of each individual used for fertilisation was dissected and immediately preserved in absolute ethanol (Table 2). After a few hours, the tainted ethanol was replaced by new absolute ethanol and samples were then stored at $-20°C$ until extracted. Total DNA was extracted using the REDExtract-N-Amp Tissue PCR Kit (Sigma-Aldrich). The universal primers LCO1490 and HCO2198 described in Folmer et al. [78] were used to amplify a fragment of the *COI* gene (maternally inherited). Amplifications were performed in a final volume of 20 µL using 10 µL of REDExtract-N-amp PCR reaction mix (Sigma-Aldrich), 0.8 µL of each primer (10 µM), and 2 µL of template DNA. The PCR program consisted of an initial denaturing step at 94°C for 2 min, 30 amplification cycles (denaturing at 94°C for 45 seconds, annealing at 50°C for 45 seconds and extension at 72°C for 50 seconds), and a final extension at 72°C for 6 min, on a PCR System 9700 (Applied Biosystems). PCR products were sent for purification and sequencing to Macrogen Inc. (Seoul, Korea). Sequences were edited and aligned using BioEdit® v.7.0.5.3 [79]. Number of alleles (*Nh*), gene diversity (*Hd*), and nucleotide diversity (π) were computed with DnaSP v.5 [80]. Pairwise genetic distances (F_{ST}) using allele frequencies were calculated with Arlequin v.3.1 [81] and their significance was assessed by performing 10,000 permutations. Note that because each fertilization attempt involved a combination of gametes from ten different donors, offspring resulted from a random combination of these genotypes.

Results

Experimental trials

The results of the ANOVA on the different logit-transformed rates are presented in Tables 3 and 4. The results of post-hoc site comparisons using relative success rates are presented in Fig. 2, where these rates are depicted. All abiotic conditions analyzed: temperature at 30°C, salinity values of 22‰ (22S) and 26‰ (26S), and copper at a concentration of 25 µg/L (Cu25), 50 µg/L (Cu50) and 100 µg/L (Cu100), produced important effects on the relative success ratio of each developmental stage considered, with differences due to both species and the site of adult collection. There was no consistent trend of one of the sites having higher or lower success rates, although many outcomes differed significantly between sites (Tables 3,4; Fig. 2).

S. plicata. There were significant interactions of treatment and site for all dependent variables. *S. plicata* showed significantly reduced fertilisation rates (FR) in most treatments (Table 3). Knysna gametes seemed somewhat less affected by the treatments than Port Elizabeth (Fig. 2a). All treatments had significant effects in Port Elizabeth, while gametes from Knysna were unaffected by temperature and Cu25 (Table 3). Significant site differences were found for 26S and Cu25, where fertilization relative to controls was significantly higher in Knysna (Fig. 2a).

The development of viable larvae (DR) was probably the most sensitive stage in the early development of this species (Fig. 2a) and was significantly impaired by all treatments, except for Cu25 and Cu50 (Table 3). Notably, the presence of Cu25 increased DR relative to the controls (thus relative success rates were above 1), although the effect is significant only in Knysna. Significant inter-site differences in relative success rates were found only for Cu50, with embryos from Port Elizabeth being more resistant.

Settlement rate (SR) tended to show higher relative success values (Fig. 2a) than the previous variables, indicating that this stage is somewhat more tolerant. All treatments except 26S yielded significantly low values for Port Elizabeth larval settlement, while no significant effect was detected for Knysna (Table 3). Relative success values in Port Elizabeth were significantly lower for 22S, and significantly higher for Cu50. Although the effect of salinity (26S) on SR was not significant, low salinities did appear to accelerate settlement within 24 hours (Fig. 3a). On the other hand, Cu100 seemed to accelerate settlement for larvae from Port Elizabeth adults but to delay it for Knysna (Fig. 3a).

As for settlement, metamorphosis in *S. plicata* (MR) was also a relatively tolerant process under most treatments and for both sites. The strongest inhibition effect on MR occurred at 22S and Cu100 for both sites (Fig. 2a), and these treatments yielded significantly lower metamorphosis than the controls at both sites (Table 3). In addition, the metamorphosis of Knysna larvae was also impaired at 26S, Cu25 and Cu50 (Table 3). Site differences were significant in the three copper treatments, with relative success rates higher in Port Elizabeth. Increased temperature accelerated the metamorphosis of the settled individuals within 72 hours, although low salinities had the opposite effect, causing a delay in metamorphosis (Fig. 3a). Most of the larvae from the 22S

Table 2. Diversity measures and population differentiation values (F_{ST}) for the mtDNA sequences (*COI* gene).

Species	*Pop.*	*N*	*Nh*	*Hd*	±SD	π	±SD	*Haplotypes*	*Lineage*	*Fst*	*p-value*
S. plicata	PE	20	2	0.100	(±0.088)	0.00292	(±0.00257)	Hap 2 (0.5)	*I*	0.7278	<0.001
								Hap 5 (0.95)	*II*		
S. plicata	KN	10	2	0.556	(±0.075)	0.00095	(±0.00013)	Hap 1 (0.5)	*I*		
								Hap 2 (0.5)	*I*		
M. squamiger	PE	13	6	0.769	(±0.103)	0.0035	(±0.00173)	Hap 7 (0.08)	*I*	−0.048	0.991
								Hap 53 (0.08)	*I*		
								Hap 1 (0.46)	*II*		
								Hap 5 (0.23)	*II*		
								Hap 9 (0.08)	*II*		
								Hap 23 (0.08)	*II*		
M. squamiger	KN	10	6	0.844	(±0.103)	0.00495	(±0.00257)	Hap 14 (0.1)	*I*		
								Hap 1 (0.40)	*II*		
								Hap 5 (0.20)	*II*		
								Hap 54 (0.1)	*II*		
								Hap 55 (0.1)	*II*		
								Hap 56 (0.1)	*II*		

Mitochondrial lineages according to Rius et al. [44] and Pineda et al. [45]. Number of individuals analyzed per population (*N*).
Number of haplotypes per population (*Nh*), Haplotypic (*Hd*) and nucleotidic (π) diversity, and their corresponding standard deviations in brackets. Pairwise genetic distances (F_{ST}).
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and Cu100 treatments never achieved complete metamorphosis within 96 hours, and none did so in Port Elizabeth at Cu100 concentration (Fig. 3a).

M. squamiger. All treatments except Cu25 significantly reduced the fertilisation rates (Fig. 2b, Table 4) at both sites combined (no significant interaction term), and the most drastic reduction was observed after exposure to 26S, 22S, Cu50 and Cu100 (Fig. 2b). For the relative success rates (RS), the interaction term was not significant, and there was an overall effect of site, with mean success rates higher in Port Elizabeth.

As for *S. plicata*, larval development was the most sensitive stage (Fig. 2b). The interaction was not significant and, combining sites, all treatments significantly reduced DR, especially high temperature, 26S, 22S, Cu50 and Cu100 (Table 4, Fig. 2b). When analysing relative success rates, the interaction proved significant, and this was due to the outcome of the Cu25 treatment, being significantly higher in Knysna.

Settlement was also less affected by temperature and salinity treatments than the previous processes (Fig. 2b). The three copper concentrations resulted in significantly lower SR than the controls in Knysna, while only Cu50 and Cu100 reduced settlement of larvae from Port Elizabeth (Table 4). High temperatures and low salinities increased the number of settlers relative to the controls (values above 1, Fig. 2b), with a significant positive effect for Knysna larvae kept at 22S and Port Elizabeth larvae at 26S (Table 4). Significant site differences in relative success rates were found for temperature and 22S (higher rates in Knysna), and Cu25 (higher rates in Port Elizabeth, Fig. 2b). Moreover, Settlement was accelerated at higher temperature (Fig. 3b), while Cu50 and Cu100 delayed settlement of larvae from Knysna but not from Port Elizabeth (Fig. 3b).

All treatments except 26S significantly decreased the MR from Port Elizabeth larvae, while only 22S, Cu50 and Cu100 impaired metamorphosis of Knysna larvae (Table 4). On the other hand, more larvae metamorphosed at high temperature and Cu25 than in the controls in Knysna (leading to relative rates higher than one, Fig. 2b), although this outcome was not significant. The relative success rates were significantly higher in Knysna for temperature and for Cu25, and in Port Elizabeth for 26S (Fig. 2b). Cu25 also accelerated the timing of metamorphosis at Knysna (Fig. 3b). No metamorphosis was observed for larvae from Port Elizabeth subjected to the 22S treatment, larvae from Knysna at Cu50, or larvae from either sampled site at Cu100 (Fig. 3b).

***S. plicata* and *M. squamiger* comparison.** When the whole developmental sequence was considered, from fertilisation of the egg to post-metamorphic juveniles, clear differences in cumulative success were found between the species, with *S. plicata* being overall more tolerant of harsh conditions than *M. squamiger* (Fig. 4). As previously stated, the development of larvae seems to be the most sensitive stage for both species, acting as a bottleneck that results in a sharp reduction in the number of viable larvae in most treatments (Fig. 4).

It is particularly relevant that the complete process of reproduction and recruitment only occurred in non-negligible numbers in the controls and the treatments with the lower copper concentrations assayed (Cu25, Cu50) in *S. plicata* (Fig. 4a), and only for the controls in the case of *M. squamiger* (Fig. 4b). In all other treatments, failure of one step or another (particularly development of larvae) prevented successful completion of the early life-history stages completely or almost so.

Genetic screening

All adults used for the fertilization experiments were sequenced (Tables 1, 2), except for two individuals of *M. squamiger* that failed to amplify. Three haplotypes were obtained for *S. plicata*, corresponding to haplotypes already described by Pineda et al. [45]. For *M. squamiger*, we found ten haplotypes. Six of these had previously been reported [44], while the sequences of the remaining four haplotypes (Hap 53–56) were new and were deposited in GenBank with accession numbers JQ815436-

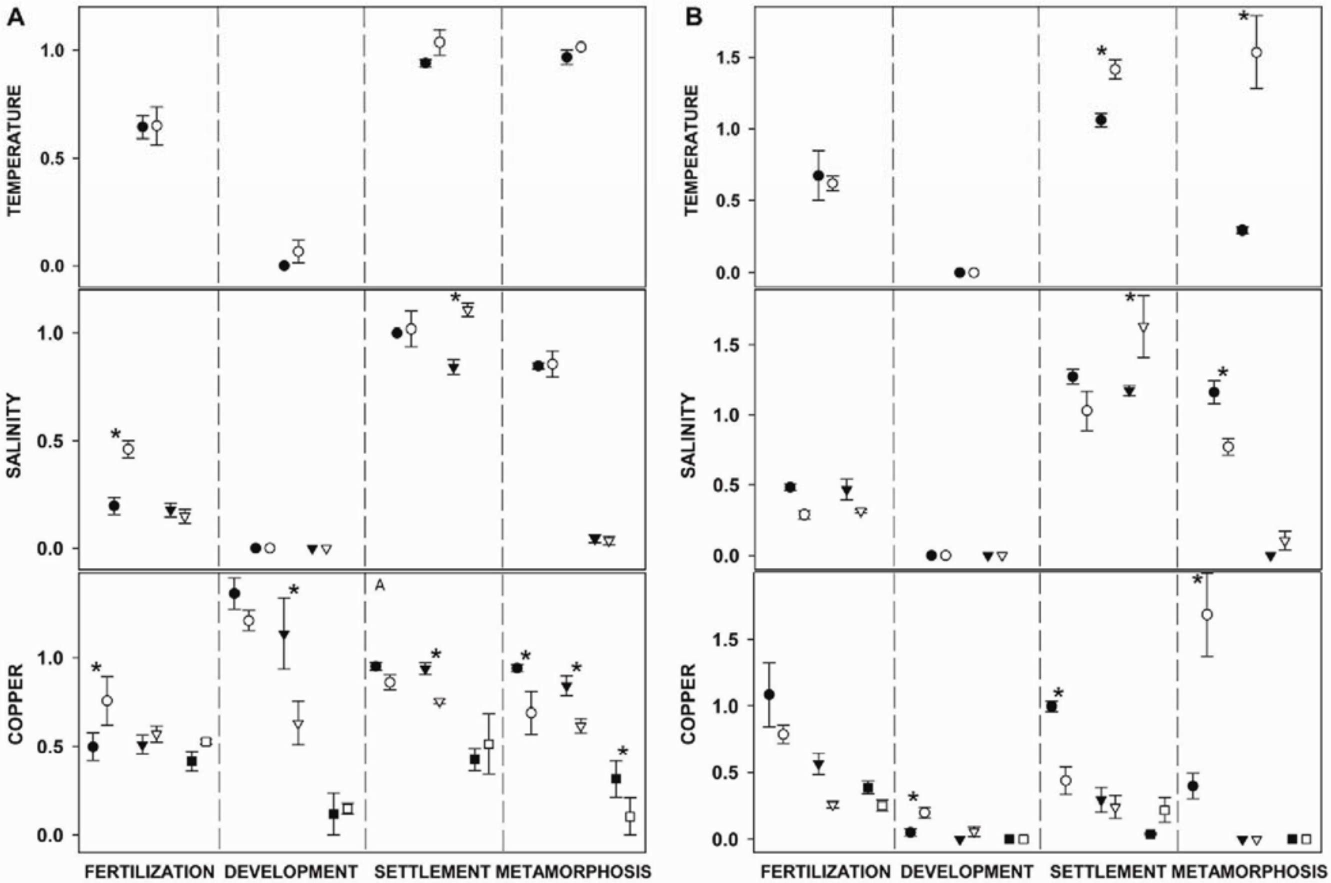

Figure 2. Relative success ratios of each developmental stage. a) *S. plicata* and b) *M. squamiger*. Values are relative to the controls (i.e., values<1 indicate less success than in the corresponding controls). Treatments include: temperature (30°C), salinity (circles for 26‰ salinity, triangles for 22‰) and copper (circles for copper concentration of 25 µg/L, triangles for 50 µg/L and squares for 100 µg/L). Black symbols correspond to the population at Port Elizabeth, while white symbols correspond to the population at Knysna. Values are means ±standard errors. Asterisks indicate significant differences between locations.
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JQ815439 (Table 2). *S. plicata* showed two clear groups of haplotypes, with Knysna composed entirely of Lineage I (50% Hap 1 and 50% Hap 2) and Port Elizabeth mainly represented by Lineage II (95% Hap 5, 5% Hap 2), *sensu* [45]. Thus, although these three haplotypes are globally distributed [45], Port Elizabeth and Knysna were highly differentiated ($F_{ST}=0.728$, $P<0.001$) (Table 2). Regarding *M. squamiger*, the two most frequent haplotypes were Haps 1 and 5 (Table 2) for both populations, and together represented ca. 60% of the genetic pool. Haplotypes corresponding to Lineage II, *sensu* [44], represented around 90% of each population, and the two populations did not differ significantly ($F_{ST}=0.048$, $P=0.991$) (Table 2).

Discussion

Increased temperature, decreased salinity and elevated copper concentrations affected several life-history stages of the introduced ascidians *Styela plicata* and *Microcosmus squamiger* at the two studied sites. Differences according to sensitivity to abiotic stressors and life-history stages were observed but overall, fertilisation and larval development were the most sensitive stages for both species. Thus, although later stages (settlement and metamorphosis) seemed in general more tolerant, the initial stages (fertilisation and development) must necessarily happen under more benign conditions.

A few of the stressors had apparent positive effects on some stages (resulting in the corresponding rates being greater than in the controls, or accelerating processes). It has been reported that moderate concentrations of pollutants can enhance some early life-history stages of marine invertebrates but eventually lead to detrimental effects (e.g. [82,83]). Similarly, our combined rates show that, notwithstanding these rare positive effects, the overall effect through the developmental stages considered is negative in all cases. Therefore, considering a single stage independently can lead to misleading conclusions about the ability of a species to overcome stressful conditions during the early life-history stages.

In general, *S. plicata* was more resistant to copper pollution, and both species coped similarly with increased temperature. Decreased salinity prevented complete development in both cases; however, some stages of *M. squamiger* (e.g. fertilization, settlement) are less affected or enhanced by low salinities. These tolerances correlate well with the types of environments where these species are commonly found. *S. plicata* is often found in harbours, which are known to accumulate copper [15,45], and *M. squamiger* in estuaries, which are characterized by frequent salinity changes [84]. In fact, Lowe [46] found that adults of *M. squamiger* could withstand reduced salinity levels (15–25‰) for extended periods of time, outcompeting native species such as *Molgula manhattensis* in southern California harbours. Similarly, estuarine sites along the southeast coast of South Africa (e.g. Port Alfred, Bushman's River

Table 3. ANOVA examining the effects of site and treatment at four developmental stages for *S. plicata*.

Source	*df*	MS	*F*	*P*
Effect on the FERTILISATION Rate				
Site	1	0.149	1.276	0.263
Treatment	6	4.063	34.903	<0.001
Site x Treatment	6	0.37	3.179	0.009
Error	56	0.116		
Comparisons for factor Treatment within site (Dunnett test, p = 0.05)				
Port Elizabeth	T, 26S,22S,Cu25,Cu50,Cu100<Control			
Knysna	26S,22S,Cu50, Cu100<Control			
Effect on the DEVELOPMENT Rate				
Site	1	31.451	20.438	<0.001
Treatment	6	135.769	88.230	<0.001
Site x Treatment	6	5.332	3.465	0.006
Error	56	1.539		
Comparisons for factor Treatment within site (Dunnett test, p = 0.05)				
Port Elizabeth	T,26S,22S,Cu100<Control			
Knysna	T,26S,22S,Cu100<Control<Cu25			
Effect on the SETTLEMENT Rate				
Site	1	115.035	74.075	<0.001
Treatment	6	17.786	11.453	<0.001
Site x Treatment	6	9.873	6.358	<0.001
Error	42	1.553		
Comparisons for factor Treatment within site (Dunnett test, p = 0.05)				
Port Elizabeth	T,22S,Cu25,Cu50,Cu100<Control			
Knysna	No differences			
Effect on the METAMORPHOSIS Rate				
Site	1	3.256	1.246	0.271
Treatment	6	86.937	33.256	<0.001
Site x Treatment	6	12.731	4.870	<0.001
Error	42	2.614		
Comparisons for factor Treatment within site (Dunnett test, p = 0.05)				
Port Elizabeth	22S,Cu100<Control			
Knysna	.26S, 22S,Cu25,Cu50,Cu100<Control			

T: temperature at 30°C; 22S: 22‰ salinity; 26S: 26‰ salinity, Cu25: copper concentration of 25 μg/L; Cu50: 50 μg/L; and Cu100: 100 μg/L.

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Mouth and East London) are dominated by *M. squamiger* while *S. plicata* is consistently absent in estuarine conditions but found in nearby harbours (M.R., *pers. obs.*).

Sensitivity differences according to development stages and stressors have been observed across phyla for other marine invertebrates, including molluscs [1,14], echinoderms [85] and ascidians [86–88]. Our results indicate that complete development, from fertilisation to metamorphosis, is impaired by all studied stressors, affecting several early life-history stages. In fact, we recorded completion of early stages only in *S. plicata* if copper concentrations were at/below 50 μg/L. Thus, the wide distribution of these species in environments where high temperature, low salinity or extreme pollutant concentrations are present cannot be inferred from laboratory or manipulative studies, but must be explained by novel strategies or behaviours in nature that increase overall reproductive success [89]. In this sense, Bellas et al. [65] suggested that the ascidian *Ciona intestinalis* could probably detect trace metals in the water with the adhesive papillae and delay or inhibit attachment. Although increasing the swimming period may decrease the probability of post-settlement survival due to the high metabolic cost required for the latter [10,90–92], the successful settlement and survival of a few individuals could result in successful introductions to new habitats. Even if recruitment failures were a common outcome, the prolonged reproductive period observed for both species [50–52] would increase the chances of a propagule finding favourable temporal windows of tolerable conditions.

The sensitivity of *S. plicata* embryos and larvae to temperature and salinity changes was in accordance with Thiyagarajan and Qian [10], who studied *S. plicata* in Hong Kong and reported recruitment failure when seawater temperature reached values of 26–30°C and salinities of 22–30‰ in summer. In our study, these conditions prevented both *S. plicata* and *M. squamiger* from completing development, with the earlier stages (embryo fertilisa-

Table 4. ANOVA examining the effects of site and treatment at four developmental stages for *M. squamiger*.

Source	*df*	MS	*F*	*P*
Effect on the FERTILISATION Rate				
Site	1	0.062	0.881	0.353
Treatment	6	1.912	27.295	<0.001
Site x Treatment	6	0.156	2.231	0.059
Error	42	0.070		
Comparisons for factor Treatment (Dunnett test, p = 0.05)				
	T,26S,22S,Cu50,Cu100<Control			
Effect on the DEVELOPMENT Rate				
Site	1	7.379	7.300	0.010
Treatment	6	69.155	68.415	<0.001
Site x Treatment	6	2.108	2.086	0.075
Error	42	1.011		
Comparisons for factor Treatment (Dunnett test, p = 0.05)				
	T,26S,22S,Cu25,Cu50,Cu100<Control			
Effect on the SETTLEMENT Rate				
Site	1	10.900	21.621	<0.001
Treatment	6	28.538	56.610	<0.001
Site x Treatment	6	3.789	7.517	<0.001
Error	56	0.504		
Comparisons for factor Treatment within site (Dunnett test, p = 0.05)				
Port Elizabeth	Cu50,Cu100<Control<26S			
Knysna	Cu25,Cu50,Cu100<Control<22S			
Effect on the METAMORPHOSIS Rate				
Site	1	0.782	1.362	0.248
Treatment	6	100.818	175.607	<0.001
Site x Treatment	6	5.648	9.839	<0.001
Error	56	0.574		
Comparisons for factor Treatment within site (Dunnett test, p = 0.05)				
Port Elizabeth	T,22S,Cu25,Cu50,Cu100<Control			
Knysna	22S,Cu50,Cu100<Control			

T: temperature at 30°C; 22S: 22‰ salinity; 26S: 26‰ salinity, Cu25: copper concentration of 25 μg/L;
Cu50: 50 μg/L; and Cu100: 100 μg/L.
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tion and larval development) being especially sensitive, while settlement was hardly affected. The lowest salinity tested (22‰), however, prevented most larvae of either species to complete metamorphosis even after successful settlement, as previously described for other ascidians [63,93]. High temperatures and low salinities also tended to accelerate development, with most larvae of both species settling within 24 h, which would limit options for escape to more favourable sites. Thus, the current climate change predictions of increasing temperatures and decreasing salinities [94] suggest that these species, particularly *S. plicata* [48], would find it difficult to cope with these predicted stresses [95].

Copper has been shown to inhibit embryo development, reduce successful settlement and metamorphosis, and reduce growth in many marine invertebrates, including ascidians (e.g. [17,96–98]). Elevated copper concentrations also negatively affected the early life-history stages of *S. plicata* and *M. squamiger*, with more dramatic effects on developmental stages of the latter species. Even at copper concentrations similar to those found in highly polluted harbours (25–50 μg/L), fertilisation success of *S. plicata* was still around 50% that of the controls for both populations, and development through metamorphosis was possible. At the highest concentration (100 μg/L), though, there was no development of the larvae and the metamorphosis of settled individuals was seriously impaired. In contrast, even the lowest concentration of copper assayed (25 μg/L) had detrimental effects on early development of *M. squamiger*. This suggests that *S. plicata* will continue to perform better in polluted habitats than *M. squamiger* and has important implications for understanding the distributions of the two species across overlapping ranges.

The genetic patterns found were clear-cut: genetic differentiation was high between populations of *S. plicata*, while it was negligible for *M. squamiger*. However, we could not detect a clear correlation of this pattern with differential responses to abiotic stress. In general, although some particular outcomes were significantly different, all populations responded similarly to the tested stressors. Genetic diversity within populations was lower for *S. plicata* than for *M. squamiger*, but again this has no clear connection with our results as, if any, the low genetic diversity species *S. plicata* was overall more tolerant to stress than *M. squamiger*. The only emerging pattern was found when comparing the responses to low salinity and high copper concentrations between populations of *S. plicata*. For instance, fertilization rates at low salinities (26‰) were considerably higher for the eggs from Knysna than for the eggs from Port Elizabeth. Adult samples from Knysna exclusively displayed haplotypes from Lineage I, which is the most widespread haplogroup in the world [45]. In contrast, adults from Port Elizabeth mainly belonged to Lineage II, which is also found in salt marsh habitats [48]. Thus, the slightly different response of these two populations of *S. plicata* may be related to differences in their genetic composition. Differential adaptation to environmental factors (e.g. temperature, salinity) of mitochondrial sequences within one species has been previously described in marine invertebrates [99–103]. Of course this adaptive capability need not be directly linked to the studied gene, but can be related to other genes that vary between lineages. In order to assess whether there is any genetic basis in the responses featured by both species, a more precise genetic characterization (for example, using microsatellites), together with controlled crossings and transplant experiments are necessary.

In conclusion, we found that several early life-history stages of the ascidians *S. plicata* and *M. squamiger* were seriously impaired by exposure to realistic scenarios of abiotic stressors, independent of the haplogroup tested. Moreover, abiotic factors do not affect animals in isolation but will normally combine as multiple stressors, often resulting in additive or synergistic effects. Thus, our results are likely to overestimate the resilience of the life-history processes studied here, a surprising fact given the abundance of these species in habitats such as harbours where such stressors are the norm. Behavioural strategies that can only be observed in the field (e.g. delay in spawning until suitable conditions are restored, strong propagule pressure with arrival of larvae from more benign environments, extended reproductive periods) seem plausible explanations for the presence of adults in these localities. Basic knowledge of reproduction, larval development and survival of these species in new habitats coupled with further information on their genetic variability is therefore essential to predict possible areas of establishment and spread worldwide.

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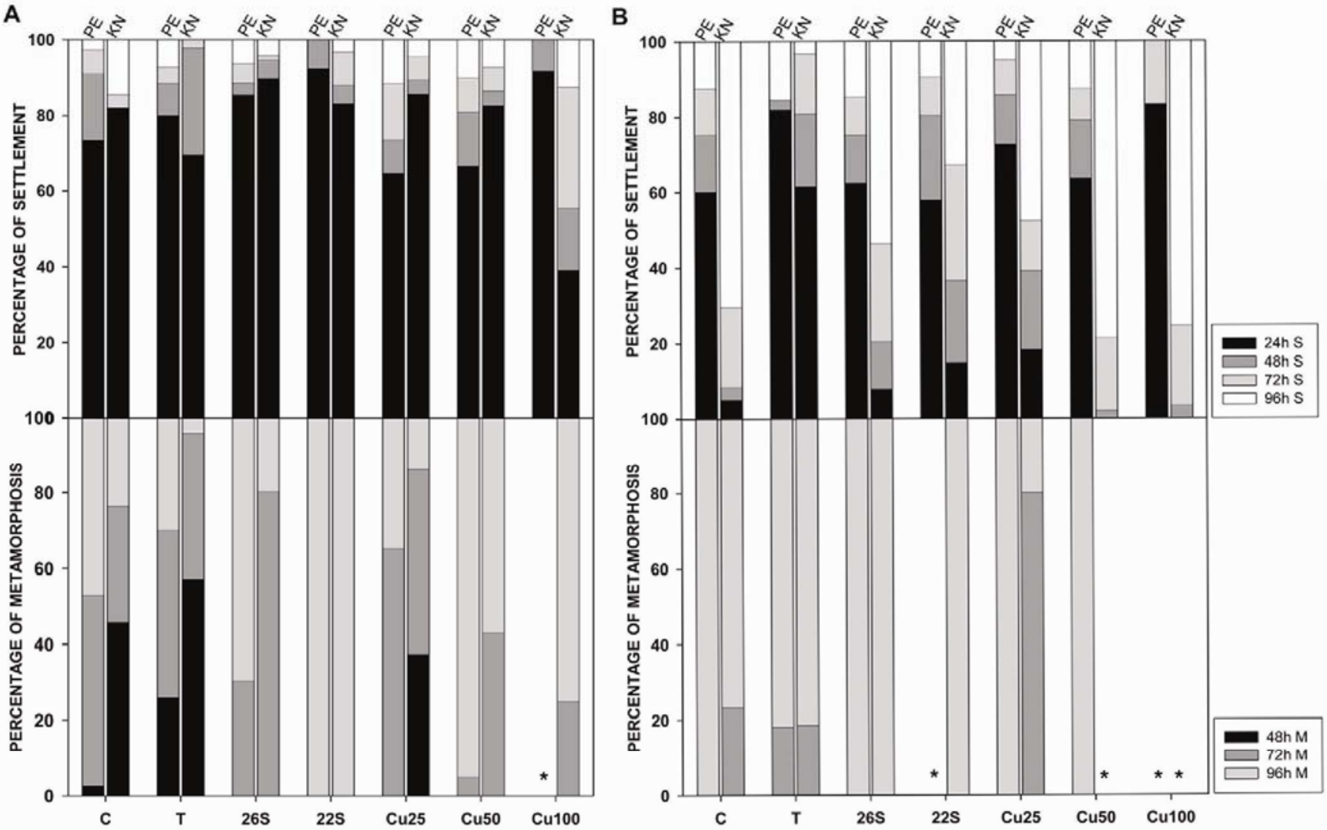

Figure 3. Percentage of total settled (above) and metamorphosed (below) individuals each 24-hour interval. a) *S. plicata* and b) *M. squamiger*. Left bars for Port Elizabeth (PE); right bars for Knysna (KN). Asterisks indicate zero success.
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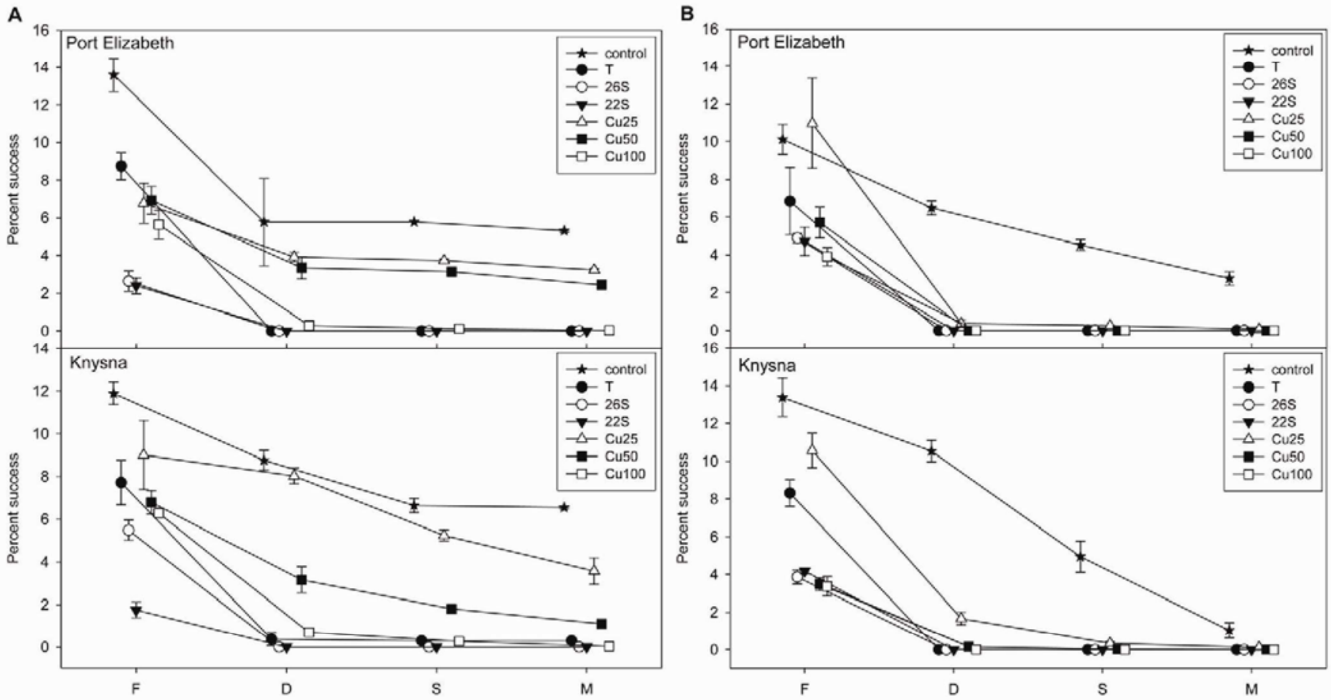

Figure 4. Cumulative success over fertilisation (F), development (D), settlement (S), and metamorphosis (M) for each treatment. a) *S. plicata* and b) *M. squamiger*. Legend: T: temperature at 30°C; 22S: 22‰ salinity; 26S: 26‰ salinity, Cu25: copper concentration of 25 μg/L; Cu50: 50 μg/L; and Cu100: 100 μg/L.
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Author Contributions

Conceived and designed the experiments: MCP CDM XT SL-L MR. Performed the experiments: MCP VO MR. Analyzed the data: MCP XT SL-L MR. Contributed reagents/materials/analysis tools: CDM XT SL-L MR. Wrote the paper: MCP CDM XT SL-L VO MR.

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