Nuevas perspectivas en el uso del mejillón cebra (*Dreissena polymorpha*) en estudios toxicológicos del sistema acuático

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„Die hohe Verehrung, die unsere Vorfahren dem reinen Wasser als "Lebensquell" entgegenbrachten, ist nur noch in der verblaßten Erinnerung an Nymphen und Flußgötter in vergilbten Märchen und Sagen erhalten. Kein Naturvolk hätte seine Abfälle in die eigene Trinkwasserversorgung oder in diejenige anderer geschüttet. Auf dem tückischen Verbrechen der "Brunnenvergiftung" stand die Todesstrafe.“

(The high appreciation our ancestors showed for clean water as a “source of live” only exists in the pale memory of nymphs and river gods from fairy tales and ancient legends. No native people would have thrown their waste into their water supplies or in those of others. The treacherous crime of “well poisoning” was penalized by capital punishment.)

Otmar Wassermann, German Toxicologist

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ACRONYM LIST

Acronym List

3β-HSD: 3β-hydroxysteroid dehydrogenase
5α-R: 5α-reductase
5-HT: Serotonin (5-hydroxytryptamine)
17β-HSD: 17β-hydroxysteroid dehydrogenase
AEAT: Acyl-coenzyme A:estradiol acyltransferase
AOX: Acyl-coenzyme A oxidase
ATAT: Acyl-coenzyme A:testosterone acyltransferase
CA: Clofibric acid
Cd: Cadmium
Cu: Copper
CYP: Cytochrome P450
DDT: Dichlorodiphenyl trichloroethane
DHA: Docosahexaenoic acid
DHEA: Dehydroepiandrosterone
DHT: 5α-dihydrotestosterone
ED: Endocrine disruptor
EPA: Eicosapentaenoic acid
FA: Fatty acid
FAME: Fatty acid methyl ester
HCB: Hexachlorobenzene
HCH: Hexachlorocyclohexane
HDL: High-density lipoprotein
Hg: Mercury
HUFA: Highly unsaturated fatty acid
MCR-ALS: Multivariate curve resolution alternating least-squares
MUFA: Monounsaturated fatty acid
NADPH: β-nicotinamide adenine dinucleotide phosphate
Ni: Nickel
OTC: Organotin compound
P450-scc: P450 side-chain cleavage
PAH: Polycyclic aromatic hydrocarbon
Pb: Lead
PCB: Polychlorinated biphenyl
PPAR: Peroxisome proliferator-activated receptor
PUFA: Polyunsaturated fatty acid
RXR: Retinoid X receptor
SFA: Saturated fatty acid
SSRI: Selective serotonin reuptake inhibitor
STP: Sewage treatment plant
SULT: Sulfotransferase
TBT: Tributyltin
TPT: Triphenyltin
UGT: UDP-glucuronosyltransferase
VLDL: Very low-density lipoprotein
Zn: Zinc

1. GENERAL INTRODUCTION

1. GENERAL INTRODUCTION

Background

The massive increase in human population has led to an exponential development in technological and industrial advancements all around the world. This outburst has greatly increased the manufacturing of synthetic substances at a global scale. Those man-made chemicals include industrial, agricultural and pharmaceutical compounds that are used on a daily basis and which have become an inseparable aspect of modern human existence. Even though being of great importance and providing indisputable benefits in various areas, those compounds once released into the environment may represent a serious hazard to exposed organisms (Fleeger et al., 2003). Aquatic environments are the ultimate sink for many of those chemicals released from anthropogenic activities, either due to direct discharges from industrial and domestic effluents (Coogan et al., 2007), or diffuse discharges from urban and agricultural runoff and industrial spills (Boreham and Birch, 1987; Vitaliano et al., 2002) or atmospheric processes (Lovett, 1994). Many persist in the environment for long periods of time, since they resist photolytic, chemical and biological degradation. Others, due to their low water solubility, bond strongly to particulate matter in the water column and as a result, get deposited in sediments which then serve as a reservoir or "sink". Society has become aware of the potential long-term adverse effects of synthesized chemicals and the risk they pose for aquatic ecosystems, since these compounds may rapidly bioaccumulate in tissues of resident organisms and biomagnify through the food chain (Fu et al., 2003). Moreover, many of those chemicals and/or their by-products may act as endocrine disruptors (EDs) by interfering with natural hormones in the body that are responsible for the maintenance of homeostasis, reproduction, development and behaviour in organisms. One of the best documented cases of chemically-induced endocrine disruption in aquatic wildlife has been reported in the mid-80s with the detection of imposex development in gastropods exposed to the biocide tributyltin (TBT) (Bryan et al., 1988). Since then, a wide variety of other potential EDs have been discovered, amongst them, biocides like atrazine and fenarimol (Hayes et al., 2002; Janer et al., 2005b), dioxins (Depledge and Billinghurst, 1999), polycyclic aromatic hydrocarbons (PAHs) like 3-methylcholanthrene (Navas and Segner, 2000), phthalates (Latini et al., 2004), and alkylphenols like the plasticizer bisphenol A (Oehlmann et al.,

2000) and the industrial surfactant 4-nonylphenol (McCormick et al., 2005). The effects of EDs are generally classified as either estrogenic (compounds that mimic or block natural estrogens) or androgenic (compounds that mimic or block natural androgens) or thyroidal (compounds with direct or indirect impact on the thyroid) (Snyder et al., 2003). More recently, focus has been placed on a new class of emerging contaminants which are those with pharmacological potential that are designed to stimulate a physiological response in humans, plants, and animals (Daughton and Ternes, 1999). Pharmaceuticals enter the aquatic environment mainly through sewage treatment plants (STPs), where they are seldom degraded during wastewater processing (Ternes et al., 1999; Nakada et al., 2006). They are considered pseudo-persistent due to their continuous release into the environment which leads to chronic exposure of aquatic organisms to a large cocktail of biologically active compounds (Daughton and Ternes, 1999; Hernando et al., 2006).

Given the fact that the aquatic ecosystem represents the habitat of many organisms and constitutes a vital resource for human society, control mechanisms are needed to guaranty its continuous preservation. Therefore, strategies have to be developed for the identification, estimation, comparative assessment and management of the risks posed by anthropogenic chemicals to the environment and biota (Gray, 1992). In the last years international organizations and environmental agencies have clearly stated that risk assessment cannot be exclusively based on chemical analysis of environmental samples because this approach does not provide any information about the deleterious effects of pollutants on living organisms. Thus, it is of great importance to evaluate the effects of environmental contaminants through toxicological investigations that give insights into their complex interactions with animal physiology (Wright and Welbourn, 2002). This requires the definition of specific biological endpoints (e.g. growth, sexual maturation, reproductive cycle, and biomarker measurements among other) that may allow the evaluation of physiological responses of organisms to environmental stressors. However, the physiological value of many parameters measured in organisms may vary greatly in relation to different biotic (e.g. age, sex, reproductive stage) and/or abiotic factors (e.g. temperature, seasonality). Therefore, caution should be taken in their application and interpretation which should be based on information derived from preliminary characterization studies providing the

fundamental knowledge necessary for their successful integration into toxicological investigations.

Bivalves such as mussels are often used in toxicological studies for testing pollutant effects and in monitoring programs to assess the environmental health of coastal areas (Farrington et al., 1983). Indeed, they possess several physiological attributes which render them particularly attractive as bioindicators. Those include a largely sedentary lifecycle, a widespread geographical distribution, and a very low metabolic rate for contaminants, which together with their feeding behaviour (filtrators) leads to high bioaccumulation levels (Bryan et al., 1985). Additionally, mussels can be easily cultured, maintained and handled, which makes them valuable organisms for laboratory studies (Galloway et al., 2002).

1.1 Organotins in the aquatic environment

Organotin compounds (OTCs) represent a group of organometallic moieties containing a Sn atom covalently bound to one or more organic substituents (e.g. methyl, ethyl, propyl, butyl and phenyl). The most prominent compounds of this group are TBT and triphenyltin (TPT) which were primarily introduced as biocides in preservatives for wood, cotton textiles, paper, paints, stains and later in several other industrial products like stabilizers in the manufacture of PVC plastics, and as active ingredients in antifouling paints (Fent, 1996). The introduction of the latter was highly appreciated by the shipping industry because of their high effectiveness and relative low production costs. Their overwhelming success resulted in production volumes reaching from 2000 tons in 1960 over 16000 tons in 1970 up to estimated 40000 tons in the mid-1980s (Omae, 2003). The continuous application of antifouling paints on ship hulls, docks, buoys and fishing nets caused the leaching of considerable amounts of TBT into the aquatic environment resulting in its accumulation at high levels in sediments, surface waters and tissues of aquatic organisms, especially near harbours and coastal areas (Table 1). Although TBT is a strongly regulated chemical in most European countries nowadays, it still can be detected in sediments and water samples (Fent, 1996) due to its strong persistence in the environment and presence in municipal and industrial

wastewater, sewage sludge and landfill leaches (Fent and Müller, 1991; Bancon-Montigny et al., 2004) (Figure 1).

Table 1. Typical concentrations of TBT detected near harbours and coastal areas in different environmental matrices.

Matrix/Organism	Concentration	Region	Country	Reference
Surface water	3-30 ng Sn/L	Coastal and continental	Portugal	Díez et al., 2005
	237-572 ng Sn/L	Coastal near harbour	Italy	Berto et al., 2007
Sediments	326-4702 ng/g	Coastal near harbour	Spain	Martínez-Lladó et al., 2007
	8057 ng Sn/g	Coastal near harbour	Italy	Berto et al., 2007
	55-7673 ng/g	Coastal near harbours	Iberian Peninsula	Díez and Bayona, 2009
	0.1-8.6 ng/g	Coastal	Ireland	Buggy and Tobin, 2006
	9.5 ng/g	Coastal	Korea	Choi et al., 2009
Mussels				
Digestive gland	161-3910 ng Sn/g d.w.	Coastal near harbour	England	Harino et al., 2005
Whole body	48-2800 ng Sn/g d.w.	Coastal near harbour	Korea	Shim et al., 2005a
Whole body	3-287 ng Sn/g w.w.	Coastal near harbour	Japan	Harino et al., 2007
Whole body	5-110 ng Sn/g w.w.	Coastal near harbour	Brazil	Limaverde et al., 2007
Whole body	173 ng/g w.w.	Coastal near harbour	Canada	Horiguchi et al., 2003
Whole body	800-2400 ng Sn/g d.w.	Coastal near harbour	France	Devier et al., 2005
Fish				
Whole body	323 ng Sn/g d.w.	Coastal	Korea	Shim et al., 2005b
Muscle	3-190 ng/g w.w.	Coastal	Malaysia	Sudaryanto et al., 2004
Whole body	0.1–19.5 ng/g w.w.	Coastal	Greece	Louppis et al., 2010

Organotin compounds are of lipophilic nature which enables them to easily permeate through biological membranes and epithelia and thus bioaccumulate in biota. They are seldom metabolized by organisms, even though this may vary among species depending on their capabilities to metabolise xenobiotics. Hence, in those organisms where metabolism and excretion of TBT is low, high tissue levels can be detected. Marine bacteria for example display a remarkable ability to bioaccumulate considerable amounts of TBT (Gadd, 2000) and so do bivalves, where tissue levels higher than 5 µg/g have been measured, due to their low metabolic rate (Laughlin, 1996). On the other hand, bioaccumulation of TBT in crustaceans and fish seem to be much lower because of their efficient enzymatic degradation (Laughlin, 1996).

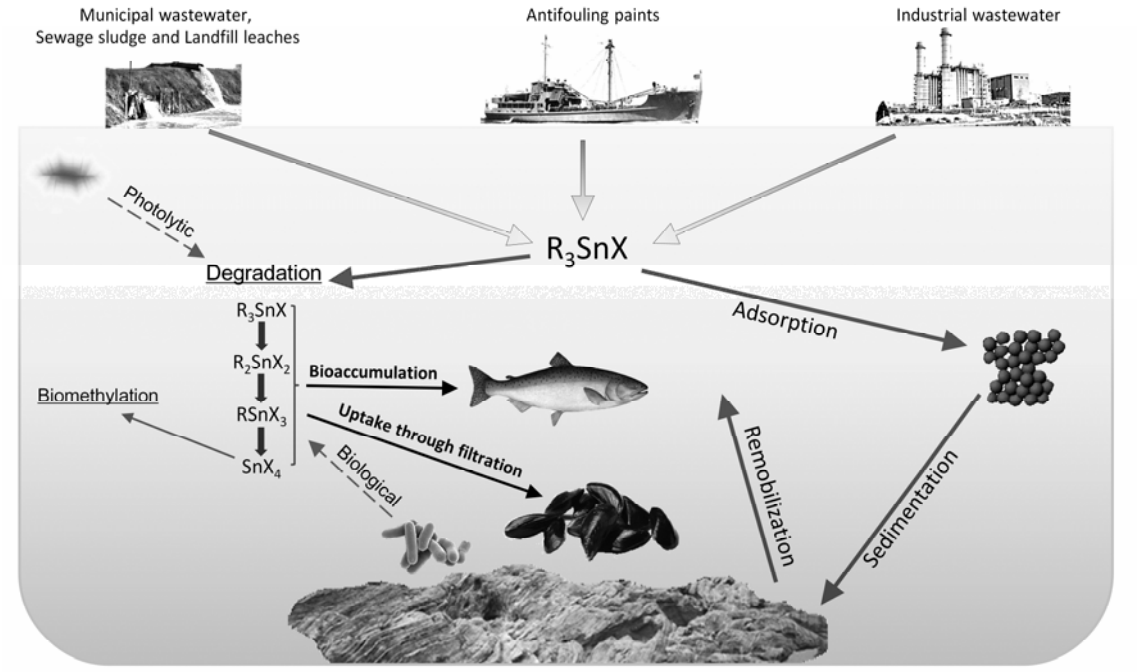

Figure 1. Distribution and fate of trialkylated organotin compounds in the aquatic environment and their biogeochemical cycling. Scheme was constructed using the following sources: Cooney (1988), Gadd (1993, 2000).

The deleterious effects of TBT on aquatic biota have been the subject of extensive studies in the past two decades covering several groups of organisms like crustaceans, echinoderms, molluscs and fish (Morcillo et al., 1998; Novelli et al., 2002; Ohji et al., 2002; Janer et al., 2007). It has become evident that certain uses of TBT can have catastrophic consequences on aquatic organisms, especially on molluscs in their sensitive stages. Indeed, first reports of detrimental effects of TBT exposure arose in the 1970s when deformation and reproductive failure were observed in an important commercial stock of oysters (*Crassostrea gigas*) in Arcachon Bay France, causing the collapse of the local oyster population (Alzieu, 1991). Concurrently, aberrations in reproductive organs in female gastropods of *Nucella lapillus* in England and of *Nassarius obsoletus* in the U.S. were reported (Blaber, 1970; Smith, 1971) and described as the superimposition of male characteristics (penis and vas deferens) on female organisms, commonly named imposex, or also known as pseudohermaphroditism (Smith, 1971; Jenner, 1979). This phenomenon is strongly correlated with the proximity to coastal areas with high shipping activities and also to Sn concentrations determined in animal tissues (Santos et al., 2002). It is generally accepted that the induction of imposex in gastropods may already occur at TBT concentrations of approximately 1 ng Sn/L (Gibbs et al., 1988). Thus, the high susceptibility of gastropods to low concentrations of organotins led to their successful integration as effective indicators for TBT pollution in environmental monitoring programs (Oehlmann et al., 1996; Ten Hallers-Tjabbes et al., 2003). Even though the negative effects of TBT on molluscs have been evidences in several studies over the last decades, the mechanisms behind the development of imposex are still unknown. Nevertheless, several theories have been formulated based on the assumption that increasing testosterone levels may be responsible for its induction which could be mediated through either: (a) inhibition of P450 aromatase (Spooner et al., 1991); (b) interference with enzymatic activity of the acyl-coenzyme A:testosterone acyltransferase (ATAT) (Gooding et al., 2003; Sternberg and LeBlanc, 2006); (c) reduced excretion of testosterone as sulfur conjugates (Ronis and Mason, 1996). Meanwhile, others have hypothesized that TBT may induce imposex by acting as an agonist of the retinoid X receptor (RXR) (Grün et al., 2006; Kanayama et al., 2005). Besides being a potent disrupter of the endocrine system, TBT may also act as a strong neurotoxin and has the potential to induce several other negative physiological effects in molluscs, such as severely reducing the viability of larvae resulting in recruitment

failure (Horiguchi et al., 1997); interfering with lipid and fatty acid homeostasis (Janer et al., 2007); reducing accumulation of testosterone as fatty acid esters (Gooding et al., 2003); inducing necrosis of digestive cells of the hepatopancreas (Chagot et al., 1990) or apoptosis in gill tissue (Mičić et al., 2001).

1.2 Pharmaceuticals in the aquatic environment

An important constituent of modern human and veterinary medicine is the use of pharmaceutical compounds for the treatment and prevention of diseases. Those chemicals share common characteristics which enable them to exert their pharmacological effect in biological systems. Drugs are therefore designed to easily pass through biological membranes so that they can perform their intended pharmacological effect. As a result, they have a high biological potency and are in many cases resistant to biodegradation during wastewater processing which results in the discharge of considerable amounts of drugs into the aquatic environment (Daughton, 2003; Bound and Voulvoulis, 2005). Besides STPs, pharmaceuticals also enter the aquatic environment through private sewage leach fields, confined animal feeding operations (Daughton, 2007), drug manufacturer effluents (Larsson et al., 2007), and by several other dispersed and diffuse discharges (Figure 2).

In the last decades, consumption and production of pharmaceuticals has increased enormously which, together with the development of new analytical techniques capable of detecting compounds at trace levels, led to an increased concern about the presence and toxicity of drugs in aquatic organisms. Indeed, several classes of drugs have been detected in surface waters (Ashton et al., 2004; Glassmeyer et al., 2005), sediments (Kwon and Armbrust, 2006) and in tissues of aquatic organisms (Chu and Metcalfe, 2007).

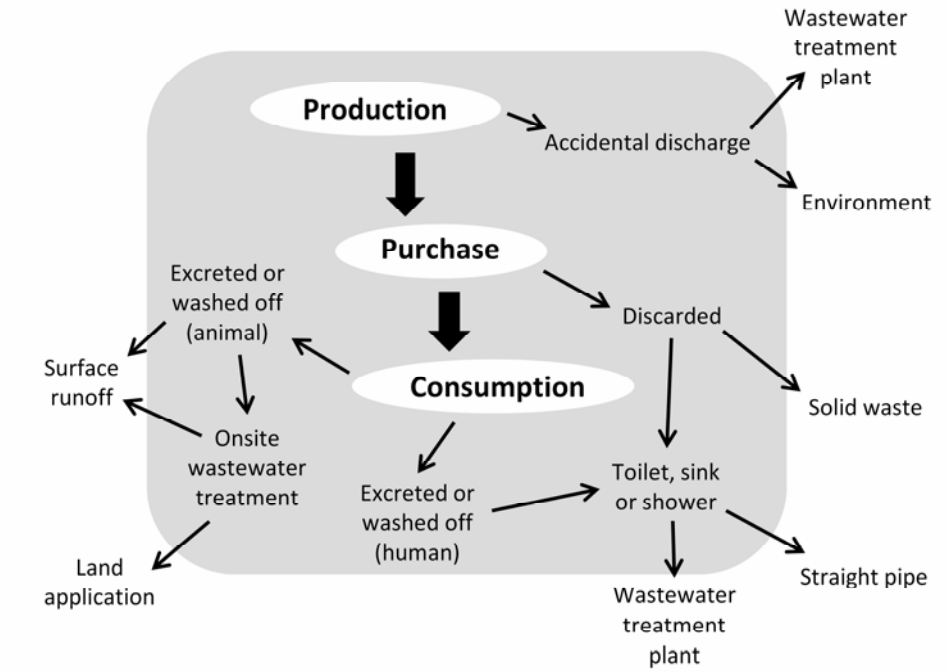

Figure 2. Most common sources (gray) and entrance pathways (white) for pharmaceuticals into the aquatic environment.

Once a drug has been consumed, it is usually conjugated or hydroxylated while a certain percentage passes through the body unchanged (Bound and Voulvoulis, 2005). The biotransformation may produce a variety of metabolites, some of which might even have greater biological activity than the parent drug (Fong and Molnar, 2008). Most of the excreted conjugates are inactive; however, liberation of the active compound within the STP is possible (Daughton and Ternes, 1999). Even though environmental concentrations of several pharmaceuticals are found in the lower part-per-billions range, effects on aquatic organisms cannot be excluded. In fact, pharmaceuticals have been designed to target specific organs, metabolic pathways, and receptors resulting in the modulation of specific physiological functions so that disease can be treated. As some of these targets are relatively common across animal phyla, it is likely that pharmaceuticals lead to undesired effects in non-target organism (Thornton, 2003). For instance, 1.5 µg/L of waterborne gemfibrozil (a lipid regulator) led to a 50% decrease of circulating testosterone in goldfish, showing its potential to act as an endocrine disrupter (Mimeault et al., 2005). Environmental relevant concentrations of ethinylestradiol (5 ng/L) caused reproductive failure and even population collapse in chronically exposed fish (Nash et al., 2004; Kidd et al., 2007). Meanwhile, 1 µg/L diclofenac (a non-steroidal anti-inflammatory drug) was shown to induce lipid peroxidation in mussels

after 96h exposure (Schmidt et al., 2011). Hence, the need to further investigate the effects of pharmaceuticals in aquatic organisms is evident, and in this context the presented thesis will focus on two widely used pharmaceuticals: fluoxetine (a selective serotonin reuptake inhibitor) and clofibrate (a lipid-lowering agent).

Selective serotonin reuptake inhibitors (SSRIs)

SSRIs are usually prescribed for the treatment of various degrees of depression as well as other mental health problems, like compulsive behaviour or eating and personality disorders. One of the most frequently prescribed SSRIs for clinical treatment is fluoxetine, the active compound in Prozac (Nentwig, 2007). Its main mode of action is the selective inhibition of the transporter responsible for serotonin reuptake at the presynaptic nerve membrane resulting in an increase in serotonin concentrations at postsynaptic receptor sites (Wong et al., 1995). After oral administration, fluoxetine is excreted 2-11% unchanged (Stanley et al., 2007); the rest is either excreted as fluoxetine glucuronide or metabolized by cytochrome P450 isoenzymes to norfluoxetine, the active metabolite in humans (Hartke and Mutschler, 1993) (Figure 3). So far, little is known about the degradation or transformation of fluoxetine in STPs. Nonetheless, concentrations in the lower ng/L range have been detected in several aquatic matrices (Table 2) and at least 1-2 ng/g of fluoxetine have been detected in tissues of different fish species collected from municipal effluent-dominated streams in the U.S. (Brooks et al., 2005; Chu and Metclafe, 2007), indicating its bioaccumulation potential.

Table 2. Environmental concentrations of fluoxetine, clofibrate and clofibric acid.

Matrix	Drug	Concentration	Reference
STP effluent			
	Fluoxetine	225 ng/L	Zorita et al., 2007
		40-73 ng/L	Batt et al., 2008
	Clofibrate	800 ng/L	Andreozzi et al., 2003
	Clofibric Acid	1600 ng/L	Ternes, 1998
		680 ng/L	Andreozzi et al., 2003
		570 ng/L	Winkler et al., 2001
		310 ng/L	Han et al., 2006
Surface water			
	Fluoxetine	99 ng/L	Metcalfe et al., 2003
		12 ng/L	Kolpin et al., 2002
	Clofibrate	40 ng/L	Richardson and Bowron, 1985
	Clofibric Acid	550 ng/L	Ternes, 1998
		98-111 ng/L	Thomas and Hilton, 2004
Ground and drinking water			
	Fluoxetine	0.25 ng/L	Schultz and Furlong, 2008
	Clofibrate	580 ng/L	Loraine and Pettigrove, 2006
	Clofibric Acid	170 ng/L	Heberer, 2002

Even though there is only a limited number of studies available investigating the effects of fluoxetine on aquatic organisms, deleterious effects have already been reported at concentrations in the range of 50-500 µg/L *viz*. interference with survival and reproduction in the daphnid *Ceriodaphnia dubia* (Henry et al., 2004); reduction in the number of offspring of the freshwater snail *Physa acuta* (Sánchez-Argüello et al., 2009); disruption of the reproductive physiology of male goldfish (*Carassius auratus*) (Mennigen et al., 2010); and reduction of the reproductive capacity of the freshwater gastropod *Potamopyrgus antipodarum* (Nentwig, 2007). In addition, fluoxetine and norfluoxetine were shown to induce gamete release and parturition in different bivalve species, however significant effects appeared only at high concentrations in the mg/L

range (Fong, 1998; Fong et al.,1998; Fong and Molnar, 2008; Bringolf et al., 2010). Thus, despite the evidence at hand, effects of fluoxetine and other SSRIs including their metabolites at environmentally relevant concentrations have not yet been sufficiently investigated (Brooks et al., 2003a,b; Foran et al., 2004).

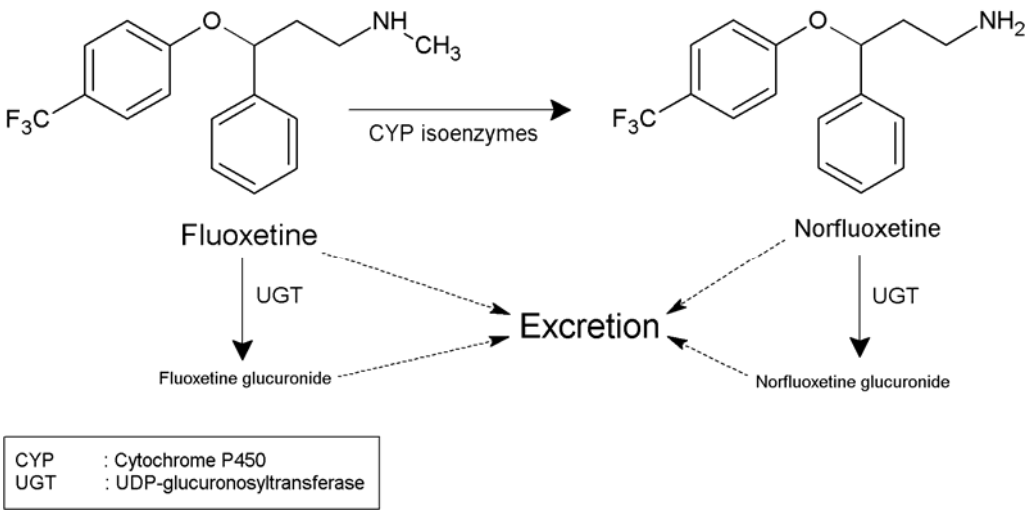

Figure 3. Fluoxetine metabolism in humans (modified after Hiemke and Härtter (2000)).

Blood lipid-lowering agents: fibrates

Fibrate drugs (clofibrate, gemfibrozil, bezafibrate) are used in human medicine as hypolipidemic agents in the treatment of cardiovascular diseases as they decrease plasma triglycerides and very low-density lipoproteins (VLDL) while increasing high-density lipoproteins (HDL) (Khetan and Collins, 2007). They act through binding of the fibrate to the peroxisome proliferator-activated receptor alpha (PPARα), which regulates genes that control lipid metabolism and inflammation (Yoon, 2009). PPARα activation results in fatty acid catabolism especially in tissues such as liver, heart, kidney, and brown adipose tissue (Figure 4). Additionally, fibrates are classified as peroxisome proliferators, as they increase to a great extend the number and size of hepatic peroxisomes leading to hepatomegaly and hepatocarcinogenesis in rodents (Rao and Reddy, 1991; Sausen et al., 1995). Peroxisomes are single membrane bound organelles present in all eukaryote cells that have a fundamental role in lipid metabolism. Although PPARs have been cloned from various fish species (Ruyter et al., 1997; Leaver et al., 2005; Kondo et al., 2007), their existence have not yet been proven

in invertebrates despite of studies reporting PPAR mediated responses like peroxisomal proliferation in mussels (Cajaraville et al., 1997).

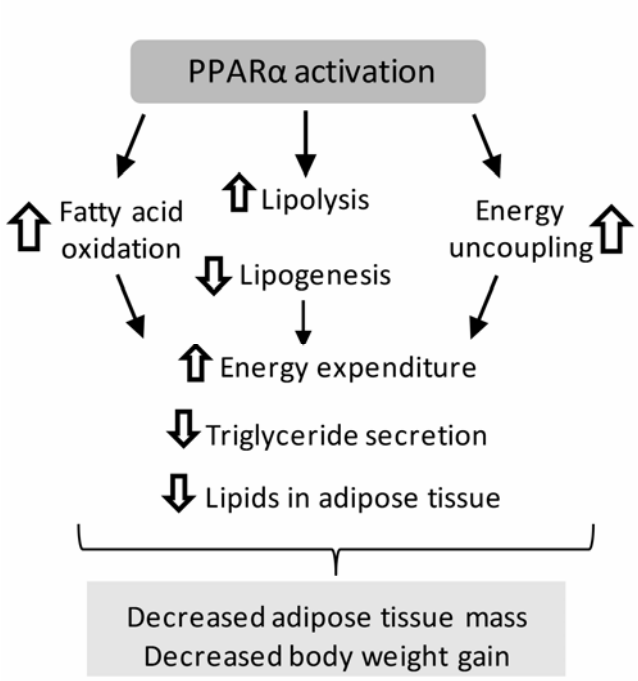

Figure 4. PPARα and its implication in lipid homeostasis. Ligand-activation of PPARα modulates the expression of several genes involved in lipid and lipoprotein metabolism as well as energy coupling (modified after Yoon (2009)).

This work will focus on clofibrate as a model contaminant of the aquatic system. It is known that after administration, clofibrate is metabolized in the liver into clofibric acid (CA) which is then excreted either unchanged (~6%) or as glucuronide (>90%) (Winkler et al., 2001). The removal efficiency of CA within a STP is about 50%, indicating a limited depuration capacity (Castiglioni et al., 2006). Moreover, the estimated half-life of CA in the environment is of 21 years which makes it one of the most persistent drug residues known (Winkler et al., 2001). Clofibrate has been detected in drinking water (Loraine and Pettigrove, 2006), surface waters (Thomas and Hilton, 2004) and in sewage treatment plant effluents (Andreozzi et al., 2003) of European countries, whereas clofibric acid is the most widely and routinely reported drug metabolite found in open waters (Khetan and Collins, 2007) (Table 2).

Despite the fact that both clofibrate and its metabolite have been detected in the environment in the upper ng/L range, studies regarding the effects of hypolipidemic drugs on the lipid composition and lipid metabolism in aquatic organisms are still

scarce, and mostly limited to fish. Hence, exposure of rainbow trout to the fibrate drug gemfibrozil disrupted lipoprotein metabolism by decreasing plasma lipoprotein levels, and modified lipid composition by reducing the abundance of long chain n−3 fatty acids (Prindiville et al., 2011). Du et al. (2004) reported that exposure to fenofibrate increased peroxisome-related activities by about 30%, resulting in a lower content of eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) in the body lipids of exposed trouts. A significant decrease of cholesterol, total lipids and triglycerides was observed in the crab *Pachygrapsus marmoratus* after several injections of 50 mg/kg clofibric acid (Lautier et al., 1986). In bivalves, peroxisome proliferation and increased peroxisomal acyl-CoA oxidase activity were demonstrated in the mussel *Mytilus galloprovincialis* after injection of 1.5 mg clofibrate per mussel (Cancio et al., 1998). Moreover, Canesi et al. (2007b) showed that concentrations of 3 to 300 ng/g dry wt. of the hypolipidemic drugs bezafibrate and gemfibrozil affected the immune function, as well as glycolysis, redox balance and peroxisomal function in the marine mussel *Mytilus galloprovincialis*. Meanwhile, exposure of zebra mussels to 1 µg/L gemfibrozil induced signs of oxidative stress in addition to elevated lipid peroxidation (Quinn et al., 2011).

1.3 Establishing new endpoints for aquatic toxicology studies in invertebrates

Even though aquatic invertebrates constitute a vast and diverse group with great ecological importance, detailed insights into their endocrinology is still lacking. This absence of knowledge hampered the progress in understanding endocrine disruption in invertebrates. On the other hand, there is evidence that environmental pollutants are interfering with the endocrine system of these organisms and disturb reproduction (Porte et al., 2006). Thus, a better understanding of invertebrate physiology is needed especially on the endogenous/exogenous factors controlling reproduction as well as the biosynthesis and metabolism of steroids including the enzymatic pathways involved. This knowledge will ultimately allow the establishment of new endpoints for aquatic toxicology studies and will help to estimate effects of endocrine disruption of environmental contaminants in these organisms.

1.3.1 Reproductive cycle

Reproductive success and population dynamics of aquatic organisms are often highly sensitive to environmental perturbations, and disturbance of reproduction can exert negative changes in community structure (Lowe and Pipe, 1987; Chu et al., 2003). The reproductive cycle can be used as a good indicator for the general fitness of an organism, as it integrates on several physiological levels, the effects of environmental stressors, including the presence of pollutants (Kime, 1995). Indeed, several studies have already demonstrated the potential of contaminants to interfere with the reproductive system of aquatic organisms. Thus, sperm and testicular abnormalities have been reported in wild flounder populations from estuaries receiving discharges of industrial and domestic waste (Gill et al., 2002). Mixture of industrial contaminants including organochlorinated compounds and DDTs induced arrest of spermatogenesis in wild carps (Lavado et al., 2004). Polychlorinated biphenyls (PCBs) negatively affected the embryological success in the starry flounder (Spies and Rice, 1988). Exposure of zebra fish (*Danio rerio*) to ethinylestradiol caused inhibition of reproduction (Segner et al., 2003), arrest in development of eggs and induction of vitellogenesis in males (Kime and Nash, 1999), whereas exposure of fathead minnows (*Pimephales promelas*) to bisphenol A resulted in inhibition of gonad growth (Sohoni et al., 2001).

Alterations on reproductive events have also been evidenced in a wide range of aquatic invertebrates, including several species of bivalve molluscs (Table 3). For instance, cadmium exposure inhibited follicle development in *Mytilus edulis* (Kluytmans et al., 1988). TBT is known to interfere with gonadal development and sexual maturation in mussels and clams (Regoli et al., 2001; Siah et al., 2003). Whereas studies on wild bivalve populations from contaminated sites demonstrated modification of spawning behaviour, oocyte degeneration, and alterations of the female reproductive apparatus (Binelli et al., 2001).

Table 3. Alterations of reproductive events in bivalves.

Species	Source	Pollutant(s)	Observed effects	Reference
Cerastoderma edule	Exposure	Pulverized fuel ash	Reduced oocyte maturation	Bowmer et al., 1994
Donax trunculus	Impacted river	N.A.	Shortened spawning period	Tlili et al., 2011
Dreissena polymorpha	Polluted lake	DDT and DDT homologues	Delay in oocyte maturation and spawning, and degeneration of oocytes	Binelli et al., 2004 Mantecca et al., 2003
Macoma balthica	Exposure	PAHs, PCBs and heavy metals in sediments	Reduction of spawning, fertilization and gonadal development	Timmermans et al., 1996
Mizuhopecten yessoensis	Polluted bay	N.A.	Retardation of gametogenesis and larval growth, oocyte resorption, autolysis of spermatozoa, decrease in fertilization success	Vaschenko et al., 1997
Modiolus kurilensis	Polluted bay	HCH, DDT and heavy metals in sediments	Alterations in spermatogenic and somatic cells in the testes	Yurchenko and Vaschenko, 2010
Mya arenaria	Impacted fjord	N.A.	Delayed gametogenesis	Gauthier-Clerc et al., 2002
Mytilus edulis	Exposure	DDT, copper and diesel oil	Oocyte degeneration, reduction in spawning frequency	Lubet et al., 1978 Strømgren and Nielsen, 1991
Mytilus galloprovincialis	Polluted estuary	PAHs in tissues	Gonadic neoplastic disorders	Ruiz et al., 2011
Potamocorbula amurensis	Polluted estuary	Silver in tissues	Reduced reproductive activity	Brown et al., 2003
Ruditapes decussatus	Polluted sea region	Cadmium in tissues	Perturbations of sexual maturation	Smaoui-Damak et al., 2006
Scrobicularia plana	Polluted estuary	TBT in tissues	Interference with embryonic development	Ruiz et al., 1995

N.A.: No contaminant analysis was performed.

Hence, studies clearly demonstrate that the reproductive axis of aquatic organisms is a target for a wide range of pollutants and therefore the assessment of their impact on wild populations should be in the focus of toxicological investigations. Many effects ultimately result in the alteration of individual reproductive processes (gonad development, gamete production and maturation, spawning etc.) whose analysis may therefore serve as marker of contaminant exposure. However, to make the assessment of reproduction in bivalves a reliable tool for toxicological studies, knowledge about invertebrate reproduction has to be improved; especially the natural progression and variation of individual reproductive events as well as the mechanisms involved in their control.

Bivalve species undergo an annual reproductive cycle which comprises a gametogenic phase, a spawning event (single or various consecutive) and a period of gonadal reconstitution (Harvey and Vincent, 1989; Juhel et al., 2003). Several environmental factors, such as temperature, salinity, photoperiod and food as well as physiological factors, like nutrient reserves, hormonal cycles and genotype may determine the onset and duration of the various phases and by this control reproduction in bivalves (Ruiz et al., 1992a; Cano et al., 1997; Osada et al., 2003; Darriba et al., 2004; Gooding and LeBlanc, 2004).

Temperature has already been linked to gametogenic development in bivalves and to the regulation of spawning (Ruiz et al., 1992b). For example, Bayne (1975) reported a linear relationship between the rate of gametogenesis and temperature changes in the mussel *Mytilus edulis*. Sastry and Blake (1971) showed that temperature influences the initiation of oocyte growth in the scallop *Argopecten irradians* by regulating the transfer of nutrient reserves to the gonads. Initiation of spawning strongly depends on the presence of adequate water temperatures. Several studies suggested that for some bivalves, a certain "temperature threshold" has to be reached so that gamete release can be initiated, which in addition can be highly variable between species and geographical region (McMahon, 1996; Honkoop et al., 1999a; Rajagopal et al., 1998; Vélez et al., 1990). Besides temperature, food quantity and quality has been reported to be another important factor with impact on gonadal development (Delgado and Camacho, 2005), egg quality, and reproductive investment (Wacker et al., 2002; Wacker and Von Elert, 2003). Hence, food availability exerts a significant influence upon production of ripe gametes during spawning (Bayne, 1976) while it also

synchronizes the release of gametes and guaranties the survival and development of larvae and juveniles (Himmelman, 1975). This is not surprising, since those processes are energy demanding and therefore dependent on the available food supply, stored energy reserves, or both. Experiments with scallops showed that starving during early gametogenesis resulted in decrease in digestive gland tissue, gonad index and also resorption of oogonia (Sastry and Blake, 1971).

Although several works elucidated the importance of environmental factors in bivalve reproduction, only few studies have investigated the potential endogenous factors involved. Nevertheless, evidence exists demonstrating the importance of the neuroendocrine system in the control of reproductive functions in bivalves. As demonstrated through *in vitro* studies with *Mytilus edulis*, neurohormones control gonial mitosis and the reinitiation of meiosis in males, and previtellogenesis and vitellogenesis in females, through directly acting on the gonad (Mathieu et al., 1991). Moreover, field studies showed a relation between annual variations of monoamines such as serotonin, dopamine and noradrenaline as well as several prostaglandins, and reproductive processes like gonad development, gametogenesis and spawning, which may indicate their involvement in these events (Makoto et al., 1989; Martínez and Rivera, 1994; Martínez et al., 1999).

Even though these studies present useful information on the possible involvement of exogenous and endogenous factors in invertebrate reproduction, the available knowledge can still be considered marginal if a comparable complexity as seen in the vertebrate reproductive system can be assumed. More research is needed in order to uncover the physiological mechanisms involved in the endogenous regulation of reproduction in invertebrates. This requires a better knowledge of their reproductive system and also of the potential factors involved in its control, such as variations in annual hormone levels and lipid composition. This may help to give a better understanding of the natural variability of the reproductive cycle and open the possibility to use hormone and lipid analysis as potential new tools in toxicological studies.

1.3.2 Sex steroids in molluscs

Over the last decades the presence of vertebrate-like sex steroids has been demonstrated in several mollusc species with the gonads and the digestive gland being the main site of steroid production (De Longcamp et al., 1974; Matsumoto et al., 1997; Janer et al., 2005b). Among the identified steroids are several forms of estrogens such as 17β-estradiol, estrone and estriol; androgens like 3α-androstanediol, androsterone, dehydroepiandrosterone (DHEA), androstenedione, testosterone and 5α-dihydrotestosterone; and progestins such as pregnenolone, 17α-hydroxypregnenolone, progesterone and 17α-hydroxyprogesterone (Le Guellec et al., 1987; Lupo di Prisco et al., 1973; Reis-Henriques and Coimbra, 1990; Reis-Henriques et al., 1990) (Table 4). Despite the fact that their existence has been demonstrated in molluscs, their origin is still a controversial subject which has been in the focus of scientific discussion for a long period (Scott, 2012). Initially it was thought that sex steroids were accumulated by molluscs though their uptake from the environment due to their presence in several plant species (Janeczko and Skoczowski, 2005). However, several studies have demonstrated that molluscs are able to synthesize sex steroids from precursors such as cholesterol or pregnenolone (Janer and Porte, 2007; Lafont and Mathieu, 2007) (Figure 5). Moreover, most of the steroidogenic pathways described for vertebrates have been demonstrated to occur in molluscs, either by directly exposing the organisms to steroid precursors or by incubating homogenates with those steroid precursors. Most of the enzymes involved in steroid metabolism can convert a variety of steroids and, some of them (e.g. hydroxylases, phase II enzymes), can also metabolize a wide range of structurally unrelated molecules (Figure 5).

Table 4. Steroids detected in tissues of molluscs.

Species	Matrix	Steroid(s)	Reference
Bivalves:			
Dreissena polymorpha	Whole animal	Estradiol	Peck et al., 2007
Mya arenaria	Gonad	Progesterone	Siah et al., 2002
Mytilus edulis	Whole animal	Progesterone, androstenedione, androsterone, androstanediol, testosterone, 5α-dihydrotestosterone, estradiol, estrone	Reis-Henriques et al., 1990
Mytilus galloprovincialis	Whole animal	Testosterone, estradiol	Janer et al., 2005a
Pecten hericius	Ovaries	Progesterone, estradiol	Botticelli et al., 1961
Ruditapes decussatus	Gonad	Progesterone, testosterone, estradiol	Ketata et al., 2007
Sinonovacula constricta	Gonad	Testosterone, estradiol	Yan et al., 2011
Gastropods:			
Aplysia depilans	Gonad	Pregnenolone, progesterone, 17α-hydroxyprogesterone, testosterone, estradiol, estrone	Lupo Di Prisco et al., 1973
	Hepatopancreas	Pregnenolone, 17α-hydroxypregnenolone	
Bolinus brandaris	Visceral coil	Testosterone, estradiol	Morcillo and Porte, 1999
Helix aspersa	Gonad	Androsterone, dehydroepiandrosterone, androstenedione, 3α-androstanediol, estradiol, estrone, estriol	Le Guellec et al., 1987
Marisa cornuarietis	Digestive gland / gonad complex	Testosterone, estradiol	Janer et al., 2006
Nucella lapillus	Whole animal	Progesterone, testosterone, estradiol	Spooner et al., 1991
Thais clavigera	Gonad	Androstenedione, testosterone, estradiol, estrone	Goto et al., 2012
Cephalopods:			
Octopus vulgaris	Gonad (Testis)	Progesterone, testosterone, estradiol	D'Aniello et al., 1996
	Gonad (Ovary)	Progesterone, estradiol	Di Cosmo et al., 2001

5α-reductases

These enzymes are membrane-bound and act upon steroids containing a Δ^4-3-keto configuration by reducing the double bond in the A ring. Thus, testosterone can be converted to 5α-dihydrotestosterone (DHT), its most potent metabolite. 5α-reductases reduce all Δ^4-3-ketosteroids (including progesterone, androstenedione and corticosterone) but with different kinetics and specificity according to the steroidogenic tissue (Normington and Russell, 1992; Poletti et al., 1998). The action of these enzymes is fundamental since type II 5α-reductase deficiency in mammals produces a syndrome termed male pseudohermaphroditism which is characterised by ambiguous external genitalia and decreased levels of plasma dihydrotestosterone (Imperato-McGinley et al., 1974). In addition, 5α-reduction precludes the aromatization of androgens to estrogens and promotes intracellular accumulation of androgen (Wilson, 2001). 5α-reductase activity has been reported in echinoderm, mollusc, and crustacean species. In molluscs, 5α-reductase activity has been demonstrated when exposing the gastropods *Clione antarctica* or *Ilyanassa obsoleta* to progesterone, androstenedione or testosterone *in vivo* (Hines et al., 1996; Oberdörster et al., 1998). Similarly, incubations of testosterone or androstenedione with digestive gland microsomes from the bivalves *Ruditapes decussata* (Morcillo et al., 1998) and *Mytilus* sp. (Janer et al., 2005a), the gastropods *Littorina littorea* (Ronis and Mason, 1996) and *Marisa cornuarietis* (Janer et al. 2005b), and with gonad homogenates of the sea urchin *Paracentrotus lividus* (Lavado et al., 2006b) and the gastropod *Helix aspersa* (Le Guellec et al., 1987) demonstrated the presence of a 5α-reductase pathway. In contrast to many vertebrate and invertebrate species, 5α-reductase plays a more important role than 17β-hydroxysteroid dehydrogenases in the metabolism of androstenedione in molluscs.

Hydroxysteroid dehydrogenases

17β-hydroxysteroid dehydrogenase (17β-HSD) enzymes are found in steroidogenic cells, either membrane bound or soluble, where they catalyse the final step in the synthesis of gonadal steroid hormones, i.e. the transformation of inactive 17-keto steroids into their active 17β-hydroxy forms or *vice versa*,. Those include the interconversion of androstenedione and testosterone, estrone and 17β-estradiol, or androstanedione and dihydrotestosterone. The presence of the 17β-HSD pathway has already been reported in several bivalves like the mussels *Mytilus galloprovincialis*

(Janer et al., 2005a) and *Mytilus edulis* (De Longcamp et al., 1974), the oyster *Crassostrea gigas* (Le Curieux-Belfond et al., 2001) and the clam *Ruditapes decussata* (Morcillo et al., 1998). In addition, annual variations of 17β-HSD activity have been linked to different gonadal maturation stages in bivalves (Mori et al., 1966; Matsumoto et al., 1997) which, as suggested, may reflect the dynamics of steroid metabolism during reproduction.

Cytochrome P450 dependent biotransformations: aromatization and hydroxylation

Cytochrome P450 monooxygenase enzymes (CYP) comprise an ancient and widely distributed protein superfamily. P450-type enzymatic activities have been reported in crustaceans, molluscs, and echinoderms (Livingstone et al., 1989; Livingstone, 1991; James and Boyle, 1998), mainly in digestive gland and pyloric caeca (Snyder, 2000). Typically, total P450 protein and associated enzymatic activities in invertebrates are found to be tenfold lower than in mammals (Livingstone, 1991).

Aromatase

The conversion of the C19 steroids to estrogens is catalyzed by the CYP19 isoform. This enzyme requires NADPH as a cofactor and involves hydroxylations and dehydrations that culminate in the aromatization of the A ring of the androgens (Simpson and Davis, 2001). Aromatase activity has been reported in molluscs (Matsumoto et al., 1997; Morcillo et al., 1999; Le Curieux-Belfond et al., 2001; Horiguchi et al., 2004) and in echinoderms (Lavado et al., 2006b). A possible involvement of aromatase activity in reproduction has been suggested to exist in *Patinopecten yessoensis* where highest levels were measured in the gonads during the growing and mature stage followed by a strong decrease at spawning (Osada et al., 2004).

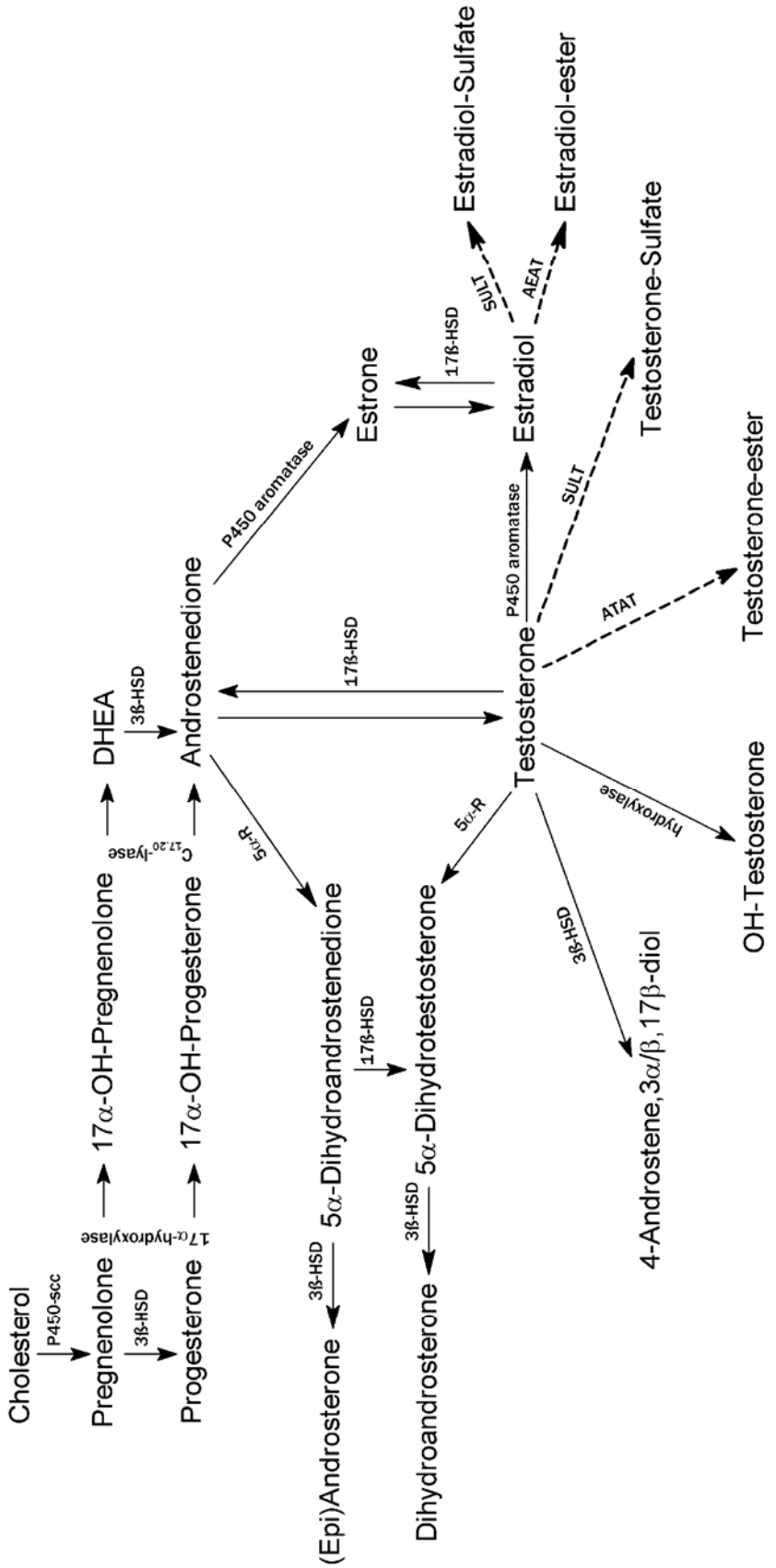

Figure 5. Pathways of steroid biosynthesis and metabolism in molluscs. P450-scc: P450 side-chain cleavage; 3ß- or 17ß-HSD: 3ß- or 17ß-hydroxysteroid dehydrogenases; 5α-R: 5α–reductase; ATAT: fatty acid acyl-CoA testosterone acyltransferase; AEAT: fatty acid acyl-CoA estradiol acyltransferase; SULT: sulfotransferase; DHEA: dehydroepiandrosterone; ------→: phase II metabolism of steroids (according to Fernandes et al. (2011)).

Hydroxylases

Hydroxylation is one of the possible pathways to render sex steroids inactive. Testosterone serves as a substrate for many P450s, and is hydroxylated in a regio specific and stereospecific manner by many different P450 isozymes (Waxman et al., 1983). In molluscs and echinoderms, testosterone hydroxylation activity has been demonstrated in digestive gland/digestive tube microsomes (Ronis and Mason, 1996; Janer et al., 2005b). The metabolic rates recorded for invertebrate enzymes and pathways involved in these hydroxylations were much lower than those usually found in vertebrates.

Sulfotransferases

Sulfate conjugation is a major pathway of detoxification or bioactivation of foreign compounds and it is important in modulating the metabolism and biological activity of endogenous substances, including steroids (Strott, 1996). Sulfonation of low-molecular weight compounds such as hydroxysteroids, estrogens, and catecholamines is catalyzed by cytosolic sulfotransferases belonging to a gene superfamily designated as SULT (Weinshilboum et al., 1997). These cytosolic enzymes utilize 3′-phosphoadenosine 5′-phosphosulfate as the sulfonate (SO_3^-) donor (Strott, 1996). The sulfation of steroids is considered to have an important role in inhibiting their biological activity and increasing their excretion. Sulfate conjugates have been frequently identified as the major metabolites formed following hydroxylation of xenobiotics (James, 1987). In addition, sulfate conjugates of steroid hormones have been observed in crustaceans (Swevers et al., 1991; Baldwin et al., 1998) and in molluscs (Hines et al., 1996; Ronis and Mason, 1996).

Fatty acid acyl-CoA acyltransferases

Fatty acid conjugation (or esterification) transforms steroids into an apolar form, which can be retained in the lipoidal matrices of the body, and reduces their bioactivity, bioavailability, and susceptibility to elimination (Borg et al., 1995). The esterification of steroids with fatty acids is facilitated by the acyl-coenzyme A:steroid acyltransferases. Conjugated steroids do not bind to steroid receptors, but can be hydrolysed when needed by esterases liberating the active steroid. They are considered to be long-acting steroids (Hochberg, 1998) and esterification is known to occur in both vertebrate and invertebrate species. Esterified steroids have been detected in several mollusc species

including the bivalves *Mytilus edulis* (Labadie et al., 2007), *Mytilus galloprovincialis* (Fernandes et al., 2010) and *Crassostrea virginica* (Janer et al., 2004). Moreover, esterification may represent the major biotransformation pathway for testosterone in molluscs, based on studies showing that exogenously provided testosterone or estradiol is converted to fatty acid esters and retained in the tissues of the mud snail *Ilyanassa obsoleta* (Gooding and LeBlanc, 2001) or the mussel *Mytilus galloprovincialis* (Janer et al., 2005a). Furthermore, decreases of fatty acid esterification of testosterone were reported during the onset and end of the reproductive season in *Ilyanassa obsoleta* (Gooding and LeBlanc, 2004) indicating their possible role in reproductive processes, however, still little is known what precise role they play in mollusc physiology.

The interference of contaminants with steroid synthesis and metabolism may alter the bioavailable amounts of active steroids within the organism, and can be a potential mechanism of endocrine disruption. This has already been evidenced in studies on aquatic organisms including molluscs. Hence, contaminants like TBT for example inhibit P450 aromatase activity in bivalves (Morcillo et al., 1998; Le Curieux-Belfond et al., 2001) and reduce ATAT activity and accumulation of testosterone fatty acid esters in gastropods (Gooding et al., 2003; Janer et al., 2005c). Exposure of *Mytilus edulis* to a mixture of North Sea oil and alkylphenols resulted in a significant increase in levels of esterified testosterone and estradiol which was accompanied by an increased sulfation of estradiol (Lavado et al., 2006a). Furthermore, 4-nonylphenol was shown to reduce testosterone and estradiol levels in zebra mussels (Riva et al., 2010). Even though those studies provide evidence that contaminants can interfere with sex steroids in molluscs, much more information is needed on their impact on the endocrine system and its potential consequences for reproductive processes. Indeed, disturbance of sexual maturation and sexual differentiation have already been associated to changes in hormone levels in clams exposed to aquatic contaminants (Gagné et al., 2003; Siah et al., 2003) showing that alterations of steroid titer may negatively affect reproduction.

The role of steroids in mollusc reproduction

Despite the existence of sex steroids in several mollusc species, their role in reproductive endocrinology has yet to be established. Nevertheless, there are several studies relating annual variations of sex steroids to reproductive events in wild bivalves. Reis-Henriques and Coimbra (1990) for example showed that the progression of progesterone levels in the whole body tissue of *Mytilus edulis* followed the reproductive cycle with highest values appearing during the spawning season. Similar observations were made with *Mya arenaria* where increases in gonadal progesterone levels occurred during the ripe stage in males and during the spawning stage in females (Siah et al., 2002); meanwhile 17β-estradiol and testosterone showed highest concentrations during the onset of vitellogenesis in females and during spawning in both sexes (Gauthier-Clerc et al., 2006). Moreover, in the clam *Ruditapes decussatus* progesterone and testosterone increased at the end of gametogenesis in both sexes while highest 17β-estradiol levels were recorded at the beginning of vitellogenesis in females (Ketata et al., 2007). In addition, results of several laboratory studies have provided critical evidence to support a possible implication of sex steroids in bivalve reproduction. Thus, injections of estradiol, testosterone, and progesterone stimulated gamete development in adult scallop *Mizuhopecten yessoensis*, leading to increases in gonad weight and oocyte diameters (Varaksina and Varaksin, 1991; Varaksina et al., 1992). Whereas, injections of estradiol in oysters *Crassostrea gigas* at early stages of maturation induced sex reversal of males to females (Mori et al., 1969). In addition, exposure experiments with the pectinids *Patinopecten yessoensis* and *Placopecten magellanicus* showed the involvement of sex steroids in vitellogenesis, gamete release, gonadal differentiation, sex determination and oocyte growth (Osada et al., 2003; Wang and Croll, 2003, 2004, 2006).

1.3.3 The importance of lipids and fatty acids

Lipids are of great nutritional value and fulfil vital functions in bivalve physiology, especially because metabolism and transport of lipids is of particular importance in reproduction and larval development (Barber and Blake, 1981; Mclean and Bulling, 2005). They represent a substrate with the highest energy yield per unit weight and are the major reserves in the digestive gland of mussels which are depleted when food availability is low (Thompson et al., 1974). The major components of lipids are phospholipids, sterols, triglycerides and free fatty acids whose variations follow seasonal cycles in mussels and which have been associated with reproductive events like gametogenesis and spawning (Pazos et al., 1996; Soudant et al., 1999). Lipids are usually accumulated during gametogenesis, especially during higher food abundance in spring, where they get mobilized and gradually passed on to the developing gametes, leading to their subsequent loss during spawning (Kluytmans et al., 1985; Napolitano et al., 1992; Nalepa et al., 1993). *In vivo* studies with the Atlantic bay scallop *Aequipecten irradians* have demonstrated that reserves stored in the digestive tissue (mostly triglycerides and phospholipids) are transferred to the gonad during gametogenesis and used for the production of ova (Sastry and Blake, 1971; Barber and Blake, 1985). This process guaranties the supply of eggs with the quality and quantity of lipid material that provides a highly concentrated form of energy reserve for the planktonic stage, a crucial requirement for larval survival and successful metamorphosis (Gallager and Mann, 1986; Helm et al., 1991).

As a constituent of lipids, fatty acids (FAs) play a major role in several important physiological mechanisms like cell structure and function, energy metabolism and storage, bioactive signaling and synthesis of various compounds involved in physiological regulation e.g. steroids, eicosanoids among others (Benatti et al., 2004). FAs can generally be divided into three major groups, saturated fatty acids (SFAs), monounsaturated fatty acids (MUFAs) and polyunsaturated fatty acids (PUFAs) whose abundances largely depend on dietary input. Most of those fatty acids derive from phytoplankton (e.g. diatoms, flagellates), bacteria and detritus which are the major nutritional sources of suspension feeding bivalves (Bastviken et al., 1998; Enright et al., 1986). Hence, species that mainly feed on phytoplankton usually show a high abundance of palmitic acid (16:0), palmitoleic acid (16:1n-7), vaccenic acid (18:1n-7),

octadecatetraenoic acid (18:4n-3), eicosapentaenoic acid (20:5n-3) and docosahexaenoic acid (22:6n-3) (Orban et al., 2002; Pazos et al., 2003; Alkanani et al., 2007).

Essential fatty acids such as arachidonic acid (20:4n-6), 20:5n-3 and 22:6n-3 are reported to have a key role in bivalve physiology like membrane functioning, development and reproduction. FAs often show typical annual variations which are mostly attributed to environmental factors (food scarcity, changes in plankton composition, temperature variations, and habitat changes), however there is also evidence showing that their annual fluctuations parallel reproductive events which may imply their involvement in reproduction itself (Kluytmans et al., 1985; Freites et al., 2002; Prato et al., 2010). A series of studies with the great scallop *Pecten maximus* for instance investigated the potential role of essential fatty acids in reproduction, which showed that (a) 22:6n-3 represents an important fatty acid on structural and functional levels of cellular making which also implies its necessity during oogenesis and embryogenesis (Soudant et al., 1996a); (b) higher dietary levels of 22:6n-3 resulted in a faster rate of gametogenesis, higher hatching rates and a smaller percentage of larvae with abnormalities (Soudant et al., 1996b); (c) annual variations of 22:6n-3 and 20:5n-3 followed reproductive phases like the gametogenic cycle (Pazos et al., 1997) as well as oocyte maturation and spawning (Besnard et al., 1989). Similarly, 20:4n-6 which is involved in membrane lipid metabolism has been found in high levels in the gonads of female scallops, indicating its potential role in oogenesis (Soudant et al., 1997). Another characteristic of 20:4n-6 is its function as precursor for a group of signal molecules called eicosanoids which control several important physiological processes in invertebrates. Eicosanoids include prostaglandins, thromboxanes, leukotrienes, hydroxy fatty acids and lipoxins, which are involved in the regulation of egg-production, egg-laying, spawning and hatching, among other processes (Stanley-Samuelson, 1987; Stanley and Howard, 1998). The available information on the possible implication of FAs in reproductive processes is scarce but sufficient evidence has been provided that shows that they may be an important factor for the better understanding of bivalve reproductive physiology.

Knowledge about the effects of environmental contaminants on fatty acid composition and lipid levels in molluscs is limited; however, some studies have already demonstrated the lipid altering potential of some compounds. TBT and TPT exposure caused changes in total lipid levels and fatty acid profiles in the ramshorn snail *Marisa*

cornuarietis (Janer et al., 2007; Lyssimachou et al., 2009). In field studies, changes in total lipid levels and lipid composition (triglycerides, phospholipids) in *Mytilus edulis* were associated with the body burdens of aromatic hydrocarbons and polychlorinated biphenyls of organisms from contaminated sites (Capuzzo and Leavitt, 1988). Mechanisms behind the alterations reported in these studies are still unknown and further research is needed to shed light on the physiological processes involved in the maintenance of lipid homeostasis. Therefore characterization of basal lipid levels and fatty acid profiles of natural populations have to be performed and their dependency on external and internal factors understood. Once established, this knowledge may offer a novel and complementary tool to assess endocrine disruption in organisms living in polluted environments.

1.4 The use of zebra mussel (*Dreissena polymorpha*) as sentinel organism

The zebra mussel (*Dreissena polymorpha*) (Pallas), (Mollusca, Bivalvia, Veneroida, Dreissenidae) is an epifaunal species which lives byssaly attached to all types of solid substrates like breakwalls, rocks, wood and organic and inorganic debris. The life cycle consists of a relatively sessile adult phase and a planktonic, free-swimming larval phase. Most adult mussels are dioecious, although a small proportion of the population can be hermaphroditic (Nichols, 1996). Fertilization occurs externally in the water, where especially females show exceedingly high fecundities reaching up to one million eggs per individual during one season (Ram et al., 1996a). Its strong proliferation, rapid growth and short highly adaptive biological cycle in combination with the highly motile veliger larvae contribute to its success as invasive species (Mackie, 1991). Moreover, zebra mussels show a high resistance to brusque changes in water temperature and salinity and are also capable to survive several days outside the water (Pollux et al., 2003). The planktonic veliger stage can remain suspended in the water column for about 2-4 weeks, and may travel downstream several hundred kilometers before settling and founding new populations (Horvath and Lamberti, 1999).

Its biological properties made it possible for the zebra mussel to colonize several new aquatic ecosystems in the last century. Majorly driven by human-mediated dispersal mechanisms, it spread from its origins the Black and Caspian Seas over most

of Europe and reached as far as the Great Lakes of North America (Mackie and Schloesser, 1996). Adults attach themselves to boats and ships, mostly of commercial or recreational nature like fishing boats, pleasure crafts and sport boats whereas veliger larvae travel in the ballast water of larger ships which allows them to be introduced into remote areas after being discharged into rivers and lakes (Hallstan et al., 2010). The arrival of the zebra mussel in Spain was firstly reported in the Ebro River in 2001 with the colonization of the Riba Roja reservoir (Ruiz-Altaba et al., 2001).

As previous studies demonstrated, the zebra mussel represents a potential good candidate for the evaluation of contaminant toxicity in laboratory experiments and freshwater monitoring studies (Table 5). The main characteristics that make it a good sentinel organism are (1) its sedentary nature, long lifespan and the fact that it can be easily collected and handled; (2) its high tolerance to environmental contaminants (Dauberschmidt et al., 1997; Hendriks et al., 1998); and (3) its high filtration rates which guaranty the uptake of pollutants suspended within the surrounding water column combined with a high accumulation potential for both inorganic and organic contaminants (Cope et al., 1999).

Table 5. Environmental toxicology studies using zebra mussel as bioindicator.

Compound Class	Compound Species	Reference
Heavy metals	Ni, Cd, Pb, Hg, Cu, Zn	Kraak et al., 1994, 1999 Navarro et al., 2011
Industrial chemicals	Chrysene, Hexachlorobiphenyl, Nonylphenol, Pentachlorophenol, Aroclor 1260, DDT, TBT	Gossiaux et al., 1998 Regoli et al., 2001 Pavlica et al., 2001 Binelli et al., 2006, 2008 Riva et al., 2010
Pharmaceuticals	Imipramine, Desipramine, Clomipramine, Fluvoxamine, Fluoxetine, Paroxetine, Gemfibrozil, Diclofenac, Paracetamol, Ibuprofen	Hardege et al., 1997 Fong, 1998 Parolini et al., 2010, 2011 Quinn et al., 2011

1.5 Study area

The Ebro River with a length of 910 km runs through the North-East section of Spain flowing from its source in the Cantabric Mountains south-eastwards into the Mediterranean Sea. It comprises the most extensive catchment area (85,362 km^2) of Spain with a vast network of tributary rivers (12,000 km) forming an area of 33,000 ha of wetlands called the Ebro Delta (Tockner et al., 2009). Even though the main climate along the river basin is Mediterranean-continental, the Ebro also traverses other climate zones that range from mountain climate in the North to semi-arid in the center to Atlantic in the headwaters. The average temperatures range from lowest levels between 0-12 °C near the Pyrenees to highest between 7-25 °C in the semi-arid area. The Ebro basin, even though not being a highly populated area in comparison to Spanish means, is affected by multiple impacts with industrial, urban and agricultural origins (Terrado et al., 2006). Close to 190 large damns have been constructed impounding more than half of the annual runoff which is used for hydropower generation and irrigation (Vericat and Batalla, 2006). The Ebro basin is also of high ecological and economic value because it harbors a great biodiversity of organisms (~3% of the catchment area) which are protected through biosphere reserves and national parks.

The two sampling sites of this study are located in the lower section of the Ebro River in the Catalan region (Figure 6). The sampling site at the city of Flix (S2) is within a highly polluted area of the Ebro River. The main source of contamination is the mercury cell chlor-alkali plant (ERCROS) situated at the base of the Flix reservoir, which has been dumping wastewater into the river system for almost a century. According to Bosch (2009), an estimation of 200,000–360,000 tons of industrial waste containing heavy metals (mostly mercury, but also cadmium and zinc) and various organochlorine compounds including penta-/hexachlorobenzenes, DDT and its metabolites, polychlorobiphenyls, polychlorostyrenes and also radioactive ^{210}Pb have accumulated in the riverbed. On the other hand, the sampling site close to the town of Riba Roja (S1) is located approx. 7-8 km upstream of Flix and was chosen as an uncontaminated reference site since it has been described as a region with low pollution levels (Faria et al., 2009).

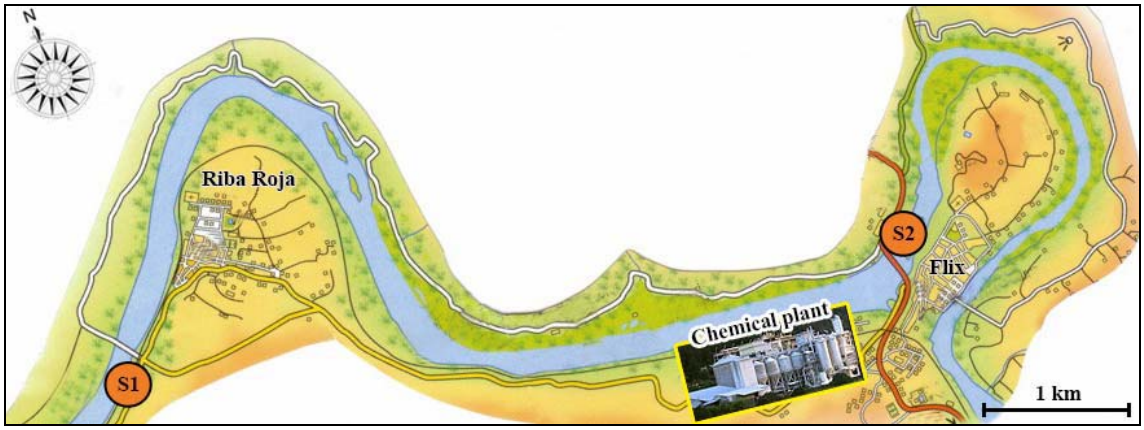

Figure 6. Map of the studied area (Ebro River) indicating the sampling sites S1 (Riba Roja) and (S2) Flix where zebra mussels were collected.

2. OBJECTIVES OF THE THESIS

2. OBJECTIVES OF THE THESIS

The overall objective of the thesis was to develop new toxicological endpoints to be applied in biomonitoring studies of freshwater systems by using the zebra mussel (*Dreissena polymorpha*) as a sentinel species. Among the investigated endpoints, special emphasis was placed on lipids (triglycerides and fatty acids), steroids (testosterone and estradiol) and steroid metabolizing enzymes, and their relationship with the zebra mussel reproductive cycle and key reproductive events. Furthermore, the effects of three model contaminants namely, the pharmaceuticals fluoxetine and clofibrate, and the biocide tributyltin were investigated following laboratory exposures at environmentally relevant concentrations with the aim of detecting alterations either in zebra mussel endocrine system or significant changes in lipids. Finally, a field study was performed to assess the health status of a zebra mussel population from a contaminated area (Flix) in comparison to a reference site (Riba Roja) by looking at changes in gonads, gonad maturation stages, endogenous steroid levels and lipid homeostasis.

The results of this research are presented as a collection of papers addressing specific aims of the work:

1. To investigate the reproductive cycle of a zebra mussel population from a relatively unpolluted area and to uncover the possible involvement of steroids and steroid metabolizing enzymes in key reproductive events, such are gametogenesis and spawning.

Paper 1. Lazzara, R., Fernandes, D., Thibaut, R., Porte, C. Study of the reproductive cycle of *Dreissena polymorpha* from the Ebro River: Insights into the involvement of endogenous and exogenous factors (In preparation).

2. To characterize the lipid content and fatty acid profiles along the reproductive cycle of the zebra mussel and to investigate the effects of the lipid regulator clofibrate on total lipids, triglycerides and fatty acid profiles following exposure to a wide range of concentrations of clofibrate.

Paper 2. Lazzara, R., Fernandes, D., Faria, M., López, J.F., Tauler, R., Porte, C., 2012. Changes in lipid content and fatty acid composition along the reproductive cycle of the freshwater mussel *Dreissena polymorpha*: Its modulation by clofibrate exposure. Science of the Total Environment, 432, 195-201.

3. To investigate the potential effects of exposure to low levels of fluoxetine (20-200 ng/L) on male and female gonad structure and gamete release, as well as on the levels of endogenous steroids (testosterone and estradiol) in the zebra mussel.

Paper 3. Lazzara, R., Blázquez, M., Porte, C., Barata, C., 2012. Low environmental levels of fluoxetine induce spawning and changes in endogenous estradiol levels in the zebra mussel *Dreissena polymorpha*. Aquatic Toxicology, 106-107, 123-130.

4. To investigate the ability of TBT to act as an endocrine disrupter in zebra mussel as well as to act as an obesogen by altering lipid homeostasis, and to investigate the different sensitivity of males and females to TBT exposure.

Paper 4. Lazzara, R., Fernandes, D., Porte, C. Short-term exposure to tributyltin modulates steroid levels and lipids in the zebra mussel *Dreissena polymorpha* (In preparation).

5. To assess the health status of a zebra mussel population from a historically polluted area of the Ebro River by determining endogenous levels of testosterone and estradiol, lipids and gonadal development as markers of endocrine alteration.

Paper 5. Lazzara, R., Fernandes, D., Porte, C. Evidence of endocrine disruption in a zebra mussel population from the Ebro River (In preparation).

3. IMPACT FACTOR OF PUBLISHED ARTICLES

CONSEJO SUPERIOR DE INVESTIGACIONES CIENTÍFICAS
Instituto de Diagnóstico Ambiental y Estudios del Agua
(IDÆA)

Cinta Porte Visa, directora de la Tesis doctoral presentada por Raimondo Lazzara, expone que el doctorando ha participado activamente en la realización de todos los artículos presentados en la memoria, tal y como se refleja en la distribución de autores, siendo primer autor de los 5 artículos presentados. En general ha participado en la discusión del diseño experimental, y ha contribuido de manera significativa al análisis de muestras, elaboración y redacción de trabajos.

A continuación se detallan los artículos que forman parte de esta tesis, el grado de participación del doctorando y el Factor de Impacto (F.I.) de las revistas en los cuales los artículos han sido publicados:

1**. Raimondo Lazzara**, Denise Fernandes, Rémi Thibaut, Cinta Porte. Study of the reproductive cycle of *Dreissena polymorpha* from the Ebro River: Insights into the involvement of endogenous and exogenous factors (en preparación).

Grado de participación: Realización del trabajo experimental, con excepción de la medida de la actividad enzimática ATAT. Discusión de resultados y elaboración del manuscrito.

2. **Raimondo Lazzara**, Denise Fernandes, Melissa Faria, Jordi F. López, Romà Tauler, Cinta Porte, 2012. Changes in lipid content and fatty acid composition along the reproductive cycle of the freshwater mussel *Dreissena polymorpha*: Its modulation by clofibrate exposure. Science of the Total Environment, 432, 195-201.

F.I.: 3.286

Grado de participación: Realización del trabajo experimental, con la excepción de la identificación de los perfiles de ácidos grasos. Discusión de resultados y elaboración del manuscrito.

3. **Raimondo Lazzara**, Mercedes Blázquez, Cinta Porte, Carlos Barata, 2012. Low environmental levels of fluoxetine induce spawning and changes in endogenous estradiol levels in the zebra mussel *Dreissena polymorpha*. Aquatic Toxicology, 106-107, 123-130.

F.I.: 3.761

Grado de participación: Realización del trabajo experimental, con la excepción de las preparaciones histológicas. Discusión de resultados y preparación del manuscrito.

CONSEJO SUPERIOR DE INVESTIGACIONES CIENTÍFICAS
Instituto de Diagnóstico Ambiental y Estudios del Agua
(IDÆA)

4. **Raimondo Lazzara**, Denise Fernandes, Cinta Porte. Short-term exposure to tributyltin modulates steroid levels and lipids in the zebra mussel *Dreissena polymorpha* (en preparación).

Grado de participación: Realización del trabajo experimental. Discusión de resultados y elaboración del manuscrito.

5. **Raimondo Lazzara**, Denise Fernandes, Cinta Porte. Evidence of endocrine disruption in a zebra mussel population from the Ebro River (en preparación).

Grado de participación: Realización del trabajo experimental. Discusión de resultados y elaboración del manuscrito.

Barcelona, 21 de diciembre 2012

Dra. Cinta Porte Visa
Directora de la Tesis

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4. SUMMARY OF RESULTS AND DISCUSSION

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Environmental pollutants like pharmaceuticals and OTCs are frequently detected in different matrices of the aquatic environment as a result of their widespread use in industrialized countries. However, knowledge about their effects on aquatic invertebrates is still limited and therefore the ecological consequences of their presence in the environment difficult to predict. Hence, there is the need to develop sensitive tools to assess the toxic potential of those contaminants, especially in freshwater organisms. In this context, the present thesis investigated new approaches to detect the toxicological impact of three frequently reported environmental contaminants namely fluoxetine, clofibrate and TBT on *Dreissena polymorpha*. First, the annual reproductive cycle of a wild population of zebra mussels from the Ebro River was investigated in terms of seasonal changes of sex hormones, lipid levels and fatty acid composition, and their relationship with reproductive events assessed (paper 1 and paper 2). This research provided background knowledge to understand the role of steroids and lipids in reproduction. Additionally, laboratory exposure experiments were undertaken to analyze the effects of contaminant exposure on lipid homeostasis (lipid levels, fatty acid composition, and triglyceride levels), steroid levels and on gonadal tissue (paper 2, 3-4). Finally, a comparative study between a heavily impacted site and a reference site was performed using two geographically close zebra mussel populations from the Ebro River. The study was aimed to investigate the effects of environmental contaminants on the reproductive cycle, sex steroid levels, lipids and gonad development (paper 5). The main results and conclusions obtained in this dissertation are summarized below.

4.1 Reproductive cycle, annual variations of sex steroids and changes in lipid levels and fatty acid composition in the zebra mussel

In paper 1 the reproductive cycle of a zebra mussel population from the lower course of the Ebro River was investigated together with the analysis of annual variations of free and esterified sex steroids (testosterone and estradiol). Histological analysis allowed the establishment of five different gonadal stages: stage 1 (early gametogenesis) and stage 2 (late gametogenesis) together comprised the cycle of gamete development and maturation, which were characterized by strong changes of the germinal epithelium and the connective tissue, as well as by the presence of gametes in different maturation stages. Stage 3 (spawning) was the period where the gonad reached its maximum extension and most of the lumen was occupied by the follicles (females) and tubules (males) which were filled with matured gametes. Stage 0 (resting phase) and stage 4 (reabsorbing) together formed the period of gonadal rest where the gonad was reduced to its lowest size and the internal structure assumed a partially empty and disorganized appearance. The gonadal maturation stages defined in this thesis followed those typically described in other works with bivalves (Pouvreau et al., 2000; Alfaro et al., 2003; Juhel et al., 2003).

Analysis of the different reproductive stages showed that gametogenesis started during late autumn/early winter and comprised a total of approx. 4-5 month which was then followed by a highly synchronized spawning event in spring. Spawning lasted another 4-5 months and was followed by a gonadal resting phase that started in early autumn. In detail, gamete development progressed during winter with the majority of animals being in gametogenesis (60-100%) between January and March, the months with the lowest water temperatures measured, hence showing an inverse relation to temperature (Figure 7). Gamete release initiated as a highly synchronized event in March, coinciding with increasing water temperatures with 90-100% of the animals being in spawning in the month between April and July. Results also demonstrated that the gonadal development between both genders was very similar. As several works have indicated, the reproductive cycle in zebra mussels depends on environmental factors and can show significant variations between different geographical regions (Nichols, 1996). Water temperature was reported to exert a strong influence on the reproductive development in zebra mussels by controlling gametogenesis and spawning. Thus, earlier works on European populations indicated that gamete development occurs during winter

and that cold water temperatures have an important regulating function on the reproductive cycle (Borcherding, 1991; Mackie, 1991). It has also been suggested that for some bivalve species gamete release is mainly controlled by temperature in the way that a certain threshold has to be reached to initiate gamete release (Borcherding, 1991; Jantz and Neumann, 1998; Bacchetta et al., 2001). Hence, our results with the population from the Ebro region would corroborate these observations showing a strong influence of water temperature on the onset and progression of important reproductive events in zebra mussels.

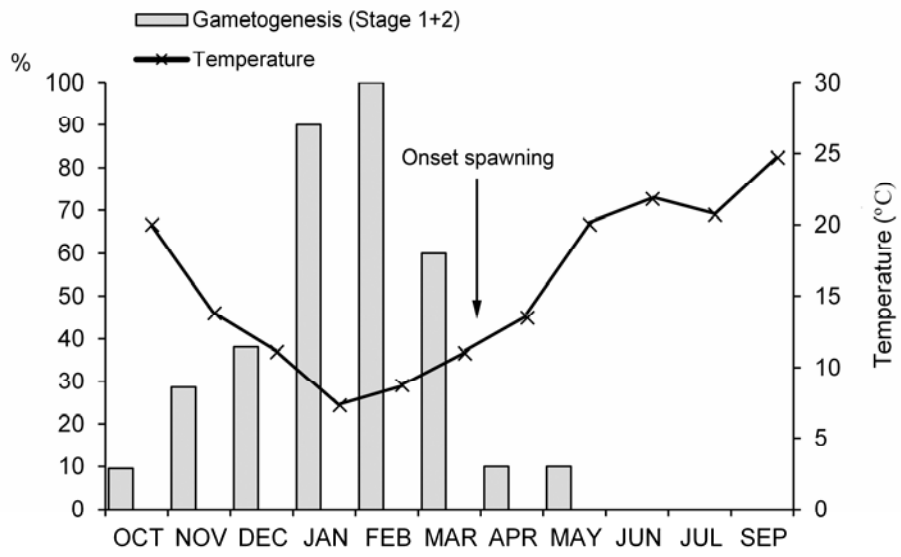

Figure 7. The relation between water temperature and progression of gametogenesis in zebra mussels.

Investigations on hormone levels clearly showed the presence of two vertebrate-like sex steroids in the zebra mussel, namely testosterone and estradiol. Due to the small size of the organisms, an extraction of individual organs was not feasible and therefore analyses of whole body extracts (without gills) were performed. The detected levels of free testosterone and estradiol along the annual cycle were in the range of those reported in previous studies with other mollusc species (Table 6). However, most of the testosterone and estradiol was found in the esterified form with free (unesterified) testosterone and estradiol comprising only 3-8% and 2-7% of total testosterone and estradiol, respectively. During the seasonal sampling, a separation of males and females was not performed, thus differences of steroid variations between genders were not assessed.

Table 6. Levels of free testosterone and estradiol in molluscs.

Steroid	Species	Matrix	Levels (ng/g)	Reference
Testosterone	*Marisa cornuarietis*	Digestive gland/Gonad	0.1-3	Janer et al., 2006
	Bolinus brandaris	Visceral coil	0.7-1.2	Morcillo and Porte, 1999
	Ruditapes decussata	Whole animal	0.1	Morcillo et al., 1998
	Mytilus galloprovincialis	Whole animal	0.1	Morcillo et al., 1999
		Whole animal	0.9-1.8	Janer et al., 2005a
	Mytilus edulis	Whole animal	0.1-0.7	Reis-Henriques et al., 1990
	Ilyanassa obsoleta	Whole animal	1-3	Gooding et al., 2003
	Mya arenaria	Gonad	0.03-0.05	Gauthier-Clerc et al., 2006
	Dreissena polymorpha	Whole body without gills	0.1-0.8	Present work
Estradiol	*Marisa cornuarietis*	Digestive gland/Gonad	0.03-0.2	Janer et al., 2006
	Bolinus brandaris	Visceral coil	0.1-0.3	Morcillo and Porte, 1999
	Mytilus galloprovincialis	Whole animal	0.07-0.4	Morcillo et al., 1999
		Whole animal	1.1-2.6	Janer et al., 2005a
	Mytilus edulis	Whole animal	0.02-0.05	Reis-Henriques et al., 1990
		Whole animal	0.5-1.0	Labadie et al., 2007
	Mya arenaria	Gonad	0.2-0.4	Gauthier-Clerc et al., 2006
	Dreissena polymorpha	Whole body without gills	0.2-1.8	Present work

Significant seasonal variations were observed for both testosterone and estradiol showing patterns indicating an annual cycle. In the case of free testosterone, two major peaks were detected, one in October and another in January/February during mid-winter, whereas the lowest levels were reported during late autumn (November) and late spring/early summer (May/June). Free estradiol on the other hand showed less pronounced but still significant annual variations with a visible peak appearing in November and another, less apparent, in February. Furthermore, annual profiles of esterified testosterone and estradiol presented a significant peak in autumn while lowest levels appeared in mid-spring, which indicates a significant increase in steroid esterification during the months of September and October followed by comparable lower and rather constant levels during the rest of the year. Curiously, no negative correlation was observed between free and esterified steroid levels. Hence, higher levels of esterified testosterone for example were not associated with lower levels of free testosterone, a phenomenon which has also been described in previous studies with *Marisa cornuarietis* (Janer et al., 2006; Lyssimachou et al., 2008). This may show that

free steroid regulation is not directly bound to steroid esterification. However, considering that free steroids only comprise a minor fraction of total steroids while the majority appears in conjugated form (~ 90-95%), changes in free steroids may not significantly affect esterified levels. Consequently, increases in esterified steroids are equivalent to increases in total steroid amount and therefore indicate a higher steroid production in the organisms, which in the zebra mussel occurred during autumn. This may show that steroid esterification is closely associated to steroid production and storage, indicating that newly produced steroids do not primary contribute to the circulating level of steroids but are rather conjugated with fatty acids and stored in the lipid matrix. The esterified form could serve as a pool from which the active steroid gets liberated by esterases during times when steroid producing tissues are quiescent (Hochberg, 1998). This could be regulated through some kind of feedback mechanism which allows zebra mussels to modulate circulating steroid levels independently from steroid production. Indeed, studies with *Ilyanassa obsoleta* and *Mytilus galloprovincialis* showed that external administration of steroids led to their absorption but not to a significant increase in free forms. The assimilated hormone was rapidly conjugated with fatty acids and stored in the lipoidal matrix of the organism (Gooding and LeBlanc, 2001; Fernandes et al., 2010). The hypothesis may further be supported by the fact that steroid producing and metabolizing organs in bivalves, mainly gonad and hepatopancreas, are also tissues where the major part of lipids and fatty acids are stored, thus providing the material for rapid steroid conjugation (Varaksina and Varaksin, 1988; Reis-Henriques and Coimbra, 1990; Matsumoto et al., 1997; Lavado et al., 2006a; Canesi et al., 2007a).

Notably, free testosterone levels closely followed the annual progression of gametogenesis (both peaking in January/February) and the transient increase in esterified forms coincided well with the period of gonadal reconstitution (Figure 8). These results suggest a potential relation between annual steroid variations and reproductive events in the zebra mussel. Similarly, Gooding and LeBlanc (2004) showed that marked seasonal variations in free and esterified testosterone levels paralleled the reproductive cycle in the gastropod snail *Ilyanassa obsoleta*. Moreover, associations of free testosterone levels and gamete development have been mentioned for *Theba pisana* where testosterone accelerated spermatogenesis (Sakr et al., 1992), and for *Ilyanassa obsoleta* which showed increases in free testosterone levels in males

during period of sexual recrudescence (Sternberg et al., 2008). However, due to the fact that no gender separation was performed in this study, it is not possible to know if the temporal increase in free testosterone occurred only in males or in both sexes; an aspect which requires further investigations.

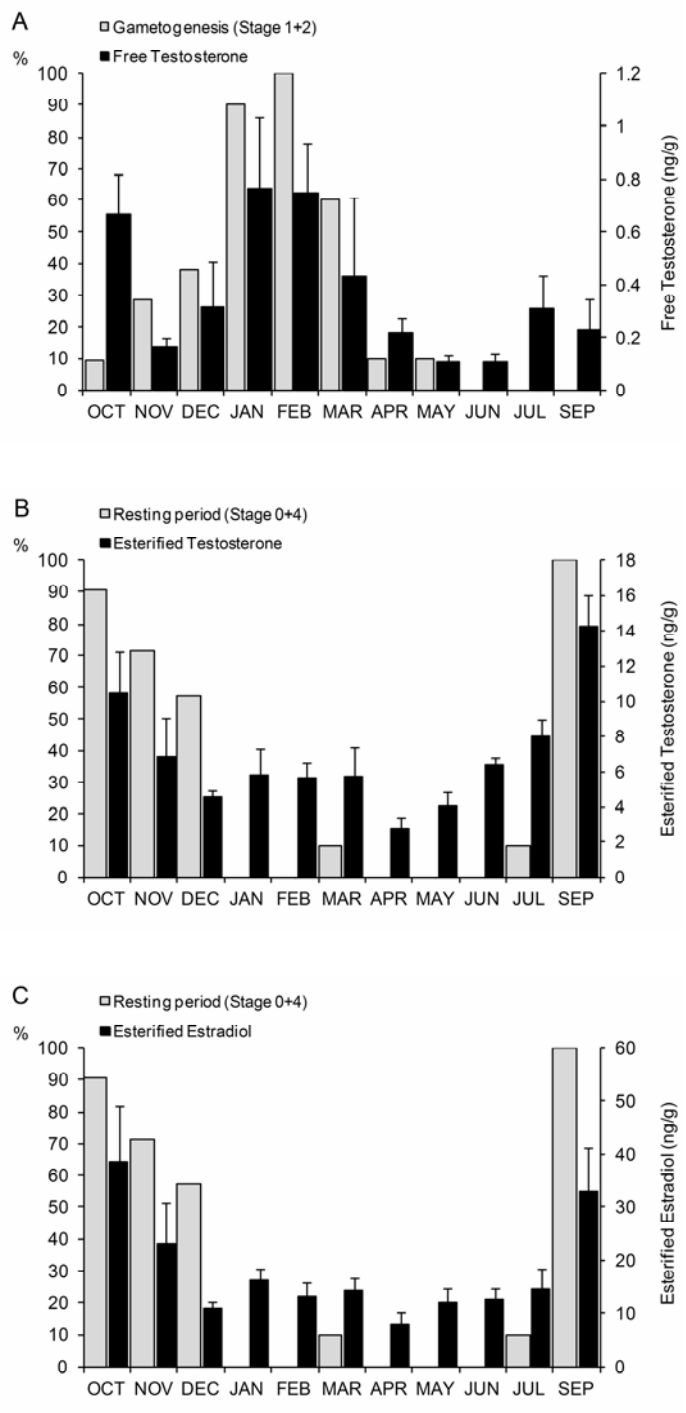

Figure 8. Relation of annual steroid levels with gonadal maturation stages in *Dreissena polymorpha*. (A) Levels of free testosterone and gametogenesis, (B) Levels of esterified testosterone and the resting period (C) Levels of esterified estradiol and the resting period.

A relationship between esterified steroids and reproduction has been reported in studies with *Marisa cornuarietis* where levels of esterified steroids were higher in organisms collected in autumn (high spawning season) than in those collected in spring (low spawning season) (Janer et al., 2006). In contrast, increased levels of esterified steroids in zebra mussels occurred during the period of gonadal rest which may show a different regulation between mollusc species. However, knowledge on the physiological role of esterified steroids in molluscs is still lacking, which makes it difficult to explain the differences observed. Nevertheless, as aforementioned, steroid esterification may fulfill a crucial storage function and the observed peak of esterified steroids may therefore indicate the period of annual steroid production and storage which occurs during the resting phase (Figure 8B, C). This could be explained by the fact that reproduction and steroidogenesis are energy demanding processes and therefore their temporal separation could be of great importance for the organism in order to balance the energy budget.

In contrast, ATAT activity was significantly higher during late gametogenesis (stage 2) and the onset of spawning. Comparable results have been reported by Janer et al. (2006) for *Marisa cornuarietis* where organisms collected during the high spawning season, showed up to 5-fold higher ATAT activities than those collected in the low spawning season. Notably, the study also showed that decreases in esterified steroid levels were not directly linked to a decrease in ATAT activity; this further coincides with the results in this study which showed no agreement between annual level of esterified steroids and variations in ATAT activity. The reason for the lack of agreement between both parameters represents an intriguing puzzle, pointing towards a potentially more complex regulatory mechanism of steroid esterification as initially assumed.

The activity of 17β-HSD which was determined by measuring the conversion of labelled androstenedione to testosterone showed a significant decrease during late winter/early spring. Annual variations of 17β-HSD activity followed a similar pattern as that of esterified testosterone, with highest levels appearing in autumn and lowest in spring (Figure 9). In addition, no relation with free testosterone levels were seen, on the contrary, both parameters showed a rather inverse annual progression. This may further confirm the link between sex steroid production and steroid esterification and that free steroid forms are not those that are newly produced by the organism but rather those that are liberated from the conjugated pool. More importantly, the decrease of

enzymatic activity would coincide with the period of late gametogenesis and early spawning, thus indicating a possible relation with reproduction in zebra mussel. Indeed, a similar connection has already been reported in *Crassostrea gigas* and *Patinopecten yessoensis* where a decrease in 17β-HSD activity was measured during the post-spawning period, which was significantly lower in comparison to the early differentiation stage (Matsumoto et al., 1997). Moreover, Varaksina and Varaksin (1988) showed that in *Patinopecten yessoensis* and Gray's mussel (*Crenomytilus grayanus*) 17β-HSD activity was higher in growing oocytes than in those completely matured. A similar relation could exist in the zebra mussel where 17β-HSD plays an important function especially during stages of early gametogenesis. In light of the presented evidence and the studies performed by other authors it seems possible that the two enzymes, ATAT and 17β-HSD, which are involved in steroid biosynthesis and metabolism, play a role in bivalve reproduction. However, more studies are needed to clarify their significance in the control of reproductive events.

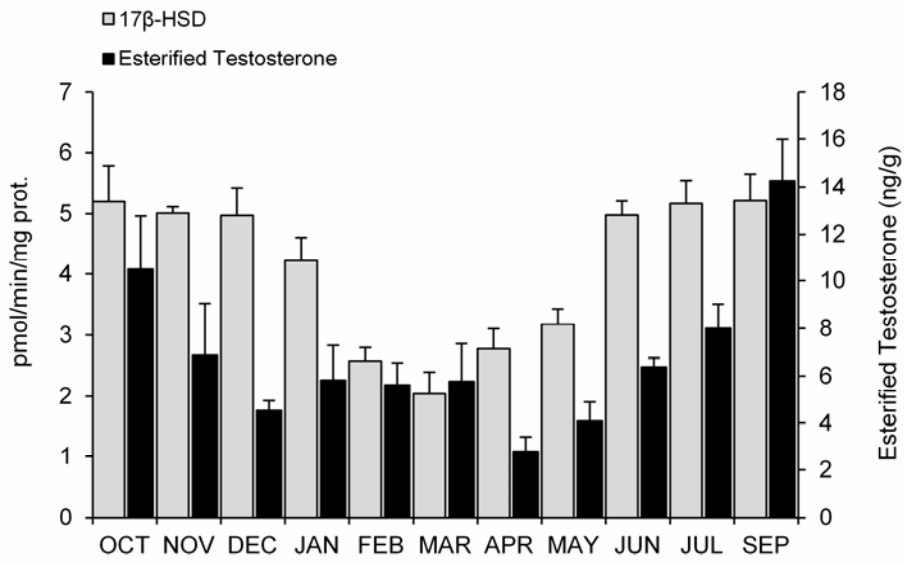

Figure 9. Relation of 17β-HSD activity with levels of esterified testosterone in *Dreissena polymorpha.*

In paper 2 lipid content and fatty acid profiles were characterized along the reproductive cycle of zebra mussels. Lipid levels varied significantly during the study period showing a clear annual cycle with a significant peak appearing in April. Lipid levels measured in the whole body tissue (0.7-2.0% of wet weight) are in the range of those previously reported for zebra mussels (Cope et al., 1999) and other bivalves (De Moreno et al., 1980; Tanabe et al., 1987; Murphy et al., 2002). Seasonality of lipid

content is a common phenomenon in bivalves which is influenced by environmental factors (temperature, food), but also depends on important physiological processes (reproduction, larval development) (Honkoop et al., 1999b; Lin et al., 2003). Bivalves are known to accumulate reserves of energy in body tissues which are of great importance for the production of gametes (Nalepa et al., 1993). In the present work, the annual lipid levels in zebra mussels were strongly associated with the reproductive cycle. A strong accumulation of lipids was detected during gametogenesis which was then followed by a significant decrease during spawning. This considerable loss of lipids can be explained by the translocation of large amounts of lipids from the lipid-rich digestive gland to the maturing gonad, where they get integrated into the developing gametes (Vassallo, 1973). This leads to their subsequent loss during gamete release and to lower lipid levels in mussels in the post-spawning period.

Fatty acids are an important constituent of lipids and their vital physiological role has become more recognized in recent years. In agreement with total lipids, annual levels of total FAME in zebra mussels varied significantly, reaching the highest levels in April. The fatty acid profile showed a high percentage of HUFAs which is quite common for bivalves feeding mainly on phytoplankton (Pirini et al., 2007). The most abundant fatty acids detected were palmitic acid (16:0), docosahexaenoic acid (DHA; 22:6n−3), eicosapentaenoic acid (EPA; 20:5n−3), palmitoleic acid (16:1n−7) and *cis*-9-eicosenoic acid (20:1n-11). A similar fatty acid profile has also been reported for the bivalves *Pecten maximus* and *Crassostrea gigas* (Pazos et al., 1996, 2003). In addition, a higher amount of the n-3 fraction in comparison to the n-6 fraction was seen; a common aspect of mussel lipids to which most of their high nutritional value for human diet is attributed. Interestingly, a strong negative correlation between the n-3 and n-6 fractions was measured indicating opposite annual trends in abundances with n-3 peaking in April and n-6 in September. Similar changes in seasonal abundances, of n-3 and n-6 fatty acid were reported in *Mytilus galloprovincialis* (Orban et al., 2002).

The majority of fatty acids followed clear annual trends with peaks appearing during distinct periods of the year, thus indicating possible influences of environmental and/or physiological factors. Analysis of annual profiles revealed groups of fatty acids showing similarities in their seasonal progression. Hence, fatty acids like 16:0, 16:1n-7, 18:1n-7 and 20:5n-3 showed highest levels during late winter/spring while fatty acids like 18:1n-9, 20:1n-11, 20:4n-6 and 22:5n-6 demonstrated highest abundances in late

summer/autumn. Annual cycles of fatty acid abundances have already been mentioned for other bivalves which may reflect changes in dietary intake but may also indicate their involvement in different physiological processes (Freites et al., 2002; Orban et al., 2002). Even though, functions of many fatty acids are still unknown, studies point towards their role in structural and functional processes in biological membranes in addition to the control of survival, growth and reproduction (Chu and Greaves, 1991; Fang et al., 1993; Delaunay et al., 1993).

In order to detect possible relations between fatty acid variations and endogenous and exogenous factors, the multivariate curve resolution alternating least-squares method (MCR-ALS) was used to reduce the dataset to a smaller number of components which allows the uncovering of common trends. The results clearly showed the identification of three temporal fatty acid patterns that explained around 99% of the total variance of the data set, and which strongly coincided with the three main events of reproduction. MCR-ALS analysis also allowed the identification of those fatty acids which mainly contributed to the three identified patterns. Thus, the 16:0, 16:1n−7, 20:5n−3 and 22:6n−3 fatty acids showed a major contribution to patterns 1 (late gamete development/spawning period) and 3 (gametogenic period); while several PUFAs, among them 20:1n−11 were the major contributors to pattern 2 (resting period). Interestingly, 16:0 contributed to all three patterns but was significantly more abundant during spawning, while palmitoleic acid (16:1n−7), the most abundant MUFA, was significantly higher during gametogenesis. A special focus was placed on the essential fatty acids (20:4n−6, 20:5n−3 and 22:6n−3) which are reported to have a key role in bivalve development, reproduction, and also in membrane functioning (Soudant et al., 1996a,b). In the present work, 20:5n−3 (EPA) showed strong seasonal variations reaching highest levels at the end of gamete development, when the gonad reaches its maximum volume, while 22:6n−3 (DHA) was rather constant during the sampling period, with the exception of the months of September and October where the relative abundance decreased. These results agree with Besnard et al. (1989) who reported high levels of 20:5n−3 in female gonads of *Pecten maximus* during sexual maturity, while levels of 22:6n−3 showed no clear seasonal trend and significantly declined after the spawning period. Hatching success and larval survival in bivalves is related to egg lipid content, and supplementation with 20:5n-3 and/or 22:6n-3 has been proved to enhance fecundity and hatching rates in different species (Utting and Millican, 1997, Caers et al.,

2002, Hendriks et al., 2003, Nevejan et al., 2003). In fact, 22:6n-3 has been suggested to play a structural and functional role in the maintenance of membranes in bivalves while 20:5n-3 is often related to energetic-type functions (Marty et al., 1992; Freites et al., 2002). Furthermore, the annual variations of 20:4n-6 coincided with the progression of the gonadal resting period, with highest levels between September and December. Comparable variations have been reported for *Crassostrea gigas* where abundance of 20:4n-6 strongly increased in the period when gonadal tissue was inactive (Pazos et al., 1996). As precursor for several cell signal molecules, 20:4n-6 might be necessary for the synthesis of certain eicosanoids such as prostaglandins which could be of special importance during different periods of the year. Several types of series 2 prostaglandins have already been identified in *Patinopecten yessoensis* and their involvement in bivalve reproduction already suggested (Osada and Nomura, 1990). Indeed, works with the scallop *Argopecten purpuratus* showed an inverse relationship between gonadal maturity and levels of the prostaglandins PGE_2 and $PGF_{2\alpha}$ (Martínez et al., 1999). A similar relation between prostaglandin synthesis and the reproductive cycle could exist in the zebra mussel which may explain the seasonal variation of 20:4n-6 levels observed in this thesis.

Variations of fatty acids like 20:4n-6 could also be explained by its relation to sex steroid production and esterification as seen in our work. The strongest agreement was detected for the 20:1n-11 and the 20:4n-6 fatty acids as indicated in figure 10. The crucial role of 20:4n-6 in the regulation of steroidogenesis is well-documented in vertebrates (Wang et al., 2000; Maloberti et al., 2002). In the goldfish (*Carassius auratus*) for example, 20:4n-6 was shown to stimulate ovarian testosterone production by way of its conversion to PGE_2 (Mercure and Van Der Kraak, 1996). Furthermore, in the oyster *Crassostrea virginica*, Janer et al. (2004) demonstrated the formation of arachidonoyl-estradiol by microsomes from the digestive gland. In addition, Fernandes et al. (2010) reported significant increases of 20:4n-6 and other PUFAs after testosterone exposure of *Mytilus galloprovincialis*, however, a significant relation was only seen for the lower concentrations. Those studies in addition to our results may prove that annual fatty acid abundance may play an important role in steroid production and esterification and that this processes may be influenced by variations of important fatty acids like 20:4n-6.

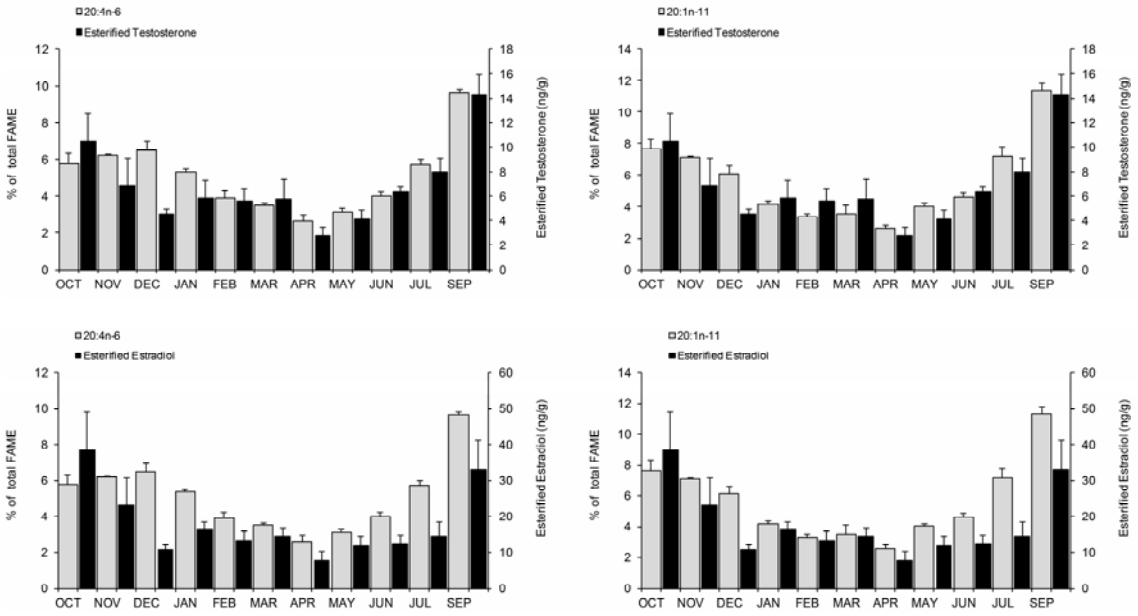

Figure 10. Showing relation between the annual progression of the 20:4n-6 and 20:1n-11 fatty acids and esterified testosterone and estradiol.

4.2 Exposure effects of pharmaceuticals and TBT in zebra mussels

Paper 3-4, assessed the effects of short-term exposure to fluoxetine (6 days) and TBT (7 days), respectively. Both compounds were shown to induce significant changes in endogenous steroid levels. Fluoxetine caused increased levels of esterified estradiol, with no changes of esterified testosterone levels or free steroid levels. TBT induced a significant increase of total testosterone titers in females while no effect was observed in males. Levels of total estradiol remained unchanged in males and females. Hence, both compounds are capable of altering steroid levels and by this interfere with the endocrine system in zebra mussels, although with distinct modes of action.

Besides its effects on the hormone system, fluoxetine also effectively induced spawning in zebra mussels. A reduction in the number of oocytes per follicle, and spermatozoa density within the tubules was detected in exposed organisms. Significant reduction of gamete content in the gonads of male and female zebra mussels was already detected at concentrations of 20 ng/L thus, at levels in the range of those detected in environmental samples (Kolpin et al., 2002; Zorita et al., 2007). As shown in paper 1, spawning in zebra mussels is a highly synchronized event which is of great importance because fertilization occurs externally in this species. Thus, artificial

induction of spawning may significantly disturb reproduction because viability of gametes in the water column is limited (Quinn and Ackerman, 2012). Therefore, consecutive studies are needed to estimate the potential impact of these compounds on the reproductive success of wild bivalve populations.

It is generally believed that SSRIs may exert their effects through modulation of endogenous serotonin (5-HT) levels (Fong et al., 1998). Studies with *Patinopecten yessoensis* and *Placopecten magellanicus* demonstrated that estradiol potentiates the effect of 5-HT on the gonad (Osada et al., 1992; Wang and Croll, 2003). It was also reported that estradiol stimulated the proliferation of 5-HT surface receptors in oocytes of *Patinopecten yessoensis* and *Crassostrea gigas* which increased their sensitivity towards 5-HT (Osada et al., 1998). Thus, there is strong evidence of a regulatory link between estradiol and 5-HT with estradiol modulating 5-HT receptors in bivalves, hence controlling effects of 5-HT on target tissues. Therefore, the increased esterification of estradiol observed in this work may be a countermeasure of the organism to increased receptor stimulation by 5-HT, because conjugated steroids demonstrate a lower bioactivity and bioavailability.

Induction of spawning triggered by SSRIs after *in vivo* exposure of bivalves has already been reported in several studies (Fong, 1998; Cunha and Machado, 2001). The mechanism behind the induction of spawning by SSRIs is unknown but 5-HT may play a central role in its mediation. Indeed, 5-HT being a potent neurohormone exerts several physiological effects like muscle relaxation (York and Twarog, 1973), siphon movement (Ram et al., 1999), ciliary activity (Stefano et al., 1977) and induction of spawning in bivalves (Gibbons and Castagna, 1984). Furthermore, 5-HT receptors have been found on the membranes of gametes, and their involvement in oocyte maturation and gamete release has been demonstrated in bivalve species (Fong et al., 1993; Ram et al., 1996b; Osada et al., 1998). Considering the evidence, it is possible that changes of endogenous 5-HT titers may have been the cause for the induction of gamete release. Hence, both effects seen after fluoxetine exposure (increases in esterified estradiol and induction of gamete release) could be explained through altered 5-HT levels.

In regard to the observed effects of TBT on sex steroids, the results of this thesis would agree with previous works on molluscs. Hence, organotins like TBT or TPT were shown to significantly alter testosterone and estradiol titers by interfering with their

production or metabolism as demonstrated for the gastropods *Ilyanassa obsoleta* (Gooding et al., 2003), *Marisa cornuarietis* (Janer et al., 2006; Lyssimachou et al., 2008) and the bivalve *Ruditapes decussata* (Morcillo et al., 1998). Even though these studies presented evidence of the endocrine-disrupting potential of organotins, the mechanistic background has not been elucidated yet. Generally, endocrine disruption induced by organotins is primarily attributed to their action on key steroid regulatory enzymes (e.g. aromatase activity) or toxicity mediated via damage to mitochondrial functions and subsequent cellular stress responses (Powers and Beavis, 1991; Gennari et al., 2000; Heidrich et al., 2001). *In vitro* experiments showed that butyltins exhibit structure related inhibition of catalytic activity of human aromatase protein from human placenta (Heidrich et al., 2001) or transferred cells (Cooke, 2002). However, organotins may also act as high affinity ligands for several nuclear receptors including the peroxisome proliferator-activated receptor γ (PPARγ) and the retinoid X receptor (RXR) (Kanayama et al., 2005; Grün et al., 2006). In humans for example, activation of the PPARγ:RXR nuclear receptor system has been shown to interfere with steroid biosynthesis through the modulation of P450 aromatase gene expression (Mu et al., 2001). This may indicate that effects of organotins are not caused by direct interference with enzymatic activity but are rather mediated on the transcriptional level which then transcends to protein and enzymatic levels (Mortensen and Arukwe, 2007). Moreover, activation of RXR through its endogenous ligand 9-*cis*-retinoic acid was shown to inhibit androgen receptor activity (Chuang et al., 2005). Furthermore, RXRs are highly conserved in evolution (Bouton et al., 2005) and studies have already shown TBT and TPT to be potent ligands for RXR homologues found in the rock shell *Thais clavigera* (Nishikawa et al., 2004). Vertebrate-like RXR receptors have also been found in gastropods like *Biomphalaria glabrata* (Bouton et al., 2005) and *Nucella lapillus* (Castro et al., 2007), which bind the natural RXR ligand 9-*cis*-retinoic acid as seen in vertebrates. Hence, similarly to vertebrates, organotins may exert their effects though interference with the RXR pathway in molluscs, however further research is needed to confirm this hypothesis.

Due to the high toxicity of TBT, possible cytotoxic effects on the hepatopancreas were investigated. Hence, short-time exposure of zebra mussels to TBT caused significant histopathological effects in the hepatopancreas of treated organisms as presented in paper 4. The effects were dose-dependent and already detectable within

the lowest exposure group. Tissue lesions appeared in the hepatopancreas tubules, the intertubular connective tissue and the primary and secondary ducts. Those included shrinkage of digestive cells, vacuolization of the digestive and duct cells and damage of the intertubular connective tissue. A smaller but significant percentage of animals showed severe effects, mostly at higher exposure concentrations (500 ng/L as Sn), which included the partial lysis of intertubular connective tissue, increased vacuolization of digestive and duct cells accompanied by cell apoptosis and abnormal formation of tubulus lumen. Alterations like marked shrinkage of digestive cells (enlargement of lumen) and strong vacuolization have already been described in other studies with zebra mussels and seen as general reaction to compounds with high toxicological potential (Quinn et al., 2004). Moreover, the hepatopancreas as primary target organ of TBT has been reported in *Crassostrea gigas* where significant histopathological alterations were detected after short-time exposure (Chagot et al., 1990). The study showed significant effects already at concentrations as low 13 ng/L (approx. 4 times lower than the lowest concentrations used in this work), which included shrinkage of the epithelia of digestive tubules, vacuolization, and appearance of necrotic tubules. Thus, effects of TBT exposure as seen in this thesis and also reported in other studies (vacuolization and shrinkage of digestive cells) could be considered as a general molluscan response to toxic compounds and have been interpreted as a physiological survival mechanism of bivalves subjected to stress (Lowe et al., 1981). In addition, the digestive gland as primary energy source is of great importance during gametogenesis and it is therefore probable that its degradation could have negative effects on reproduction (Gabbott and Bayne, 1973).

The presented thesis tried to investigate possible effects on lipid homeostasis after exposure of zebra mussels to environmental contaminants. In this context, two compounds have been selected based on their lipid altering capacity. Besides TBT, which has been shown to alter lipids and fatty acids in molluscs (Janer et al., 2007), clofibrate was chosen for its proven lipid lowering effects in humans (Goldberg et al., 1979). After 7 days exposure to (20 ng/L to 2 mg/L) of clofibrate, a marked decrease in total triglycerides was detected together with an increase in the total amount of fatty acids per gram wet weight (Paper 2). In zebra mussels significant alterations were already detected at environmental relevant concentrations i.e. 200 ng/L of clofibrate (Richardson and Bowron, 1985). Triglycerides have also been reported to decrease in

fish and crabs exposed to fibrates like gemfibrozil and clofibric acid (Lautier et al., 1986; Prindiville et al., 2011), thus being in agreement with this work. Fibrates decrease serum triglycerides by stimulating the removal of triglyceride rich lipoproteins by lipoprotein lipases that extracellularly hydrolyze triglycerides into glycerol and fatty acids (Schoonjans et al., 1996). In vertebrates, these effects are mediated through the activation of PPARα which regulates genes that control lipid oxidation, lipoprotein metabolism and vascular inflammation (Fruchart et al., 1999). PPARα has also been identified as the key regulator of the genes involved in peroxisomal, mitochondrial, and microsomal fatty acid oxidation systems including sets of β-oxidation enzymes (Reddy, 2001). Even though PPAR mediated mechanisms have been detected in molluscs after exposure to contaminants with known potential for PPAR activation (Cancio et al., 1998; Cajaraville and Ortiz-Zarragoitia, 2006; Lyssimachou et al., 2009) their involvement in the observed effects on lipid homeostasis in zebra mussels can only be suspected at the moment.

Acyl-CoA oxidase (AOX) is the first and rate limiting enzyme of β-oxidation and is therefore involved in the catabolism of fatty acids. Analysis showed no significant difference of AOX activity between control and clofibrate exposed organism (data not published). The increase in fatty acids could have been the result of the significant liberation of fatty acids from triglyceride catabolism without a concomitant stimulation of their degradation as evidenced by the lack of AOX induction.

Similar to clofibrate, exposure to TBT caused significant alterations of lipid homeostasis (paper 4). Thus, a marked increase of total lipids in females exposed to the highest concentration (500 ng/L Sn) was seen, whereas no effects were observed in males. Concurrently, triglyceride content increased in a dose-dependent manner by 32% in males and of up to 45% in females. This was accompanied by changes in fatty acid composition which showed a significant increase in n-6 highly unsaturated fatty acids (HUFAs) in both sexes whereas total FAMEs in females showed a visible, but not significant, upwards trend. A similar increase in total lipids and total FAMEs has been reported in females of *Marisa cornuarietis* after 100 days exposure to TBT (Janer et al., 2007). Furthermore, gender-specific changes in abundances of individual fatty acids were detected, namely 18:1n-9 and 20:5n-3 in males, and 18:1n-5 in females whereas changes of 18:2n-6 and 20:4n-6 were observed in both sexes. Recent studies with *Marisa cornuarietis* showed similar changes in 18:1n-9, 20:4n-6 and the n-6 HUFAs

group after TPT exposure, although alterations appeared only in female organisms (Lyssimachou et al., 2009). Thus, the presented study with zebra mussels, in agreement with other studies on molluscs, shows that organotin exposure significantly interferes with lipid and fatty acid homeostasis, and that females are more susceptible to those effects than males.

The underlying mechanisms through which TBT causes the observed alterations of lipid homeostasis are still not understood. Several studies with vertebrates have shown that TBT exposure stimulates adipogenesis, lipid storage and triglyceride accumulation through activation of PPARγ and RXR (Kanayama, 2005; Nakanishi et al., 2005). In a similar manner, natural fatty acids, especially PUFAs, are endogenous ligands for several PPARs and RXR (Keller et al., 1993). In regard to PPARs, ligand dependent activation of the different subtypes can either induce lipogenesis or lipolysis (Willson and Wahli, 1997; Reddy and Hashimoto, 2001; Kota et al., 2005; Li and Glass, 2004). However, little is known about the possible presence of PPARs in invertebrates, even though their existence can be expected since these receptors mediate basic processes in lipid homeostasis, cell proliferation, and cell differentiation (Lemberger et al., 1996). In addition, PPAR receptors may have evolved prior to the divergence of vertebrates and invertebrates which suggests the universal nature of the nuclear hormone receptor superfamily (Dreyer et al., 1993; Mangelsdorf et al., 1995). Nevertheless, recent studies show that besides the PPARγ signalling pathway there is also the possibility that adipogenesis is induced through RXR homodimers (Ziouzenkova and Plutzky, 2008) for which TBT may act as ligand (Inadera and Shimomura, 2005). In a similar way, several fatty acids like palmitoleic acid (16:1n-7), oleic acid (18:1n-9), linoleic acid (18:2n-6), arachidonic acid (20:4n-6), and docosahexaenoic acid (22:6n-3) (De Urquiza et al., 2000; Goldstein et al., 2003) can mediate their effects directly through activation of RXR dimers, including the homodimeric form (Lengqvist et al., 2004). Interestingly, some of the fatty acids identified as RXR ligands (18:1n-9, 18:2n-6 and 20:4n-6) have undergone significant changes in our study which may indicate their modulation due to the interference of TBT with the RXR pathway. This may suggest that changes in RXR homodimer activation cloud be a possible explanation for the detected changes in lipid homeostasis in zebra mussels. Thus, the observed modulation of lipid regulation by TBT and their

agreement with vertebrate studies may indicate the possibility of a transcriptional mechanism involving the RXR pathway which has been conserved across phyla.

As shown, common environmental pollutants like clofibrate and TBT demonstrate the potential to significantly alter lipid homeostasis in zebra mussels. Due to the great importance of lipids for many physiological processes, significant changes in lipid composition as seen after clofibrate exposure may present serious problems for organisms, especially during sensitive stages like growth and reproduction. Of similar concern are significant alterations in fatty acid composition as detected in TBT exposed organisms. Fatty acids are of great importance for the production and development of gametes in bivalves (Soudant et al., 1996a,b) and also play a critical multi-functional role in cell structure and function, energy metabolism and storage, cell signalling and biosynthesis of several compounds involved in regulatory processes (e.g. steroids, eicosanoids etc) (Willson and Wahli, 1997). Eicosanoids derive from three HUFAs namely linolenic (18:3n-3), arachidonic (20:4n-6) and eicosapentaenoic (20:5n-3) acid and play an important role in invertebrate physiology. They are involved in the regulation of egg-production, egg-laying, spawning and hatching, mediation of immunological responses to infections, regulation of epithelial ion and water flux, temperature set points, and neurophysiology (Stanley-Samuelson, 1994a,b; Stanley and Howard, 1998). More important, two of the three fatty acids involved in eicosanoid production have undergone significant alterations in zebra mussels after TBT exposure. Therefore, TBT induced changes of lipid levels in combination with changes in fatty acid composition may potentially have serious effects on reproduction and development of zebra mussels through alterations of physiological concentrations of important fatty acids as well as eicosanoid synthesis.

4.3 Field study assessing the health status of a zebra mussel population from a historically polluted site of the Ebro River

The study presented in paper 5 aimed to investigate possible endocrine disrupting effects in a zebra mussel population situated only a few hundred meters downstream of a chlor-alkali plant at the city of Flix (lower course of the Ebro River). The long operation of the plant caused a considerable pollution of the adjacent river section including several kilometers of the downstream area (Eljarrat et al., 2008; Faria et al.,

2010). Toxic effects on zebra mussels were assessed in comparison to a reference site (Riba Roja) located in close vicinity (7-8 km upstream). The alterations observed in this study included changes of the reproductive cycle, annual variations of hormone and fatty acid levels as well as alterations in the development of the gonad and the hepatopancreas.

The zebra mussel population from the Flix area showed marked changes in the progression of key reproductive events in comparison to the Riba Roja population. Hence, the onset of gametogenesis was delayed for 2-3 months and the period of gonadal arrest was significantly prolonged (2 months) while the spawning period was strongly shortened (2 months). Thus, besides being in disagreement with the population at Riba Roja, the reproductive cycle of the population from Flix also showed characteristics which strongly deviated from those reported for most zebra mussel populations. More specifically, as an important regulating factor, temperature directs the reproductive cycle in zebra mussels by determining the onset and progression of reproductive events. The water temperature measured at both sites was not significantly different (range 7-25 ºC). In European populations gametogenesis usually restarts and progresses during winter when water temperatures are lowest and the onset of spawning is triggered by increasing temperatures in spring which for German populations have been determined to be about 12 °C (Borcherding, 1991; Jantz and Neumann, 1998), while Sprung (1992) established a temperature range of 10-17 °C for most European populations. In contrast, gametogenesis in the population from Flix started during a period when temperatures were already increasing and the major part of the animals spawned when temperatures had already reached 20 ºC; hence the expected agreement with water temperatures was not seen. More importantly, spawning at Flix comprised little over 2 months which is not reconcilable with most works on zebra mussels. Indeed, Juhel et al. (2003) working with Irish populations reported that animals of different size classes all showed a spawning period of at least 4-5 months. Furthermore, Bacchetta et al. (2001) showed periods of 4-6 months for Italian populations, and Gist et al. (1997) even reported a spawning period of up to 7 months for North American populations.

Due to the detected alterations in reproduction, histological analysis of the gonad and hepatopancreas were undertaken to reveal possible changes which would explain the observed effects. Interestingly, no significant effects at the histological level were

observed; however significant differences in gonadal development and hepatopancreas size were seen between both sites. Thus, in comparison to the reference site, the Flix population showed a significantly delayed gonadal development reaching maximum extension at a later reproductive stage while concomitantly demonstrating a reduced total size, which was 22-30% less then seen at Riba Roja. Interestingly, gonadal development showed no difference between genders at Riba Roja whereas males at Flix showed a generally lower gonad size than females.

Volume changes in hepatopancreas are normally negatively correlated to gonadal development in bivalves (Sastry, 1966; Fuji and Hashizume, 1974). Therefore, as expected, the population from Riba Roja showed a significant reduction in hepatopancreas size during late gametogenesis and spawning, the period when the gonad had significantly increased its volume. However, at Flix changes in hepatopancreas size only agreed with the development of the gonad in females during the spawning stage, whereas non agreement was seen in males; rather an unexplained significant increase was detected during the late development stage. Moreover, no gender specific differences were seen at Riba Roja while at Flix, hepatopancreas size was generally higher in males than in females. Our results would indicate that especially in males, size development of the hepatopancreas in mussels from Flix was different from what would be expected in an unaffected bivalve population.

The molluscan hepatopancreas is responsible for nutrient storage and the transfer of assimilated food to the body tissues (Sastry and Blake, 1971). Digestive gland lipid content reaches storage levels early in the gametogenic cycle which then serves as main nutrient reserve for gamete production (Barber and Blake, 1981). The cause for the anomalous increase of the hepatopancreas observed in males from Flix during late gametogenesis is not known, but may represent a pathological alteration.

Throughout the reproductive period, sexual maturation and the energy status of bivalves are likely to respond to numerous environmental conditions such as changes in temperature, food availability but also contamination levels. Therefore, changes in gonad development may occur due to reduced nutritional intake; however, this should also be reflected in reduced development of storage organs like the hepatopancreas and also in lower amounts of total lipids (Cartier et al., 2004). Those effects were not observed in zebra mussels from the Flix region. On the contrary, highest total annual

volume of the hepatopancreas was observed in males from Flix and lipid levels were even higher during most months of the sampling period when compared to Riba Roja. This would indicate that lack of food may not be the main cause for the altered gonadal development observed at Flix.

Annual variations of testosterone and estradiol were measured in order to investigate a possible relation between changes in steroid levels and alterations in reproduction. Results show that testosterone levels were significantly depressed (2-3 fold) in the population from Flix which concomitantly resulted in a significant alteration of the estradiol/testosterone ratio which was in average 2-3 at Riba Roja and 3-6 at Flix even reaching a ratio of 10 in the months of October and November. Estradiol levels on the other hand were quite similar between both sites for most months. This agrees with previous studies on the Flix region where male carps showed depressed plasma testosterone levels (up to 5-fold) in comparison to the reference site (an area downstream of the city of Lérida) which were accompanied by an increased E2/T ratio (2-4 times higher than reference site) (Lavado et al., 2004). Studies have also reported the presence of several endocrine disrupters in water and sediment samples from the Flix region *viz*. polychlorinated biphenyls (PCBs) (Fernández et al., 1999), hexachlorobenzenes (HCBs) (Lacorte et al., 2006; Bosch et al., 2009) as well as DDT (Lavado et al., 2004; Bosch et al., 2009). Thus, there is the possibility that the presence of estrogenic compounds in the environment may have altered levels of testosterone in mussels from Flix.

Analysis of annual fatty acid composition showed strong similarities in annual levels of total FAME between both populations which would further indicate similar nutritional conditions. However, significant changes were seen for some individual fatty acids including the essential forms. Thus, differences appeared for the 20:5n-3 fatty acid which showed significant higher levels in the months of June and July in the Flix population. On the other hand 22:6n-3 demonstrated significant lower levels at Flix during most of the sampling season in comparison to Riba Roja. Both fatty acids are involved in gamete production and larvae survival and the observed differences could be associated to changes in gametogenesis and spawning as well as general lower production of gametes due to a decreased development of the gonad.

Analyses of factors with a strong regulatory function like temperature, lipid levels and fatty acid composition were strikingly similar between Flix and the reference site. Despite that, strong changes in reproduction and the endocrine system were observed which may indicate interference by anthropogenic compounds. Moreover, comparable alterations as seen in this work have been reported in other studies on bivalves from contaminated sites showing effects like delay in oocyte maturation (Binelli et al., 2004), retardation in sexual maturation of the gonad (Siah et al., 2003), delayed gametogenesis (Gauthier-Clerc et al., 2002), and interference with sperm release as well as disturbance in the gametogenic phases of the ovary (Bacchetta and Mantecca, 2009).

5. CONCLUSIONS

5. CONCLUSIONS

I. Levels of testosterone and estradiol in *Dreissena polymorpha* tissue were clearly associated to reproductive events. Free testosterone levels followed the progression of gamete development while levels of esterified testosterone and estradiol peaked during the period of gonadal rest. A peak of ATAT activity was observed at the onset of gamete release.

II. Total lipids as well as total fatty acid levels in zebra mussels showed a strong increase in April at the end of the gametogenic cycle which was followed by their gradual decrease during spawning until reaching lowest levels in September. The analysis of annual fatty acid variations using the MCR-ALS method revealed three temporal fatty acid patterns which were strongly related to the three key reproductive events namely, gametogenesis, spawning and resting period; demonstrating the key role that lipids and fatty acids play in zebra mussel reproduction.

III. Exposure to clofibrate significantly altered lipid homeostasis in zebra mussels by decreasing triglyceride levels and concomitantly increasing total fatty acids. This effects were observed at rather low concentrations (200 ng/L) and considering the involvement of lipids in physiological functions such energy processes, membrane formation and reproduction, results may indicate that clofibrate might potentially interfere with those functions in exposed mussels.

IV. Fluoxetine exposure induced gamete release in both males and females of *Dreissena polymorpha* and significantly increased levels of esterified estradiol. Effects were already seen at concentrations as low as 20 ng/L which raises concerns about the ability of fluoxetine to interfere with reproduction in wild mussel populations.

V. TBT interfered with lipid homeostasis and steroid metabolism in zebra mussels with female organisms being more susceptible than males. Increases in total testosterone levels were already detected at the lowest concentration tested (20 ng/L TBT as Sn) in females whereas no effects were observed in males. Triglyceride levels significantly increased at concentrations of 200 and 500 ng/L TBT as Sn in females and at 500 ng/L TBT as Sn in males while total lipids increased at the highest concentration only in

females. These findings support the ability of TBT to act as an obesogen in *Dreissena polymorpha.*

VI. TBT demonstrated toxic effects on the hepatopancreas of zebra mussels. The observed alterations (cell vacuolization, apoptosis and damage of the connective tissue) were dose-dependent and appeared in all exposure groups.

VII. Evidences of endocrine disruption were detected in a zebra mussel population from Flix which showed depressed levels of testosterone resulting in a skewed estrogen/androgen ratio, delay in gametogenesis, reduction of the spawning period, a significant prolongation of the resting phase, reduced gonad size, and several changes in fatty acid profiles, among them a significant decrease of the essential fatty acid 22:6n-3.

6. RESUMEN DE LA TESIS

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1. Introducción

El aumento masivo de la población humana ha llevado consigo un aumento exponencial en el desarrollo tecnológico e industrial en todo el mundo. Este fuerte crecimiento ha producido un incremento de la manufacturación de compuestos sintéticos a nivel global. Estos productos antropogénicos incluyen entre otros residuos industriales, compuestos de uso agrícola y productos farmacéuticos entre muchos otros compuestos que se utilizan diariamente y que se han convertido en inseparables de la existencia humana. Estos compuestos, una vez liberados en el medio ambiente, pueden representar un peligro grave para organismos expuestos (Fleeger et al., 2003). Así, el sistema acuático es el depósito final para muchos de estos compuestos químicos liberados por actividades antropogénicas, por emisiones directas de efluentes industriales y domésticos (Coogan et al., 2007), por emisiones difusas de escorrentías superficiales urbanas y agrícolas, derrames industriales (Boreham y Birch, 1987; Vitaliano et al., 2002) o por procesos atmosféricos (Lovett, 1994). Muchos compuestos persisten en el ambiente por periodos largos porque resisten a la degradación fotolítica, química y biológica. Otros, por su baja solubilidad en agua, se adhieren fuertemente a partículas en la columna de agua y, como consecuencia, se acumulan en sedimentos que sirven como almacén o depósito. La sociedad ha tomado conciencia de los potenciales efectos adversos a largo plazo de los productos químicos sintetizados y sus subproductos y del riesgo para los ecosistemas acuáticos, puesto que estos compuestos se pueden bioacumular rápidamente en tejidos de organismos residentes y biomagnificar a través de la cadena trófica (Fu et al., 2003).

Además, muchos de estos compuestos químicos y/o sus subproductos pueden actuar como disruptores endocrinos (EDs) e interferir con hormonas naturales en los organismos; hormonas indispensables para el desarrollo de funciones fisiológicas tales como la reproducción, desarrollo y comportamiento de los animales. En las últimas décadas se ha descubierto una amplia variedad de EDs potenciales, entre ellos, biocidas como la atrazina y el fenarimol (Hayes et al., 2002; Janer et al., 2005b), dioxinas (Depledge y Billinghurst, 1999), hidrocarburos aromáticos policíclicos, como el 3-metilcolantreno (Navas y Segner, 2000), ftalatos (Latini et al., 2004), y fenoles alquilados como el plastificante bisfenol A (Oehlmann et al., 2000) y el detergente

industrial 4-nonilfenol (McCormick et al., 2005). Los EDs son generalmente clasificados como estrogénicos (compuestos que imitan a los estrógenos naturales), androgénicos (compuestos que imitan a los andrógenos naturales) o tiroidales (compuestos que directamente o indirectamente afectan la glándula tiroidea) (Snyder et al., 2003). Recientemente, muchos estudios investigan una nueva clase de contaminantes emergentes, los fármacos, diseñados para inducir una respuesta biológica específica en humanos, plantas y animales (Daughton y Ternes, 1999). Estos compuestos farmacéuticos entran al medio acuático principalmente a través de plantas de tratamiento de aguas residuales, donde raramente son degradados durante el tratamiento del agua residual (Ternes et al., 1999; Nakada et al., 2006). Son considerados pseudo-persistentes por su liberación continuada al ambiente que emula la exposición crónica de organismos acuáticos a un cóctel de substancias con actividad biológica (Daughton y Ternes, 1999; Hernando et al., 2006).

Dado que el ecosistema acuático es el hábitat de muchos organismos y constituye un recurso importante por la sociedad, son necesarios mecanismos de control para garantizar su preservación. Por esto, hay que desarrollar estrategias para la identificación, estimación, evaluación comparativa y detección de los riesgos que presentan los productos químicos de origen sintético para el ambiente y la biota (Gray, 1992). En los últimos años, organizaciones internacionales afirmaron claramente que la evaluación de riesgo no se puede basar exclusivamente en el análisis químico de muestras ambientales porque este enfoque no proporciona información acerca de los efectos nocivos de los contaminantes sobre los organismos vivos. Así, es de suma importancia evaluar los efectos de contaminantes medioambientales a través de investigaciones toxicológicas que proporcionen información acerca de su interacción con la fisiología del organismo. Esto requiere la definición de criterios de valoración biológicos específicos (por ejemplo, el crecimiento, la maduración sexual, el ciclo reproductivo y la medida de biomarcadores, entre otros) que permitan la detección de respuestas fisiológicas a la contaminación. Sin embargo, las respuestas fisiológicas pueden variar, en gran medida, en relación a diferente factores bióticos (por ejemplo, la edad, el sexo, el estadio reproductivo) y/o abióticos (por ejemplo, la temperatura, la estacionalidad). Por consiguiente, su aplicación e interpretación debería tratarse con cautela, basándose en información obtenida de estudios preliminares de caracterización,

que proporcionan el conocimiento básico necesario para la integración de las respuestas fisiológicas y/o biomarcadores en investigaciones toxicológicas.

Algunos bivalvos, como los mejillones, se utilizan a menudo en estudios toxicológicos para investigar los efectos de contaminantes y en programas de monitoreo para evaluar la salud ambiental de zonas costeras. Ciertamente, los bivalvos poseen varias cualidades que los convierten en particularmente atractivos para su uso como bioindicadores. Son sedentarios, con una extensa distribución geográfica, y una tasa metabólica muy baja para contaminantes, que, juntamente con su régimen alimenticio (filtradores), resulta en altos niveles de bioacumulación. Adicionalmente, los mejillones se pueden cultivar, mantener y manipular fácilmente, lo que los hace organismos valiosos para estudios de laboratorio.

TBT, fluoxetina y clofibrato: contaminantes del ecosistema acuático

Muchos estudios han demostrado la existencia de un número creciente de contaminantes que afectan la salud de los ecosistemas. Algunos de ellos, tienen la capacidad de alterar funciones fisiológicas importantes y causar efectos adversos para la salud de los organismos a concentraciones muy bajas (Lavado et al., 2004; Faria et al., 2010). Uno de los más conocidos e investigados es el TBT, extensivamente estudiado en las últimas décadas. Los primeros estudios surgieron en la década de 1970, cuando se observaron deformaciones y fallo reproductivo en cultivos comerciales de ostra (*Crassostrea gigas*) en la bahía de Arcachon, Francia, causando el colapso de la población local de ostras (Alzieu, 1991). Al mismo tiempo, se describieron alteraciones en órganos reproductivos en hembras del gasterópodo *Nucella lapillus*, en Inglaterra, y de *Nassarius obsoletus,* en EEUU (Blaber, 1970; Smith, 1971); estas alteraciones consistían en la superimposición de características sexuales masculinas en hembras, y se le dio el nombre de imposex (Smith, 1971; Jenner, 1979). Desde entonces, se han encontrado diversos efectos negativos de TBT en moluscos, tales como la reducción significativa de la viabilidad de larvas (Horiguchi et al., 1997); cambios en lípidos y ácidos grasos (Janer et al., 2007); reducción de los niveles endógenos de testosterona esterificada con ácidos grasos (Gooding et al., 2003); inducción de necrosis en células digestivas del hepatopáncreas (Chagot et al., 1990) o apoptosis del tejido branquial (Mičić et al., 2001).

Los productos farmacéuticos son compuestos ampliamente utilizados. Miles de moléculas activas diferentes se utilizan actualmente en el mundo para combatir o prevenir enfermedades, y cientos de nuevos productos se sintetizan cada año para reemplazar otros ya obsoletos. En los últimos 15 años, diferentes estudios señalan a los fármacos como una nueva clase de contaminantes ambientales emergentes (Kolpin et al., 2002). Estos productos incluyen antibióticos, hormonas, analgésicos, anti-depresivos, reguladores de lípidos y productos de quimioterapia, entre otros. Los productos farmacéuticos, habitualmente llegan al medio acuático a través de vertidos de plantas depuradoras, y pueden en ocasiones terminar en los abastecimientos de agua (Zuccato et al., 2005). En las plantas de depuración, no todos los productos farmacéuticos se eliminan con los sistemas de tratamiento actuales, por lo que pueden estar presentes en los efluentes de las plantas de tratamiento, en distintas masas de agua e incluso en el agua potable a muy bajas concentraciones. Entre los productos farmacéuticos frecuentemente detectados en el medio acuático se encuentran los inhibidores selectivos de la recaptación de serotonina (ISRS), prescritos para el tratamiento de depresión u otros problemas de la salud mental. Uno de los más importantes de este grupo de productos farmacéuticos es la fluoxetina, detectada en diferentes matrices del medio acuático. También se han descrito concentraciones de 1-2 ng/g en tejidos de peces muestreados en aguas superficiales en EEUU (Brooks et al., 2005; Chu y Metcalfe, 2007), mostrando su capacidad de bioacumularse en biota. Aunque el número de estudios que han investigado los efectos de fluoxetina en organismos acuáticos es limitado, ya se han descrito algunos efectos negativos. Como por ejemplo, alteraciones en la reproducción en *Ceriodaphnia dubia* (Henry et al., 2004); reducción de la fertilidad en el gasterópodo *Physa acuta* (Sánchez-Argüello et al., 2009); alteraciones en la reproducción en machos del carpín (*Carassius auratus*) (Mennigen et al., 2010); y reducción de la capacidad reproductiva del caracol de agua dulce *Potamopyrgus antipodarum* (Nentwig, 2007).

Otra clase de productos farmacéuticos que han despertado el interés de la comunidad científica son los reguladores de lípidos, que se utilizan fundamentalmente para bajar los niveles de colesterol en sangre. Las formas utilizadas más frecuentes son los fibratos, entre ellos el clofibrato, que permiten reducir los niveles de triglicéridos y aumentan los niveles de HDL (lipoproteína de alta densidad, *High-Density Lipoprotein HDL*) (Khetan y Collins, 2007). Tras su administración, el clofibrato es metabolizado

por el hígado en ácido clofíbrico, que es excretado inalterado (~6%), o como glucurónido (>90%) (Winkler et al., 2001). La eficiencia de la eliminación del ácido clofíbrico en las plantas de tratamiento de agua residuales es aproximadamente del 50% (Ternes, 1998). Con una vida media estimada en el medio ambiente de 21 años, representa uno de los residuos farmacéuticos conocidos más persistentes (Winkler et al., 2001). El clofibrato ha sido detectado en agua potable (Loraine y Pettigrove, 2006), aguas superficiales (Thomas y Hilton, 2004) y en los efluentes de las plantas de tratamiento de agua residuales (Andreozzi et al., 2003) de países europeos, mientras que el ácido clofíbrico es el metabolito de un fármaco más frecuentemente reportado en aguas abiertas (Khetan y Collins, 2007). A pesar de que ambos, clofibrato y su metabolito, han sido detectados en el medio ambiente en el rango superior de ng/L, los estudios estimando los efectos de drogas hipolipidémicas sobre la composición lipídica y el metabolismo de lípidos en organismos acuáticos, son todavía escasos y en su mayor parte limitados a peces. Así, la exposición de la trucha arcoíris (*Oncorhynchus mykiss*) al producto farmacéutico gemfibrozil, perturbó el metabolismo de la lipoproteína, disminuyendo niveles de lipoproteína en plasma, y modificando la composición de las lipoproteínas reduciendo la abundancia de los ácidos grasos del grupo n-3 (Prindiville et al., 2011). Du et al. (2004) reportó que la exposición a fenofibrato aumentó la actividad peroxisomal en un 30%, resultando en un contenido reducido del ácido eicosapentaenoico (20:5n-3) y ácido docosahexaenoico (22:6n-3) en los lípidos de truchas tratados. En bivalvos, se detectó proliferación peroxisomal y un incremento de actividad del enzima acil-coenzima A oxidasa en el mejillón *Mytilus galloprovincialis* después de inyecciones de 1.5 mg de clofibrato por mejillón (Cancio et al., 1998). Además, Canesi et al. (2007b) mostraron que una concentración de 3 a 300 ng/g peso seco de los fármacos hipolipemiantes bezafibrato y gemfibrozil, afectó la función inmunológica, además de la glicolisis, el equilibrio de los reacciones redox y la función peroxisomal en el mejillón *Mytilus galloprovincialis*. Así mismo, la exposición del mejillón cebra a 1 μg/L de gemfibrozil, indujo síntomas de estrés oxidativo y un aumento de la peroxidación lipídica (Quinn et al., 2011).

Nuevas herramientas de evaluación en estudios toxicológicos del sistema acuático

Aunque los invertebrados acuáticos constituyen un grupo de organismos diverso y numeroso con gran importancia ecológica, aún faltan conocimientos detallados de su endocrinología. Esta ausencia de conocimiento ha impedido progresar en el conocimiento del fenómeno de disrupción endocrina en invertebrados. A pesar de ello, existen numerosas evidencias de que los contaminantes medioambientales interfieren con el sistema endocrino de estos organismos perturbando la reproducción, lo que es especialmente preocupante por sus consecuencias a nivel individual y, especialmente, poblacional. Así, es necesario un mejor conocimiento del papel de los esteroides en la reproducción de invertebrados y los factores endógenos/exógenos involucrados en su control, conjuntamente con las vías enzimáticas implicadas en su síntesis y metabolismo. Este conocimiento, permitirá el establecimiento de nuevos criterios de evaluación que permitirán estimar los efectos de la disrupción endocrina de contaminantes medioambientales en estos organismos.

El ciclo reproductivo

El éxito reproductivo y la dinámica poblacional de organismos acuáticos, muchas veces es altamente sensible a perturbaciones ambientales, y la interferencia de contaminantes con sus funciones reproductivas puede resultar en cambios negativos en la estructura de la comunidad, abundancia, diversidad y composición (Lowe y Pipe, 1987; Chu et al., 2003). El ciclo reproductivo como un elemento importante de la fisiología animal, puede representar un buen indicador general sobre el estado físico porque integra a diferentes niveles fisiológicos los efectos de cambios ambientales, incluyendo la presencia de contaminantes (Kime, 1995). De hecho, muchos estudios ya demostraron el potencial de contaminantes de interferir con el sistema reproductivo de organismos acuáticos. Así, se han reportado anomalías testiculares en poblaciones salvajes de platijas de un estuario que recibe desagües industriales y domésticos (Gill et al., 2002). Mezclas de DDT y de contaminantes industriales conteniendo compuestos organoclorados, indujeron bloqueo de espermatogénesis en carpas salvajes (Lavado et al., 2004). También se detectaron alteraciones de eventos reproductivos en bivalvos, como la inhibición del desarrollo folicular en gónadas de *Mytilus edulis* después de la exposición a cadmio (Kluytmans et al., 1988). Además, el TBT es conocido por interferir con el desarrollo gonadal y la maduración sexual en mejillones y almejas

(Regoli et al., 2001; Siah et al., 2003). Asimismo se reportó un impacto sobre la reproducción en poblaciones salvajes de bivalvos recogidos en áreas contaminadas que mostraron la modificación de la puesta, degeneración de los oocitos y alteraciones en el sistema reproductivo de hembras (Binelli et al., 2001). Estos estudios demostraron claramente que el sistema reproductivo es diana para un amplio rango de contaminantes. Cambios en el desarrollo gonadal, producción y maduración de gametos, puesta etc. pueden servir como un indicador viable y fácil para investigar la exposición a contaminantes. No obstante, para realizar una evaluación exhaustiva del efecto de los contaminantes en la reproducción en bivalvos es necesario mejorar de manera significativa el conocimiento de su ciclo reproductivo y los factores endógenos (hormonas, lípidos, ácidos grasos) y exógenos (variaciones de temperatura, etc.) implicadas.

La implicación de las hormonas sexuales en la reproducción de los moluscos

Varios estudios ya han relacionado variaciones de esteroides sexuales con eventos reproductivos en poblaciones de bivalvos salvajes. Reis-Henriques y Coimbra (1990), por ejemplo, demostraron que los niveles de progesterona en homogenados del organismo entero de *Mytilus edulis*, seguían el ciclo reproductivo con niveles más altos durante la época de puesta. Observaciones similares se hicieron con *Mya arenaria*, donde aumentos en niveles gonadales de progesterona, aparecieron durante el estadio de la maduración gonadal en machos y durante el estadio de la puesta en hembras (Siah et al., 2002); mientras estradiol y testosterona mostraron las concentraciones más altas al inicio de la vitelogénesis en hembras y durante la época de puesta en ambos sexos (Gauthier-Clerc et al., 2006). También en la almeja *Ruditapes decussata*, los niveles de progesterona y testosterona aumentaron al final de la gametogénesis en ambos sexos, mientras los niveles más altos del estradiol se registraron al inicio de la vitelogénesis en hembras (Ketata et al., 2007). Además, diversos experimentos de exposición en el laboratorio han confirmado una posible implicación de los esteroides sexuales en la reproducción de bivalvos (Varaksina y Varaksin, 1991; Osada et al., 2003; Wang y Croll, 2003, 2004, 2006).

La importancia de los lípidos y ácidos grasos

Del mismo modo que los esteroides sexuales, se han asociado variaciones significativas del contenido de lípidos y ácidos grasos con el proceso de reproducción en moluscos. En general, la abundancia de lípidos sigue una variación anual que está fuertemente relacionada con los procesos reproductivos; los lípidos se acumulan durante la gametogénesis, sobre todo durante tiempos de abundancia nutricional, y se transfieren gradualmente a los gametos en desarrollo, resultando ulteriormente en su pérdida durante la puesta (Kluytmans et al., 1985; Napolitano et al., 1992; Nalepa et al., 1993). Estudios *in vivo* con la vieira *Aequipecten irradians* demostraron que las reservas almacenadas en el tejido gonadal (en su mayor parte triglicéridos y fosfolípidos) se transfieren a la gónada durante la gametogénesis y se utilizan para la producción de los huevos (Sastry y Blake, 1971; Barber y Blake, 1985). Esto garantiza el abastecimiento de los huevos con una alta concentración de reserva energética para el estadio planctónico, requisito importante para la sobrevivencia larval y la metamorfosis (Gallager y Mann, 1986; Helm et al., 1991).

Por otra parte, los ácidos grasos constituyen una fracción importante de los lípidos cuyo papel en procesos fisiológicos, como la estructuración y función celular, el metabolismo y el almacenamiento energético, el señalamiento celular y la síntesis de varios compuestos implicados en la regulación fisiológica, como los esteroides y los eicosanoides, entre otros, ha sido demostrada (Benatti et al., 2004). Además, se ha sugerido su implicación en procesos reproductivos en bivalvos. Se ha determinado que, sobre todo los ácidos grasos esenciales, como el ácido araquidónico (20:4n-6), el 20:5n-3 y el 22:6n-3, juegan un papel clave en el control de eventos reproductivos. Una serie de estudios con la vieira *Pecten maximus*, por ejemplo, investigaron la posible implicación de ácidos grasos esenciales en la reproducción, y mostraron que: (a) el 22:6n-3 juega un papel importante a nivel funcional y estructural en la célula y que podría tener una función crucial durante la ovogénesis y la embriogénesis (Soudant et al., 1996a); (b) niveles altos dietéticos de 22:6n-3 dieron como resultado una tasa más alta de la gametogénesis, un aumento de la tasa de eclosión y un porcentaje más bajo de larvas con anormalidades (Soudant et al., 1996b); (c) variaciones anuales de 20:5n-3 y 22:6n-3 siguieron fases reproductivas como el ciclo gametogénico (Pazos et al., 1997) y la maduración de los oocitos y la puesta (Besnard et al., 1989) que confirmaría su implicación en la reproducción de los bivalvos. Asimismo, 20:4n-6, que está implicado

en el metabolismo lipídico de las membranas, se detectó en niveles altos en la gónada de hembras de vieiras, indicando su papel en la ovogénesis (Soudant et al., 1997). Aunque la función de muchos ácidos grasos en procesos fisiológicos no está bien establecida, sus implicaciones en la reproducción de moluscos son muy probables. Por ello existe la necesidad de caracterizar niveles basales de los lípidos y de perfiles de los ácidos grasos en poblaciones naturales que permitan establecer su dependencia de factores externos e internos. Esta información puede ofrecer una herramienta nueva y complementaria para detectar el fenómeno de disrupción endocrina en estudios toxicológicos.

2. Objetivos

El objetivo general de esta tesis es desarrollar nuevas herramientas toxicológicas que puedan ser aplicadas en estudios de biovigilancia del medio acuático utilizando el mejillón cebra (*Dreissena polymorpha*) como organismo centinela. Se ha prestado especial atención al estudio de lípidos (triglicéridos y ácidos grasos), esteroides (testosterona y estradiol) y de enzimas implicados en el metabolismo de esteroides y su relación con el ciclo reproductivo del mejillón cebra. Además, se ha investigado los efectos de los tres contaminantes modelo, fluoxetina, clofibrato y TBT, y sus efectos en el sistema endocrino y en los lípidos de mejillones expuestos a concentraciones ambientalmente relevantes. Por último, se realizó un estudio de campo para detectar el estado de salud de una población de mejillones cebra de una zona contaminada (Flix) en comparación con una estación de referencia (Riba Roja), realizando un estudio histológico de las gónadas, y determinando niveles endógenos de esteroides y lípidos.

Los resultados de esta tesis se presentan como una colección de artículos con los siguientes objetivos específicos:

1. Investigar el ciclo reproductivo de una población de mejillón cebra de una zona no impactada para detectar el papel de esteroides y enzimas implicadas en el metabolismo de esteroides en el ciclo reproductivo del mejillón y su potencial asociación con eventos reproductivos clave, como la gametogénesis y la puesta.

Artículo 1. Lazzara, R., Fernandes, D., Thibaut, R., Porte, C. Study of the reproductive cycle of *Dreissena polymorpha* from the Ebro River: Insights into the involvement of endogenous and exogenous factors (en preparación).

2. Caracterizar el contenido lipídico y los perfiles de los ácidos grasos al largo del ciclo reproductivo del mejillón cebra e investigar los efectos del regulador de lípidos clofibrato sobre los lípidos totales, triglicéridos y los perfiles de los ácidos grasos de organismos expuestos a un amplio rango de concentraciones.

Artículo 2. Lazzara, R., Fernandes, D., Faria, M., López, J.F., Tauler, R., Porte, C., 2012. Changes in lipid content and fatty acid composition along the reproductive cycle of the freshwater mussel *Dreissena polymorpha*: Its modulation by clofibrate exposure. Science of the Total Environment, 432, 195-201.

3. Investigar los efectos de la exposición a niveles ambientalmente relevantes de fluoxetina (20-200ng/L) sobre la estructura gonadal de machos y hembras, la inducción de la puesta y los niveles endógenos de los esteroides (testosterona y estradiol) en el mejillón cebra.

Artículo 3. Lazzara, R., Blázquez, M., Porte, C., Barata, C., 2012. Low environmental levels of fluoxetine induce spawning and changes in endogenous estradiol levels in the zebra mussel *Dreissena polymorpha*. Aquatic Toxicology, 106-107, 123-130.

4. Investigar la capacidad de TBT de actuar como disruptor endocrino y obesógeno y su capacidad de alterar la homeostasis lipídica en el mejillón cebra además de investigar diferencias en la susceptibilidad de machos y hembras a la exposición de TBT.

Artículo 4. Lazzara, R., Fernandes, D., Porte, C. Short-term exposure to tributyltin modulates steroid levels and lipids in the zebra mussel *Dreissena polymorpha* (en preparación).

5. Establecer el estado de salud de una población de mejillón cebra de una zona históricamente contaminada del río Ebro a través la determinación de los niveles endógenos de la testosterona y del estradiol, lípidos y del desarrollo gonadal como marcadores de alteración endocrina.

Artículo 5. Lazzara, R., Fernandes, D., Porte, C. Evidence of endocrine disruption in a zebra mussel population from the Ebro River (en preparación).

3. Resumen y discusión general

Contaminantes ambientales como compuestos farmacéuticos y organoestánnicos se detectan frecuentemente en ecosistemas acuáticos como consecuencia de su amplio uso en los países industrializados. No obstante, el conocimiento de sus efectos sobre la biota acuática es limitado aún y por eso es difícil evaluar sus efectos ecológicos. Por lo tanto, el desarrollo de métodos sensibles para la investigación de efectos tóxicos de estos contaminantes es necesario, especialmente por los ecosistemas de agua dulce. En este contexto, la presente tesis pretende investigar nuevos criterios de evaluación para determinar el impacto toxicológico de tres contaminantes ambientales frecuentemente detectados en el agua, fluoxetina, clofibrato y TBT, sobre el mejillón de agua dulce *Dreissena polymorpha* (mejillón cebra). En primer lugar se determinó el ciclo reproductivo anual de una población natural de mejillón cebra del río Ebro evaluando cambios en los niveles de lípidos y perfiles de ácidos grasos además de las variaciones anuales de las hormonas sexuales, y su papel fisiológico en la reproducción de bivalvos (artículo 1 y 2). Esta investigación proporcionó nuevos conocimientos sobre la variabilidad anual de esteroides y ácidos grasos en relación al ciclo reproductivo del mejillón cebra. En segundo lugar se estudiaron los efectos de la exposición a contaminantes sobre la homeostasis de lípidos (niveles de lípidos, composición de los ácidos grasos y niveles de triglicéridos), esteroides sexuales además de alteraciones histológicos (artículos 2, 3-4). Finalmente, se realizó un estudio comparativo en el campo entre una estación fuertemente impactada por compuestos organoclorados y metales pesados y una estación de referencia usando dos poblaciones de mejillones cebra geográficamente cercanas del río Ebro (artículo 5). Este estudio tuvo como objetivo la investigación de los efectos de contaminantes ambientales sobre el ciclo reproductivo, los niveles de esteroides sexuales y la homeostasis lipídica además de efectos en el desarrollo anual de la gónada y del hepatopáncreas.

Los principales resultados y las conclusiones obtenidas en esta tesis se resumen a continuación.

3.1 Ciclo reproductivo, variaciones anuales de esteroides sexuales y cambios en los niveles de los lípidos y en la composición de ácidos grasos del mejillón cebra

En el artículo 1 se investigó el ciclo reproductivo de una población de mejillón cebra del río Ebro junto con el análisis de los esteroides sexuales libres y esterificados. Mediante un análisis histológico se determinaron cinco estadios de maduración gonadal: estadio 1 (gametogénesis temprana) y estadio 2 (gametogénesis avanzada) ambos implicados en el desarrollo y maduración de los gametos que conlleva cambios significativos del epitelio germinal y del tejido conjuntivo además de la presencia de gametos en diferentes estadios de maturación. Estadio 3 (puesta), periodo en el que la gónada llega a su extensión máxima y cuando la mayor parte del volumen gonadal es ocupado por gametos maduros. Estadio 0 (descanso) y estadio 4 (reabsorción) forman el periodo dónde el tejido gonadal es quiescente y la gónada llega a su volumen mínimo con una estructura interna de aspecto vacío y desorganizado.

El análisis del ciclo reproductivo demostró que la gametogénesis, dura próximamente 4-5 meses, comenzando a finales de otoño/principios de invierno y es seguida de la puesta que dura otros 4-5 meses. Además, el desarrollo gonadal entre sexos es muy parecido. Los resultados obtenidos coincidieron con trabajos realizados sobre poblaciones europeas de mejillones cebra que demostraron que la gametogénesis normalmente comienza durante el invierno cuando las temperaturas ambientales son bajas (Borcherding, 1991; Mackie, 1991). Respecto a la puesta, diversos estudios indican que ésta puede ocurrir como un evento aislado o también consecutivamente durante un ciclo (Jantz y Neumann, 1998; Juhel et al., 2003). En los mejillones del río Ebro, la puesta comienza como evento de alta sincronización en marzo, coincidiendo con el aumento de las temperaturas ambientales. Estudios recientes han demostrado que la temperatura tiene un papel importante sobre el ciclo reproductivo de bivalvos regulado el desarrollo de la gametogénesis y el comienzo de la puesta (Borcherding, 1991; Jantz y Neumann, 1998; Bacchetta et al., 2001). Así, los resultados presentados en este trabajo confirman el papel importante de la temperatura en la regulación del ciclo reproductivo en mejillones cebra.

El estudio del ciclo hormonal reveló la presencia de los dos esteroides sexuales, testosterona y estradiol. Los niveles de los esteroides libres determinados en mejillón cebra se asemejaron al rango encontrado en estudios realizados con otros moluscos

(Janer y Porte, 2007). Sin embargo, la mayoría de los esteroides se encontraron en forma esterificada, constituyendo los libres solo el 3-8% y 2-7% de testosterona y estradiol total, respectivamente. Los esteroides mostraron variaciones significativas durante el año según un ciclo anual. En el caso de testosterona libre se detectaron dos picos significativos, uno en el octubre y el otro en enero/febrero. Estradiol libre presentó picos menos marcados en el noviembre y febrero.

Estudios recientes indican un papel importante de los esteroides sexuales en la reproducción de bivalvos (Gooding y LeBlanc, 2004; Janer et al., 2006). El trabajo presentado en el artículo 1 tuvo como objetivo la investigación del papel de esteroides en la reproducción del mejillón cebra. Los resultados demostraron que la gametogénesis seguía las variaciones anuales de la testosterona libre. Observaciones comparables se han encontrado en otros estudios con gasterópodos demostrando una relación entre la testosterona y el desarrollo de gametos en machos (Sakr et al., 1992; Sternberg et al., 2008). Adicionalmente, se detectó un pico en la producción y esterificación de esteroides durante el periodo de descanso gonadal que demuestra que la producción y almacenamiento de hormonas ocurre cuando el organismo no está en el periodo reproductivo. Esto puede indicar que en bivalvos existe una separación entre estos dos eventos (reproducción y la esteroidogénesis) que, desde el punto de vista energético, puede ser de gran importancia para el organismo. Por lo tanto, el presente trabajo ofrece información adicional que reforzaría la probable relación entre hormonas sexuales y el ciclo reproductivo en bivalvos.

La actividad de la acil-CoA:testosterona aciltransferasa (ATAT) mostró picos durante el periodo de la gametogénesis avanzada (estadio 2) y el comienzo de la puesta. Sorprendentemente, la esterificación anual de los esteroides no coincidió con las variaciones anuales de ATAT que cataliza la esterificación de esteroides con ácidos grasos. Esto coincide con trabajos de Janer et al. (2006) que demostraron que no existe una relación entre la esterificación de esteroides y la actividad de ATAT en moluscos. La falta de concordancia entre estos dos parámetros (esterificación esteroides, actividad ATAT) indica que la esterificación de los esteroides en bivalvos es más compleja que asumido y requiere de más estudios.

La actividad de la 17β-hidroxiesteroide deshidrogenasa (17β-HSD), determinada a partir de la conversión de la androstenediona a testosterona, demostró una

disminución significativa que coincidió con la fase de la gametogénesis avanzada y el comienzo de la puesta. Niveles altos de 17β-HSD durante las fases iniciales de la maturación de los gametos se han podido observar también en otros bivalvos como *Crassostrea gigas* y *Patinopecten yessoensis* (Matsumoto et al., 1997) y *Crenomytilus grayanus* (Varaksina y Varaksin, 1988). A pesar de esto, los resultados presentados en este trabajo junto con estudios precedentes muestran una relación entre las actividades de ATAT/17β-HSD y la reproducción en bivalvos. No obstante, se requiere más información para determinar su significado en el control de la reproducción en bivalvos.

En al artículo 2 se caracterizó el contenido de lípidos y de los perfiles de ácidos durante el ciclo reproductivo del mejillón cebra. Los niveles de lípidos determinados en este trabajo (0.7-2.0% peso fresco) se encuentran en el rango descrito en trabajos previos realizados en el mejillón cebra (Cope et al., 1999) y también en otros bivalvos (De Moreno et al., 1980; Tanabe et al., 1987; Murphy et al., 2002). Los niveles de lípidos variaron significativamente durante el periodo del estudio, mostrando un ciclo anual con un pico en abril cuando comenzó la puesta seguido de una reducción significativa durante los meses sucesivos. La relación entre las variaciones de lípidos y la reproducción en bivalvos ya se ha puesto de manifiesto en otros trabajos con una acumulación de lípidos durante el desarrollo de los gametos y una disminución gradual durante la puesta (Nalepa et al., 1993). Por tanto, las variaciones de lípidos determinadas en esta tesis seguían un ciclo típico de otras especies de bivalvos que está fuertemente relacionado con la reproducción de los mismos.

El perfil de los ácidos grasos observado en nuestro estudio corresponde al que se encuentra en mejillones con una dieta basada en fitoplancton con un alto porcentaje de formas insaturadas (Pirini et al., 2007). Los ácidos grasos más abundantes fueron el ácido palmítico (16:0), el ácido docosahexaenoico (DHA; 22:6n-3), el ácido eicosapentaenoico (EPA; 20:5n-3), el ácido palmitoleico (16:1n-7) y el ácido *cis*-9-eicosenoico (20:1n-11). Las formas n-3 y n-6 mostraron tendencias inversas durante el año con los n-3 presentando un pico en abril y los n-6 en septiembre. Adicionalmente, los FAME totales siguieron la progresión anual de los lípidos demostrando un pico significativo en el abril.

El análisis de los perfiles de ácidos grasos reveló grupos que mostraron pautas anuales distintas. Éstos incluyeron el 16:0, 16:1n-7, 18:1n-7 y 20:5n-3 que llegaron a

niveles máximos durante inverno/primavera mientras otros como el 18:1n-9, 20:1n-11, 20:4n-6 y 22:5n-6 mostraron abundancias más altas en verano/otoño. La implicación de ácidos grasos en procesos fisiológicos importantes como la formación de membranas, el control del crecimiento y la reproducción es conocida desde más de tres décadas (Chu y Greaves, 1991; Fang et al., 1993; Delaunay et al., 1993). El trabajo presentado en el artículo 2 pretendió estudiar y relacionar los patrones de ácidos grasos con el ciclo anual. Por esto se aplicó el análisis MCR-ALS (Multivariate curve resolution alternating least-squares) que identificó tres pautas temporales que explicaban 99% de la variación del conjunto de datos. Estas pautas coincidieron bien con los tres eventos de la reproducción. Así, la primera pauta coincidió con el periodo de la gametogénesis avanzada/puesta, la secunda con el periodo de descanso gonadal y la tercera se relacionó con el periodo del desarrollo de los gametos. Los ácidos grasos que contribuyeron mayormente a las pautas 1 y 3 fueron 16:0, 16:1n-7, 20:5n-3 y 22:6n-3 mientras varios ácidos grasos poliinsaturados como el 20:1n-11 contribuyeron a la pauta 2. Se prestó especial énfasis en la investigación de los ácidos grasos esenciales por sus probables implicaciones en la reproducción. El 20:5n-3 (EPA) mostró un pico de abundancia significativo que coincidió con el final de la gametogénesis mientras el 22:6n-3 (DHA) fue bastante constante durante el periodo de investigación. La importancia de los mencionados ácidos grasos en procesos reproductivos confirmarían otros estudios dónde se observaron niveles altos del 20:5n-3 en las gónadas de *Pecten maximus* (Besnard et al., 1989) y en los que la administración de 20:5n-3 y 22:6n-3 aumentó la fecundación y la sobrevivencia de larvas en diferente especies de bivalvos (Utting y Millican, 1997; Caers et al., 2002; Nevejan et al., 2003). Tomando en cuenta los resultados de estos estudios, se puede concluir que las variaciones anuales de EPA y DHA indican su implicación en la reproducción, teniendo el EPA importancia en procesos energéticos y el DHA jugando un papel estructural general, especialmente durante el desarrollo de gametos (Marty et al., 1992; Freites et al., 2002). Además, la variación anual de 20:4n-6, con niveles altos entre septiembre y diciembre, estuvo bien relacionado con el periodo de descanso gonadal. El 20:4n-6 es un precursor de una serie de mediadores celulares como las prostaglandinas que se determinó en bivalvos como el *Patinopecten yessoensis* y que son implicadas en muchos procesos fisiológicos, entre ellos la reproducción (Osada y Nomura, 1990). Por esto, una relación entre la síntesis de prostaglandinas y el ciclo reproductivo puede existir en el mejillón cebra que podría bien explicar las variaciones del 20:4n-6 observadas en este trabajo.

3.2 Efectos de exposición a farmacéuticos y TBT sobre el mejillón cebra

En los artículos 3 y 4 se investigó los efectos de exposición a corto plazo a fluoxetina y TBT. Ambos contaminantes indujeron cambios significativos en los niveles de los esteroides endógenos. La fluoxetina aumentó significativamente los niveles del estradiol esterificado (1-2 veces) y el TBT provocó alteraciones en los niveles de la testosterona en hembras, aumentándolos por 2-3 veces. Esto demostró el potencial de los dos compuestos investigados de interferir con el sistema endocrino en el mejillón cebra aunque a través de diferentes mecanismos fisiológicos.

Se asume que los inhibidores selectivos de la recaptación de serotonina (ISRS) actúan aumentando los niveles de la serotonina (5-HT) (Fong et al., 1998). Estudios con *Patinopecten yessoensis* y *Placopecten magellanicus* demostraron que el estradiol aumentó la sensibilidad de los tejidos diana de la serotonina a través del incremento de receptores 5-HT (Osada et al., 1992; Wang y Croll, 2003; Osada et al., 1998). Por tanto, el estradiol ejerce un mecanismo de regulación sobre los efectos fisiológicos de la serotonina en bivalvos. Por eso, el aumento de la esterificación del estradiol puede ser un modo de reducir la actividad de la hormona, y compensar así los efectos de niveles alterados de serotonina inducidos por exposición a fluoxetina.

La exposición a fluoxetina indujo una disminución significativa de la densidad de espermatozoides y oocitos que se detectó en animales maduros, que demostraron una reducción en el número de oocitos por folículo y de la densidad de espermatozoides en los túbulos. La inducción de la puesta por los ISRS en bivalvos es un efecto conocido que se documentó ya en otros trabajos (Fong, 1998, Cunha y Machado, 2001). Aunque el mecanismo involucrado no es conocido, se postuló alteraciones en la regulación de la serotonina como explicación más probable (Fong et al., 1998). Los resultados indican que la exposición a corto plazo a fluoxetina puede inducir una reducción de gametos a concentraciones ambientalmente relevantes (Brooks et al., 2003a; Zorita et al., 2007). Así, la exposición a fluoxetina puede causar problemas graves en poblaciones naturales de mejillones por su probable interferencia con eventos reproductivos importantes como la puesta. No obstante, son necesarios estudios para estimar el impacto de los ISRS sobre el éxito reproductivo de bivalvos naturales, especialmente aquellos con fertilización externa como el mejillón cebra.

Los efectos del TBT sobre el sistema hormonal del mejillón cebra coinciden con estudios precedentes con moluscos. Por tanto, se demostró que compuestos organoestánnicos pueden alterar significativamente los niveles de la testosterona y del estradiol, interfiriendo en su producción y metabolismo como reportado por los gasterópodos *Ilyanassa obsoleta* (Gooding et al., 2003), *Marisa cornuarietis* (Janer et al., 2006; Lyssimachou et al., 2008) y el bivalvo *Ruditapes decussata* (Morcillo et al., 1998). A pesar de que muchos estudios se han ocupado de la investigación de los efectos de los organoestánnicos sobre organismos no dianas, el mecanismo todavía no se clarificó. Muchos estudios indicaron un efecto sobre enzimas claves de la regulación de esteroides (Powers y Beavis, 1991; Gennari et al., 2000; Heidrich et al., 2001) pero también existe evidencia en vertebrados que demostró que los organoestánnicos pueden alterar el metabolismo de esteroides a nivel de la transcripción genética (Kanayama et al., 2005; Grün et al., 2006; Mu et al., 2001). Aquél mecanismo puede ser mediado por la vía metabólica del receptor X retinoide (RXR), un tipo de receptor nuclear que es activado por el ácido retinoico. De hecho, existen estudios que mostraron TBT y TPT como ligandos potentes de homólogos del RXR que se detectó en el gasterópodo *Thais clavigera* (Nishikawa et al., 2004). Por esto, existe la posibilidad que los organoestánnicos ejercen sus efectos a través la activación del RXR en moluscos en el mismo modo como demostrado en vertebrados.

Por su alta toxicidad, se investigó los efectos citotoxicos del TBT sobre el hepatopáncreas del mejillón cebra. La exposición a corto plazo a TBT provocó efectos histopatológicos significativos en el hepatopáncreas de los organismos expuestos que fueron concentración-dependientes y que se presentaron en todos las concentraciones. Se detectó lesiones en las células digestivas, el tejido conjuntivo y en las células de los ductos primarios y secundarios. Aquellos incluyeron contracción de las células digestivas, vacuolización de las células digestivas y de los ductos hepáticos así como daños del tejido conjuntivo. En los casos extremos, que se observó a concentraciones altas, se detectó la lisis parcial del tejido conjuntivo además de una fuerte vacuolización de las células digestivas y de los ductos, que fue acompañado de la apoptosis y la formación anormal del lumen de los túbulos. Estudios de exposición a TBT con *Crassostrea gigas* revelaron efectos sobre el hepatopáncreas comparables a aquellos observado en esta tesis, demostrando vacuolización y retracción de las células digestivas (Chagot et al., 1990). Lesiones similares en el hepatopancreas se describió también en

otros trabajos investigando efectos de exposición a desaguas municipales (Quinn et al., 2004) que se describió como una respuesta general de los moluscos al estrés celular inducido por contaminantes ambientales (Lowe et al., 1981).

Este trabajo pretende detectar efectos de contaminantes ambientales sobre la homeostasis lipídica en el mejillón cebra. Por esto se elijó dos compuestos frecuentemente detectados en el medio ambiente para investigar su potencial de interferir con la regulación de lípidos, a saber, el clofibrato y el TBT. La exposición de 7 días a clofibrato (20 ng/L a 2 mg/L) provocó una reducción marcada de los triglicéridos que fue acompañado por un aumento significativo de niveles de los ácidos grasos totales. Estas alteraciones se detectaron a concentraciones ambientalmente relevantes de 200 ng/L (Richardson y Bowron, 1985). La reducción de triglicéridos inducido por la exposición a fibratos como gemfibrozil y ácido clofibrico se reportó también en organismos acuáticos como peces y cangrejos (Prindiville et al., 2011). En general, se atribuye la disminución de los triglicéridos a la actividad aumentada de la lipoproteinlipasa que hidroliza a los triglicéridos y los descompone a glicerol y ácidos grasos (Schoonjans et al., 1996). Aún no se sabe mucho sobre la existencia de enzimas que catalizan la hidrólisis de lípidos en moluscos pero un proteína con función similar ya se describió en el pectínido *Patinopecten yessoensis* (Kim et al., 2008). Además, en vertebrados los efectos de los fibratos son mediados por los receptores activados por proliferadores de peroxisomas (PPARs) que no se detectó ayún en invertebrados. Por esto el mecanismo mediando los efectos del clofibrato observado en este trabajo no es conocido y tiene que ser clarificado en estudios sucesivos.

La acil-CoA oxidasa es el enzima limitante de la beta oxidación y por esto implicada en el catabolismo de los ácidos grasos. Durante los análisis de los lípidos se determinó también la actividad de la acil-CoA oxidasa que no demostró una inducción por la exposición a clofibrato en organismos expuestos. Por tanto, el aumento de los ácidos grasos puede ser explicado por su liberación durante la hidrólisis de los triglicéridos sin una concomitante estimulación de su degradación en seguido, como indicado por la falta de la activación de la acil-CoA oxidasa.

Los resultados presentados en el artículo 4, demuestran que la exposición a la concentración más alta de TBT aumentó significativamente los niveles de los lípidos totales en hembras sin demonstrar efectos en machos. Al mismo tiempo, el contenido de

los triglicéridos aumentó en ambas sexos con efectos más marcados en las hembras. Además, se detectó cambios en la composición de los ácidos grasos que indicaron un aumento en ambos sexos de la fracción n-6 de los ácidos grasos con alta insaturación así como alteraciones en ácidos grasos individuales como el 18:1n-9, 20:4n-6 y 20:5n-3. Se reportó efectos comparables de TBT y TPT sobre la homeostasis lipídica en gasterópodos que demostraron un aumento en los lípidos y los ácidos grasos totales (Janer et al., 2007) y un cambio significativo de los perfiles de los ácidos grasos (Lyssimachou et al., 2009) en hembras de *Marisa cornuarietis*. Por esto, el estudio presentado confirmaría el potencial de los compuestos organoestánnicos de interferir con la homeostasis de los lípidos y de los ácidos grasos en moluscos, siendo más susceptible las hembras.

El mecanismo responsable de las alteraciones lipídicas causadas por TBT se desconoce. Estudios con vertebrados demostraron que el TBT puede estimular la adipogenesis, el almacenamiento de lípidos y la acumulación de triglicéridos al actuar como ligando de los receptores PPARγ y RXR (Kanayama, 2005; Nakanishi et al., 2005). Los ligandos endógenos de los PPAR son varios ácidos grasos que a través de la activación de los receptores nucleares controlan la lipogénesis así como la lipolisis (Willson y Wahli, 1997; Reddy y Hashimoto, 2001; Kota et al., 2005; Li y Glass, 2004). Además de actuar como ligando de receptores PPAR, el TBT puede también inducir la adipogénesis activando homodimeros de RXR (Inadera y Shimomura, 2005; Ziouzenkova y Plutzky, 2008). En modo similar, varios ácidos grasos como el ácido palmitoleico (16:1n-7), el ácido oleico (18:1n-9), el ácido linoleico (18:2n-6), el ácido araquidónico (20:4n-6) y el ácido docosahexaenoico (22:6n-3) (De Urquiza et al., 2000; Goldstein et al., 2003) pueden mediar sus efectos a través la activación de los dímeros de RXR, e incluso de la forma homodímerica (Lengqvist et al., 2004). Acidos grasos identificados como ligandos de RXR (18:1n-9, 18:2n-6 y 20:4n-6) mostraron cambios significativos tras la exposición a TBT, lo que pude indicar una interferencia con la ruta de señalización del RXR. Por esto, cambios en la activación de los homodímeres RXR pueden ser el mecanismo que medió los efectos de las alteraciones en la homeostasis lipídica observados en el mejillón cebra.

Los resultados demuestran la capacidad del clofibrato y del TBT de interferir con la regulación de lípidos y ácidos grasos en el mejillón cebra a concentraciones ambientalmente relevantes. Considerando el papel importante de los lípidos en muchos

procesos fisiológicos, cambios significativos en la composición lipídica pueden representar problemas serios para los organismos expuestos, especialmente durante fases sensibles como el crecimiento o la reproducción. Además, los ácidos grasos juegan un papel importante en procesos fisiológicos como la estructura y función de las células, el metabolismo energético y en el caso de los eicosanoides están involucrados en las redes de comunicación celular (Willson y Wahli, 1997). Los eicosanoides origen de tres ácidos grasos, específicamente el ácido linolenico, el ácido araquidónico y el ácido eicosapentaenoico. Estas moléculas regulan procesos importantes de la reproducción como la producción de huevos, la puesta y la salida del cascaron (Stanley-Samuelson, 1994a,b; Stanley y Howard, 1998). Por consiguiente, las alteraciones detectadas en este trabajo como las alteraciones de los niveles de lípidos y de triglicéridos además de los cambios en la composición de ácidos grasos podrían indicar el potencial de compuestos como el clofibrato y el TBT de perturbar procesos fisiológicos importantes en moluscos como la homeostasis energética y la reproducción.

3.3 Estudio del campo investigando el estado de salud de una población de mejillón cebra situada en un área contaminada

En el artículo 5 se investigaron los efectos de disrupción sobre el sistema endocrino en mejillones muestreados en un área altamente contaminada en el tramo bajo del río Ebro (Flix). Los efectos se evaluaron en relación a una estación de referencia situada en un área cercana, a unos 7-8 kilómetros río arriba (Riba Roja). Los efectos observados incluyeron alteraciones en el ciclo reproductivo, cambios en los niveles anuales de las hormonas sexuales y ácidos grasos, además de cambios en el desarrollo gonadal y del hepatopáncreas.

Los cambios en el ciclo reproductivo se presentaron como un retraso temporal de la gametogénesis (2-3 meses) y una prolongación del periodo de descanso gonadal (2 meses), mientras que el periodo de la puesta se redujo significativamente (2 meses). A parte de no coincidir con la población de referencia, el ciclo reproductivo de los mejillones cebra de Flix presentaba también algunas alteraciones respecto a lo que se describía en otros trabajos con mejillones cebra. En este contexto, se reportó la temperatura como factor importante en la reproducción que controla la progresión y duración de los eventos reproductivos. De este modo, el comienzo y la progresión de la

gametogénesis y de la puesta es determinada por cambios en la temperatura acuática. En poblaciones europeas, la gametogénesis suele empezar durante el invierno cuando las temperaturas alcanzan niveles más bajos, mientras que el aumento de la temperatura indica el inicio de la puesta que se estableció en el rango de 10-17 °C por poblaciones europeas del mejillón cebra (Sprung, 1992). Las temperaturas medidas en ambas estaciones no mostraron diferencias significativas (rango 7-25 ºC). Sin embargo, la gametogénesis en la población de Flix empezó durante el periodo cuando las temperaturas ya alcanzaron niveles más altos durante la primavera, y por consecuencia, la puesta empezó a temperaturas en el rango de 20-22 °C. Esto demostró un ciclo reproductivo que no sigue los cambios en la temperatura como establecida en otros trabajos sobre poblaciones europeas del mejillón cebra. Aún más importante, la duración de la puesta no coincidió con lo que se describió en la literatura, constando solo 2 meses. La duración de la puesta en mejillones cebra normalmente no dura menos de 4 meses e incluso puede durar 7 meses (Gist et al., 1997; Bacchetta et al., 2001; Juhel et al., 2003). Por tanto, los resultados presentados demuestran que el ciclo reproductivo de la población de Flix desvió mucho del que se esperaría en una población no impactada.

Las observaciones histológicas no demostraron alteraciones histopatológicas en la gónada o en el hepatopáncreas en la población de Flix pero se detectaron cambios en el tamaño relativo de ambos órganos. En comparación con Riba Roja, los organismos de Flix presentaron un retraso temporal en el desarrollo gonadal además de una reducción del tamaño total de la gónada que fue 22-30% más bajo del que se observó en Riba Roja. Además no se detectó diferencias entro los sexos en Riba Roja, mientras que los machos de Flix mostraron un desarrollo gonadal más bajo que las hembras.

Normalmente, los cambios en el hepatopáncreas muestran una correlación negativa con el desarrollo gonadal en bivalvos (Sastry, 1966; Fuji y Hashizume, 1974). Los cambios en el hepatopáncreas en Riba Roja estaban bien relacionados con el desarrollo de la gónada, mostrando una disminución significativa durante la fase final del desarrollo de los gametos y el comienzo la puesta que es el periodo cuando el tamaño gonadal aumenta significativamente. En los organismos de Flix, el hepatopáncreas no siguió un desarrollo acorde con los cambios gonadales, sobre todo en machos, donde se detectó un aumento significativo en el volumen del hepatopáncreas durante el estadio de la gametogénesis avanzada. Además, en Flix el tamaño del

hepatopáncreas fue en general más grande en machos que en hembras, un efecto que no se observó en los organismos de Riba Roja. Como consecuencia, los resultados demuestran que, particularmente en machos, el desarrollo gonadal del hepatopáncreas se desvió mucho del normal. Esto es particularmente preocupante dato que en moluscos, el hepatopáncreas juega un papel importante durante la reproducción, donde tiene la función de recurso nutricional para el desarrollo de la gónada (Barber y Blake, 1981). La causa del desarrollo anómalo del hepatopáncreas observado en los machos de Flix no es conocido pero puede bien ser una alteración patológica.

Durante el periodo de la reproducción, la maduración sexual y el estado energético en bivalvos pueden responder a varias condiciones ambientales, como cambios en la temperatura, disponibilidad de nutrientes y también niveles de contaminación. Por esto, los cambios en el desarrollo gonadal pueden estar relacionados con una escasez alimentaria; sin embargo esto se debería también reflejar en los órganos de almacenaje como el hepatopáncreas y también en los niveles de los lípidos. Este efecto no se observó en los mejillones cebra de Flix. Al contrario, el volumen anual más alto del hepatopáncreas se observó en los machos de Flix y los niveles anuales de los lípidos también fueron más altos durante la mayoría de los meses en comparación con Riba Roja. Esto indicaría que la escasez alimentaria no fue la causa principal de las alteraciones observadas en Flix.

Se investigaron las variaciones anuales en los niveles de testosterona y estradiol para detectar las posibles conexiones entre los cambios hormonales y las alteraciones observadas en la reproducción. Los resultados indican una reducción significativa de la testosterona (2-3 veces) en los organismos de Flix que fue acompañado por un cambio en el ratio estradiol/testosterona. Otros autores obtuvieron resultados similares en carpas capturadas en la zona de Flix que también presentaron niveles de testosterona más bajos y una alteración en el ratio de las hormonas sexuales (Lavado et al., 2004). Además, estudios ambientales en la zona de Flix demostraron la presencia de disruptores endocrinos en muestras de agua y sedimentos que incluyeron bifenilos policlorinados (BPC) (Fernández et al., 1999), hexaclorobencenos (Lacorte et al., 2006; Bosch et al., 2009) y DDT (Lavado et al., 2004; Bosch et al., 2009). Como consecuencia, existe la posibilidad que la presencia de compuestos estrogénicos en el medio ambiente indujo alteraciones en los niveles de testosterona en los mejillones de Flix.

Los análisis de los niveles de lípidos y de los perfiles de los ácidos grasos mostraron diferencias en perfiles de unos ácidos grasos individuales. En comparación con Riba Roja, se detectaron cambios en el 20:5n-3 que mostró niveles más altos en junio y julio en los organismos de Flix. Además, el 22:6n-3 mostró niveles anuales generalmente más bajos en animales de Flix. Como se indicó en otros trabajos, ambos ácidos grasos juegan un papel importante en la reproducción, como la producción de gametos y la tasa de eclosión. Por tanto, las alteraciones observadas pueden estar relacionadas con los cambios en la gametogénesis y la puesta así como con la reducción de la producción de gametos debido al desarrollo reducido de la gónada.

Considerando que no se realizaron análisis químicos durante el muestreo, no es posible relacionar los efectos observados con la contaminación ambiental. Sin embargo, factores ambientales importantes como la temperatura, los niveles de lípidos y la composición de los ácidos grasos fueron notablemente similares entre las dos estaciones. Además, se observaron cambios significativos en la reproducción y en el sistema endocrino que pueden indicar una interferencia de compuestos antropogénicos. Otros autores ya habían documentado cambios similares con bivalvos muestreados en áreas contaminadas donde se encontraron efectos como alteraciones en la maduración de los oocitos (Binelli et al., 2004), retraso en la gametogénesis (Gauthier-Clerc et al., 2002) y de la maduración sexual de la gónada (Siah et al., 2003) e interferencias con la puesta y las fases gametogénicas del ovario (Bacchetta y Mantecca, 2009).

4. Conclusiones

I. Los niveles de testosterona y estradiol mostraron variaciones claramente asociadas con los eventos reproductivos. La variación en los niveles endógenos de testosterona libre se relacionó estrechamente con el proceso de gametogénesis mientras que los niveles de testosterona y el estradiol esterificado mostraron valores máximos durante el periodo del descanso gonadal. El máximo de la actividad de la ATAT, implicada en la esterificación de testosterona con ácidos grasos, se detectó al inicio del periodo de puesta o expulsión de gametos.

II. Los lípidos y los niveles de FAME totales mostraron un fuerte aumento en el mes de abril, coincidiendo con el final de la gametogénesis; esto fue seguido por una disminución gradual durante la puesta hasta legar a los niveles más bajos en el mes de septiembre. El análisis de las variaciones de los ácidos grasos usando MCR-ALS reveló tres pautas temporales de ácidos grasos fuertemente relacionadas con los tres eventos principales de la reproducción (gametogénesis, puesta, descanso gonadal) demostrando un papel importante de los lípidos y de los ácidos grasos en la reproducción.

III. El fármaco clofibrato alteró de modo significativo el contenido de lípidos de mejillones expuestos, reduciendo los niveles de triglicéridos y aumentando simultáneamente los FAME totales. Estos efectos se observaron a concentraciones relativamente bajas, de 200 ng/L. Dada la implicación de los lípidos en procesos energéticos, formación de las membranas y en la reproducción, las alteraciones detectadas indican la capacidad del clofibrato de interferir con procesos claves de la fisiología del mejillón.

IV. La exposición a fluoxetina indujo la expulsión de gametos en machos y hembras de *Dreissena polymorpha* y también provocó un aumento significativo de la esterificación de estradiol. Los efectos se detectaron a concentraciones ambientalmente relevantes que sugieren la posibilidad de que fluoxetina pueda interferir con la reproducción en poblaciones de mejillones naturales.

V. El TBT interfirió con los niveles endógenos de lípidos y el metabolismo de esteroides en el mejillón cebra, siendo las hembras más susceptibles que los machos. Se detectó un aumento de los niveles de la testosterona en hembras expuestas a 20 ng

TBT/L (como Sn) pero no se observó dicho efecto en machos. Los niveles de triglicéridos aumentaron significativamente en hembras expuestas a 200 y 500 ng/L TBT como Sn, y en machos expuestos a 500 ng/L TBT como Sn; mientras que los lípidos totales aumentaron únicamente en hembras expuestas a la concentración más alta. Estos resultados muestran la capacidad del TBT de actuar como obesógeno en *Dreissena polymorpha*.

VI. El TBT también demostró efectos tóxicos sobre el hepatopáncreas del mejillón cebra. Las alteraciones observadas (vacuolización, apoptosis celular y daños en el tejido conjuntivo) eran concentración-dependientes y aparecieron en todos los grupos de exposición.

VII. Se detectaron evidencias de disrupción endocrina en la población de mejillones cebra de Flix que mostraron una reducción de los niveles de testosterona, un cambio en el ratio andrógenos/estrógenos, retraso en la gametogénesis, reducción del periodo de la puesta y prolongación de la fase de descanso gonadal, una reducción del tamaño gonadal y cambios en los perfiles de ácido grasos, entre ellos una reducción significativa del ácido graso 22:6n-3.

7. REFERENCES

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8. PUBLICATIONS

Paper 1

STUDY OF THE REPRODUCTIVE CYCLE OF *DREISSENA POLYMORPHA* FROM THE EBRO RIVER: INSIGHTS INTO THE INVOLVEMENT OF ENDOGENOUS AND EXOGENOUS FACTORS

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In preparation

Resumen

Se ha estudiado el ciclo reproductivo de una población de mejillón cebra (*Dreissena polymorpha*) del curso bajo del río Ebro (España). Además, se determinaron variaciones en los esteroides sexuales y en las actividades de los enzimas involucrados en la síntesis y metabolismo de andrógenos. La gametogénesis empezó en noviembre y terminó en abril, y fue directamente seguida de un periodo de puesta altamente sincronizada que duró hasta julio. La producción de gametos alcanzó su punto máximo durante los meses con temperatura del agua más baja (7-9 ºC) mientras que el comienzo de la puesta coincidió con las temperaturas más altas (11-14 ºC). Los niveles anuales de testosterona y estradiol libre estaban en el rango de 0.10-0.76 ng/g y 0.16-1.78 ng/g, respectivamente. La testosterona esterificada estaba en el rango de 2.79-14.26 ng/g, y el estradiol esterificado estaba en el rango de 7.72-38.46 ng/g. Los niveles de la testosterona libre seguían bien la progresión de la gametogénesis y se encontró una relación entre un pico significativo de la esterificación de esteroides y el periodo de regresión gonadal. Coincidiendo con el comienzo de la puesta, la actividad de la acil-CoA:testosterona aciltransferasa (ATAT) mostró un pico en marzo y abril mientras que la actividad de la 17β-hidroxiesteroide deshidrogenasa (17β-HSD) disminuyó significativamente entre febrero y mayo, con los niveles más bajos en marzo. En general, el estudio describe el ciclo reproductivo del mejillón cebra durante un periodo de 12 meses y además presenta información sobre el papel que desempeñan la producción y regulación de hormonas sexuales en la reproducción de bivalvos.

Abstract

The reproductive cycle of a zebra mussel (*Dreissena polymorpha)* population from the lower course of the Ebro River (Spain) was studied. In addition, seasonal variations of sex steroids were determined together with activities of enzymes involved in the synthesis and metabolism of androgens. Gametogenesis started in November and was completed in April, which then was directly followed by a highly synchronized spawning period that lasted until July. Gamete production peaked during months of lowest water temperatures (7-9 ºC) while onset of spawning coincided with higher temperatures (11-14 ºC). Annual levels of free testosterone and free estradiol were in the range of 0.10-0.76 ng/g and 0.16-1.78 ng/g, respectively. Esterified testosterone was in the range of 2.79-14.26 ng/g and esterified estradiol in the range of 7.72-38.46 ng/g. Levels of free testosterone closely followed progression of gametogenesis and a significant peak in steroid esterification was well related with the gonadal resting period. Coinciding with the onset of spawning, activity of acyl-CoA:testosterone acyltransferase (ATAT) showed a peak in March and April whereas 17β-hydroxysteroid dehydrogenase (17β-HSD) activity significantly decreased between February and May with lowest levels in March. Overall, the study depicts the reproductive cycle of the zebra mussel over a period of 12 months and also provides further evidence for the involvement of sex steroid production and regulation in bivalve reproduction.

Introduction

The zebra mussel *Dreissena polymorpha* is an invasive freshwater species with origins in the Caspian Sea region which has spread over Europe and North America (Mackie and Schloesser, 1996; Nichols, 1996), and has recently established colonies in the lower Ebro River (NE, Spain). Since its arrival, the zebra mussel expanded rapidly colonizing the entire axis including 7 rivers in the Ebro basin (Durán et al., 2010). It is a dioecious species with external fertilization and a free swimming planktonic larva showing an r-selected reproductive strategy. Once established it quickly becomes the most dominant species posing a considerable threat to the native benthic fauna (Aldridge et al., 2004). Studies have already been undertaken on the reproductive cycle of *Dreissena* on different populations worldwide showing the different phases of reproduction and also their variability between geographical locations (Borcherding, 1991; Nichols, 1996; Jantz and Neumann, 1998; Juhel et al., 2003).

Several environmental factors controlling the reproductive process have already been suggested, with the most important being temperature and food availability which together determine the onset and duration of gametogenesis and spawning (Pieters et al., 1980; Vélez and Epifano, 1981; Martínez et al., 2000). However, little information is available on the endogenous factors involved. Recent studies suggest that sex-steroids may play a similar important role in invertebrate reproduction as they have been demonstrated to do in vertebrates. Indeed, presence of several vertebrate-like steroids has already been reported in various mollusc species (Croll and Wang, 2007; Janer and Porte, 2007) and their annual variations have been linked to the reproductive cycle. Progesterone (P4) levels for example were associated with the spawning season in the mussel *Mytilus edulis* (Reis-Henriques and Coimbra, 1990) and increases in ovarian 17β-estradiol (E2) levels paralleled sexual maturation in the Pacific oyster *Crassostrea gigas* and the scallop *Patinopecten yessoensis* (Matsumoto et al., 1997). Besides, studies by Gauthier-Clerc et al. (2006) with the soft-shell clam *Mya arenaria* demonstrated that highest concentrations of E2 and testosterone (T) occurred during the previttelogenic stage in females and during spawning in both sexes. Finally, Wang and Croll (2003, 2004) investigated effects of steroids on the gonad of the scallop *Placopecten magellanicus* showing that (i) E2 and P4 potentiated gamete release in both sexes while T facilitated spawning in males only; (ii) E2, T, P4 and dehydroepiandrosterone (DHEA) all accelerated gonadal differentiation and shifted sex ratios towards more

males; (iii) E2 stimulated oocyte growth while T appeared to induce degeneration of oocytes. Thus, even though information is still too fragmented to allow a full comprehension of the physiological role which steroids play in mollusc reproduction, their possible involvement cannot be dismissed.

Biosynthesis of steroids has already been well described in mollusc species through several studies showing the presence of key steps of steroidogenesis leading to androgen and estrogen synthesis (Janer and Porte, 2007). In bivalves, several key enzymes have already been reported including 3β-hydroxysteroid dehydrogenase (3β-HSD), 5α-reductase (De Longcamp et al., 1974), P450-aromatase (Morcillo et al., 1999) and 17β-hydroxysteroid dehydrogenase (17β-HSD) (Mori et al., 1965). More importantly, annual variations of some of these enzymes have also been related to the reproductive cycle. Hence, presence of 17β-HSD was demonstrated in the ovaries of *C. gigas* and *P. yessoensis* (Matsumoto et al., 1997) and annual activity corresponded with reproductive events, thus being lower during post spawning stage than during early differentiation stage. Furthermore, in the ovotestis of the snail *Helix pomatia*, 3β-HSD showed higher activity before oviposition than after (Krusch et al., 1979). All these variations may reflect the dynamics of steroid metabolism in molluscs and probably other invertebrates.

Together with steroidogenesis, steroid metabolism plays an important role in the regulation of endogenous steroid levels. Major metabolic pathways in molluscs include steroid hydroxylation (Morcillo et al., 1998), conjugation (Janer et al., 2005c) and the formation of steroid-esters (Gooding and LeBlanc, 2001; Janer et al., 2004). The latter are catalyzed by acyl-CoA:steroid acyltransferases which conjugate steroids with fatty acids to form apolar metabolites which then can be stored in the lipid matrices of the organism. In gastropods, esterification represents the most important metabolic pathway for sex steroids like T and E2, enabling them to control free steroid levels. Indeed, exogenously provided T had almost none or only little impact on free steroid levels of the mud snail *Ilyanassa obsoleta* because excessive T was rapidly conjugated to fatty acid esters and then stored in the fat tissue of the organism (Gooding and LeBlanc, 2001, 2004). Conjugation of steroids with fatty acids has also been demonstrated in several bivalves like the eastern oyster *Crassostrea virginica* where the formation of sex steroid esters of E2 and DHEA was reported (Janer et al., 2004) as well as the mussel *Mytilus galloprovincialis* where exposure to T or E2 resulted in the formation of

testosterone or estradiol fatty acid esters (Janer et al., 2005a; Fernandes et al., 2010). Although several studies on the esterification of steroids have been performed in molluscs, the understanding of their physiological function and especially their role in reproduction is still lacking.

The following study aims to investigate the reproductive cycle of the fresh water mussel *Dreissena polymorpha* by assessing the seasonal gonadal development over a period of twelve months. In addition, annual changes in the activity of two important enzymes (17β-HSD and ATAT) involved in androgen biosynthesis and metabolism were measured together with endogenous levels of T and E2. Possible relations between endogenous and exogenous parameters and reproduction are presented. Our study will therefore provide basal knowledge on annual biosynthesis and metabolism of selected sex-steroids in addition to new insights into bivalve reproduction.

Material and Methods

Chemicals

Unlabeled steroids (testosterone, androstenedione), hematoxylin, eosin-y and paraplast embedding medium were purchased from Sigma (Steinheim, Germany). [1β-^{3}H] androstenedione (15-30 Ci/mmol) was obtained from Perkin-Elmer Life Sciences (Boston, MA, USA) and [4-^{14}C] testosterone (50-60 mCi/mmol) from Amersham (Buckinghamshire, UK). Radioimmunoassay (RIA) kits for the analysis of testosterone and 17β-estradiol were purchased from Radim Inc. (Pomezia, Italy). All solvents and reagents were of analytical grade from Merck (Darmstadt, Germany).

Seasonal sampling

The sampling site chosen for this study is situated in the lower section of the Ebro River, approx. 100 km before the river discharges into the Ebro Delta (41° 14' 53.93 N/ 0° 28' 40.47 E). The sampling was conducted monthly, beginning in October 2008 until September 2009. Mussels were collected near the river bed at a depth of approximately 0.5 meter where they normally stay attached to small rocks. After collection, mussels were transported in buckets filled with river water to a nearby laboratory where they were immediately dissected. Tissue samples were deep frozen in liquid nitrogen and

then kept in a freezer at -80 ºC until analysis. For histology, entire animals were put into cassettes and fixed in 10% buffered formalin (0.1 M phosphate buffer, pH 7.4) for 48 h. Samples were subsequently rinsed with water and stored in 70% ethanol. During the sampling, the shells of about 80-100 animals were measured each month with a vernier caliper to the nearest 0.1 mm. Furthermore, measurements of the water temperature, pH, conductivity and concentration of dissolved O_2 (CRISON-Oxi 45P/CRISON-CM 35) were taken.

Histological analyses

Tissue was dehydrated with ethanol, embedded in paraplast, sectioned (7 μm) and stained with hematoxylin and eosin-y. For each animal a series of 10-15 histological cuts was prepared and analyzed with a Leica DMLS light microscope. The number of animals processed for each month ranged from 10 to 20. For statistical analysis males and females were pooled. To determine gonadal maturation in *D. polymorpha* a descriptive staging system was devised based on previous methods used for mussels (Villalba, 1995; Toro et al., 2002). Five main stages were applied as follows:

In males:

- Resting (Stage 0): In this stage the gonad appears empty; the few developing gametes are mostly situated at the periphery of the tubules. Few hemocytes can be found within the gonadal tissue. The center of the tubules is mostly filled with connective tissue.

- Early development (Stage 1): The germinal epithelium forms a thick bond at the edges of the tubules and several developing gametes are already visible surrounding the central lumen. The connective tissue has redeveloped to thin filaments which are loosely dispersed through the gonad.

- Late development (Stage 2): The connective tissue has been reduced to a thin layer separating the round shaped tubules which occupy most of the gonad, and are evenly filled with reproductive cells of different maturation stages.

- Spawning (Stage 3): This stage is characterized by the star shaped structure formed by the spermatozoa in the center of the tubules. The tails of the

spermatozoa are visible as fine filaments within the tubules. Most of the germinal epithelium is constituted by thick reproductive cells.

- Reabsorbing (Stage 4): Gonadal tissue appears empty and disorganized. Residual gametes can be found within the tubules which have lost most of their round shape structure. Connective tissue is loosely scattered through the gonad. Many hemocytes are present inside and outside of the follicles.

In females:

- Resting (Stage 0): The gonad appears empty, only a few follicles contain residual ova. Connective tissue is present throughout the entire gonad. Few hemocytes can be found within the ovaries interstitial tissue.

- Early development (Stage 1): Thick germinal epithelium cells surround the central lumen of the round shaped follicles which are filled with connective tissue. Developing oocytes appear in different sizes at the periphery.

- Late development (Stage 2): Germinal epithelium is reduced to a small band at the edge of the follicles. The lumen of the gonad is occupied by oocytes in different development stages. Small filaments of the remaining connective tissue are visible in the center.

- Spawning (Stage 3): The peripheral area and parts of the central lumen of the follicle is occupied by mature oocytes. Most follicles have a flabby appearance and several pedunculated oocytes are visible at the periphery. Germinal epithelium has reduced to a one germ cell lining at the periphery of the follicle, and the strongly reduced connective tissue is scattered loosely throughout the gonad forming discontinuous layers.

- Reabsorbing (Stage 4): The gonadal tissue appears disorganized and the lumen is mostly empty. Connective tissue is present inside as well as outside of the follicles which have a drawn-out or concaved appearance. The few remaining ova are dispersed through the entire lumen. Hemocytes can only occasionally be found.

Illustrations of the five different gonadal stages in males and females of *Dreissena polymorpha* are given in figure 1 & 2.

Determination of tissue steroid levels

Tissue samples (0.3–0.4 g w.w.) were homogenized in ethanol and frozen overnight at -80 ºC. The homogenates were then thawed and extracted three times with 2 ml of ethyl acetate. The organic extract was separated into two aliquots for determination of free and total steroids and then dried under a nitrogen flow. For determination of free steroids the method described by Janer et al. (2005a) was adapted. Briefly, dried residues were re-dissolved in a solution of 80% methanol, washed three times with petroleum ether to remove the lipid fraction and then evaporated to dryness under a nitrogen stream. For the determination of total steroids (free and esterified) the method described by Gooding et al. (2003) was applied with some modifications. Thus, samples were re-dissolved in 1.0 ml methanol containing 1% KOH and incubated for 3 hours at 45 ºC. After the saponification step, 4 ml of Milli-Q water was added and the samples then extracted three times with 3 ml of dichloromethane. The efficiency of the extraction and delipidation was 74 ± 3 % for testosterone and 80 ± 3 % for estradiol (Morcillo et al., 1999). Samples were resuspended in 50 mM potassium phosphate buffer pH 7.6 containing 0.1% gelatin and assayed for testosterone and estradiol concentration by radioimmunoassay using a ^{125}I-RIA kit. The detection limits of the assay were 25 pg/g for testosterone and 20 pg/g for estradiol.

Preparation of tissue homogenate (S9 fraction) for enzymatic assay

A pool of 2-3 animals was homogenized in 1:7 w/v of ice-cold 100 mM potassium phosphate buffer pH 7.6, containing 100 mM KCl, 1 mM ethylenediaminetetraacetic acid (EDTA), 1 mM dithiothreitol (DTT) and 1 mM phenylmethylsulfonylfluoride (PMSF). Homogenates were first centrifuged at 500 x g for 15 min, fatty layer removed and then centrifuged at 10 000 x g for 20 min at 4 ºC and the supernatant (S9-fraction) freshly used for the biochemical assays. Proteins were measured by the method of Bradford (1976) using bovine serum albumin as standard.

Determination of 17β-hydroxysteroid dehydrogenase (17β-HSD) activity

Androstenedione metabolism was assessed by a modification of the method previously introduced by Janer et al. (2005b). S9 fraction (0.2 mg protein) was incubated with 0.2 μM [^{3}H] androstenedione (150 000 dpm), 320 μM NADPH, 10 mM $MgCl_2$ and 50 mM Tris–HCl pH 7.4 in a final volume of 250 μl. Reaction was initiated by adding NADPH and samples then incubated under constant shaking for 30 min in an air heated incubator at 30 °C. Incubations were stopped by adding 250 μl of acetonitrile and after centrifugation (1500 x *g*, 15 min), 100 μl of supernatant was injected into a RP-HPLC column.

Determination of acyl-CoA:testosterone acyltransferase (ATAT) activity

Acyl-CoA:testosterone acyltransferase activity was determined using the method described by Lavado et al. (2006) following some modifications. S9 fraction (0.2 mg protein) was incubated with 0.5 M sodium acetate buffer pH 6.0, 11 μM [^{14}C] testosterone (150 000 dpm), 1 mM palmitoyl-CoA and 100 mM $MgCl_2$ in a final volume of 500 μl. The reaction was initiated by the addition of palmitoyl-CoA and samples were incubated under constant shaking for 60 min in an air heated incubator at 30 ºC. The reaction was stopped by adding 2 ml of ethyl acetate; samples were then extracted twice and then evaporated to dryness under a nitrogen flow. The dry residue was re-dissolved in 500 μl methanol and 100 μl injected into a RP-HPLC column.

HPLC system

HPLC analysis was performed on a Agilent Technologies 1200 Series system equipped with a 250 mm x 4 mm LiChrospher 100 RP-18 (5 μm) reverse-phase column (Merck, Darmstadt, Germany) protected by a guard column LiChrospher 100 RP-18 (5 μm). Separation of [^{3}H] androstenedione metabolites was performed at 1 ml/min with a mobile phase composed of (A) 75% water and 25% acetonitrile, and (B) 25% water and 75% acetonitrile. The run consisted of a linear gradient from 100% A to 100% B (0-30 min), isocratic mode at 100% B (5 min), linear gradient from 100% B to 100% A (5 min), and isocratic mode at 100% A (5 min). Separation of [^{14}C] testosterone and its palmitoyl-ester was performed at 1.2 ml/min with a mobile phase composed of (A) 56% water containing 0.1% acetic acid (pH 3), 13% acetonitrile and 31% methanol and (B)

100% methanol. The run consisted of 9 min isocratic 100% A, 6 min of linear gradient from 100% A to 100% B, and 25 min isocratic 100% B. Chromatographic peaks were monitored by on-line radioactivity detection with a Radioflow detector LB 509 (Berthold Technologies, Bad Wildbad, Germany) using Flo-Scint 3 (Packard BioScience, Groningen, Netherlands) as scintillation cocktail. Metabolites were quantified by integrating the area under the radioactive peaks.

Statistical analyses

Data is reported as mean ± SEM. Statistical treatment of data was performed by using the SPSS statistical package (SPSS v.17.0, IBM Company, Chicago, USA). Differences between months for steroid levels and enzymatic activities were determined using analyses of variance (one-way ANOVA) with Tukey's post-hoc multiple comparison test ($p<0.05$). Prior to any analysis, data was tested for normality and variance homoscedasticity, and when necessary, log-transformed to meet the assumptions of parametric tests. Bivariate Pearson's correlation analysis was performed to detect significant correlations between different parameters.

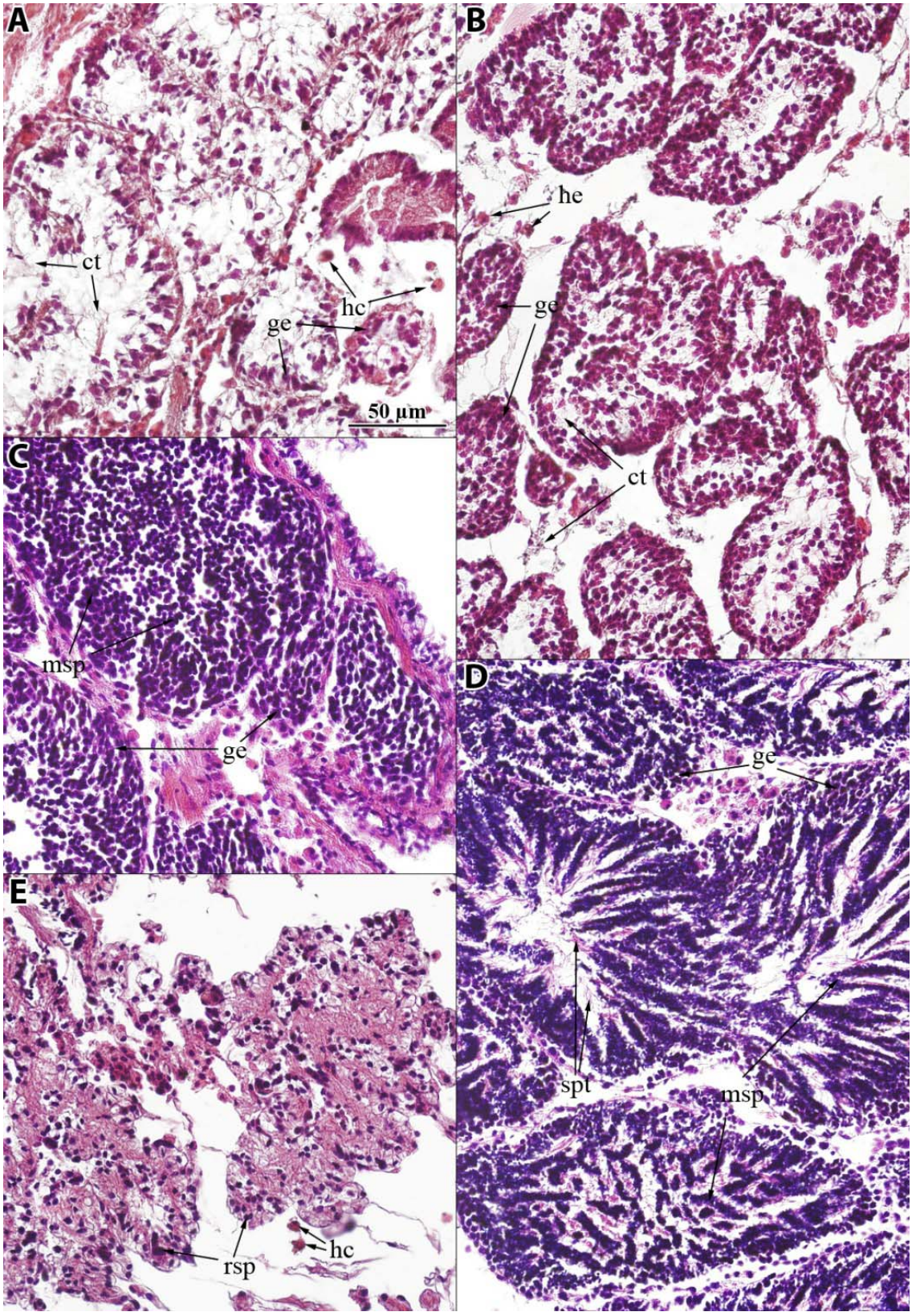

Figure 1. Different stages of gonadal development in males of *Dreissena polymorpha*. A: Stage 0 (resting), B: Stage 1 (early development), C: Stage 2 (late development), D: Stage 3 (spawning), E: Stage 4 (reabsorbing). Abbr.: ct: connective tissue, ge: germinal epithelium, hc: hemocytes, msp: mature spermatozoa, rsp: residual spermatozoa, spt: spermatozoon tails. Scale bar on A applies to all photographs.

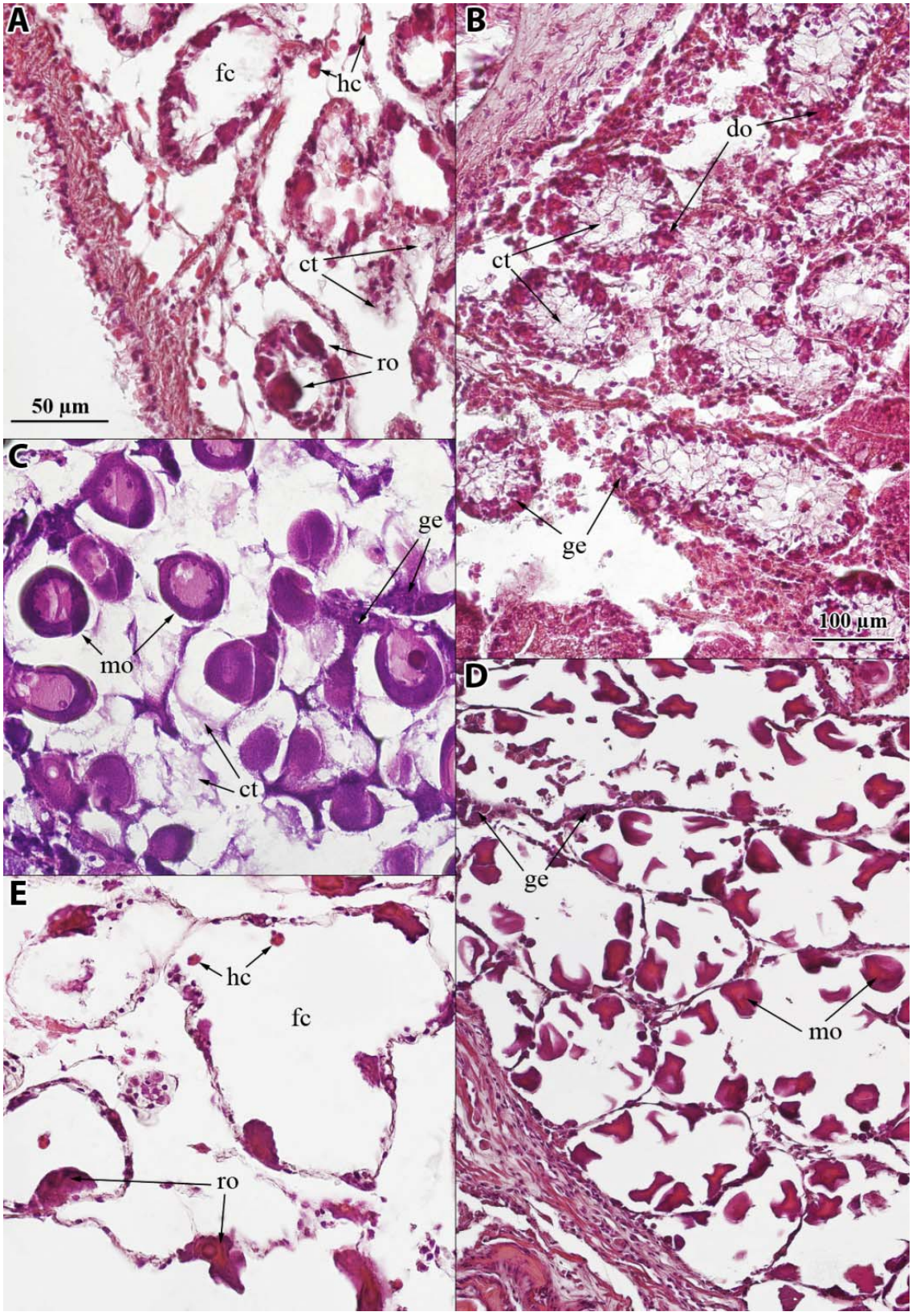

Figure 2. Different stages of gonadal development in females of *Dreissena polymorpha*. A: Stage 0 (resting), B: Stage 1 (early development), C: Stage 2 (late development), D: Stage 3 (spawning), E: Stage 4 (reabsorbing). Abbr.: ct: connective tissue, do: developing oocytes, fc: follicle center, ge: germinal epithelium, hc: hemocytes, mo: mature oocytes (ova), ro: residual ova. Scale bar on A applies to photographs A, C, E; scale bar on B to photographs B and D.

Results

Water temperature

In figure 3 water temperatures from October 2008 to September 2009 are depicted. A clear cycle of water temperature was observed with temperatures decreasing from October to January and then gradually increasing to peak in September. Temperature range varied from lowest levels of 7.3 °C in January to highest levels of 24.7 °C in September.

Reproductive cycle

To assure that only mussels of the same size class were used for the study, shell length was measured which showed an average size between 19.3–22.5 mm and no significant difference between sampling months. Given the fact that *Dreissena polymorpha* grows approximately 1.5–2.0 cm per year with an average lifespan of 2-3 years and a maximum length of 2.3–2.5 cm (Mackie, 1991), all organisms were considered adults at more or less the same age and sexual maturity. Hence, for the assessment of the annual reproductive cycle of *Dreissena polymorpha*, the gonadal tissue was examined to determine the individual maturation stage of each organism. In figure 3 the monthly percentages of individual gonadal stages during the sampling period are illustrated. Gametogenesis started around November when water temperatures began to decline, and in January, the month with the lowest annual water temperatures, most of the population (80% of animals) was found in early development (stage 1). Already in February, a significant amount of animals (30%) entered late development (stage 2) which for the major part of the population ended with the full development on the gonad in April, hence completing the gametogenic cycle. Gamete development in both genders was quite similar. Thus, in males collected in November spermatogenesis was already apparent and the amount of mature spermatozoa increased throughout winter. A large number of lobes of the testis were completely filled with mature gametes by March. In females, even though follicles of the ovaries were less developed in the months of October and November, a considerable amount of gametes were already present. Follicles and gametes then grew significantly throughout winter and early spring, and at the beginning of April the follicles of the ovary were filled with matured ova. Gametogenesis was followed by a highly synchronized spawning event which started in

March when temperatures began to increase, with 90-100% of the animals being in spawning (stage 3) from April until July corresponding with water temperatures in the range of 14-22 °C. The beginning of the spawning season was indicated by ripe oocytes (and sperm) in females (and males) and the maximum extension of the gonad, which was filled with matured gametes which then gradually got released during the progression of the spawning period. From our results we would suggest that both gamete development and spawning comprised a period of approx. 4-5 months. The reproductive cycle ended with the period of gonadal rest (stages 0 and 4) which started in September and ended in December. During this stage the gonads have a disorganized structure and the gonadal tissue becomes inactive and reaches its lowest size volume.

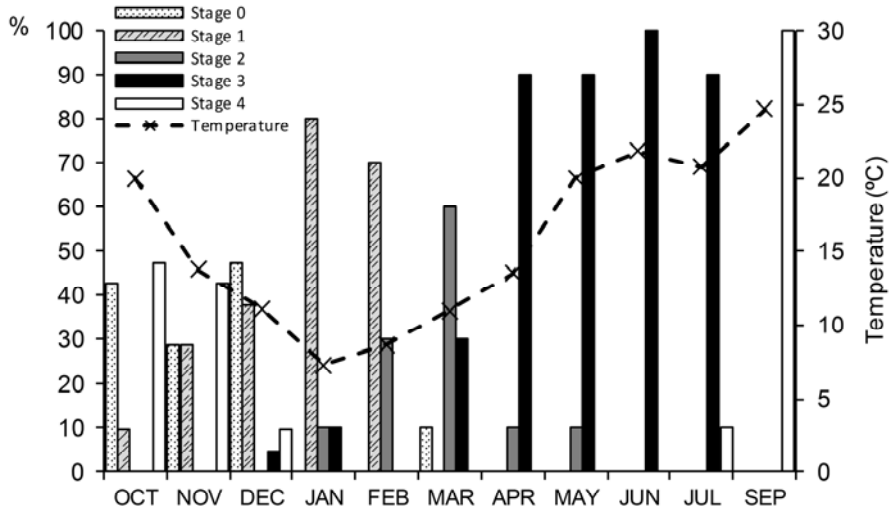

Figure 3. Combined presentation of annual water temperatures at study site and sexual maturation stages of the gonad of *Dreissena polymorpha* during a sampling period of 12 months (n=10-20). 0. Resting, 1. Early development, 2. Late development, 3. Spawning and 4. Reabsorbing.

Tissue steroid levels

Levels of testosterone and 17β-estradiol were measured in tissue homogenates of zebra mussels. To determine the total steroid amount (free and esterified), the protocol included a saponification step prior to extraction which served to cleave the ester bonds of the steroid esters. For the determination of free steroids the saponification step was omitted. The amount of esterified steroids was calculated by subtraction of the free from the total steroid content. Endogenous levels of free testosterone and estradiol showed relatively high variability between individuals. Free testosterone was in the range of 0.10-0.76 ng/g and free estradiol between 0.16-1.78 ng/g. Both steroids were found predominately in their esterified form; esterified testosterone was 93.4 ± 1.3 % of total testosterone and esterified estradiol was 96.0 ± 0.6 % of total estradiol. Both free and esterified estradiol was in average 2-3 times higher than free and esterified testosterone. The range of measured esterified testosterone was 2.79-14.26 ng/g and estradiol 7.72-38.46 ng/g.

Both free testosterone and estradiol showed statistically significant variations along the seasonal cycle (Figure 4). Free testosterone showed peaks in October and in the months of January and February. Significant peaks in free estradiol were observed in November and in February. The highest level of free testosterone occurred in January (0.76 ± 0.26 ng/g) and the lowest level in June (0.10 ± 0.02 ng/g) whereas free estradiol was highest in November (1.78 ± 1.07 ng/g) and lowest in May (0.16 ± 0.01 ng/g). In contrast, levels of esterified testosterone (T) and estradiol (E2) showed great similarity during the sampling period (Figure 4). Maximum levels appeared in autumn (T, 14.26 ± 1.72 ng/g/ E2, 38.46 ± 10.65 ng/g) and both reached their lowest levels in April (T, 2.79 ± 0.60 ng/g / E2, 7.72 ± 2.52 ng/g).

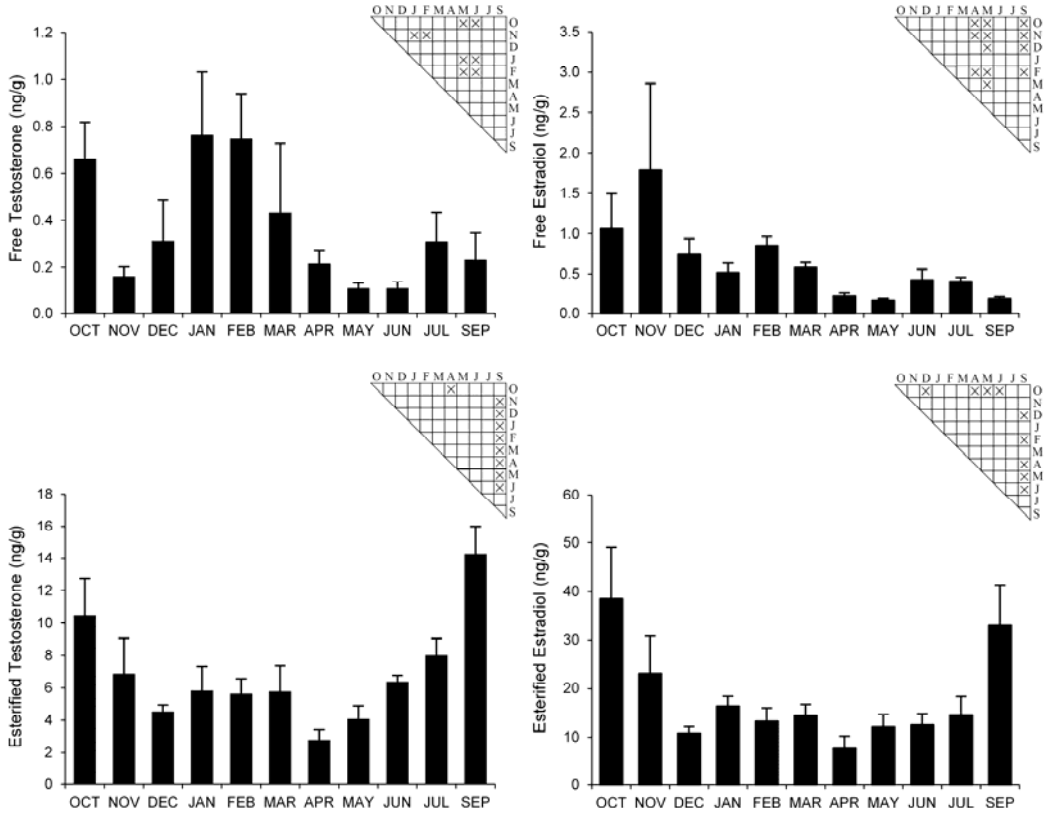

Figure 4. Tissue levels of free and esterified testosterone and estradiol in *Dreissena polymorpha*. Data presented as mean ± SEM (n=5). [x]denotes significant differences ($P<0.05$) between months according to Tukey's test.

Testosterone biosynthesis and metabolism

Both 17β-HSD and ATAT were measured in the isolated S9 fraction extracts of body homogenates of *Dreissena polymorpha*. 17β-hydroxysteroid dehydrogenase readily converted androstenedione to testosterone in the presence of NADPH. The specific activity ranged from 2.04 to 5.21 pmol/min/mg protein. A significant decrease in the enzyme activity was observed between February and May with lowest activity in March (Figure 5A).

ATAT conjugates testosterone with fatty acids to form an apolar metabolite which then can be stored in the lipoidal matrices of the organism. This phase II enzyme had a specific activity ranging from 1.47 to 21.18 pmol/min/mg protein. A pronounced increase in enzymatic activity was observed in March and April which in comparison to other months was up to 12-14 times higher. Maximum annual activity was measured in March while lowest activities occurred in June (Figure 5B). The increase in ATAT activity would coincide with the end of gametogenesis and also with period where free testosterone levels began to decrease.

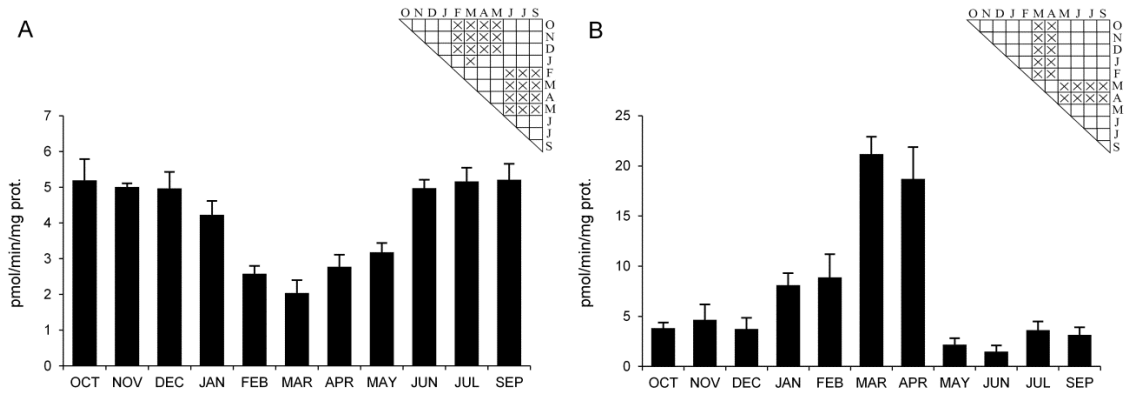

Figure 5. Enzymatic activity of (A) 17β-HSD and (B) ATAT measured in S9 fractions isolated from body tissue of *Dreissena polymorpha*. Data expressed as mean ± SEM (n=6). [x]denotes significant differences (P<0.05) between months according to Tukey's test.

Discussion

The main focus of this work was to assess the reproductive cycle of a zebra mussel population from the lower course of the Ebro River, including the investigation of possible regulating factors. Those comprised sex steroids, temperature and enzymatic activities which have already been associated with bivalve reproduction in previous studies (Cáceres-Martínez and Figueras, 1998; Jantz and Neumann, 1998; Janer et al., 2006; Ciocan et al., 2010). Hence, according to our results, both gametogenesis and spawning may be influenced by changes in water temperature. Our data showed that the major part of the population was in gametogenesis (60-100%) between January and March, thus coinciding with the period of lowest annual water temperatures. In addition, gamete release began within the months where temperatures were increasing (11-14 °C) (Figure 3). These results are consistent with reports on zebra mussel populations from Germany and North America (Borcherding, 1991; Haag and Garton, 1992; Nichols, 1996) showing that gametogenesis usually progresses during period of low temperatures and that adequate water temperatures are necessary for the onset of spawning. Our study with the zebra mussel would therefore consolidate the crucial importance of temperature in the control of key reproductive events in bivalves.

Testosterone and 17β-estradiol were predominantly found in their esterified form all over the reproductive cycle. Similar observations were made in previous mollusc studies by Janer et al. (2006) and Lyssimachou et al. (2008) for *Marisa cornuarietis* and also in *Dreissena polymorpha* by Peck et al. (2007). Free testosterone levels (0.10-0.76 ng/g) as well as free estradiol levels (0.16-1.78 ng/g) were well within the range

measured in other molluscs with free estradiol being generally two to three times higher (Morcillo et al., 1999; Janer and Porte, 2007). Observation of the annual variations revealed that free forms of both steroids demonstrated two distinct peaks during the year with estradiol showing its highest peak in late-autumn and testosterone in winter. Furthermore, progression of gametogenesis was well related to variations of free testosterone levels. This would suggest that this event may to some extend be controlled by testosterone which would confirm earlier studies done on the land snail *Theba pisana* by Sakr et al. (1992) who was able to demonstrate that testosterone accelerates spermatogenesis in males. However, due to the fact that no separation between genders was performed it is unknown if this peak is mainly attributed to male individuals of the population.

In contrast to the free forms, esterified steroids seem to show very similar seasonal variations. Esterified testosterone and estradiol with ranges between 2.79-14.26 ng/g and 7.72-38.46 ng/g respectively, are comparable to levels measured in the digestive gland/gonad complex of *M. cornuarietis* (Janer et al., 2006). A significant increase in the esterification of testosterone and estradiol was detected in autumn with a peak between September and October. This would coincide with the period of gonadal rest (stage 0+4). The esterification with fatty acids is considered a major biotransformation pathway for sex steroids in molluscs (Gooding and LeBlanc, 2001; Janer and Porte, 2007) and several species of echinoderms as well as arthropods where fatty acid conjugation of ecdysteroids have been demonstrated (Slinger et al., 1986; Slinger and Isaac, 1988; Janer et al., 2005c). Indeed, considerable biotransformation of steroids to fatty acid conjugates have been reported for the gastropod *I. obsoleta* and the bivalve *C. virginica* (Gooding and LeBlanc, 2001; Janer et al., 2004). More importantly, esterification of steroids is a crucial mechanism for the control of seasonal free steroid levels in gastropods (Gooding and LeBlanc, 2004). The conjugation of steroid molecules with fatty acids significantly increases their fat solubility, thus facilitating their storage in lipoidal matrices and greatly decreases the bioactivity, bioavailability and excretion of those conjugates (Borg et al., 1995). The role of the esterification process on the ability of those metabolites to exert biological effects is not completely understood. Studies on rodents led to the suggestion that the esterification largely increases the half-live of a steroid and prevents its systemic clearance because the hydrophobic ester shields the steroid nucleus from catabolic enzymes making it more

resistant to enzymatic hydrolysis. This extends the activity of the steroid ester and significantly increases its potency through the prolonged release of the active hormone from its fat tissue store by hydrolytic enzymes. Those can be activated in times when steroidogenic organs are quiescent and circulating steroid levels are low (Hochberg et al., 1991, Hochberg, 1998).

The metabolism of androstenedione indicated the presence of a 17β-HSD catalyzed hydroxylation pathway in *Dreissena* like it has been reported in several other molluscs (Matsumoto et al., 1997; Janer et al., 2005b). In our study we saw that the activity of 17β-HSD followed a clear annual progression with relatively stable levels between June and September as well as October and December interrupted by a significant decrease in enzymatic activity between February and May with lowest levels in March. A connection between 17β-HSD activity and reproductive events has already been reported for other bivalves suggesting an involvement in reproduction (Matsumoto et al., 1997; Mori et al., 1966). Our study clearly shows that the activity of the 17β-HSD significantly drops during the last stages of gametogenesis and the early stages of the spawning process. Our results would coincide with works on other bivalve molluscs which showed higher 17β-HSD activity in growing oocytes than in oocytes which had completed their growth (Varaksina and Varaksin, 1988). Interestingly, a significant negative correlation with ATAT was also detected (r^2=-0.608; $p<0.01$) which showed a strong increase in the months of March and April where 17β-HSD activity was lowest. This might indicate increased esterification of steroids when androgen production is low. However, no clear relation between ATAT activity and levels of esterified steroids was detectable. Similar observations have been made with the gastropod *M. cornuarietis* where a decrease in esterified steroids by TBT could not be directly linked with a decrease in microsomal ATAT activity (Janer et al., 2006). Nevertheless, peak in ATAT activity would coincide with decrease in free testosterone levels and also with the onset of spawning. Thus, inability to detect increases in testosterone-esters may be due to their integration into gametes and their loss during subsequent release. This would also explain why lowest levels of both esterified estradiol and testosterone were detected in April, the month were 90% of the population began to spawn. Indeed, transfer of nonpolar hormone derivates to the ovaries was already reported in other invertebrates like the tick *Ornithodoros moubata* (Connat et al., 1984) which were also detected in freshly laid eggs. Significant amounts of apolar ecdysteroid conjugates were

also found in newly-laid eggs of the house cricket *Acheta domesticus* which may indicate their important role during embryogenesis (Whiting and Dinan, 1989). However, further studies are necessary to give a definite explanation why no clear correlation between ATAT activity and steroid production and esterification exists in molluscs.

The present study investigated the reproductive cycle in zebra mussels and the possible involvement of physiological and environmental factors. Results would confirm the important role of temperature in the control of zebra mussel reproduction as it has been seen in other bivalves. In addition, new evidence is provided that would support the involvement of sex steroids in the control of reproductive events including important enzymes of steroid regulation. Therefore, our work may provide useful information which may help to gain a better understanding of bivalve reproduction and the regulating mechanisms involved.

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Paper 2

CHANGES IN LIPID CONTENT AND FATTY ACID COMPOSITION ALONG THE REPRODUCTIVE CYCLE OF THE FRESHWATER MUSSEL *DREISSENA POLYMORPHA*: ITS MODULATION BY CLOFIBRATE EXPOSURE

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Resumen

Se han determinado los lípidos totales y perfiles de ácidos grasos durante el ciclo reproductivo del mejillón cebra (*Dreissena polymorpha*). Se han detectado un total de 33 ácidos grasos de entre 14 y 22 átomos de carbono: El ácido palmítico (16:0) fue el más abundante (13-24%) seguido del ácido docosahexaenoico (22:6n-3), ácido eicosapentaenoico (20:5n-3) y ácido palmitoleico (16:1n-7). Los ácidos grasos individuales (16:0, 16:2n-4, 18:1n-7, 18:2n-6, 18:3n-4, 18:4n-3, 20:4n-3, 20:5n-3) se variaron en función de los procesos reproductivos mientras otros ácidos grasos implicados en funciones de tipo estructural (18:0 y 22:6n-3) permanecieron relativamente estables durante el periodo del estudio. El análisis multivariante del conjunto de datos confirmó la fuerte relación entre los perfiles de ácidos grasos y el ciclo reproductivo del mejillón cebra. Además, se evaluaron los efectos del fármaco clofibrato sobre la composición lipídica y los perfiles de los ácidos grasos tras una exposición de 7 días a un amplio rango de concentraciones (20 ng/L a 2 mg/L). Se observó una reducción significativa en los triglicéridos totales (38%-48%) junto a un aumento de la cantidad de ácidos grasos por gramo de peso fresco (1.5 a 2.2 veces) en mejillones expuestos. Este trabajo revela la capacidad de clofibrato de inducir cambios en el lipidoma del mejillón cebra a concentraciones de 200 ng/L o superiores.

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Changes in lipid content and fatty acid composition along the reproductive cycle of the freshwater mussel *Dreissena polymorpha*: Its modulation by clofibrate exposure

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ABSTRACT

Total lipids and fatty acid profiles were determined along the reproductive cycle of the zebra mussel (*Dreissena polymorpha*). A total of 33 fatty acids with carbon atoms from 14 to 22 were identified: palmitic acid (16:0) was the most abundant fatty acid (13–24%) followed by docosahexaenoic acid (DHA; 22:6n−3), eicosapentaenoic acid (EPA; 20:5n−3) and palmitoleic acid (16:1n−7). Some individual fatty acids (16:0, 16:2n−4, 18:1n−7, 18:2n−6, 18:3n−4, 18:4n−3, 20:4n−3, 20:5n−3) were strongly related to reproductive events, while others having structural-type functions (18:0 and 22:6n−3) were rather stable during the study period. Multivariate analysis of the whole data set using the multivariate curve resolution alternating least squares method confirmed the strong relationship of fatty acid profiles with the reproductive cycle of zebra mussel. Additionally, the effects of the pharmaceutical clofibrate on lipid composition and fatty acid profiles were assessed following 7-day exposure of zebra mussels to a wide range of concentrations (20 ng/L to 2 mg/L). A significant reduction in total triglycerides (38%–48%) together with an increase in the amount of fatty acids per gram wet weight (1.5- to 2.2-fold) was observed in the exposed mussels. This work highlights the ability of clofibrate to induce changes on the lipidome of zebra mussels at concentrations as low as 200 ng/L.

1. Introduction

The zebra mussel (*Dreissena polymorpha*) has colonized waters of different trophic statuses and with various temperature conditions all over Europe (Stanczykowska, 1977). In Spain, the lower course of the Ebro River was first colonized in 2001, and zebra mussels have now established permanent colonies that due to their strong numerical increase poses a considerable threat to the native benthic fauna (Durán et al., 2010). Due to its status of invasive sp-ecies, studies have been undertaken in different areas to give a better understanding of zebra mussel biology especially of the reproductive cycle, which is the potential target of pest control strategies (Ram et al., 1992). Its success in colonizing new environments may be attributed to high fecundity, efficient larval dispersal and few natural controls, among others. Due to its widespread distribution, zebra mussels have often been used to investigate the effects of organic compounds, including endocrine disrupting chemicals (Quinn et al., 2004, 2006) and pharmaceuticals (Binelli et al., 2009; Parolini et al., 2010). However, a detailed characterization of zebra mussel lipidome as well as its modulation by biotic (e.g. reproductive events) and abiotic (e.g. pollutants) factors is still lacking.

Lipids have been reported to play a key role in mollusk reproduction and to support energy requirements for priority functions (i.e. basal metabolism) in periods of food scarcity when carbohydrate reserves are depleted (Abad et al., 1995). A strong relationship between the reproductive state of individuals and the lipid content of the gonads has been reported for several bivalve and prosobranch species (Pazos et al., 1997; Morais et al., 2003). Lipids are usually mobilized for gametogenesis and constitute a very important energy reserve in the oocytes, which assures future viability of the larvae (Gallager et al., 1986). Therefore, an interference of pollutants with the synthesis, metabolism and mobilization of lipids may consequently alter energy storage and reproduction in mollusks, with potential consequences at higher biological organization levels.

Fibrates are among the most commonly detected pharmaceuticals in the aquatic environment where they have been detected at concentrations up to μg/L in surface waters (Corcoran et al., 2010). Concentrations in the ng/L range of clofibric acid (0.4–18 ng/L), bezafibrate (0.3–25 ng/L) and gemfibrozil (0.7–76 ng/L) have been reported in surface waters from the Ebro River (Gros et al., 2009). The mode of action of fibrates involves the activation of lipoprotein lipase activity which results in an effective reduction of very-low-density lipoproteins (VLDL) through increased clearing rates. Fibrates can also reduce plasma triglyceride levels while raising levels of high-density lipoproteins (HDL) (Zimetbaum et al., 1991), and stimulate hepatic fatty acid uptake and conversion to acyl-CoA derivatives, leading to an induction of the β-oxidation pathway with a concomitant decrease in fatty acid synthesis. This results in a lower availability of fatty acids for triglyceride synthesis (Staels et al.,

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1998). The expression of these lipid-regulating proteins and enzymes is controlled by the peroxisome proliferator-activated receptors (PPARs α, β and γ) for which fibrates are known to be transcriptional activators (Schoonjans et al., 1996).

Interestingly, in bivalves, plasma lipoproteins of different densities, similar to those found in humans, have been described. Free fatty acids and triglycerides are transported within the hemolymph to different organs by those lipoproteins, while triacylglycerols circulate in hemolymph preferably associated with hematic cells (Pollero and Heras, 1989). Also, a pancreatic lipase-related protein (PY–PLRP) was described in the ovary of the scallop *Patinopecten yessoensis* (Kim et al., 2008). Nonetheless, PPARs have not been demonstrated in mollusks and little is known on the effects and modes of action of PPAR agonists in these organisms. Cancio et al. (1998) reported peroxisome proliferation and increased peroxisomal acyl-CoA oxidase activity in the mussel *Mytilus galloprovincialis* after injection of 1.5 mg clofibrate per mussel. Canesi et al. (2007) showed that concentrations of 3 to 300 ng/g dry wt. of the hypolipidemic drugs bezafibrate and gemfibrozil affected the immune function, as well as glycolysis, redox balance and peroxisomal function in the marine mussel *M. galloprovincialis*. Also zebra mussels *D. polymorpha* exposed to 1 μg/L gemfibrozil showed signs of oxidative stress with elevated lipid peroxidation (Quinn et al., 2011). However, studies regarding the effect of hypolipidemic drugs on lipid composition and lipid metabolism in aquatic organisms are still scarce, and mostly limited to fish. The fibrate drug gemfibrozil disrupted lipoprotein metabolism in rainbow trout by decreasing plasma lipoprotein levels, and modified lipoprotein composition by reducing the abundance of long-chain n−3 fatty acids (Prindiville et al., 2011). Du et al. (2004) showed that exposure to fenofibrate increased by about 30% peroxisome-related activities, and this was responsible for the lower contents of eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) in the body lipids of fenofibrate-treated trout. A significant decrease of cholesterol, total lipids and triglycerides was observed in the crab *Pachygrapsus marmoratus* after several injections of 50 mg/kg clofibric acid (Lautier et al., 1986).

Thus, the aim of this work was to elucidate the potential action of clofibrate in zebra mussel lipids. Our hypothesis was that exposure to the lipid regulator clofibrate might lead to changes in triglyceride levels and fatty acid concentrations/patterns, similar to those that have been reported in vertebrates. More specifically this work aimed at (a) characterizing the lipid content and fatty acid profiles along the reproductive cycle of zebra mussels collected from the lower course of the Ebro River, and (b) assessing the effects of the lipid regulator clofibrate on total lipids, triglycerides and fatty acid profiles following exposure of zebra mussels to a wide range of concentrations (0.020 to 2000 μg/L) for 7 days. The obtained data will hopefully provide baseline information for further assessment of the disruption of zebra mussel lipidome following exposure to hipolipidemic drugs or other pollutants.

2. Material and methods

2.1. Seasonal sampling

Zebra mussels (*D. polymorpha*) were collected monthly (October 2008 to September 2009) from the lower course of the Ebro River (NE Spain) in a relatively unpolluted site right after Riba-Roja dam (41° 14′ 53.93 N/0° 28′ 40.47 E). Individuals (16 to 22 mm in length; n = 90) were collected in an area close to the river bed. Mussels were kept in buckets filled with water and transported to a nearby laboratory where they were immediately dissected. The gills were removed and the rest of the tissue was frozen in liquid nitrogen and stored at −80 °C until analyzed. The entire body of 20 individuals per month was dissected and placed in 10% buffered formalin (0.1 M potassium phosphate, pH 7.4) for histological examination.

2.2. Clofibrate exposure experiments

Zebra mussels collected in February (Experiment 1) and May 2010 (Experiment 2) were transported in buckets filled with water to the animal-holding facilities (IDAEA, Barcelona) and kept in 20 L tanks at 20 ± 1 °C and 12:12 dark:light photoperiod. Mussels were acclimatized for a period of 3 days to ASTM-reconstituted water of similar hardness (170 mg/L $CaCO_3$) and conductivity (600 μS/cm) as the Ebro River water. Animals were fed daily with a suspension 1:1 of algae *Scenedesmus subspicatus* and *Chlorella vulgaris* (10^6 cells/mL). After acclimatization, ~450 mussels were randomly selected for the experiments. They were placed on fishing nets suspended in 20 L glass aquaria (~90 individuals per aquaria). Dissolved oxygen was maintained constant by continuous aeration using filtered compressed air through glass diffusers.

Mussels were exposed to different concentrations of clofibrate (Experiment 1: 20, 200 and 2000 ng/L; Experiment 2: 20, 200 and 2000 μg/L) added to the rearing water. There were two sets of controls: non-exposed mussels (C) and mussels exposed to 0.002% triethyleneglycol, used as a carrier solvent (SC). Water was changed daily and a fresh dose of clofibrate was added; mussels were fed every 48 h adding food 15–30 min before water renewal. Mortality was of 5 to 8 individuals per tank for the first experiment, and 4 to 9 individual per tank for the second experiment. After a 7-day exposure, the mussels were dissected, and the whole body without gills was stored at −80 °C.

2.3. Total lipid and fatty acid analyses

Lipids were extracted by a modification of the method of Folch et al. (1957). Each sample, which consisted in a pool of 2–3 individuals without gills, was homogenized in an ice-cold solution of chloroform:methanol (2:1 v/v) containing 0.01% butylated hydroxytoluene (BHT) as antioxidant. After centrifugation, 0.88% KCl was added to the obtained supernatant (1:4 v/v) and then thoroughly mixed. The chloroform layer was collected, filtered (Whatman filter paper) and the solvent evaporated under nitrogen. The dried lipid content was determined gravimetrically (Sartorius, BP210D, 0.01 mg) and kept at −20 °C for fatty acid analysis.

For the analysis of fatty acids, 1 mg of total lipids dissolved in chloroform:methanol was mixed with nonadecanoic acid (19:0) (internal standard) and dried under a gentle stream of nitrogen. The dried residue was saponified with 2 N KOH/MeOH/10% Milli-Q water for 24 h at room temperature, and afterward neutral lipids were removed by vigorous shaking with 1 mL of n-hexane (×3). The samples were further acidified to pH ~ 3 with HCl and fatty acids extracted with 1 mL of n-hexane (×3) and concentrated by vacuum rotary evaporation. The fatty acid fraction was then methylated with a solution of 10% boron trifluoride-methanol (Fluka) for 24 h at room temperature. The methylated fraction was resuspended in 10% NaCl and fatty acid methyl esters (FAME) extracted with 1 mL of n-hexane (×3), transferred to vials, evaporated to near dryness under a gentle stream of nitrogen, and analyzed by gas chromatography–flame ionization detector (GC–FID). The instrument was a Thermo Finnigan Trace GC. The column, a DB-23 fused silica 30 m × 0.25 mm i.d., film thickness 0.25 μm (Agilent J&W), was programmed from 60 to 150 °C at 15 °C/min, then from 150 to 230 °C at 3 °C/min, keeping the final temperature for 10 min. The carrier gas was helium at a constant flow-rate of 1.5 mL/min. The injector and the detector temperatures were set at 280 and 300 °C, respectively. FAME profile was first identified through gas chromatography–mass spectrometry (GC–MS); the location of the double-bonds was determined by derivatizing FAME extracts with 2-amino-2-methylpropanol 95% according to Zhang et al. (1988). The reaction was carried out under argon atmosphere in 2 mL glass-sealed ampules at 180 °C overnight, and the 4,4-dimethyloxazoline (DMOX) derivatives of fatty acids were extracted with dichloromethane. The extract was dried by passing through a Pasteur pipette filled with sodium sulfate,

the solvent evaporated under nitrogen and dissolved in n-hexane for GC–MS analysis.

2.4. Determination of triglycerides

Tissue concentrations of triglycerides were determined using a commercial kit (Spinreact S.A., Sant Esteve De Bas, Spain) based on a peroxidase coupled method for the colorimetric detection of triglycerides following hydrolysis, phosphorylation of glycerol and oxidation to produce hydrogen peroxide (McGowan et al., 1983). Briefly, 20 μL of the lipid extract was dried under a gentle nitrogen stream and then redissolved in 20 μl methanol. The extract was then mixed with the assay reagents, incubated in the dark for 10 min and measured spectrophotometrically at 505 nm according to the manufacturer's protocol.

2.5. Histological analysis

Individuals fixed in 10% buffered formalin were dehydrated with ethanol, cleared with Histo-Clear II (National Diganostics, Hessle Hull, England), and embedded in paraplast Sigma-Aldrich (Steinheim, Germany). Sections (7 μm) were stained with hematoxylin–eosin Y, mounted and examined by light microscopy. Individuals were classified in five different reproductive stages as described by Juhel et al. (2003). Briefly, stage 0: sexual rest, inactive or undifferentiated; stage 1: immature gametes (spermatogonia/oogonia) visible, early development; stage 2: follicles contain mainly ripe gametes, late development; stage 3: fully mature gonads, almost no reserve cells, spawning; stage 4: reabsorbing gonads, disorganized and mostly empty, few remaining gametes.

2.6. Statistical analysis

Data are reported as means + SEM. Statistical treatment of data was performed by using the SPSS statistical package (SPSS v.17.0, IBM Company, Chicago, USA). Differences between months or between control and exposure groups were determined using analyses of variance (one-way ANOVA) with either Tukey's post-hoc multiple comparison test or Dunnett's post-hoc test ($p<0.05$), respectively. Prior to analysis, data was tested for normality and homoscedasticity of variance. Bivariate Pearson's correlation analysis was performed ($p<0.01$) to detect significant correlations between individual fatty acids and fatty acid groups.

For multivariate data analysis, experimental data was arranged in a single data matrix containing all 33 analyzed fatty acids represented as relative percentage of total fatty acids over a time period of 11 months. The data matrix consisted of 11 rows (sampling months) and 33 columns (relative percentages of the different fatty acids). Scaling was not needed because the data were already in the same scale as the percentage values. Data analysis was performed using MATLAB (Mathworks, MA, USA, 2010) and MCR-ALS Toolbox (www.mcrals.info/). Principal component analysis (PCA) was used in this study to identify the main trends in the data set and to estimate the main fatty acid patterns. Further analysis of the data matrix was performed using the multivariate curve resolution alternating least-squares method, MCR-ALS (Tauler et al., 1995; 1998). A detailed description of the method and its application in MATLAB can be found in Jaumot et al. (2005). Just like PCA, the MCR-ALS method is based on a bilinear model which performs a data matrix decomposition that can be described as follows:

$$D = CS^T + E.$$

The S^T matrix contains the loadings which, in our case, represent the fatty acid composition of the profiles resolved by MCR-ALS analysis. C corresponds to the matrix of scores representing the temporal distribution of the resolved fatty acid patterns. E is the error matrix containing data variance residuals which are not explained by the model. The bilinear model described through the equation above assumes that a large part of the variation found in the data set can be explained by a small number of components represented by the reduced scores (temporal distribution) and loadings (fatty acid composition) matrices. Likewise, it was assumed that the measured fatty acid concentrations (variables) of each month are largely influenced by a limited number of unknown environmental and physiological factors. By reducing the data set to a smaller number of components, principal trends/patterns can be discovered more easily which may allow the identification of the most important influential factors. Since the solutions provided by MCR-ALS analysis are ambiguous, the data analysis has to be performed under some constraints. The constraints used in this study during bilinear matrix decomposition were non-negativity for the loadings and scores profiles and the closure of the scores profile, where the closure constant was set to be equal to 100. The last constraint was applied because the data set was represented in percentages. Initial estimates of the matrices C and S^T which are necessary for the MCR-ALS implementation were extracted using the purest variables like those in the SIMPLISMA method (Windig and Guilment, 1991).

3. Results

A total of 33 fatty acids with carbon atoms from 14 to 22 were identified in the lipid fraction of zebra mussel (*D. polymorpha*). A detailed description of the fatty acid composition during the sampling period is shown in Table 1. Palmitic acid (16:0) was the most abundant fatty acid (13–24%) followed by docosahexaenoic acid (DHA; 22:6n−3), eicosapentaenoic acid (EPA; 20:5n−3) and palmitoleic acid (16:1n−7) with a relative abundance of 8–12%, 6–13% and 6–11%, respectively. Saturated fatty acids (SFA) represented 22.3% to 31.6% of the total fatty acids. Monounsaturated fatty acids (MUFA) varied between 19.9% and 27.3% of the total fatty acids: 16:1n−7, 18:1n−7 and 20:1n−11 contributed with the largest percentages to this fraction. Polyunsaturated fatty acids (PUFA) were the most abundant fatty acids (46.2% to 52.9%), particularly 22:6n−3 and 20:5n−3; the amount of n−3 PUFA being higher than that of n−6 PUFA. Total lipids represented 0.6 to 2.0% wet weight in the soft tissue of *D. polymorpha*; the lowest levels were observed between September and January (0.6–0.9%), and the highest between April and June (1.5–2.0%) (Table 1).

Fig. 1 summarizes the distribution of sexual maturation stages during the sampling period. Several distinctive patterns were observed. In October, gonads of most organisms examined were in reabsorbing (47%) or resting (42%) stages. The reabsorbing stage (stage 4) is characterized by a disorganized follicle structure with very few or none remaining ova or spermatozoa; following this stage, the gonad reaches its lowest size volume and the gonadal tissue becomes temporarily inactive (stage 0). Organisms with gonads in stages 0 and 4 were detected from September to December. In January, 80% of the individuals examined had gonads in stage 1; this stage corresponded to early gametogenesis and was characterized by an increase in gonad volume. Two months later, in March, 60% of the individuals were classified as having gonads in stage 2 (late gametogenesis) while 30% had already fully mature gonads (stage 3). Finally, from April to July, 90 to 100% of the individuals examined had fully mature gonads, often characterized by the presence of a star-shaped lumina of the follicles with spermatozoa tails visible in the center of the tubule in males, and pedunculated oocytes present in the ovaries. At the beginning of September, all the animals had gonads in the reabsorbing stage (Fig. 1).

Regarding seasonal variation of fatty acids, 16:0, the most abundant fatty acid, showed the highest relative abundance from April to June (23–24%) when mussels had fully mature gonads, and the lowest

Table 1
Seasonal variation of the fatty acid profile, total fatty acids and total lipids in the soft tissue of *Dreissena polymorpha* collected from the lower course of the Ebro River. Data are expressed as % of total fatty acid methyl esters as mean ± SEM ($n = 3$). Different letters indicate statistically significant differences (Tukey's test, $p < 0.05$).

Fatty acid	Oct.	Nov.	Dec.	Jan.	Feb.	Mar.	Apr.	May	Jun.	Jul.	Sep.
14:0	1.41 ± 0.12^{bcd}	1.70 ± 0.05^{de}	1.63 ± 0.12^{cde}	2.37 ± 0.28^{f}	2.18 ± 0.20^{ef}	1.65 ± 0.12^{cde}	0.95 ± 0.08^{ab}	0.71 ± 0.05^{a}	0.59 ± 0.01^{a}	1.00 ± 0.08^{abc}	1.08 ± 0.08^{abcd}
15:0	0.26 ± 0.02^{abc}	0.37 ± 0.05^{c}	0.29 ± 0.03^{abc}	0.30 ± 0.04^{abc}	0.19 ± 0.03^{ab}	0.35 ± 0.06^{bc}	0.14 ± 0.02^{a}	0.17 ± 0.02^{a}	0.14 ± 0.02^{a}	0.22 ± 0.02^{abc}	0.34 ± 0.03^{bc}
16:0	17.54 ± 1.17^{bc}	15.27 ± 0.37^{ab}	14.29 ± 0.99^{ab}	14.77 ± 0.70^{ab}	16.38 ± 0.95^{abc}	17.55 ± 0.67^{bc}	23.05 ± 0.83^{de}	24.39 ± 0.29^{e}	24.13 ± 0.63^{de}	20.12 ± 0.40^{cd}	13.27 ± 1.29^{a}
16:1n−7	7.05 ± 0.42^{ab}	8.17 ± 0.30^{bc}	8.30 ± 0.49^{bcd}	10.09 ± 0.13^{cde}	10.69 ± 0.24^{e}	10.41 ± 0.24^{de}	10.18 ± 0.93^{cde}	8.14 ± 0.10^{bc}	6.52 ± 0.07^{ab}	6.28 ± 0.11^{ab}	5.92 ± 0.72^{a}
16:1n−5	0.41 ± 0.06^{a}	0.52 ± 0.01^{a}	0.53 ± 0.06^{ab}	0.93 ± 0.03^{c}	0.93 ± 0.04^{c}	0.82 ± 0.02^{c}	0.73 ± 0.09^{bc}	0.54 ± 0.00^{ab}	0.40 ± 0.01^{a}	0.41 ± 0.02^{a}	0.33 ± 0.03^{a}
17 iso	1.75 ± 0.15^{ab}	1.40 ± 0.06^{a}	1.74 ± 0.11^{ab}	1.94 ± 0.26^{ab}	2.35 ± 0.16^{bc}	2.81 ± 0.17^{c}	1.57 ± 0.13^{a}	1.50 ± 0.03^{a}	1.48 ± 0.02^{a}	1.82 ± 0.20^{ab}	2.84 ± 0.16^{c}
16:2n−4	0.34 ± 0.02^{b}	0.30 ± 0.01^{b}	0.30 ± 0.01^{b}	0.32 ± 0.02^{b}	0.56 ± 0.03^{c}	0.28 ± 0.04^{b}	0.59 ± 0.07^{c}	0.38 ± 0.03^{b}	0.30 ± 0.04^{b}	0.26 ± 0.01^{b}	0.08 ± 0.01^{a}
17:0	1.30 ± 0.02^{a}	1.12 ± 0.01^{a}	1.21 ± 0.03^{a}	1.34 ± 0.17^{a}	1.09 ± 0.02^{a}	1.42 ± 0.02^{a}	1.16 ± 0.12^{a}	1.27 ± 0.04^{a}	1.31 ± 0.06^{a}	1.35 ± 0.04^{a}	1.40 ± 0.11^{a}
16:3n−4	0.16 ± 0.04^{ab}	0.18 ± 0.02^{b}	0.15 ± 0.02^{ab}	0.16 ± 0.01^{b}	0.50 ± 0.04^{c}	0.54 ± 0.06^{c}	0.56 ± 0.01^{c}	0.22 ± 0.00^{b}	0.10 ± 0.01^{ab}	0.10 ± 0.01^{ab}	0.03 ± 0.01^{a}
18:0	3.51 ± 0.17^{ab}	3.06 ± 0.20^{a}	3.13 ± 0.11^{a}	2.99 ± 0.29^{a}	3.42 ± 0.26^{ab}	3.25 ± 0.13^{a}	3.03 ± 0.53^{a}	3.55 ± 0.31^{ab}	4.00 ± 0.32^{ab}	4.09 ± 0.30^{ab}	4.84 ± 0.25^{b}
18:1n−9	4.55 ± 0.05^{e}	4.03 ± 0.10^{cde}	3.70 ± 0.07^{bcd}	3.59 ± 0.05^{abc}	3.04 ± 0.10^{a}	3.56 ± 0.13^{abc}	3.19 ± 0.11^{ab}	3.10 ± 0.09^{ab}	3.01 ± 0.17^{a}	3.64 ± 0.12^{abcd}	4.25 ± 0.26^{de}
18:1n−7	2.90 ± 0.19^{ab}	3.15 ± 0.10^{abc}	3.36 ± 0.43^{abc}	4.19 ± 0.23^{bcde}	4.77 ± 0.29^{de}	4.36 ± 0.25^{cde}	4.86 ± 0.30^{e}	4.28 ± 0.29^{cde}	3.49 ± 0.27^{abcd}	3.35 ± 0.17^{abc}	2.22 ± 0.18^{a}
18:1n−5	0.24 ± 0.02^{bcd}	0.25 ± 0.01^{bcd}	0.25 ± 0.00^{cd}	0.32 ± 0.02^{d}	0.24 ± 0.03^{bcd}	0.16 ± 0.02^{abc}	0.17 ± 0.01^{abc}	0.14 ± 0.00^{ab}	0.10 ± 0.00^{a}	0.17 ± 0.02^{abc}	0.12 ± 0.05^{a}
18:2n−6	2.31 ± 0.12^{c}	1.84 ± 0.05^{ab}	2.08 ± 0.11^{bc}	2.01 ± 0.09^{abc}	2.26 ± 0.10^{bc}	2.21 ± 0.11^{bc}	2.34 ± 0.06^{c}	2.16 ± 0.05^{bc}	2.01 ± 0.11^{abc}	2.11 ± 0.10^{bc}	1.60 ± 0.08^{a}
18:2n−4	0.12 ± 0.01^{ab}	0.12 ± 0.01^{ab}	0.11 ± 0.00^{ab}	0.10 ± 0.01^{a}	0.19 ± 0.01^{bc}	0.18 ± 0.02^{ab}	0.30 ± 0.03^{d}	0.27 ± 0.03^{cd}	0.19 ± 0.02^{bc}	0.15 ± 0.01^{ab}	0.10 ± 0.01^{a}
18:3n−4	0.23 ± 0.01^{ab}	0.30 ± 0.01^{bc}	0.23 ± 0.03^{ab}	0.24 ± 0.00^{ab}	0.20 ± 0.00^{ab}	0.19 ± 0.01^{ab}	0.37 ± 0.03^{c}	0.30 ± 0.03^{abc}	0.21 ± 0.02^{ab}	0.26 ± 0.01^{abc}	0.16 ± 0.07^{a}
18:3n−3	4.13 ± 0.43^{d}	2.25 ± 0.15^{a}	2.11 ± 0.21^{a}	2.13 ± 0.12^{a}	2.44 ± 0.27^{ab}	2.32 ± 0.08^{ab}	3.33 ± 0.20^{bcd}	3.54 ± 0.18^{cd}	3.35 ± 0.13^{bcd}	2.92 ± 0.21^{abc}	1.88 ± 0.13^{a}
18:4n−3	2.22 ± 0.28^{bc}	1.68 ± 0.08^{ab}	2.14 ± 0.25^{bc}	2.89 ± 0.09^{bcd}	3.79 ± 3.38^{d}	3.46 ± 0.09^{cd}	4.17 ± 0.48^{d}	3.32 ± 0.12^{cd}	2.16 ± 0.23^{bc}	0.80 ± 0.38^{a}	0.47 ± 0.06^{a}
20:1n−11	7.66 ± 0.62^{c}	7.13 ± 0.06^{c}	6.12 ± 0.17^{abc}	4.17 ± 0.17^{ab}	3.34 ± 0.19^{a}	3.50 ± 0.58^{a}	2.61 ± 0.22^{a}	4.05 ± 0.16^{a}	4.62 ± 0.28^{ab}	7.16 ± 0.60^{c}	11.36 ± 0.47^{d}
20:1n−9	1.71 ± 0.06^{bc}	1.57 ± 0.04^{abc}	1.58 ± 0.17^{abc}	1.16 ± 0.05^{ab}	0.99 ± 0.06^{a}	1.14 ± 0.02^{ab}	1.06 ± 0.19^{a}	1.48 ± 0.30^{abc}	1.27 ± 0.08^{ab}	1.78 ± 0.04^{bc}	1.94 ± 0.08^{c}
20:1n−7	0.77 ± 0.08^{bcde}	0.87 ± 0.02^{def}	0.98 ± 0.13^{ef}	0.70 ± 0.05^{abcde}	0.47 ± 0.04^{abc}	0.42 ± 0.15^{ab}	0.34 ± 0.06^{a}	0.45 ± 0.01^{ab}	0.53 ± 0.03^{abcd}	0.85 ± 0.09^{cdef}	1.18 ± 0.01^{f}
20:2NMID	0.37 ± 0.03^{abcd}	0.42 ± 0.01^{cd}	0.43 ± 0.01^{d}	0.46 ± 0.03^{d}	0.38 ± 0.01^{abcd}	0.27 ± 0.04^{ab}	0.25 ± 0.02^{a}	0.29 ± 0.03^{abc}	0.29 ± 0.02^{abc}	0.39 ± 0.01^{bcd}	0.62 ± 0.05^{e}
20:2n−6	0.54 ± 0.03^{c}	0.43 ± 0.01^{abc}	0.44 ± 0.03^{abc}	0.32 ± 0.03^{a}	0.32 ± 0.01^{a}	0.50 ± 0.02^{bc}	0.37 ± 0.03^{ab}	0.44 ± 0.05^{abc}	0.40 ± 0.02^{abc}	0.51 ± 0.01^{bc}	0.39 ± 0.05^{abc}
20:3n−6	0.23 ± 0.01^{ab}	0.25 ± 0.01^{ab}	0.26 ± 0.02^{ab}	0.24 ± 0.00^{ab}	0.21 ± 0.01^{ab}	0.29 ± 0.00^{b}	0.19 ± 0.04^{a}	0.20 ± 0.02^{a}	0.21 ± 0.01^{ab}	0.26 ± 0.01^{ab}	0.40 ± 0.01^{c}
20:4n−6	5.77 ± 0.54^{c}	6.22 ± 0.07^{c}	6.51 ± 0.47^{c}	5.36 ± 0.16^{bc}	3.91 ± 0.36^{ab}	3.53 ± 0.08^{a}	2.63 ± 0.30^{a}	3.11 ± 0.21^{a}	4.02 ± 0.21^{ab}	5.70 ± 0.28^{c}	9.66 ± 0.15^{d}
20:4n−3	0.66 ± 0.03^{bcd}	0.60 ± 0.03^{bc}	0.56 ± 0.01^{b}	0.59 ± 0.01^{bc}	0.65 ± 0.03^{bcd}	0.61 ± 0.03^{bc}	0.75 ± 0.04^{cde}	0.86 ± 0.07^{e}	0.80 ± 0.01^{de}	0.65 ± 0.03^{bcd}	0.34 ± 0.03^{a}
20:5n−3	8.40 ± 0.46^{b}	8.99 ± 0.13^{bc}	9.43 ± 0.20^{bcd}	11.07 ± 0.26^{def}	12.50 ± 0.16^{f}	12.82 ± 0.46^{f}	12.04 ± 0.52^{ef}	10.31 ± 0.36^{cde}	10.15 ± 0.54^{bcd}	8.99 ± 0.34^{bc}	6.29 ± 0.15^{a}
22:2NMID	2.57 ± 0.28^{cd}	2.67 ± 0.08^{cd}	2.53 ± 0.31^{cd}	1.92 ± 0.07^{bc}	1.41 ± 0.18^{ab}	1.40 ± 0.11^{ab}	0.70 ± 0.05^{a}	1.23 ± 0.14^{ab}	1.42 ± 0.05^{ab}	2.05 ± 0.04^{bc}	3.07 ± 0.26^{d}
22:3NMIT	0.61 ± 0.06^{a}	0.76 ± 0.01^{ab}	0.75 ± 0.06^{ab}	0.92 ± 0.03^{ab}	1.48 ± 0.07^{c}	1.48 ± 0.03^{c}	1.18 ± 0.05^{bc}	1.22 ± 0.05^{bc}	1.17 ± 0.01^{bc}	0.52 ± 0.02^{a}	0.85 ± 0.31^{ab}
22:4n−6	1.07 ± 0.14^{c}	1.18 ± 0.04^{c}	1.30 ± 0.17^{c}	1.07 ± 0.03^{c}	0.61 ± 0.09^{ab}	1.08 ± 0.04^{c}	0.27 ± 0.06^{a}	0.38 ± 0.03^{a}	0.57 ± 0.02^{ab}	0.92 ± 0.06^{bc}	2.06 ± 0.12^{d}
22:5n−6	5.18 ± 0.49^{cde}	5.53 ± 0.10^{de}	6.04 ± 0.44^{ef}	5.08 ± 0.27^{cde}	3.76 ± 0.30^{abc}	2.16 ± 0.18^{a}	2.21 ± 0.28^{a}	2.85 ± 0.18^{ab}	3.35 ± 0.17^{ab}	4.34 ± 0.37^{bcd}	7.27 ± 0.42^{f}
22:5n−3	4.91 ± 0.41^{abc}	5.76 ± 0.07^{c}	5.74 ± 0.50^{c}	4.74 ± 0.20^{abc}	3.47 ± 0.28^{ab}	3.13 ± 0.07^{a}	3.15 ± 0.76^{a}	4.11 ± 0.12^{abc}	5.42 ± 0.13^{c}	5.74 ± 0.41^{c}	5.14 ± 0.35^{bc}
22:6n−3	9.10 ± 0.37^{ab}	11.86 ± 0.30^{f}	11.79 ± 0.09^{f}	11.53 ± 0.32^{bc}	11.31 ± 0.46^{bc}	12.16 ± 0.11^{c}	11.55 ± 1.22^{bc}	11.05 ± 0.50^{abc}	12.27 ± 0.39^{c}	11.09 ± 0.42^{bc}	8.48 ± 0.46^{a}
$\sum$ SFA	25.77 ± 1.08^{abc}	22.93 ± 0.53^{ab}	22.30 ± 0.89^{a}	23.71 ± 1.13^{ab}	25.60 ± 0.99^{abc}	27.03 ± 0.74^{bcd}	29.90 ± 1.30^{cd}	31.58 ± 0.20^{d}	31.65 ± 0.91^{d}	28.59 ± 0.73^{cd}	23.78 ± 1.22^{ab}
$\sum$ MUFA	25.29 ± 0.37^{bcd}	25.70 ± 0.31^{cd}	24.81 ± 0.25^{bcd}	25.15 ± 0.14^{bcd}	24.48 ± 0.24^{bcd}	24.37 ± 0.66^{bcd}	23.15 ± 1.42^{abc}	22.17 ± 0.72^{ab}	19.94 ± 0.52^{a}	23.65 ± 0.90^{bc}	27.31 ± 0.60^{d}
$\sum$ PUFA	48.94 ± 1.21^{a}	51.37 ± 0.48^{a}	52.90 ± 1.14^{a}	51.14 ± 1.20^{a}	49.92 ± 1.22^{a}	48.61 ± 0.43^{a}	46.96 ± 2.55^{a}	46.25 ± 0.87^{a}	48.41 ± 1.13^{a}	47.76 ± 1.56^{a}	48.91 ± 1.56^{a}
$\sum$ n−3	29.43 ± 0.63^{b}	31.14 ± 0.38^{b}	31.76 ± 0.09^{bc}	32.95 ± 0.68^{bcd}	34.15 ± 0.22^{cd}	34.50 ± 0.53^{d}	34.99 ± 1.86^{d}	33.19 ± 0.52^{bcd}	34.17 ± 0.92^{cd}	30.18 ± 0.80^{b}	22.60 ± 0.48^{a}
$\sum$ n−6	15.71 ± 1.03^{de}	16.23 ± 0.14^{e}	17.38 ± 0.96^{e}	14.99 ± 0.46^{cde}	12.53 ± 0.76^{abcd}	11.24 ± 0.27^{ab}	9.20 ± 0.69^{a}	10.37 ± 0.33^{a}	11.72 ± 0.33^{abc}	14.36 ± 0.73^{bcde}	22.24 ± 0.89^{f}
Total fatty acids (mg/g w.w.)	3.63 ± 0.39^{ab}	3.16 ± 0.20^{ab}	3.03 ± 0.50^{ab}	3.33 ± 0.55^{ab}	6.00 ± 1.12^{ab}	6.55 ± 0.59^{ab}	13.11 ± 2.66^{c}	7.13 ± 0.72^{b}	7.59 ± 0.63^{b}	4.34 ± 0.21^{ab}	1.89 ± 0.17^{a}
Total lipids (% w.w.)	0.83 ± 0.09^{a}	0.94 ± 0.04^{ab}	0.79 ± 0.05^{a}	0.87 ± 0.06^{a}	1.19 ± 0.12^{ab}	1.01 ± 0.09^{ab}	2.07 ± 0.21^{c}	1.50 ± 0.05^{bc}	1.51 ± 0.05^{bc}	1.02 ± 0.01^{ab}	0.66 ± 0.06^{a}

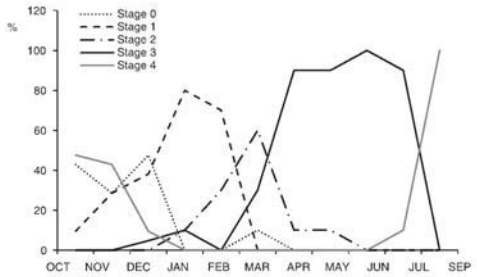

Fig. 1. Distribution of sexual maturation stages in the zebra mussel *Dreissena polymorpha* collected from the Ebro River during the annual sampling; 20 individuals examined per month.

relative abundance in September (13%), right after the spawning period (Table 1). Besides, these variations were well in agreement with the lipid content of the organisms (Table 1). n−3 PUFA, which accounted for one-third of the total fatty acids in zebra mussel, also showed maximum abundance in April (34.9% of the total fatty acids) coinciding with the onset of the spawning season (90% of individuals examined having gonads in stage 3), and minimum levels in September (22.6%), when 100% of the individuals had gonads in stage 4 (reabsorbing period) (Fig. 1). However, the opposite trend was recorded for n−6 PUFA which had minimum levels in April (9.2%) and maximum abundance in September (22.2%) (Table 1). A negative correlation between n−3 and n−6 PUFA levels ($p<0.01$, $r^2=-0.792$) was observed during the study period. Nonetheless, a close look to n−3 PUFA shows that 22:6n−3 (DHA) was rather constant over the studied period (11–12%), with the exception of a significant decrease in September and October, during the reabsorbing and resting periods of the gonads.

Apart from differences in relative abundance, significant changes in total fatty acid levels during the sampling period were observed (Table 1). Total fatty acid levels ranged from 1.8 to 13.1 (mg/g wet weight) with the highest levels in April and the lowest in September. As expected, seasonal variations of total fatty acids closely matched levels of total lipids ($p<0.001$, $r^2=0.929$).

In order to assess the potential interference of the lipid regulator clofibrate in zebra mussel lipids, two different exposure experiments were performed in February (Experiment 1: 0.02, 0.2, 2 μg clofibrate/L) and May (Experiment 2: 20, 200, 2000 μg clofibrate/L). Fig. 2A shows the effect of clofibrate exposure on total fatty acids; results from the two experiments are shown together and fatty acid levels are expressed as percentage of their respective controls (SC). A statistically significant increase in total fatty acids was observed in the whole body of mussels exposed at a concentration of clofibrate as low as 0.2 μg/L; fatty acid levels increased in a concentration-dependent manner up to 200% in those mussels exposed to 20 and 200 μg/L clofibrate. The increase in fatty acids was not observed in zebra mussels exposed to the lowest concentration (0.02 μg/L). In addition to the increase in total fatty acids, exposure to clofibrate caused a significant decrease in the levels of triglycerides in the range of 38 to 48% (Fig. 2B). Only, zebra mussels exposed to the lowest concentration of clofibrate (0.02 μg/L) showed no significant alteration in the levels of triglycerides. In spite of the significant effect of clofibrate exposure on total fatty acids and triglycerides, no significant alteration on fatty acid profiles was observed in the exposed mussels (data not shown).

4. Discussion

According to our results, one main spawning period could be distinguished in the population of zebra mussels from the lower course of the

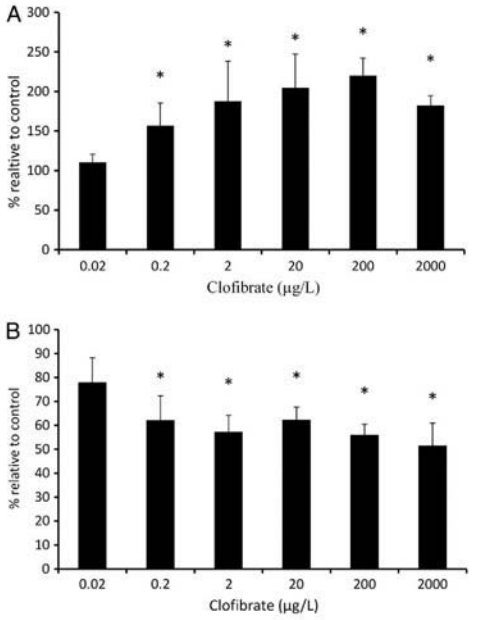

Fig. 2. Changes in (A) total fatty acids, and (B) triglycerides following exposure of the zebra mussel *Dreissena polymorpha* to different concentrations of clofibrate. Values are expressed as percentage of their respective control values as mean ± SEM (n = 3). * denotes significant differences from the controls (Dunnett's test, $p<0.05$). Fatty acids in control groups were 7.77 ± 2.28 and 5.55 ± 0.62 mg/g w.w. for the 1st and 2nd experiments, respectively. Triglycerides were 6.78 ± 0.15 and 6.32 ± 0.49 mg/g w.w.

Ebro River; this spawning period lasted for approx. 4–5 months, from April to July. During these months, 90 to 100% of the individuals examined were in stage 3; the release of gametes was therefore a highly synchronized event. The spawning process had completely finished at the beginning of September when gonads were mostly empty and only few residual gametes could be observed (stage 4). Interestingly, lipids and total fatty acids showed a peak in April, at the beginning of the spawning season, and gradually declined through the summer, to reach the lowest levels in September (post-spawning) (Table 1), showing that in zebra mussel, as in other bivalves, lipids are first stored during gametogenesis and then lost during spawning.

MCR-ALS analysis was used to identify what physically interpretable principal trends were present in the fatty acids data set. It allowed the identification of three temporal patterns (components) that explained around 99% of the total variance of the data set (Fig. 3A). These distribution patterns closely matched the distribution of sexual maturation stages of the organisms during the annual sampling (Fig. 1). The first identified pattern showed a maximum in April to June, coinciding with the late gametogenesis/spawning period; the second identified pattern displayed maximum values in September–October (100% of the individuals had resting/reabsorbing gonads) and a minimum in February to April, and coincides with the resting period; while the third pattern showed highest levels between January and March, during the gametogenic period. Therefore, MCR-ALS analyses reinforced the key role that reproductive events have in the fatty acid profile of zebra mussel.

The loadings profile demonstrates the contribution of each of the 33 fatty acids to the 3 temporal patterns (components) resolved by MCR-ALS analyses (Fig. 3B). Due to the fact that we worked with percentage unscaled data, the contribution of the fatty acids in the loadings profile largely corresponds to their relative abundance found in the mussel tissue. Thus, fatty acids 16:0, 16:1n−7, 20:5n−3 and 22:6n−3 showed a major contribution to patterns 1 and 3; while 16:0 and several PUFAs, among them 20:1n−11 were the major contributors to pattern 2. Interestingly, palmitic acid (16:0), the first fatty acid produced during lipogenesis and from which longer fatty acids are formed, was more abundant during spawning, while palmitoleic acid (16:1n−7), the most abundant MUFA, was significantly higher during gametogenesis (January to April; stage 2) (Table 1). On the other hand, n−3 PUFA reached maximum abundance in March–April, while the lowest levels were detected in September. Essential fatty acids (20:4n−6, 20:5n−3 and 22:6n−3) are reported to have a key role in bivalve development and reproduction, and also in membrane functioning. In the present work, 20:5n−3 (EPA) showed a strong seasonal variation reaching the highest levels at the end of the gametogenesis, when the gonad reaches its maximum volume, while 22:6n−3 (DHA) was rather constant during the sampling period, with the exception of the months of September and October where the relative abundance slightly decreased (Table 1). Similarly, Besnard et al. (1989) reported high levels of 20:5n−3 in female gonads of *Pecten maximus* during sexual maturity, while levels of 22:6n−3 showed no clear seasonal trend and significantly declined after the spawning period. Indeed, 22:6n−3 has been suggested to play a structural and functional role in the maintenance of cell membranes in bivalves while 20:5n−3 is often related to energetic-type functions (Besnard et al., 1989; Marty et al., 1992; Freites et al., 2002).

Optimization of lipid storage and mobilization of lipid reserves are critical to the reproductive and developmental success of most organisms. Therefore, impairment of lipid metabolism following exposure to contaminants might have harmful consequences in terms of adaptive responses and reproductive success (Capuzzo and Leavitt, 1988). In the present work, we observed a significant reduction of triglyceride levels (38% to 48%) together with a concomitant increase of total fatty acids (56 to 119%) in the whole body of zebra mussels exposed to increasing concentrations of clofibrate. These effects were observed at concentrations of clofibrate in water as low as 200 ng/L. Although concentrations 10- to 50-fold lower of clofibric acid have been reported in surface waters from the Ebro River (0.4–18 ng/L), the North Sea and various Swiss lakes (1–2 ng/L) (Buser and Müller, 1998; Gros et al., 2009), other studies have reported concentrations in the μg/L range (Heberer, 2002), suggesting that the observed alterations are likely to occur in the environment.

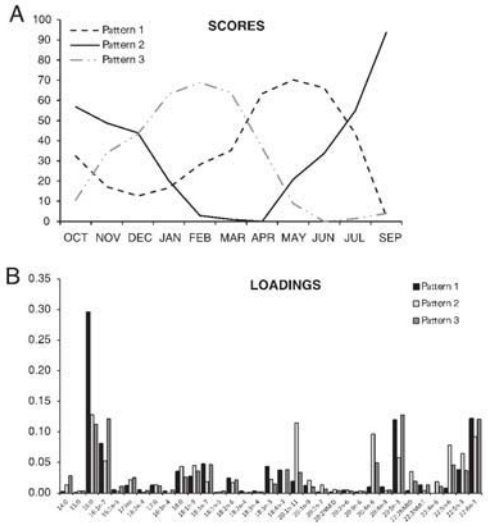

Fig. 3. Main fatty acid patterns identified by MCR-ALS analysis in the soft tissue of the zebra mussel *Dreissena polymorpha* from the Ebro River. (A) Temporal distribution of the fatty acid patterns and their contribution to the sampled months (scores); and (B) Composition of the identified patterns of fatty acids (loadings).

The observed decrease in triglycerides in exposed mussels is in agreement with other studies that reported a reduction of VLDL together with a decrease in serum triglycerides in both humans and fish after the administration of clofibrate and gemfibrozil (Kesäniemi and Grundy, 1984; Prindiville et al., 2011). The decrease in serum triglycerides has been associated to increased removal of triglyceride-rich lipoproteins by lipoprotein lipases that extracellularly hydrolyze triglycerides into glycerol and fatty acids (Schoonjans et al., 1996). Although, the existence and function of lipoprotein lipases in mollusks are still poorly known (Kim et al., 2008), our data indicates hydrolysis of triglycerides and a concomitant increase of total fatty acids (56 to 119%) in clofibrate-exposed zebra mussels, with no significant alteration of the fatty acid profile. Nonetheless, Du et al. (2004) reported a specific decrease of EPA and DHA contents in the liver of fenofibrate-treated rainbow trout, probably caused by an induction of peroxisomal activities that will oxidize very long chain fatty acids. The peroxisomal activity, acyl-CoA oxidase, was determined in clofibrate-exposed zebra mussels, but no significant induction was observed (data not shown). This enzyme catalyzes the β-oxidation of very long (C>20) and long chain (C14–C18) fatty acids, and a lack of induction of peroxisomal activities by clofibrate may explain the increase in total fatty acid levels and the unaltered fatty acid profiles detected in exposed organisms. In vertebrates, the effects of clofibrate are mediated through the transcriptional activation of PPARα receptor for which clofibrate is a well-known ligand and which is involved in fatty acid oxidation and catabolism (Forman et al., 1997). However, the existence of PPARs has not yet been proven in mollusks, despite studies reporting inducibility of peroxisomal proliferation by certain pollutants, a PPAR-mediated response in vertebrates (Cajaraville et al., 1997).

5. Conclusion

This work provides baseline information on the reproductive cycle of the zebra mussel inhabiting the lower part of the Ebro River and points out the strong relationship between reproductive events and the percentage of lipids, total fatty acids and fatty acid profiles in zebra mussels. In addition, the work evidences significant changes in triglycerides and total fatty acids following exposure of the zebra mussel to rather low concentrations of clofibrate in water (200 ng/L); the consequences of these alterations for the reproductive physiology of zebra mussel merit further research.

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Paper 3

LOW ENVIRONMENTAL LEVELS OF FLUOXETINE INDUCE SPAWNING AND CHANGES IN ENDOGENOUS ESTRADIOL LEVELS IN THE ZEBRA MUSSEL *DREISSENA POLYMORPHA*

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Resumen

El farmacéutico fluoxetina, un inhibidor selectivo de la recaptación de serotonina (ISRS), es detectado frecuentemente en el efluente de plantas depuradoras municipales y en aguas superficiales entre el rango de ng/l. No existe información suficiente respecto a los riesgos potenciales de fluoxetina para organismos acuáticos a concentraciones ambientalmente relevantes. Se detectó que los ISRS fluoxetina y fluvoxamina actúan como inductores de la puesta en bivalvos a concentraciones altas. Por lo cual este estudio pretende investigar los efectos de la exposición a fluoxetina en al mejillón cebra (*Dreissena polymorpha*) estimando su capacidad de inducir la puesta a concentraciones ambientalmente relevantes (20 a 200 ng/l), así como alteraciones en los niveles endógenos de testosterona y estradiol. El análisis histológico de las gónadas de machos y hembras mostró una disminución en la densidad de oocitos y espermatozoides dependiente de la concentración. Así se detectó una disminución del número de oocitos por folículo de 40-70% y de la densidad de espermatozoides de 21-25%, respecto al control, tras una exposición a 20 y 200 ng/l de fluoxetina durante 6 días. Hubo también un aumento significativo (1.5 veces) de los niveles endógenos del estradiol esterificado en organismos expuestos a 200 ng/l de fluoxetina. El estudio evidencia la capacidad de fluoxetina de alterar el sistema endocrino de moluscos a concentraciones ambientalmente relevantes.

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Low environmental levels of fluoxetine induce spawning and changes in endogenous estradiol levels in the zebra mussel *Dreissena polymorpha*

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ABSTRACT

The pharmaceutical fluoxetine, a selective serotonin reuptake inhibitor (SSRI), is often detected in municipal wastewater treatment plant effluents and surface waters within the ng/l range. There is, however, insufficient research evaluating potential hazards of fluoxetine in aquatic organisms at environmentally relevant concentrations. Taking into account that several SSRIs (fluoxetine, fluvoxamine) act as spawning inducers in bivalves, this study aimed at investigating the effects of fluoxetine exposure in the zebra mussel (*Dreissena polymorpha*) by assessing its potential to induce spawning at environmentally relevant concentrations (20 and 200 ng/l), as well as alterations of endogenous levels of testosterone and estradiol. Histological analyses of female and male gonads showed a concentration dependent decrease of oocyte and spermatozoan density, with a reduction in the number of oocytes per follicle of 40–70%, and spermatozoan density of 21–25%, relative to controls, following exposure to 20 and 200 ng/l of fluoxetine for 6 days, respectively. There was also a significant increase (1.5-fold) in the endogenous level of esterified estradiol in organisms exposed to 200 ng/l fluoxetine. Overall, the study shows that exposure to low levels of fluoxetine may effectively induce gamete liberation in the zebra mussel as well as alter endogenous levels of estradiol, and evidences the need of further investigating the potential of fluoxetine to alter the endocrine system of molluscs at environmentally relevant concentrations.

1. Introduction

In recent years an increased occurrence of pharmaceuticals have been reported in surface waters, seawater, effluents from municipal wastewater treatment facilities and even groundwater (Fent et al., 2006). A growing body of literature has emerged describing the potential of these compounds to adversely affect aquatic organisms. The main danger of pharmaceuticals arises from their environmental persistence and high bioactivity (Daughton and Ternes, 1999; Bringolf et al., 2010). Moreover, their continuous influx into the aquatic environment results in chronic exposure of aquatic organisms, especially of those residing in effluent-dominated ecosystems (Brooks et al., 2006). Of special concern are drugs which, even at low environmental concentrations, have a negative effect on the nervous or endocrine system of exposed organisms. One of the most studied is ethinylestradiol, an endocrine disrupter that produces adverse effects on fish, gastropod and mussel populations at concentrations between 1 and 50 ng/l (Jobling et al., 2004; Ciocan et al., 2010; Cubero-Leon et al., 2010).

Other pharmaceuticals of special environmental concern are the selective serotonin reuptake inhibitors (SSRIs), including fluoxetine, the active compound in Prozac®. Fluoxetine, like other SSRIs, is a high-prescription-volume drug in the United States and several other countries, used for the treatment of depression and certain compulsive disorders (RxList, 2009). As a consequence, many industrialized countries with large metropolitan areas have detectable quantities of SSRIs and their metabolites in their surface waters (Kwon and Armbrust, 2006). Fluoxetine is metabolized in the human body to norfluoxetine glucuronide and it is primarily excreted via urine containing approximately 2–11% of the administered dose as unchanged parent compound (Hiemke and Härtter, 2000). Kolpin et al. (2002) reported concentrations of fluoxetine in streams of the United States as high as 12 ng/l while other sources mention concentrations up to 99 ng/l in sewage effluents in Canada (Metcalfe et al., 2003). Brain tissue samples of fish had 1.58 ± 0.74 ng/g fluoxetine in an effluent-dominated stream in North Texas, United States (Brooks et al., 2005). Fluoxetine has also been detected in biosolids and sediments in the United States at average concentrations of 37.4 and 1.84 μg/kg.

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respectively (Furlong et al., 2004). Even though SSRIs and their metabolites are usually found in low concentrations (ng/l) in the aquatic environment, their biological effects at relevant environmental concentrations have seldom been assessed on aquatic organisms.

SSRIs block the reuptake of serotonin (5-HT) from the pre-synaptic nerve cleft resulting in an increased 5-HT neurotransmission in humans. As such, they can mimic the action of 5-HT (Brooks et al., 2003). Exposure of goldfish (*Carassius auratus*) to fluoxetine (540 ng/l) lead to a disruption of its reproductive physiology and energy metabolism, altering neuroendocrine hormones involved in steroidogenesis, spermiation and carbohydrate metabolism (Mennigen et al., 2010a,b). *In vitro* studies or injection of fluoxetine stimulated ovarian and testicular development and increased the size of ovaries and oocytes in the decapod crustacean *Uca pugilator* (Kulkarni and Fingerman, 1992; Sarojini et al., 1993). Such findings suggest that fluoxetine indirectly induced gonad development and oocyte maturation in crustaceans, probably via a putative 5-HT resembling ovarian and testicular stimulating factor released from the thoracic ganglia. Recently, De Lange et al. (2006) reported that 100 ng/l fluoxetine reduced the locomotion activity of the amphipod *Gammarus pulex*. In bivalve molluscs, such as *Dreissena polymorpha* and *Macoma balthica*, several reproductive events like spawning and parturition are regulated by serotonin and can be induced or potentiated by exogenous administration of SSRIs (Fong, 1998; Fong et al., 1998; Honkoop et al., 1999). Fong (1998) reported that water fluoxetine levels of 34–340 μg/l induced spawning in male zebra mussels of *D. polymorpha*. In a later study, Fong et al. (2003) showed that SSRIs induced spawning in mussels and clams by increasing serotonin activity. In addition, there is evidence that fluoxetine at concentrations of 300 and 3000 μg/l induced the release of non-viable glochidia in the endangered freshwater naiad *Elliptio complanata* (Bringolf et al., 2010). As the reproductive cycle of this species includes an obligatory parasitic form (McMahon and Bogan, 2001), the viability of the glochidia is of crucial importance (Faria et al., 2010). Thus, a spawning inducing compound like fluoxetine may potentially interfere with the reproductive cycle of endangered species and significantly reduce their reproductive success.

Nevertheless the above-mentioned studies conducted in bivalves, despite providing some mechanistic information on the mode of action of SSRIs, were not performed at realistic environmental concentrations. As in fish and crustaceans, SSRIs may also disrupt the reproductive axis in sexually mature bivalves (Kulkarni and Fingerman, 1992; Sarojini et al., 1993; Mennigen et al., 2010a,b) which share similar steroidogenic pathways with vertebrates (Porte et al., 2006). Thus, 17β-estradiol, for example, is involved in the regulation of the immune response (Canesi et al., 2004), the induction of Ca^{2+}-dependent NO production through activation of a signalling pathway at the cell surface (Stefano et al., 2003), and the modulation of the lysosomal function, as well as of lipid and glucose metabolism in mussel hepatopancreas (Canesi et al., 2007). It has also been suggested that 17β-estradiol may play an important role in reproductive events and sexual maturation and differentiation in bivalve molluscs (Janer et al., 2005; Gauthier-Clerc et al., 2006; Croll and Wang, 2007). In addition, there is evidence that mussels possess the ability to maintain stable levels of endogenous estradiol through its conjugation with fatty acids, a metabolic process that considerably reduces the biological activity of free steroids (Janer et al., 2005).

This study aimed at determining the potential effects of exposure to low levels of fluoxetine (20–200 ng/l) on female and male gonad structure and gamete release as well as on the levels of endogenous steroids (testosterone and estradiol) in the freshwater mussel *D. polymorpha*.

2. Materials and methods

2.1. Sample collection

Zebra mussels (*D. polymorpha*) with mean weight of 0.3 ± 0.05 g and mean length of 22 ± 1 cm were collected in April 2008 from the Ebro River, from an unpolluted site near the town of Riba Roja (Catalonia, Spain). Animals were transported in buckets of approx. 40 L filled with local water to the animal-holding facilities (IDAEA, Barcelona) and kept in 20 L tanks at 20 ± 1 °C. Before exposure, mussels were acclimatized for a period of 10 days to ASTM reconstituted water of similar hardness (170 mg/l $CaCO_3$) and conductivity (600 μS/cm) as local Ebro River water.

2.2. Experimental design

Environmental conditions, i.e. temperature, conductivity of ASTM water and photoperiod simulated the original conditions of mussels. Animals were fed daily with a 1:1 suspension of algae *Scenedesmus subspicatus* and *Chlorella vulgaris* (10^6 cells/ml). Water was renewed every day. After acclimatization, ~400 mussels were selected for the experiments. They were placed on fishing nets suspended in 20 L glass aquaria (~90 individuals per aquaria). Dissolved oxygen was maintained constant by continuous aeration using filtered compressed air through glass diffusers.

Mussels were exposed to different concentrations of fluoxetine (20 and 200 ng/l), added to the rearing water, for 6 days. There were two sets of controls: non-exposed mussels (C) and mussels exposed to 0.002% triethylene glycol, used as a carrier solvent (SC). Water was changed daily and a fresh dose of fluoxetine was added; mussels were fed every 48 h adding food 15–30 min before water renewal. After the 6-day exposure, mussels were dissected, the gills separated from the whole body and stored at −80 °C. For histology, 20 organisms per exposure group were dissected, placed into cassettes and fixed in 4% buffered formaldehyde (0.1 M phosphate buffer, pH 7.4) for 48 h. Samples were subsequently rinsed with water and stored in 70% ethanol. Prior to paraffin embedding, individuals were transversally cut with a scalpel and both the byssus and the foot removed since they could further interfere during the sectioning process. Both portions were then embedded in paraffin, cut at 7 μm thick sections and stained with Hematoxylin–Eosin Y.

2.3. Analysis of tissue steroid levels

Tissue levels of free testosterone and estradiol were analyzed as described in Janer et al. (2005). Briefly, tissue samples (0.25–0.35 g wet weight; $n = 12$) were homogenized in ethanol, and frozen overnight at −80 °C. Homogenates were then thawed and extracted with 2 ml of ethyl acetate (3×), the organic extracts recombined and reduced under a nitrogen stream. Dry residues were redissolved in 80% methanol. This solution was then washed with petroleum ether to remove the lipid fraction and evaporated to dryness. The dry residue was redissolved in 4 ml Milli-Q water and passed through a C18 cartridge (Isolute, International Sorbent Technology, Mid Glamorgan, UK; 1 g, 6 ml), that had been sequentially pre-conditioned with methanol (4 ml) and Milli-Q water (8 ml). After finishing the concentration step, cartridges were washed with Milli-Q water (8 ml), dried and connected to a NH_2 cartridge (Sep-Pack® Plus; Waters, Milfold, MA, USA). The C18–NH_2 system was then washed with 8 ml n-hexane and the steroids eluted with 9 ml dichloromethane:methanol (7:3). This fraction was collected and evaporated to dryness.

Total testosterone and estradiol (free + esterified) were measured as described by Gooding et al. (2003), with some modifications. Tissue, homogenized as for free steroid determination (see

above), was extracted with ethyl acetate (3 × 2 ml). The organic extract was evaporated under nitrogen, resuspended in 1.0 ml methanol containing 1% KOH, and incubated at 45 °C for 3 h. After the saponification step, Milli-Q water (4.0 ml) was added, and the sample extracted with dichloromethane (3 × 3 ml).

The efficiency of the extraction and delipidation procedure was 74 ± 3% for testosterone and 80 ± 3% for estradiol (Morcillo et al., 1999). The recovery for the purification step (SPE cartridges), evaluated with radiolabelled steroids was in the range 95–97% for both testosterone and estradiol (Janer et al., 2005). Dry extracts (tissue and water samples) were resuspended in 50 mM potassium phosphate buffer pH 7.6 containing 0.1% gelatine, and assayed for testosterone and estradiol concentration using commercial RIA kits (Radim, Rome, Italy). Standard curves with the steroids dissolved in the same phosphate buffer were performed in every run. The limits of detection were 25 pg/g for testosterone (T) and 20 pg/g for estradiol (E2). Intra-assay coefficients of variation were of 6.1% (T) and 3.3% (E2). Inter-assay coefficients of variation were of 9.3% (T) and 3.5% (E2).

2.4. Quantitative histology using image processing techniques

Histological preparations were first examined under a light microscope to determine sex and gonadal maturation stage following conventional histological procedures according to the method described by Juhel et al. (2003). For quantitative histological analysis only individuals in the spawning stage were selected, due to their high incidence (85–90%). Other stages of maturation were not considered in this study due to their low presence (10–15%) and thus the difficulty to make a separate and accurate evaluation. All tissue images were captured using a Digital Sight-Ri1 (Nikon) digital camera mounted on an Eclipse 90i (Nikon) light microscope. A photo ocular lens of 10× was used with an objective of either 4× (females) or 10× (males) resulting in a magnification of 40× and 100×, respectively. The image analysis methods used in this study are adapted from those already described by Arimoto and Feng (1983) and Heffernan and Walker (1989) as sensitive assessments of the gonadal gamete content in mussels with modifications aimed to improve accuracy and reproducibility. Gonadal gamete content was assessed in the follicle (females) and tubule lumen (males), and only follicles/tubules with a clearly distinguishable wall were selected. Gamete density in female gonads was analyzed as the number of oocytes per follicle area (μm^2) of 10 follicles in three randomly chosen sections per individual gonad (30 follicles per individual). Follicle area was determined with the aid of image analysis software (NIS-Elements AR ver. 3.0, Laboratory Imaging, Nikon). Male gonads were analyzed using ImageJ image analyzing software, using a modification of the protocol described by Toro et al. (2002). Briefly, color images were transformed to 8-bit grey scale images and pictures were then thresholded individually. Tubules were selected and the spermatozoa amount (% inside the tubule lumen) calculated as the ratio between the number of pixels occupied by spermatozoa and the total number of pixels of the tubule area. For each male a total of 10 tubules were selected from 3 randomly chosen sections of the gonad.

2.5. Water analyses

Duplicated water samples were taken from the freshly prepared solutions and after 24-h exposure to measure oxygen levels, pH and fluoxetine concentrations in water. Dissolved oxygen concentration (DO) was measured using a polarographic oxygen electrode coupled to a CyberScan DO 300 EUTECH model meter (Lab Process Distributions, Alella, Barcelona, Spain). Water pH was measured using an epoxy-body combination electrode, coupled to a Crison micro pH 2001 meter and calibrated with standard pH buffer solutions (Sigma, Madrid, Spain). Measurement of fluoxetine levels was limited to the highest tested concentration (200 ng/l) as follows: 1000 ml of water were pre-concentrated in Oasis 60 mg SPE cartridges which were conditioned with 10 ml of methanol followed by 10 ml of water; water was pre-concentrated at a flow rate of 10 ml/min and eluted with 2 × 4 ml of methanol. This extract was then reduced under nitrogen to incipient dryness and reconstituted with 500 µl of the mobile phase. Fluoxetine was analyzed by high performance liquid chromatography coupled to diode array detection (HPLC-DAD) with an Agilent Technologies 1200 series diode array detector G1315D; 50 µl of the extract were injected on a 50 mm × 4.6 mm XBridge TM C18 column of 3.5 µm particle diameter (Waters, USA). A gradient elution starting at 70:30 water (pH 4.0) acetonitrile to 100% acetonitrile in 20 min was used at a flow rate of 0.8 ml/min. Acquisition was done at a wavelength of 226 nm. Under those conditions, fluoxetine eluted at 4.15 min and had recoveries from water samples determined using an external standard of 99 ± 3% ($n = 4$).

2.6. Statistical analyses

Differences in steroid levels were determined by one-way ANOVA, followed by Tukey's post hoc multiple comparison tests ($P < 0.05$). Significant differences in the number of oocytes per follicle area and spermatozoa density of tubules across treatments were analyzed using a nested one-way ANOVA with individual mussels nested within treatments, followed by Tukey's post hoc multiple comparison test ($P < 0.05$). Prior to all analyses requirements for parametric testing were determined and if not met data was log transformed to improve normality and homoscedasticity of variance.

3. Results

3.1. Water analyses

During the entire experiment, oxygen levels were 90% of air saturation and pH was within 7.8–8.1. Measured fluoxetine levels in freshly prepared water were close to nominal values (mean ± SEM, $n = 4$) 180 ± 30 ng/l, decreasing to 110 ± 60 ng/l after 24-h exposure.

3.2. Histological analyses

Of the 80 analyzed organisms, 50 were females and 30 males. Most individuals were found in the spawning stage (stage 3). Within females 87% of individuals from the control (C), solvent control (SC) and 20 ng/l fluoxetine treatments, and 100% of those exposed to 200 ng/l of fluoxetine had gonads in stage 3. Within males 86%, 88%, 83% and 78% of individuals from the C, SC, 20 ng/l and 200 ng/l fluoxetine treatments had gonads in stage 3, respectively (Fig. 1). Female gonads were characterized by having the entire peripheral area and parts of the central lumen of the follicle occupied by mature oocytes (Fig. 1A). In addition a few pedunculated oocytes were located at the walls of the pre-spawning follicles. The connective tissue was almost completely reduced and formed a discontinuous layer surrounding the follicles. Male gonads were characterized by a high amount of spermatozoa with visible tails closely packed in the center of the star-shaped lumen of the seminiferous tubules. Larger precursor germ cells (spermatocytes) were located at the periphery while a thin layer of connective tissue served as separation between adjacent tubules (Fig. 1C).

Exposure to fluoxetine dramatically decreased the number of oocytes per follicle and spermatozoa density in tubules (Fig. 1B and D). In exposed females, the central lumen was mostly empty, only

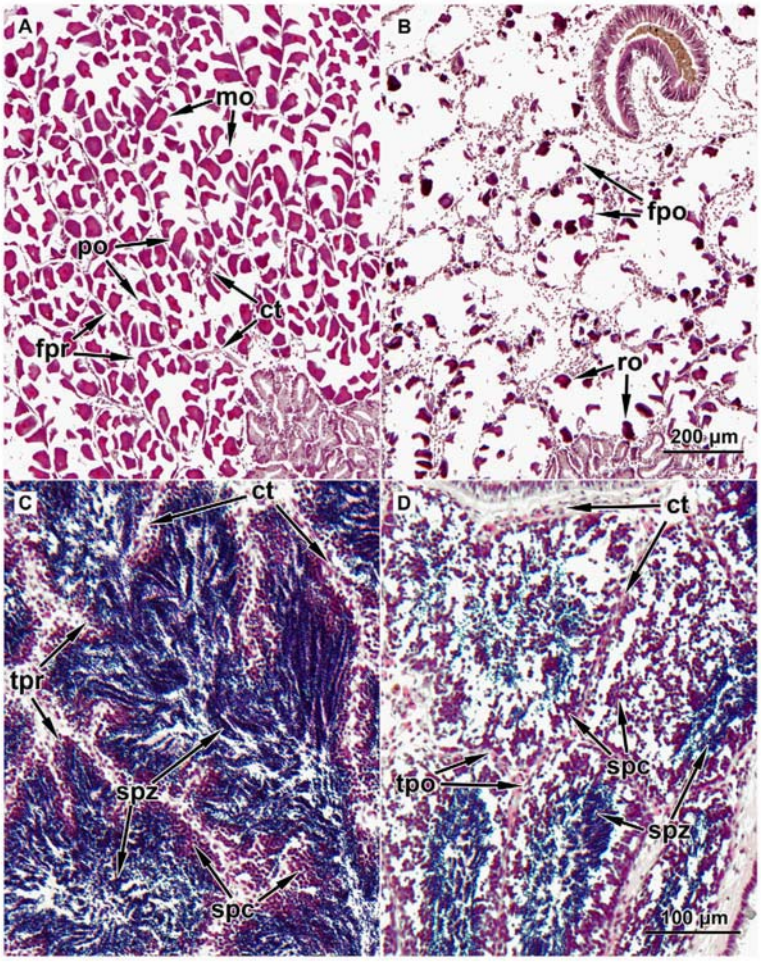

Fig. 1. Photomicrographs of male and female zebra mussel (*D. polymorpha*) gonads in control and fluoxetine-exposed treatments (200 ng/l). (A) Ovaries of a control female showing follicles filled with mature oocytes. (B) Fluoxetine-exposed female ovaries with mostly empty central lumen and only residual oocytes at the periphery. (C) Control male testes characterized by a dense amount of spermatozoa visible in the center of the star-shaped lumen of the seminiferous tubules. (D) Testes of fluoxetine-exposed male exhibiting the presence of a less dense amount of spermatozoa especially in the center of the lumen. *Abbreviations*: ct, connective tissue; fpo, follicle post-spawning; fpr, follicle pre-spawning; mo, mature oocyte; po, pedunculated oocyte; ro, residual oocyte; spc, spermatocytes; spz, spermatozoa; tpo, tubule post-spawning; tpr, tubule pre-spawning. Scale bar in B applies to photos of female gonads and D to male gonads.

a few residual oocytes were found at the periphery of the follicles (Fig. 1B). Exposed males showed a reduction in the number of spermatozoa in the center of the lumen concomitant with a thickening of the connective tissue layer (Fig. 1D). Also the typical star shaped alignment of the spermatozoa in the center of the lumen was much less apparent in males exposed to fluoxetine. In female gonads, nested one-way ANOVA results performed on 1200 follicles indicated that despite the existence of significant ($P < 0.05$) differences among individual mussels within treatments ($F_{38,1158} = 13.9$), fluoxetine exposure significantly ($F_{3,42} = 30.4$, $P < 0.05$) affected the number of oocytes per follicle area. Results depicted in Fig. 2A clearly showed decreasing numbers of oocytes per follicle with increasing exposure to fluoxetine. The decrease in the number of oocytes per follicle area was approximately 40% and 70% for 20 and 200 ng/l fluoxetine, respectively, compared to the control group. In testes, nested one-way ANOVA results performed on 250 tubules indicated also the existence of significant ($P < 0.05$) differences among individual mussels within treatments ($F_{21,225} = 25.7$), but fluoxetine exposure levels significantly ($F_{3,21} = 12.4$, $P < 0.05$) reduced the tubule area covered by spermatozoa (Fig. 2B). The decrease in spermatozoa density was of 21% and 25% for 20 and 200 ng/l fluoxetine, respectively.

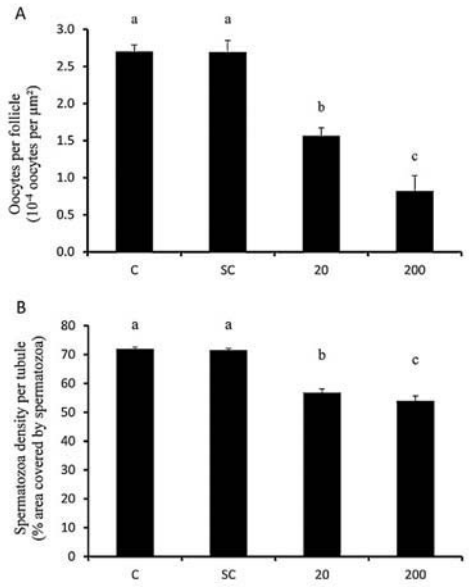

Fig. 2. Number of oocytes per follicle area in female gonads (A) and of spermatozoa density (% of tubule area covered by spermatozoa) in male gonads (B) of zebra mussels exposed to fluoxetine (20–200 ng/l) for 6 days. Different letters indicate significant ($P<0.05$) differences after nested ANOVA and post hoc Tukey's test. C and SC are control and solvent control treatments, respectively. Values are mean ± SEM ($n=7–11$).

3.3. Tissue steroid levels

Steroid levels were measured in the soft tissue of control and fluoxetine exposed organisms. Levels of free/unconjugated testosterone were in the range of 0.08–0.14 ng/g wet weight, and levels of free estradiol were of 0.04–0.10 ng/g wet weight. No significant ($P<0.05$) differences were observed between control and exposed organisms (Fig. 3A). After saponification of the samples, the ester bonds of the steroid metabolites were cleaved and total steroids (including both free and esterified forms) were determined. Esterified steroids were determined by subtraction of free steroids from total steroid content. Both testosterone and estradiol were predominately found in their esterified form (97.6–98.9%). Esterified testosterone was in the range of 3.39–4.38 ng/g wet weight and esterified estradiol ranged from 5.77 to 12.79 ng/g wet weight. After six-day exposure, esterified estradiol levels increased significantly ($P<0.05$) 1.5-fold in the group exposed to 200 ng/l fluoxetine. Interestingly, no alterations were detected in esterified testosterone levels (Fig. 3B).

4. Discussion

The histological analyses of fluoxetine-exposed zebra mussel gonads clearly showed a significant decrease in the number of oocytes within the follicles and of spermatozoa density within the male seminiferous tubules, suggesting the ability of fluoxetine to induce spawning at rather low concentrations (20–200 ng/l). The apparently lower gamete decreases reported for males than for females (Fig. 2) is related to the fact that mature testes include simultaneously several types of germinal cells (spermatocytes and spermatozoa) and that mainly spermatozoa located in the center of the star-shaped lumen of seminiferous tubules were released after exposure to fluoxetine as depicted in Fig. 1. Unfortunately, due to the small size and huge number of spermatozoa it was impossible to count them. Instead changes in germinal cell density were evaluated. On the contrary, mature ovarian mostly had mature oocytes that were released after fluoxetine exposure (Fig. 1). Fong (1998) reported that fluoxetine at concentrations of 34 and 340 µg/l induced spawning in male and female zebra mussel in just a few hours. In the present study, we demonstrated that spawning may be inducible at even much lower fluoxetine concentrations following exposure for a period of several days. The observed decrease in the number of gametes in the gonads of exposed organisms may be caused by a cumulative effect of the compound directly on the gonads or through the accumulation of fluoxetine also in other peripheral tissues other than the gonad. The concentration of fluoxetine in water markedly decreased after 24 h of exposure, suggesting an uptake of the compound by the test organisms. Several studies performed on different aquatic organisms indicated that fluoxetine bioaccumulates in significant amounts and that such accumulation is pH-dependent. Estimated bioaccumulation factors of fluoxetine at neutral pH ranged from 80 to 3100 in the Japanese medaka *Oryzias latipes* (Nakamura et al., 2008; Paterson and Metcalfe, 2008). Bringolf et al. (2010) reported bioconcentration factors of fluoxetine over 1000 in the freshwater mussel *E. complanata* inhabiting a contaminated effluent channel.

Testosterone and estradiol extracted from the whole body of *D. polymorpha* were predominantly found in their esterified form, agreeing with previous results reported for other molluscs (Gooding and LeBlanc, 2004; Janer et al., 2006). Our experiment showed that short term exposure to fluoxetine did not significantly alter free steroid levels, but led to a significant increase (1.5-fold) in esterified estradiol in zebra mussels exposed to the highest fluoxetine concentration (200 ng/l). The mechanism behind the observed increase in esterified estradiol is unknown. Generally, esterification of fatty acids significantly increases their lipophilicity, so that they can be stored in the lipoidal matrices of the organism while concurrently reducing their bioactivity and bioavailability (Borg et al., 1995). Interestingly, we observed levels of esterified estradiol of 8–14 ng/g w.w. and testosterone 3–8 ng/g w.w. in zebra mussel during the spawning stage, followed by a significant increase to 38 ng/g of esterified estradiol and 14 ng/g of esterified testosterone after the spawning phase, when gonads are in a reabsorbing stage (Lazzara et al., unpublished results). Thus, it is likely that a stimulation of gamete release by fluoxetine will consequently modulate levels of endogenous steroids; however, only estradiol was modulated whereas no effect on testosterone was detected.

Several studies have shown that serotonergic agents can modify estrogen titers and *vice versa* (Maswood et al., 1999; Raap et al., 2000). Rats injected with fluoxetine revealed that concentrations of 0.5–5 mg/kg may significantly alter circulating estrogen levels (Taylor et al., 2004). Moreover estradiol titers increased in Japanese medaka exposed to 0.1 and 0.5 mg/l fluoxetine in water, whereas testosterone levels remained unaffected (Foran et al., 2004). The mechanisms involved in the modulation of estradiol levels by fluoxetine are unclear, but Rehavi et al. (2000) speculated that in rats SSRIs may inhibit the release of the luteinizing hormone which results in decreased ovarian release of estrogens in females.

Moreover, fluoxetine can mimic the action of 5-HT which is implicated in different functions in both vertebrates and invertebrates. 5-HT regulates several biological functions, like heartbeat rhythm, feeding, and locomotion in the gastropod mollusc *Ilyanassa obsoleta* (Couper and Leise, 1996) and spawning and oocyte maturation in the bivalves *Spisula solidissima* and *S.*

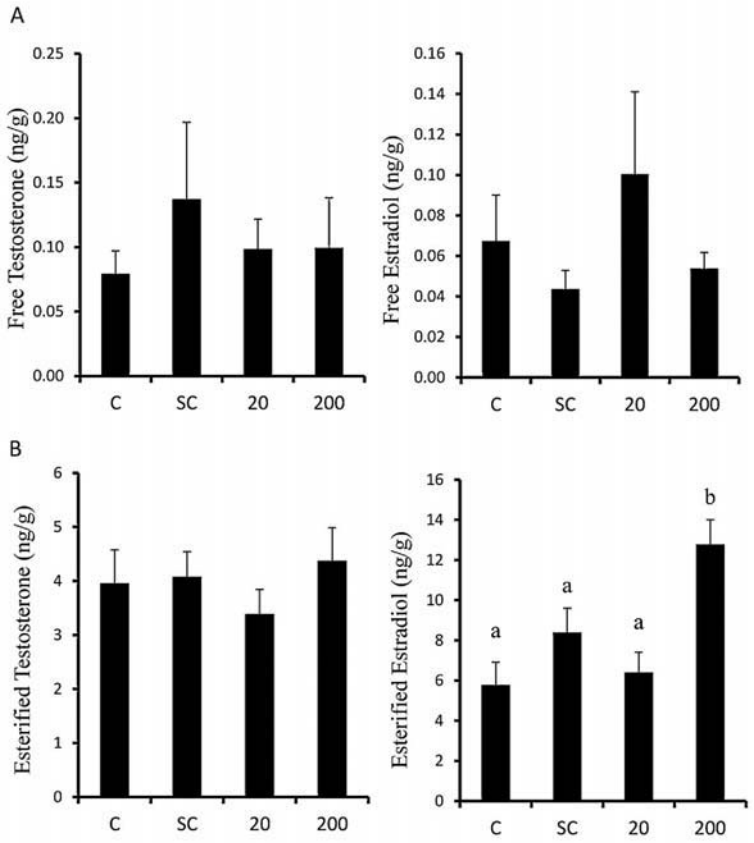

Fig. 3. Free and esterified levels of testosterone and estradiol determined in the whole body (without gills) of *D. polymorpha* exposed to fluoxetine (20–200 ng/l) for 6 days. Values are mean ± SEM ($n = 12$). C and SC are control and solvent control treatments, respectively. Different letters indicate significant ($P < 0.05$) differences after ANOVA and post hoc Tukey's test.

sachalinensis (Hirai et al., 1988). Interestingly, 5-HT receptors were found on the membrane of oocytes and spermatozoa of the clam *S. solidissima* and were involved in the release of gametes (Kadam et al., 1991). These receptors are regulated by 17β-estradiol which may potentiate 5-HT induced gamete release through increasing receptor sensitivity (Osada et al., 1998; Croll and Wang, 2007). However, it is unclear whether SSRIs directly increase 5-HT levels in bivalves or act as 5-HT receptor ligands (Fong et al., 2003). Nevertheless, an increased stimulation of 5-HT receptors could be to some extent counteracted by an increased esterification of estradiol, which would lead to reduced receptor sensitivity. Thus, one may hypothesize that the observed increase in esterified estradiol levels without a concomitant increase in esterified testosterone titers, would act as a mechanism of defense against an increased stimulation of 5-HT receptors by fluoxetine.

Overall, this study showed a concentration-dependent increase in gamete release in both females and males under fluoxetine exposure. This confirms the role of fluoxetine as a spawning inducer in zebra mussel, as reported in earlier studies with much higher fluoxetine concentrations. In the present study, spawning occurred at concentrations of fluoxetine as low as 20 ng/l. Concentrations of fluoxetine of 99–841 ng/l in waste water treated effluents and of 12–30 ng/l in contaminated surface waters have often been reported (Corcoran et al., 2010). Therefore, it is likely that exposure to fluoxetine in the low parts-per-trillion levels range may have a negative impact on zebra mussel reproduction since in this species fertilization is external and hence spawning has to be highly synchronized because of the short viability of gametes in water (Newell et al., 1982; Ram et al., 1996; Juhel et al., 2003). Thus, this study provides additional evidence that environmentally relevant levels of pharmaceuticals such as fluoxetine are biological active in aquatic organisms (Mennigen et al., 2010a,b). In particular, the present results showed that at ng/l, fluoxetine was able to alter the steroid metabolism and reproduction of mussels.

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Paper 4

SHORT-TERM EXPOSURE TO TRIBUTYLTIN MODULATES STEROID LEVELS AND LIPIDS IN THE ZEBRA MUSSEL *DREISSENA POLYMORPHA*

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In preparation

Resumen

Anti-incrustantes de alta toxicidad como tributilestaño y trifenilestaño, que se liberaron en el sistema acuático debido a su uso continuo como pinturas anti-incrustantes por barcos y redes de pesca, pueden tener consecuencias negativas por los organismos acuáticos, especialmente moluscos. Este estudio pretendió determinar los efectos de TBT sobre la homeostasis de lípidos y esteroides en el bivalvo de agua dulce *Dreissena polymorpha.* Por esto, se expusieron mejillones a concentraciones diferentes de TBT (20, 200 y 500 ng/L TBT como Sn) por un periodo de 7 días. El contenido de triglicéridos aumentó en 32% en machos expuestos a 500 ng/L TBT como Sn, y un 37% y 45% en hembras expuestas a 200 y 500 ng/L TBT como Sn, respectivamente. Se detectó un aumento en lípidos totales (34%) en hembras expuestas a la concentración más alta, mientras que aparecieron cambios significativos en los perfiles de ácidos grasos en ambos sexos. Además, los análisis de los esteroides sexuales indicaron un aumento significativo de 2.7, 2.0 y 2.5 veces de la testosterona total para las concentraciones 20, 200 y 500 ng/L como Sn en hembras, mientras que no se observaron alteraciones en machos. Además, se detectaron efectos tóxicos de TBT sobre el hepatopáncreas, como vacuolización y apoptosis de las células digestivas y de los ductos, además de daños en el tejido conjuntivo intertubular.

Abstract

Highly toxic antifouling agents such as tributyltin (TBT) and triphenyltin (TPT) have been released into aquatic ecosystems through their continuous use as antifouling paints on ships or fishing nets resulting in negative effects for aquatic organisms, especially molluscs. The purpose of this study was to determine the effects of TBT on lipid and steroid homeostasis in the freshwater bivalve *Dreissena polymorpha*. Therefore, mussels were exposed to different concentrations of TBT (20, 200 and 500 ng/L TBT as Sn) over a period of 7 days. Triglyceride content increased in males by 32% for 500 ng/L TBT as Sn and in females by 37% and 45% for 200 and 500 ng/L TBT as Sn, respectively. An increase in total lipids (34%) was seen in females exposed to the highest concentration whereas significant changes of fatty acid profiles appeared in both sexes. In addition, analysis of sex steroids indicated a significant 2.7, 2.0 and 2.5 fold increase in total testosterone for the 20, 200 and 500 ng/L as Sn exposure concentrations in females while no alterations were observed in males. Furthermore, toxic effects of TBT on the hepatopancreas resulted in vacuolization and apoptosis of digestive and duct cells as well as in damages of the intertubular connective tissue.

Introduction

Organotin compounds like TBT have been universally used as biocides, wood preservatives and antifouling agents for ships and boats (Evans et al., 1995; Haslbeck et al., 1996). Their widespread application has led to a considerable pollution of aquatic systems on a global scale. TBT is also a well-known threat to non-target organisms including a wide range of vertebrate (McAllister and Kime, 2003; Yu et al., 2003) and invertebrate species (Fent, 1996) which have shown reproductive abnormalities after exposure to TBT. Gastropod snails are within the most sensitive species to be affected by organotin compounds (OTCs) and are highly susceptible to imposex (imposition of male sexual organs on females) induction which can lead to infertility and population decline (Bryan et al., 1986; Gibbs et al., 1991).

TBT is a strong ligand of the retinoid X receptor (RXR) in a variety of invertebrate and vertebrate species at nanomolar concentrations (Le Maire et al., 2009) whose activation is probably the mechanism responsible for the imposex response and may also be the cause of metabolic and endocrine disruption processes (Nishikawa et al., 2004; Iguchi and Katsu, 2008). Belonging to the family of the ligand-activated transcription factors, RXRs are highly conserved in evolution (Bouton et al., 2005) and regulate a wide variety of biological functions in mammals including reproduction, development and general metabolism. More importantly, RXR can form heterodimers with several nuclear receptors such as the liver X receptor (LXR) and the peroxisome proliferator-activated receptor gamma (PPARγ) which are known to be involved in the regulation of lipid homeostasis (Spiegelman, 1998; Edwards et al., 2002). Furthermore, activation of the PPARγ-RXR heterodimers can result in the modulation of steroid metabolism, i.e. through inhibition of aromatase expression and activity in human granulosa cells (Mu et al., 2000; Fan et al., 2005).Therefore, ligand activation of RXR has a potentially pleiotropic effect on numerous biological functions including lipid metabolism (Szanto et al., 2004) and androgen receptor regulation (Chuang et al., 2005). Indeed, exposure experiments with organotins like TBT and TPT using the gastropod snail *Marisa cornuarietis* demonstrated a strong impact on lipid homeostasis resulting in significant alterations in total lipids and total fatty acids in females which was accompanied by changes in fatty acid profiles (Janer et al., 2007; Lyssimachou et al., 2009).

Steroid altering effects of organotins in molluscs are well-known and have already been described in several studies (Bettin et al., 1996; Gooding et al., 2003; Janer et al., 2005; Santos et al., 2005). For example, a series of exposure experiments with *M. cornuarietis* showed significant effects on important sex steroids in female organisms. More precisely, TBT reduced esterified forms of testosterone and estradiol (Janer et al., 2006a) whereas exposure to TPT led to significant increases in esterified testosterone and decreases in esterified estradiol (Lyssimachou et al., 2008) as well as inhibition of 5α-dihydroandrostenedione formation (Janer et al., 2006b). Moreover, studies on the clam *Ruditapes decussata* revealed a significant increase in the formation of 6α-hydroxytestosterone and a dose-dependent decrease in the aromatization of testosterone to estrone and estradiol as well as an increase in testosterone in the tissue of TBT-exposed organisms (Morcillo et al., 1998). Even though the mechanism behind the observed effects has yet to be explained, there is no doubt that OTCs are potent modulators of steroid and lipid metabolism in invertebrates, with female organisms being more susceptible than males.

Additionally, OTCs have been demonstrated to be highly toxic for a wide range of aquatic vertebrates and invertebrates (Bushong et al., 1988). In fish for example, TBT exposure resulted in a significant increase in apoptotic ovarian follicular cells in the marbled rockfish (*Sebastiscus marmoratus*) (Zhang et al., 2007), and in the rainbow trout (*Salmo gairdneri*) hyperplasia of liver cells and a significant increase in relative liver weights were seen (Seinen et al., 1981). In the gastropod *Haliotis diversicolor supertexta* TBT exposure resulted in structural alterations, vacuolation and partial inflammation of hepatopancreas tissue (Zhou et al., 2010). In molluscs, the hepatopancreas assumes several important physiological functions like immune control, antioxidation, lipid synthesis and storage, glycogen and mineral storage, intra-/extracellular digestion and especially detoxification (Lobo-da-Cunha, 1999, 2000). Tissue analyses of the hepatopancreas are often used in studies to evaluate toxic effects of environmental pollutants. Indeed, Vogt (1987) proposed that alterations in the hepatopancreas represent a highly sensitive overall response of organisms showing the synergistic impact of a toxicant, its metabolites and other exogenous factors.

As indicated, there is sufficient evidence showing deleterious effects of OTCs on gastropods but little is known about their effect on the biosynthesis and metabolism of lipids and steroids in freshwater bivalves. Therefore, the impact of TBT exposure on

zebra mussels to different concentrations (20, 200, 500 ng/L as Sn, nominal concentrations) in water was assessed by observing changes in lipid, fatty acid as well as steroid levels. In addition, histological observations of the hepatopancreas have been undertaken because of its important role as primary lipid storage organ and site of xenobiotic metabolism of lipophilic substances like TBT.

Material and Methods

Experimental design

Zebra mussels (*Dreissena polymorpha*) with an average shell length of 21.0 ± 0.8 cm were collected in April 2009 from the lower course of the Ebro River in a relatively unpolluted site (41° 14' 53.93 N/ 0° 28' 40.47 E). Animals were transported in buckets of approx. 40 L filled with local water to the animal-holding facilities (IDAEA, Barcelona) and kept in 20 L tanks at 20 ± 1°C and 12:12 dark:light photoperiod. Mussels were acclimatized for a period of 4 days to ASTM reconstituted water of similar hardness (170 mg/l $CaCO_3$) and conductivity (600 µS/cm) as local Ebro River water. Animals were fed with a suspension 1:1 of algae *Scenedesmus subspicatus* and *Chlorella vulgaris* (10^6 cells/ml).

After acclimatization, approx. 450 mussels were selected for the experiment. They were randomly placed on fishing nets suspended in 20 L glass aquaria (~90 individuals per aquaria). Dissolved oxygen was maintained constant by continuous aeration using filtered compressed air through glass diffusers. Animals were exposed to three different concentrations of TBT (20, 200 and 500 ng/L as Sn) for a period of 7 days. There were two sets of controls: (C) non-exposed mussels and (SC) mussels exposed to 0.002% triethylene glycol used as solvent. Water was changed daily and a fresh dose of TBT was added; mussels were fed every 48 hours adding food 15-30 min before water renewal. After 7-days exposure, mussels were separated by sex, dissected and the whole body without gills stored at −80ºC. The gender was determined *in situ* by dissecting a small part of the gonad which then was immediately examined under a light microscope for the presence of either spermatozoa or oocytes. The entire body of 12 organisms per exposure group was dissected and placed in 10% buffered formalin (0.1 M potassium phosphate, pH 7.4) for histological examination.

Analysis of tissue steroid levels

Total testosterone and estradiol were measured as described by Gooding et al. (2003), with some modifications. Briefly, tissue samples (0.4 g wet weight; n=5) were homogenized in ethanol and extracted with ethyl acetate (3×2 ml). The organic extract was evaporated under nitrogen, resuspended in 1.0 ml methanol containing 1% KOH, and incubated at 45°C for 3 h. After the saponification step, Milli-Q water (4.0 ml) was added, the sample extracted with dichloromethane (3×3 ml) and then dried under a gentle nitrogen stream. The efficiency of the extraction and delipidation procedure was 74 ± 3% for testosterone and 80 ± 3% for estradiol (Morcillo et al., 1999). Dry extracts were resuspended in 50 mM potassium phosphate buffer pH 7.6 containing 0.1% gelatine, and concentration assayed using commercial RIA kits (Radim, Rome, Italy). Standard curves with the steroids dissolved in the same phosphate buffer were performed in every run. The limits of detection of the assay were 25 pg/g for testosterone and 20 pg/g for estradiol.

Total lipids and fatty acid analysis

Lipids were extracted using a modification of the method introduced by Folch et al. (1957). Briefly, each sample which consisted in a pool of 2 individuals, was homogenized in an ice-cold solution of chloroform:methanol (2:1 v/v) containing 0.01% butylated hydroxytoluene (BHT). After centrifugation and addition of 0.88% KCl, the chloroform layer was collected, filtered and evaporated under nitrogen. The dried lipid content was determined gravimetrically (Sartorius, BP210D, 0.01 mg) and kept at -20ºC for fatty acid analysis.

For the analysis of fatty acids, 1 mg of total lipids dissolved in chloroform:methanol was mixed with nonadecanoic acid (19:0; internal standard) and saponified with 2 N KOH/MeOH/10% Milli-Q water, and neutral lipids removed with n-hexane. Samples were further acidified to pH ~3 with HCl and the fatty acids extracted and methylated with a solution of 10% boron trifluoride-methanol. The methylated fraction was resuspended in 10% NaCl, fatty acid methyl esters (FAME) extracted with n-hexane and analyzed by gas chromatography-flame ionization detector (GC-FID). The instrument was a Thermo Finnigan Trace GC. The column, a DB-23 fused silica 30 m x 0.25 mm i.d., film thickness 0.25 μm (Agilent J&W). The oven

temperature program was from 60 to 150°C at 15°C/min, then from 150 to 230°C at 3°C/min, keeping the final temperature for 10 min. The carrier gas was helium at a constant flow-rate of 1.5 ml/min. The injector and the detector temperature were set at 280 and 300ºC, respectively.

Determination of triglycerides

Tissue concentrations of triglycerides were determined using a commercial kit (Spinreact S.A., Sant Esteve De Bas, Spain) based on a peroxidase-coupled method for the colorimetric detection of triglycerides following hydrolysis, phosphorilation of glycerol and oxidation to produce hydrogen peroxide (McGowan et al., 1983). Briefly, 20 µl of lipid extract were dried under a gentle nitrogen stream and then redissolved in 20 µl methanol. The extract was then mixed with the assay reagents, incubated in the dark for 10 min and measured spectrophotometrically at 505 nm according to the manufacturer's protocol.

Histopathological analysis

Individuals fixed in 10% buffered formalin were dehydrated with ethanol, cleared with Histo-Clear II (National Diganostics, Hessle Hull, England), and embedded in paraplast Sigma-Aldrich (Steinheim, Germany). Sections (7 µm) were stained with hematoxylin-eosin y. The histopathological study was carried out by examining each preparation individually under the light microscope. Gender and gonadal maturation stage were determined following conventional histological procedures according to the method described by Juhel et al. (2003). To determine alterations of the hepatopancreas, pathological changes in digestive cells, primary and secondary hepatopancreatic duct cells as well as intertubular connective tissue were observed. The main criteria for the screening were the detection of cell vacuolization, cell apoptosis, and shrinkage of digestive cells as well as alterations of the connective tissue.

Statistical analyses

Data is reported as mean ± SEM. Statistical treatment of data was performed by using the SPSS statistical package (SPSS v.17.0, IBM Company, Chicago, USA). Differences between control and exposure groups were determined by one-way ANOVA followed

by a post-hoc Dunnett's test ($P<0.05$), and a Student's t-test was used for comparison of control and solvent control groups as well as to compare non exposed male with female organisms. Suitability for parametric testing was assessed by analyzing the dataset for normality and homoscedasticity of variance and if not met a log transformation was performed.

Results

Endogenous steroid levels

Steroid levels were determined in the soft tissue of *D. polymorpha* after exposure to different concentrations of TBT for a period of 7 days. Total steroid levels of control animals were significantly higher in males than in females (2 and 3.5 fold for testosterone and estradiol, respectively, $p<0.05$) (Figure 1). Average levels of total testosterone in non-exposed organisms were 7.0 ng/g w.w. in males and 3.6 ng/g w.w. in females, while total estradiol levels were of 13.0 ng/g w.w. in males and 3.7 ng/g w.w. in females. No significant differences between control and solvent control groups were observed. TBT exposure led to a significant alteration of testosterone levels only in females which showed a 2.7, 2.0 and 2.5 fold increase following exposure to 20, 200 and 500 ng/L TBT as Sn, respectively.

Fatty acid profiles and total lipid content

A detailed description of the fatty acid composition from the lipid fraction of control and exposed males and females of *D. polymorpha* is given in table 1. A total of 33 fatty acids with carbon atoms from 14 to 22 were identified showing different unsaturation degrees. Gender-specific differences were seen for individual fatty acids, fatty acid groups and total fatty acid levels (mg/g w.w.) as indicated in table 1. The group of saturated fatty acids (SFAs) comprised 28% and 32% of total fatty acids in males and females, respectively. However, the most abundant fraction were unsaturated fatty acids and within these monounsaturated fatty acids (MUFAs) with relative abundances (% of total fatty acids) of 23% in males and 26% in females as well as polyunsaturated fatty acids (PUFAs) comprising 10% in males and 12% in females. Highly unsaturated fatty acids (HUFAs) (20 and more carbon atoms/3 and more unsaturations) were the largest fraction of unsaturated fatty acids which contributed with 39% in males and 31% in

females to total fatty acids. Within both PUFAs and HUFAs, amount of n-3 fraction was higher than that of n-6. Palmitic acid (16:0) was the most abundant fatty acid followed by docosahexaenoic acid (DHA; 22:6n-3), eicosapentaenoic acid (EPA; 20:5n-3) and palmitoleic acid (16:1n-7). Total fatty acids were significantly higher (1.5 fold) in females than in males whereas no significant differences in total lipids (% of w.w.) were observed. In addition, females had higher levels of mono und polyunsaturated fatty acids with carbon atoms of 16 and 18, and males of those with 20 and higher carbon atoms including the highly unsaturated fatty acids.

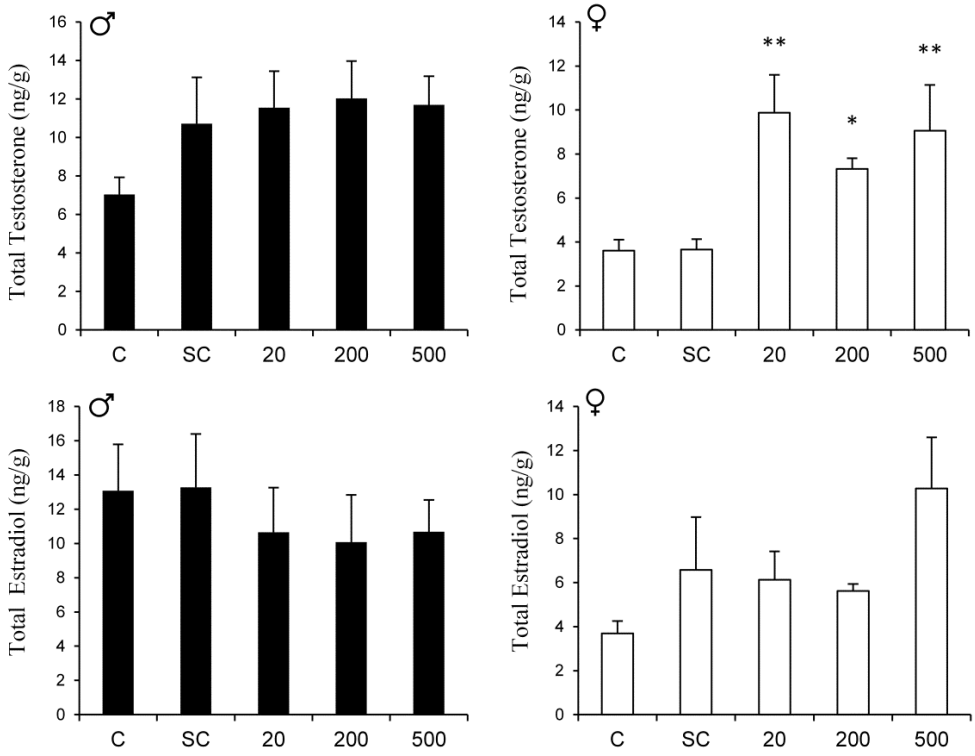

Figure 1. Total testosterone and estradiol levels determined in soft tissue homogenate of zebra mussels exposed to TBT (ng/L as Sn) for 7 days. Values are represented as mean ± SEM (n=5). */** Denotes significant differences towards solvent control (P<0.05/P<0.01 respectively).

One week exposure to TBT caused significant alterations in lipid levels and fatty acid profile in the soft tissue of *D. polymorpha*. Total lipids showed a significant increase (~34%) for the highest concentration in females (Figure 2A) accompanied by a visible, yet not significant increase (~23%) in total fatty acids (Table 1). No effects on total lipids and total fatty acid levels were seen in males. Furthermore, n-6 HUFA increased significantly in males by 22.7% (200 ng/L TBT as Sn) and 35.0% (500 ng/L TBT as Sn) as well as in females by 36.0% (200 ng/L TBT as Sn) and 33.2% (500 ng/L

TBT as Sn) (Figure 2B). Among individual fatty acids, significant changes were seen for the essential arachidonic acid (AA, 20:4n-6) and EPA. Hence, EPA significantly decreased for all concentrations (9-13%) in males whereas AA significantly increased (50-85%) for 200 and 500 ng/L TBT as Sn concentrations in both sexes (Table 1).

Triglyceride tissue levels

Our analysis showed triglyceride levels (% of total lipids) of 64% in males and 58% in females of control group. In both sexes a dose-dependent effect on triglyceride levels was seen which was more pronounced in females than in males (Figure 2C). Thus, triglyceride levels significantly increased in males by 32% for the 500 ng/L TBT as Sn group, and in females by 37% and 45% for the 200 and 500 ng/L TBT as Sn groups, respectively.

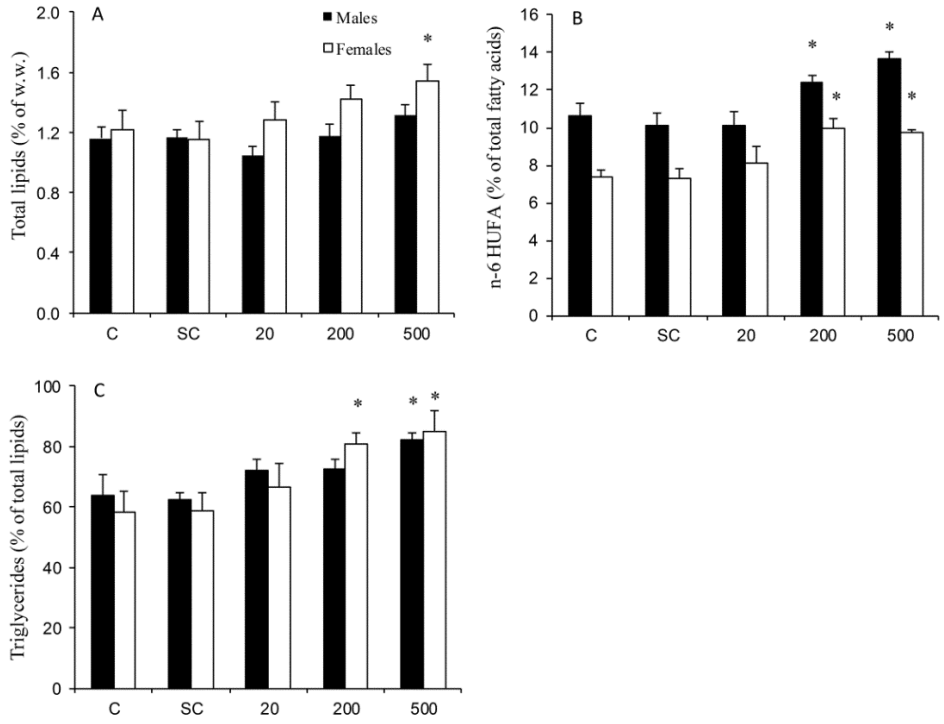

Figure 2. A. Total lipids (% of w.w.), B. n-6 HUFA (% of total fatty acids) and C. triglyceride levels (% of total lipids) in male and female zebra mussels from control and TBT exposed groups. Data is presented as mean ± SEM (n=6/6/3). * Denotes significant differences towards solvent control (P<0.05).

Histopathology

Histological analyses of treated animals showed a dose dependent effect of TBT exposure on the hepatopancreas in both sexes. There were no visible gender differences in regard of the severity of pathological effects. The gender ratio (males/females) was 1, 0.7, 0.7, 1.4 and 0.7 for the control, solvent, 20, 200 and 500 ng/L TBT as Sn groups, respectively. The analyses of the gonad revealed that all animals were in the spawning stage and that there was little individual variance in the gonadal maturation level. Major effects of TBT exposure on the hepatopancreatic tissue were seen in digestive cells, intertubular connective tissue and ciliated duct cells of the primary and secondary ducts. Already at lowest exposure concentration (20 ng/L TBT as Sn), mussels were detected showing shrinkage of digestive cells, vacuolization of the digestive and duct cells and damage of the intertubular connective tissue. With increasing concentration, degree of severity of tissue lesions augmented as well as the amount of affected animals. Those included partial lysis of intertubular connective tissue, increased vacuolization of digestive and duct cells accompanied by cell apoptosis and abnormal formation of tubulus lumen (Figure 3).

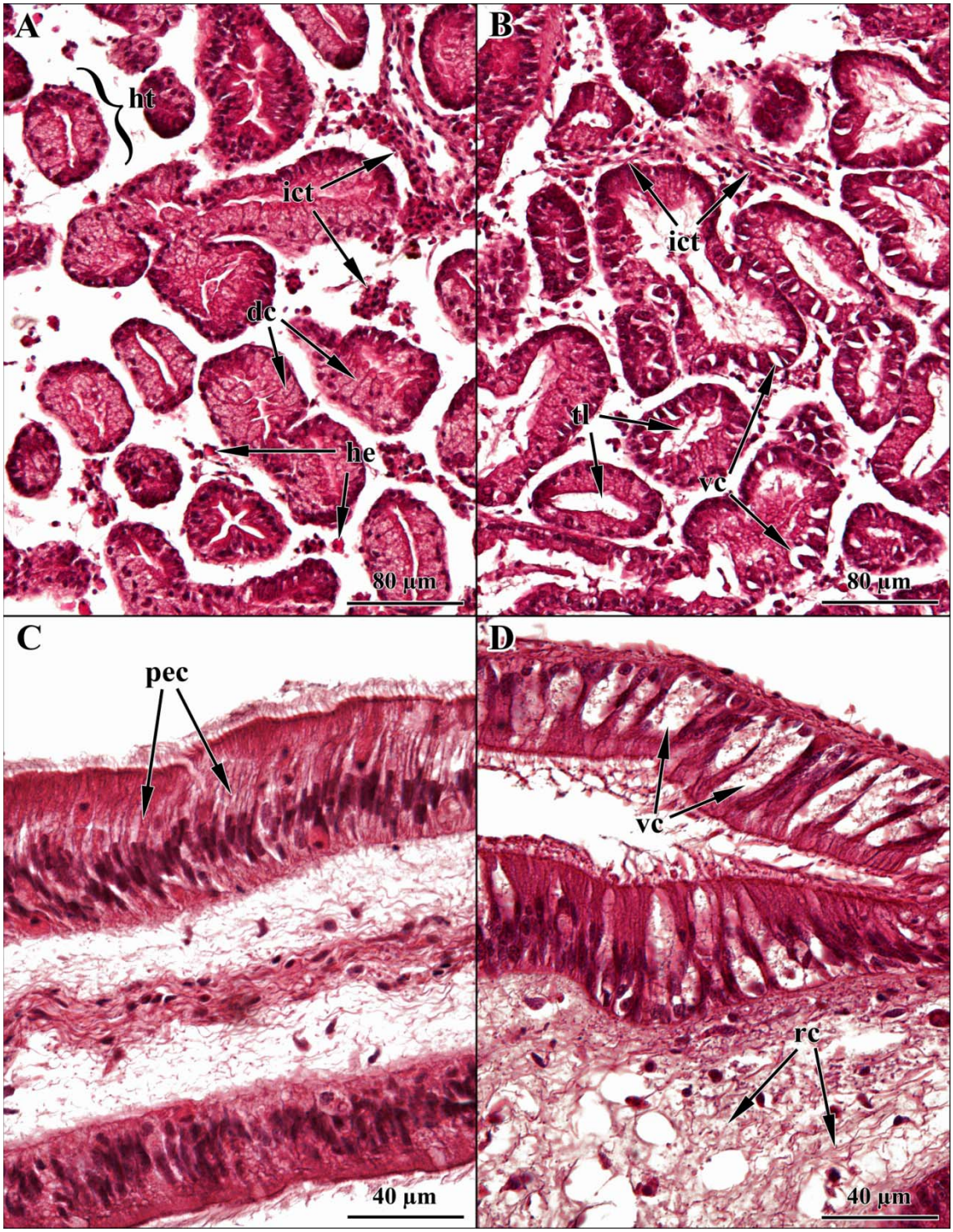

Figure 3. Photomicrographs of hepatopancreas and primary hepatopancreatic duct of zebra mussels exposed to TBT for 7 days. A. hepatopancreas of untreated control organism; B. hepatopancreas of organism treated with 500 ng/L TBT as Sn showing strong vacuolization and shrinkage of digestive cells accompanied by cell apoptosis, abnormal formation of tubulus lumen and partial lysis of intertubular connective tissue; C. primary duct of untreated control organism; D. primary duct of organism treated with 500 ng/L TBT as Sn showing strong vacuolization of prismatic epithelial cells. Abbr. dc, digestive cells; he, hemocytes; ht, hepatopancreatic tubule; ict, intertubular connective tissue; pec, prismatic epithelial cells; rc, reticulated connective tissue; tl, tubulus lumen; vc, vacuolization. Magnification levels are 200x for A&B and 400x for C&D.

Discussion

According to our results one week exposure of zebra mussels to TBT had a significant effect on lipid levels in female organisms in addition to alterations of triglyceride concentrations and fatty acid composition in both sexes. Parallel, total testosterone levels increased significantly in females whereas in males TBT exposure showed no significant effects on steroid titers. Our results would agree with other works on molluscs where after exposure to OTCs, total lipid and total FAME levels were significantly altered in female organisms of *M. cornuarietis* in addition to significant changes in fatty acid composition in both sexes (Janer et al., 2007; Lyssimachou et al., 2009). Those studies clearly showed that OTCs are potent modulators of lipid metabolism in molluscs which, as the authors indicated, may be mediated through RXR or RXR-PPAR activation. Indeed, Grün et al. (2006) already demonstrated TBT induced alterations of adipogenesis (substantial increase in the amount of triglycerides in tissue of newborn mice) *in vivo* and the RXR and PPARγ receptor mediated differentiation in adipocytes *in vitro*. PPARγ is an important transcription factor in adipose tissue where it is involved in energy storage (Auwerx, 1999); its activation increases fatty acid uptake and represses genes that induce lipolysis in adipose tissue (Ferré, 2004). Genes that are regulated by PPARγ encode the adipocyte fatty acid binding protein, lipoprotein lipase, fatty acid transport protein and acyl-CoA synthetase (Kersten et al., 2000). Hence, treatment with PPARγ activators shifts fatty acids into fat cells and also increases triglyceride uptake and storage capacity (Martin et al., 1998; Chao et al., 2000), a mechanism that was investigated in studies with rodents which after treatment with PPARγ agonists showed weight gain accompanied by adipocyte hypertrophy and hyperplasia (De Vos et al., 1996). Furthermore, the presence of the retinoid pathway in invertebrates has already been suggested after RXR homologues have been cloned from the gastropods *Thais clavigera* (Nishikawa et al., 2004), *Nucella lapillus* (Castro et al., 2007) and *Biomphalaria glabrata* (Bouton et al., 2005). Thus, taking into account the results of our work and the observed parallelism of TBT's mode of action between vertebrates (Grün and Blumberg, 2006) and invertebrates like *M. cornuarietis*, the possible involvement of a RXR mediated mechanism should be considered.

TBT exposure resulted in significant changes in abundances of individual fatty acids some of which were gender-specific like 18:1n-9, 20:5n-3 in males, and 18:1n-5 in females whereas changes of 18:2n-6, 20:4n-6 and n-6 HUFA occurred in both sexes. Our results would coincide with studies by Lyssimachou et al. (2009) where significant changes in fatty acids like 18:1n-9, 20:4n-6 and the n-6 HUFA group have been observed in females of *M. cornuarietis* after TPT exposure. Fatty acids like 16:1n-7, 18:0, 18:1n-9, 18:2n-6, 20:4n-6 and 20:5n-3 are known endogenous ligands for several nuclear receptors in vertebrates, amongst those PPARs whose activation can induce either lipogenesis or lipolysis (Willson and Wahli, 1997; Reddy and Hashimoto, 2001; Kota et al., 2005; Mochizuki et al., 2006). Moreover, other transcriptional factors have been identified to be targets of fatty acid regulation such as the liver X receptor and RXR, which are both involved in lipid regulation (Benatti et al., 2004). In this context, changes in AA may be of special interest because a significant increase (50-85%) appeared in both males and females and its potential as a regulator of lipogenesis in vertebrates has already been suggested (Yoshikawa et al., 2002). Hence, the fact that several fatty acids which are potential lipid regulators have undergone significant alterations of their abundances may indicate a regulatory response of the organism to changes in lipid levels induced by TBT.

Alternatively, alterations in fatty acid levels could also be explained by general TBT toxicity. Experiments with culture medium of the yeast *Saccharomyces cerevisiae* indicated that supplementation with PUFAs attenuated toxic effects of TBT exposure, probably through the increased lipophilicity of the membrane that would reduce the passive diffusion of TBT (Masia et al., 1998). Thus, the observed increase in n-6 HUFAs in this and other studies on invertebrates may very well be a protective mechanism of the organism to maintain cellular integrity after TBT exposure. In regard to AA, its function as precursor for important cell signaling molecules called eicosanoids which include prostaglandins, thromboxanes, leukotrienes, hydroxy fatty acids and lipoxins is well-known (Balsinde et al., 2002). In invertebrates, these signaling molecules play a role in egg-production, modulation of immunological responses to infections as well as in the regulation of stress response through the increase of heat shock proteins (HSPs) (Santoro et al., 1989; Stanley, 2006). Induction of HSP synthesis can result in stress tolerance and cytoprotection against stress-induced molecular damage (Santoro, 2000). Thus, an increase in AA may minimize the effect of

TBT exposure and maintain normal physiological functions. Another aspect of prostaglandin production is their effect on adipocyte differentiation which, depending on the species, can either be pro-adipogenic or anti-adipogenic. Prostacyclin for example has been demonstrated to be proadipogenic, whereas $PGF_{2\alpha}$ inhibits the differentiation of primary preadipocytes (Madsen et al., 2005). Thus, AA may play a versatile role in firstly attenuating toxic effects of TBT on the cellular level and secondly be involved in regulating lipid homeostasis.

Total testosterone levels showed a clear increase (2-2.7 times) in females after seven days of TBT exposure. Our results would be in agreement with previous studies on *M. cornuarietis* where exposure to TPT induced a significant increase in esterified testosterone in females while no effects were observed in males (Lyssimachou et al., 2008). In addition, the clam *Ruditapes decussata* showed increased free testosterone levels after TBT exposure while TPT exposed sea urchins *Paracentrotus lividus* demonstrated significant increases of esterified testosterone in the coelomic fluid (Morcillo et al., 1998; Lavado et al., 2006). On the other hand, long-term studies on TBT using *Ilyanassa obsoleta* showed a significant increase in free testosterone accompanied with a moderate decrease in conjugated-testosterone levels (Gooding et al., 2003). Similar results have been mentioned by Janer et al. (2006a) where 100 days exposure of *M. cornuarietis* to TBT led to a significant decrease in esterified testosterone in females. It is clear that study results may be species and/or compound specific and also be influenced by exposure period, but significant alterations of androgen metabolism in invertebrates through OTCs have been sufficiently demonstrated. The mechanism involved in the alteration of androgen levels has not been completely determined yet. Several hypotheses involving metabolic key enzymes like acyl-coenzyme A:testosterone acyltransferase (ATAT), 17β-hydroxysteroid dehydrogenase (17β-HSD) and P450 aromatase have been proposed but study results are either inconclusive, do not withstand critical scrutiny or even indicate that these enzymes may not be the primary targets of TBT and TPT (Gooding et al., 2003; Santos et al., 2005; Lyssimachou et al., 2008; Sternberg et al., 2010). Hence, the nuclear receptor theory as proposed by several authors may give a better explanation model. It has been shown that RXR-PPARγ heterodimers can regulate steroid metabolism by inhibiting aromatase expression and activity in human granulosa cells (Mu et al., 2000; Fan et al., 2005). The involvement of RXR in androgen regulation can also be explained

by its formation of heterodimers with the androgen receptor (AR) as reported by Chuang et al. (2005). Accordingly, the AR and RXR affect their transcription mutually and by this RXR activation may produce androgenic but also antiandrogenic effects. However, due to the fact that invertebrate model are still lacking, the cause of the significant changes in total testosterone observed in our study still remains unclear.

Finally, histopathological analyses showed a strong effect of TBT exposure on the hepatopancreas already at concentrations as low as of 20 ng/L as Sn, with alterations like visible retraction of digestive cells or vacuolization of digestive and duct cells in addition to an increased occurrence of apoptotic hepatocytes. The amount of affected animals increased significantly at the higher concentrations as well as the severity of the lesions. A considerable amount of animals of the 200 and 500 ng/L as Sn groups showed at least some degree of cell vacuolization and damages of the intertubular connective tissue. Among those were organisms that demonstrated severe tissue alterations like partial lysis of the intertubular connective tissue and abnormal formation of the tubular lumen which was accompanied by a strong shrinkage of digestive cells and also a considerable amount of apoptotic cells (Figure 3). TBT´s potential of inducing pathological changes in bivalves has already been demonstrated for *Mytilus galloprovincialis* where apoptosis of gill tissue was reported (Mičić et al., 2001). Several histopathological effects seen in this study like shrinkage and vacuolization of hepatocytes and damage of intertubular connective tissue have already been described for other environmental pollutants like atrazine (Zupan and Kalafatić, 2003). Some of those effects have been attributed to changes in protein metabolism. In the case of TBT, it has been suggested that toxic effects are related to the binding of TBT metabolites to cellular proteins and inhibition of enzymes involved in detoxification processes like those of the cytochrome P-450 system or the glutathione S-transferase (Lee, 1991). Molluscs also have comparable low cytochrome P-450 content and mixed function oxygenase activity in the hepatopancreas which may in addition cause a decreased metabolism of TBT, thus leading to an increased accumulation (Livingstone and Farrar, 1985).

Overall, this study presents evidence that short term exposure of *Dreissena polymorpha* to environmentally relevant concentrations of TBT caused changes in fatty acid composition as well as triglyceride levels in males and females while total lipids increased in females only. In addition, total testosterone increased significantly in

females demonstrating TBT's potential to interfere with androgen metabolism in the zebra mussel. The higher susceptibility of female organisms remains unexplained and should be addressed in further research. Furthermore, histopathological analyses demonstrated a detrimental effect on the digestive and duct cells of the hepatopancreas accompanied by apoptotic hepatocytes and deterioration of the intertubular connective tissue. Considering the important role that fatty acids exhibit as cell signaling molecules, substrates for eicosanoid synthesis and ligands of PPARs and RXR, their modification through TBT may be of special concern. Furthermore, the observed changes in triglycerides and lipid homeostasis as well as androgen metabolism may strengthen the hypothesis that TBT may exert its effects through a RXR mediated pathway in molluscs similar to what has been seen in vertebrates.

Table 1. Fatty acid profiles measured in the soft body tissue of *Dreissena polymorpha* after 7 days of TBT exposure.

	Males					Females				
Fatty acid	C	SC	20	200	500	C	SC	20	200	500
14:0	0.87 ± 0.06[a]	0.71 ± 0.09	0.96 ± 0.08	0.85 ± 0.08	0.81 ± 0.08	0.70 ± 0.05[a]	0.72 ± 0.10	0.83 ± 0.17	0.74 ± 0.07	0.85 ± 0.13
15:0	0.24 ± 0.04[a]	0.16 ± 0.03	0.14 ± 0.03	0.23 ± 0.03	0.25 ± 0.04	0.12 ± 0.01[b]	0.12 ± 0.01	0.10 ± 0.03	0.08 ± 0.02	0.05 ± 0.01*
16:0	20.24 ± 0.40[a]	21.81 ± 0.94	21.26 ± 0.89	20.36 ± 0.31	19.74 ± 0.34	23.59 ± 0.77[b]	24.35 ± 0.98	23.16 ± 0.67	23.23 ± 0.65	24.46 ± 1.71
16:1n-7	7.45 ± 0.38[a]	6.66 ± 0.36	7.02 ± 0.47	7.19 ± 0.40	6.29 ± 0.33	8.34 ± 0.31[a]	8.91 ± 0.45	9.43 ± 1.24	8.57 ± 0.27	8.40 ± 0.62
16:1n-5	0.48 ± 0.04[a]	0.48 ± 0.03	0.49 ± 0.03	0.49 ± 0.02	0.44 ± 0.02	0.61 ± 0.03[b]	0.65 ± 0.03	0.68 ± 0.06	0.60 ± 0.02	0.59 ± 0.05
17 iso	1.76 ± 0.08[a]	1.61 ± 0.09	1.75 ± 0.10	1.72 ± 0.11	1.72 ± 0.14	1.65 ± 0.07[a]	1.78 ± 0.09	1.83 ± 0.08	1.75 ± 0.07	1.87 ± 0.06
16:2n-4	0.22 ± 0.07[a]	0.19 ± 0.02	0.24 ± 0.04	0.24 ± 0.05	0.18 ± 0.05	0.31 ± 0.05[a]	0.29 ± 0.07	0.21 ± 0.08	0.28 ± 0.07	0.35 ± 0.03
17:0	1.34 ± 0.06[a]	1.49 ± 0.02	1.40 ± 0.05	1.41 ± 0.04	1.46 ± 0.03	1.18 ± 0.02[a]	1.24 ± 0.12	1.22 ± 0.08	1.19 ± 0.04	1.15 ± 0.06
16:3n-4	0.16 ± 0.05[a]	0.18 ± 0.04	0.20 ± 0.03	0.19 ± 0.02	0.15 ± 0.02	0.16 ± 0.01[a]	0.17 ± 0.03	0.20 ± 0.07	0.14 ± 0.02	0.33 ± 0.05
18:0	3.72 ± 0.16[a]	3.10 ± 0.17	3.62 ± 0.37	3.40 ± 0.17	3.09 ± 0.58	4.68 ± 0.22[b]	4.15 ± 0.11	3.98 ± 0.27	4.53 ± 0.18	4.16 ± 0.25
18:1n-9	2.95 ± 0.15[a]	3.23 ± 0.17	3.28 ± 0.15	1.87 ± 0.13*	1.67 ± 0.10*	3.27 ± 0.08[a]	3.23 ± 0.15	3.16 ± 0.24	3.17 ± 0.12	3.24 ± 0.12
18:1n-7	4.76 ± 0.22[a]	4.54 ± 0.35	4.93 ± 0.30	4.70 ± 0.32	4.61 ± 0.27	6.04 ± 0.14[b]	5.58 ± 0.17	5.51 ± 0.48	5.79 ± 0.23	6.45 ± 0.41
18:1n-5	0.19 ± 0.02[a]	0.18 ± 0.03	0.18 ± 0.03	0.21 ± 0.03	0.18 ± 0.02	0.22 ± 0.02[a]	0.22 ± 0.01	0.13 ± 0.01*	0.12 ± 0.01*	0.09 ± 0.00*
18:2n-6	2.07 ± 0.07[a]	2.37 ± 0.21	2.16 ± 0.08	1.44 ± 0.09*	1.58 ± 0.11*	2.31 ± 0.05[b]	2.27 ± 0.06	1.51 ± 0.08*	1.30 ± 0.08*	1.35 ± 0.08*
18:2n-4	0.29 ± 0.03[a]	0.24 ± 0.02	0.28 ± 0.03	0.28 ± 0.03	0.24 ± 0.01	0.36 ± 0.01[a]	0.32 ± 0.02	0.30 ± 0.04	0.35 ± 0.02	0.35 ± 0.03
18:3n-4	0.42 ± 0.05[a]	0.36 ± 0.06	0.40 ± 0.04	0.45 ± 0.04	0.37 ± 0.03	0.48 ± 0.04[a]	0.41 ± 0.04	0.42 ± 0.06	0.47 ± 0.05	0.55 ± 0.03
18:3n-3	2.05 ± 0.19[a]	2.08 ± 0.17	2.18 ± 0.20	2.15 ± 0.13	1.91 ± 0.14	2.82 ± 0.12[b]	2.81 ± 0.12	2.55 ± 0.29	2.68 ± 0.12	2.63 ± 0.25
18:4n-3	1.81 ± 0.35[a]	1.78 ± 0.18	1.89 ± 0.27	1.83 ± 0.13	1.53 ± 0.18	2.89 ± 0.14[b]	3.09 ± 0.16	2.85 ± 0.41	2.72 ± 0.19	2.90 ± 0.30
20:1n-11	5.04 ± 0.23[a]	4.83 ± 0.33	5.17 ± 0.12	5.29 ± 0.21	5.85 ± 0.13*	5.41 ± 0.19[a]	5.05 ± 0.28	4.85 ± 0.38	5.50 ± 0.21	4.83 ± 0.30
20:1n-9	1.57 ± 0.09[a]	1.47 ± 0.07	1.51 ± 0.04	1.51 ± 0.05	1.51 ± 0.01	1.36 ± 0.05[a]	1.33 ± 0.07	1.26 ± 0.08	1.23 ± 0.04	1.23 ± 0.02
20:1n-7	0.75 ± 0.07[a]	0.68 ± 0.03	0.67 ± 0.05	0.69 ± 0.04	0.72 ± 0.01	0.51 ± 0.03[b]	0.53 ± 0.04	0.53 ± 0.06	0.56 ± 0.06	0.50 ± 0.02
20:2NMID	0.44 ± 0.03[a]	0.37 ± 0.03	0.39 ± 0.03	0.39 ± 0.02	0.43 ± 0.03	0.35 ± 0.02[a]	0.36 ± 0.02	0.35 ± 0.03	0.38 ± 0.03	0.31 ± 0.02
20:2n-6	0.44 ± 0.02[a]	0.44 ± 0.01	0.43 ± 0.01	0.46 ± 0.02	0.45 ± 0.02	0.42 ± 0.02[a]	0.42 ± 0.01	0.41 ± 0.02	0.41 ± 0.01	0.40 ± 0.02
20:3n-6	0.24 ± 0.01[a]	0.22 ± 0.01	0.22 ± 0.01	0.25 ± 0.01	0.24 ± 0.00	0.19 ± 0.01[b]	0.19 ± 0.01	0.21 ± 0.01	0.19 ± 0.00	0.17 ± 0.01
20:4n-6	4.93 ± 0.38[a]	4.48 ± 0.27	4.58 ± 0.30	6.76 ± 0.28*	7.65 ± 0.34*	3.10 ± 0.21[b]	3.16 ± 0.15	3.62 ± 0.26	5.83 ± 0.44*	5.81 ± 0.27*
20:4n-3	0.64 ± 0.04[a]	0.61 ± 0.01	0.63 ± 0.03	0.67 ± 0.02	0.64 ± 0.02	0.80 ± 0.03[b]	0.75 ± 0.01	0.75 ± 0.02	0.72 ± 0.02	0.71 ± 0.05
20:5n-3	9.92 ± 0.13[a]	10.74 ± 0.23	9.50 ± 0.24*	9.68 ± 0.26*	9.82 ± 0.22*	8.99 ± 0.23[b]	9.07 ± 0.22	10.00 ± 0.48	8.77 ± 0.33	8.68 ± 0.37
22:2NMID	1.80 ± 0.13[a]	1.35 ± 0.13	1.59 ± 0.12	1.45 ± 0.08	1.71 ± 0.12	1.52 ± 0.11[a]	1.39 ± 0.16	1.34 ± 0.19	1.46 ± 0.15	1.25 ± 0.12
22:3NMIT	0.99 ± 0.16[a]	1.01 ± 0.13	1.02 ± 0.15	1.00 ± 0.15	1.05 ± 0.17	0.96 ± 0.13[a]	0.96 ± 0.13	1.03 ± 0.15	0.91 ± 0.14	0.92 ± 0.12
22:4n-6	0.70 ± 0.08[a]	0.60 ± 0.05	0.69 ± 0.06	0.70 ± 0.03	0.78 ± 0.02*	0.47 ± 0.04[b]	0.44 ± 0.05	0.46 ± 0.08	0.43 ± 0.03	0.40 ± 0.05
22:5n-6	3.78 ± 0.22[a]	3.79 ± 0.34	3.57 ± 0.30	3.70 ± 0.12	3.94 ± 0.08	2.67 ± 0.17[b]	2.56 ± 0.22	2.84 ± 0.39	2.58 ± 0.17	2.45 ± 0.21
22:5n-3	4.69 ± 0.43[a]	4.40 ± 0.36	4.82 ± 0.36	5.00 ± 0.18	5.13 ± 0.30	3.43 ± 0.18[b]	3.45 ± 0.25	3.32 ± 0.57	3.31 ± 0.11	3.09 ± 0.27
22:6n-3	13.07 ± 0.61[a]	13.86 ± 0.91	12.83 ± 0.93	13.46 ± 0.45	13.87 ± 0.64	10.09 ± 0.44[b]	10.05 ± 0.59	10.99 ± 1.24	10.05 ± 0.64	9.52 ± 0.61
SFA	28.16 ± 0.48[a]	28.88 ± 1.07	29.13 ± 1.09	27.98 ± 0.53	27.06 ± 0.86	31.91 ± 0.84[b]	32.36 ± 1.13	31.11 ± 0.89	31.51 ± 0.83	32.55 ± 1.41
MUFA	23.18 ± 0.50[a]	22.06 ± 0.76	23.26 ± 0.76	21.95 ± 0.43	21.28 ± 0.54	25.75 ± 0.25[b]	25.50 ± 0.47	25.55 ± 1.54	25.54 ± 0.50	25.32 ± 0.36
PUFA	9.71 ± 0.62[a]	9.35 ± 0.34	9.75 ± 0.53	8.88 ± 0.28	8.55 ± 0.37	11.62 ± 0.21[b]	11.52 ± 0.16	10.14 ± 0.63	10.17 ± 0.22	10.41 ± 0.54
n-3	3.86 ± 0.53[a]	3.86 ± 0.35	4.07 ± 0.46	3.98 ± 0.25	3.45 ± 0.31	5.71 ± 0.24[b]	5.89 ± 0.26	5.40 ± 0.68	5.39 ± 0.30	5.52 ± 0.55
n-6	2.51 ± 0.07[a]	2.81 ± 0.21	2.58 ± 0.08	1.90 ± 0.10	2.03 ± 0.12	2.73 ± 0.06[b]	2.70 ± 0.06	1.92 ± 0.09	1.71 ± 0.08	1.75 ± 0.09
HUFA	38.95 ± 1.46[a]	39.72 ± 1.90	37.86 ± 2.09	41.20 ± 0.80	43.11 ± 1.43	30.71 ± 0.96[b]	30.63 ± 1.39	33.21 ± 2.88	32.79 ± 1.22	31.73 ± 1.15
HUFA n-3	28.32 ± 0.89[a]	29.61 ± 1.33	27.78 ± 1.33	28.80 ± 0.50	29.46 ± 1.08	23.32 ± 0.71[b]	23.32 ± 0.94	25.06 ± 2.15	22.86 ± 0.93	21.99 ± 1.01
HUFA n-6	10.64 ± 0.62[a]	10.11 ± 0.66	10.08 ± 0.77	12.40 ± 0.35*	13.65 ± 0.41*	7.39 ± 0.36[b]	7.31 ± 0.48	8.15 ± 0.82	9.94 ± 0.55*	9.74 ± 0.18*
Total FAME (mg/g w.w.)	4.27 ± 0.54[a]	4.86 ± 0.16	4.76 ± 0.20	4.68 ± 0.44	5.05 ± 0.42	6.44 ± 0.59[b]	6.00 ± 0.67	5.78 ± 0.64	5.75 ± 0.30	7.40 ± 0.58
Total lipids (% of w.w.)	1.16 ± 0.08[a]	1.16 ± 0.06	1.05 ± 0.06	1.17 ± 0.08	1.31 ± 0.07	1.22 ± 0.13[a]	1.15 ± 0.12	1.28 ± 0.12	1.42 ± 0.09	1.54 ± 0.11*

Data is expressed as % of total fatty acids (mean ± SEM; n=6). *significant differences compared to controls using Dunnett's test($P < 0.05$). Different letters indicate significant differences between males and females using Student's t-test ($P < 0.05$).

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Paper 5

EVIDENCE OF ENDOCRINE DISRUPTION IN A ZEBRA MUSSEL POPULATION FROM THE EBRO RIVER

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Resumen

Se muestrearon mejillones cebra (*Dreissena polymorpha*) entre octubre 2008 y septiembre 2009 en dos estaciones a lo largo del tramo bajo del río Ebro (España) con el objetivo de investigar la existencia de efectos de disrupción endocrina. Se detectaron diferentes alteraciones (cambios significativos en estadios reproductivos claves, desarrollo gonadal y hepatopancreático y niveles de andrógenos) en mejillones muestreados aguas abajo de una planta de cloro-álcali que sugiere fuertemente que la presencia de contaminantes en el área fue el primer factor causal. En comparación con la estación de referencia (Riba Roja), el comienzo de la gametogénesis ocurrió 2-3 meses más tarde, y el periodo del descanso gonadal se prolongó significativamente (2 meses), mientras que el periodo de la puesta se redujo (2 meses). Simultáneamente, el análisis de las imágenes de las preparaciones histológicas demostraron un retraso en el desarrollo gonadal, una disminución significativa del tamaño gonadal y cambios anómalos del tamaño del hepatopáncreas, con efectos más fuertes en machos. Además, se detectó una reducción de la testosterona (2-3 veces) que resultó en una alteración del ratio estradiol/testosterona que fue 2-3 en Riba Roja y 3-6 en Flix incluso alcanzando 10 en los meses de octubre y noviembre. Las análisis de los ácidos grasos demostraron cambios significativos en los ácidos grasos esenciales 20:5n-3 y 22:6n-3 que son conocidos por estar relacionados con eventos reproductivos. En general, este trabajo provee la primera evidencia de la existencia de alteraciones significativas en el ciclo reproductivo de mejillones cebra de la sección río aguas abajo de la planta de Flix y puede también demostrar una relación estrecha entre la regulación de los niveles hormonales, de ácidos grasos y de la reproducción.

Abstract

Zebra mussels (*Dreissena polymorpha*) were collected between October 2008 and September 2009 from two sites along the lower course of the Ebro River (Spain) with the aim of investigating the existence of endocrine-disrupting effects. Several alterations (significant changes within key stages of the reproductive cycle, gonad and hepatopancreas development, and androgen levels) were detected in mussels collected downstream a chlor-alkali plant (Flix) which strongly suggest that the presence of contaminants in the area is a major causal factor. In comparison to reference site (Riba Roja), onset of gametogenesis occurred 2-3 months later and period of gonadal arrest was significantly increased (2 months) while spawning period was strongly shortened (2 months). Concomitantly, image analysis of histological preparations showed delay in gonad development and a significant decrease in gonad size which was accompanied by aberrant hepatopancreas size changes with effects being stronger in males. In addition, testosterone levels were significantly depressed (2-3 times) resulting in a significant alteration of the annual estradiol/testosterone ratio which was 2-3 at Riba Roja and 3-6 at Flix even reaching 10 in the months of October and November. Fatty acid analysis showed significant changes within the essential 20:5n-3 and 22:6n-3 fatty acids which are known to be related to reproductive events. Overall, this work provides the first evidence of the existence of significant alterations in the reproductive cycle of zebra mussels from the downstream section of the Flix plant and may also evidence a close relation between the regulation of hormone and fatty acid levels and reproduction.

Introduction

In recent years components of domestic sewage and several industrial chemicals were shown to be capable of disrupting the endocrine systems of a wide range of wildlife species. Those so called endocrine disruptors (EDs) may represent one of the biggest threats to the integrity of aquatic ecosystems today (Segner et al., 2003; Sumpter and Johnson, 2005). Many known EDs are estrogenic and therefore may specifically target the reproductive hormone-receptor system which seems to be particularly vulnerable. This has been demonstrated in fish, where exposure to EDs caused abnormalities in reproductive tissues like inhibition of testicular growth (Jobling et al., 1996), reduced ovarian development (Stott et al., 1983) and formation of testis-ova (intersex) (Gray and Metcalfe, 1997). In addition, effects on hormones have been reported showing altered sex steroid titers of testosterone, androstenedione and estradiol (Dubé and MacLatchy, 2000; Monteiro et al., 2000a,b). However, most data on the biological effects and mechanisms of action of EDs on aquatic organisms come from studies on vertebrates. The lack of invertebrate studies on this subject is largely due to the limited knowledge on the endocrine physiology of many invertebrate groups that represent important components of the aquatic systems. This is especially alarming considering that several compounds have already been identified as EDs in molluscs like organotins (Ronis and Mason, 1996; Oberdörster and McClellan-Green, 2002), xenoestrogens such as nonylphenol and pentachlorophenol (Blaise et al., 1999) as well as octylphenol and bisphenol A (Oehlmann et al., 2000). Studies with the ramshorn snail *Marisa cornuarietis* showed that exposure to TPT can significantly alter levels of esterified testosterone and estradiol (Lyssimachou et al., 2008). In the gastropods *Hexaplex trunculus* and *Bolinus brandaris*, organotins altered androstenedione metabolism by interfering with key enzymatic pathways involved in androgen synthesis (Lyssimachou et al., 2009). Furthermore, Duft et al. (2007) was able to demonstrate a high susceptibility of prosobranch snails to a broad spectrum of well-known androgenic/estrogenic contaminants. The study showed that responses to androgens comprised the development of imposex and the reduction of fertility or embryo production, while estrogens stimulated egg and embryo production, and increased the weight of glands. Endocrine disruption has also been described in wild bivalve populations collected in high urban and industrial areas (Pampanin et al., 2005) or streams receiving sewage and domestic effluents (Blaise et al., 2002; Quinn et al.,

2004). These and other observations indicate that endocrine disruption is already occurring in invertebrate populations, but as yet, with the exception of organotin effects in gastropods, data from systematic field investigations are still unavailable

The need to detect endocrine disruption *in situ* and the provision of an early warning system for ecologically relevant effects in invertebrates is pressing. Several biomarkers have already been developed to detect endocrine disruption in aquatic organisms like the induction of vitellogenin in fish (Purdom et al., 1994; Allen et al., 1999) or the development of ovotestis in lobsters (Sangalang and Jones, 1997) which are good indicators for exposure to xenoestrogens. In invertebrates like echinoderms (Khristoforova et al., 1984), crustaceans (McKenney and Matthews, 1990) and bivalves (Kluytmans et al., 1988) the reproductive cycle has been shown to be a potential target for EDs where impairment of gonadal follicle development, alterations of steroid metabolism in the gonads and cessation of larval development were detected. Similarly, hormones are involved in the control of biochemistry, physiology and behavior of all invertebrate species studied so far, and are also an important constituent of the endocrine system. Alterations of steroid synthesis and metabolism in field populations of mollusc species have already been attributed to environmental contaminants especially organotin compounds (Morcillo and Porte, 2000; Santos et al., 2002). Besides the latter, field studies investigating the impact of EDs on steroid levels in molluscs are lacking partially because of their strong seasonal variations which are largely controlled by environmental factors but also reproduction (Gooding and LeBlanc, 2004). Thus, the usage of steroid levels as biomarkers in field studies may require good knowledge of their characteristic annual profile which is largely species specific but may also be influenced by still unknown endogenous and exogenous factors. However, once established, the measurements of sex steroids may provide a very useful approach for the detection of endocrine disrupting chemicals in the environment.

In this context, the present work was designed to investigate two natural occurring zebra mussel populations from the lower course of the Ebro River situated in close geographical vicinity. One site is situated in a highly contaminated area of the Flix reservoir where a chlor-alkali plant is operating since over a century. Its long existence and operation led to a significant water and sediment contamination of the river including a large part of the downstream section. Water and sediment studies of this area demonstrated the presence of several organic and inorganic pollutants like

polychlorinated compounds and heavy metals (Ramos et al., 1999; Lacorte et al., 2006; Bosch et al., 2009). Furthermore, organisms collected in vicinity of the chemical plant showed high tissue levels of several contaminants including polychlorobiphenyls (PCB), hexachlorobenzenes (HCB), DDT congeners (Elijarrat et al., 2008; Faria et al., 2010) and high amounts of mercury (Carrasco et al., 2008) indicating a considerable bioaccumulation within the aquatic biota. The second study site is situated only a few kilometers upstream within a relative uncontaminated region of the river. The short distance between both sites provided a unique opportunity to study the development of two zebra mussel populations during a period of one year and to assess possible effects of environmental contaminants on hormone levels and the reproductive cycle.

Several endpoints were observed in this study which included the main reproductive events and their seasonal progression, annual volume changes of the gonad and the hepatopancreas together with expression levels of the sex steroids testosterone and estradiol. Furthermore, annual lipid levels and fatty acid composition were measured and their potential as possible markers for the assessment of environmental contaminants capable to interfere with lipid metabolism evaluated. Therefore, this work might provide new insights into the usage of specific endpoints in the sentinel species *Dreissena polymorpha* to detect effects of environmental pollution in field populations.

Material and methods

Sample collection and preparation

Mussels were collected from 2 stations along the Ebro River: (a) Riba Roja, in a relatively uncontaminated region; (b) Flix, located a few hundred meters downstream of the discharge area of the local chlor-alkali plant (Figure 1). Both stations are situated within a distance of 6-8 km of the river basin. Zebra mussels (*Dreissena polymorpha*) were sampled monthly between October 2008 and September 2009 with exceptions due to weather conditions and flooding. Mussels with a mean length of 20.9 ± 2.3 mm (Riba Roja) and 18.5 ± 1.6 mm (Flix) were harvested by hand at a water depth of approx. 0.5 meter and transported in buckets filled with river water to a nearby laboratory where they were immediately dissected. Water temperature, pH, conductivity and concentration of dissolved O_2 (CRISON-Oxi 45P/CRISON-CM 35) were measured. In the laboratory, gills were separated from the rest tissue and samples then deep frozen in

liquid nitrogen and stored at -80 ºC. For histological analysis, 20 entire animals were fixed in 10% buffered formaldehyde (0.1 M phosphate buffer, pH 7.4) for 48 h. Samples were subsequently rinsed with water and stored in 70% ethanol.

Histological analysis

Individuals were dehydrated in graded ethanol, cleared with Histo-Clear II (National Diganostics, Hessle Hull, England), and embedded in paraplast (Sigma). Sections (7 μm) were stained with hematoxylin-eosin y, mounted and examined by light microscopy. Individuals were classified in five different reproductive stages as described by Juhel et al. (2003). Briefly, Stage 0: sexual rest, inactive or undifferentiated; stage 1: immature gametes (spermatogonia/oogonia) visible, early development; stage 2: follicles contain mainly ripe gametes, late development; stage 3: fully mature gonads, almost no reserve cells, spawning; stage 4: reabsorbing gonads, disorganized and mostly empty, few remaining gametes.

Image analysis of histological cross-sections

All histological slides were first examined under a light microscope to determine sex and reproductive stage. Tissue images were then captured using a Digital Sight-Ri1 208 (Nikon) digital camera mounted on an Eclipse 90i (Nikon) light microscope using a magnification of 20x. The purpose was to determine variations in gonad and hepatopancreas size during the annual reproductive cycle at both sampling sites. Therefore, the areas occupied by the gonad and the hepatopancreas were determined quantitatively by using the method described in Kang et al. (2003, 2010). Briefly, for each mussel, gonad area and hepatopancreas area were measured at 3 randomly selected cross-sections using ImageJ image analyzing software. Percentage of gonad area (PGA) and percentage of hepatopancreas area (PHA) was determined by calculating the ratio of each area in relation to the total area of the cross-section. Average values for each mussel were calculated and animals then pooled by sex and gonadal stage. In this way an average of 80-90 animals were analyzed for each sampling site.

Tissue steroid levels

Testosterone and estradiol were analyzed as described in Janer et al. (2005). Briefly, tissue samples (0.25–0.35 g wet weight) were homogenized in ethanol, and frozen

overnight at −80 °C. Homogenates were then thawed and extracted tree times with 2 ml of ethyl acetate, the organic extracts recombined and reduced under a nitrogen stream. Dry extracts were then resuspended in 1 ml methanol containing 1% KOH, and incubated at 45 °C for 3 h. After the saponification step, Milli-Q water (4.0 ml) was added, and the samples extracted 3 times with 3 ml dichloromethane and dried under nitrogen. The efficiency of the extraction was 74 ± 3% for testosterone and 80 ± 3% for estradiol (Morcillo et al., 1999). Dry extracts were resuspended in 50 mM potassium phosphate buffer pH 7.6 containing 0.1% gelatine, and assayed for testosterone and estradiol concentration using commercial RIA kits (Radim, Rome, Italy). Standard curves with the steroids dissolved in the same phosphate buffer were performed in every run. The limits of detection of the assay were 25 pg/g for testosterone (T) and 20 pg/g for estradiol (E2). Intra-assay coefficients of variation were of 6.1% (T) and 3.3% (E2). Inter-assay coefficients of variation were of 9.3% (T) and 3.5% (E2).

Total lipid and fatty acid analysis

Lipids were extracted by a modification of the method of Folch et al. (1957). Each sample, which consisted in a pool of 2-3 individuals without gills, was homogenized in an ice-cold solution of chloroform:methanol (2:1 v/v) containing 0.01% butylated hydroxytoluene (BHT) as antioxidant. After centrifugation, 0.88% KCl was added to the obtained supernatant (1:4 v/v) and then thoroughly mixed. The chloroform layer was collected, filtered (Whatman filter paper) and the solvent evaporated under nitrogen. The dried lipid content was determined gravimetrically (Sartorius, BP210D, 0.01 mg) and kept at -20 ºC for fatty acid analysis.

For the analysis of fatty acids, 1 mg of total lipids dissolved in chloroform:methanol was mixed with nonadecanoic acid (19:0) (internal standard) and dried under a gentle stream of nitrogen. The dried residue was saponified with 2 N KOH/MeOH/10% Milli-Q water for 24 h at room temperature, and afterwards neutral lipids were removed by vigorous shaking with 1 ml of n-hexane (x3). Samples were further acidified to pH ~3 with HCl and fatty acids extracted with 1 ml of n-hexane (x3) and concentrated by vacuum rotary evaporation. The fatty acid fraction was then methylated with a solution of 10% boron trifluoride-methanol (Fluka) for 24 hours at room temperature. The methylated fraction was resuspended in 10% NaCl and fatty acid

methyl esters (FAME) extracted with 1 ml of n-hexane (x3), transferred to vials, evaporated to near dryness under a gentle stream of nitrogen, and analyzed by a gas chromatography-flame ionization detector (GC-FID). The instrument was a Thermo Finnigan Trace GC. The column, a DB-23 fused silica 30 m x 0.25 mm i.d., film thickness 0.25 μm (Agilent J&W). The oven temperature program was from 60 to 150 °C at 15 °C/min, then from 150 to 230 °C at 3 °C/min, keeping the final temperature for 10 min. The carrier gas was helium at a constant flow-rate of 1.5 ml/min. The injector and the detector temperature were set at 280 and 300 ºC, respectively.

Statistical analysis

Data is reported as mean ± SEM. Statistical treatment of data was performed by using the SPSS statistical package (SPSS v.17.0, IBM Company, Chicago, USA). Differences in individual fatty acids and fatty acid groups, PGA and PHA, as well as sex steroid levels between reference and contaminated site were determined using Student's t-test ($p<0.05$). Correlations between total lipid and total FAME content of the mussels were examined by Pearson's correlation coefficient ($p<0.001$). Significant changes in annual lipid and FAME levels, PGA and PHA as well as sex steroid levels were determined using Tukey's multiple comparison test.

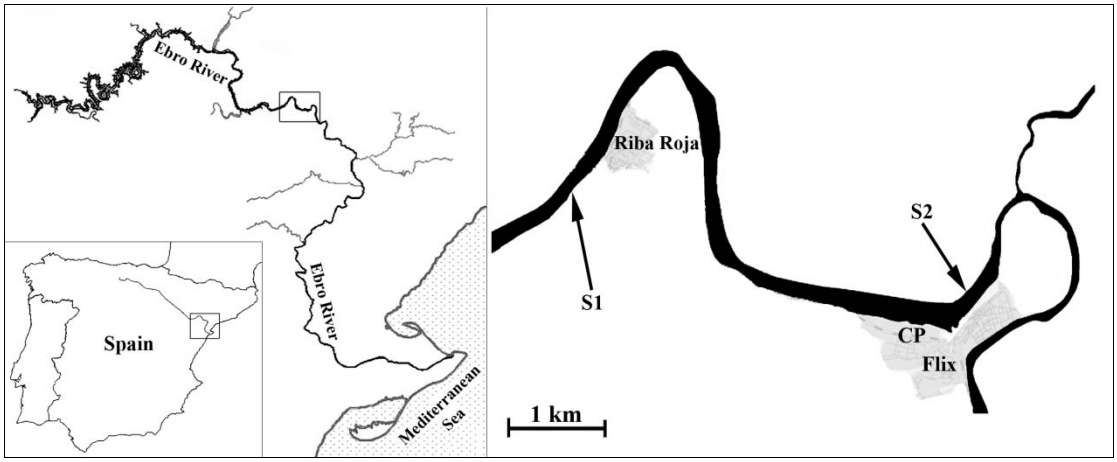

Figure 1. Map showing the sampling sites located in the lower course of the Ebro River: Riba Roja (S1), Flix (S2). CP: Chlor-alkali plant.

Results

Histological analysis of the gonad allowed the identification of 5 maturation stages which were grouped as follows: gametogenesis (stage 1+2), spawning (Stage 3) and resting phase (Stage 0+4) (Figure 2). Gametogenesis started in October and had a peak in January at Riba Roja and in April at Flix (90%-100% of animals in stage 1 and 2). The population at Riba Roja mostly concluded gamete development in April, with the major part of the individuals being in the spawning until end-July. In contrast, most of the organisms at Flix were in the gametogenic stage from January to May, which was followed by a comparatively short spawning period with 100% of individuals being in this stage in June and July. Both populations entered the gonadal resting phase in September which lasted until November at Riba Roja and until January at Flix. In October and November, a large part of the animals were still in the resting phase while a smaller portion had already restarted gamete development. Considering that water temperature was similar at both sampling sites, at Riba Roja, spawning began in spring when water temperatures reached 12 ºC whereas the population at Flix entered spawning in early-summer when water temperatures had already surpassed 20 ºC.

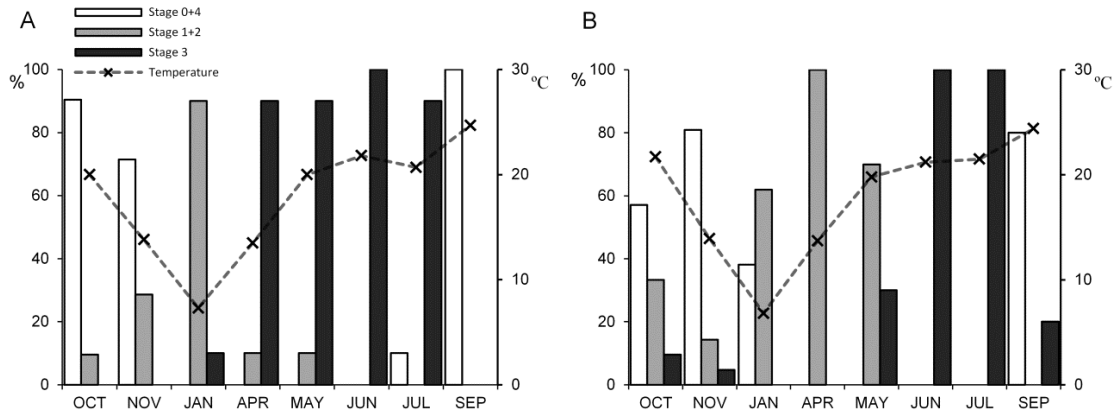

Figure 2. Different sexual maturation stages detected in the gonads of zebra mussels from Riba Roja (A) and Flix (B) over the sampling period. Stage 0+4: Indicates the percentage of individuals having gonads in the resting phase; Stage 1+2: Percentage of individuals with gonads in gametogenesis; Stage 3: Percentage of individuals in spawning. Number of individuals analyzed: 10-20.

Image analysis of PGA and PHA revealed significant variations of gonad and hepatopancreas size between different maturation stages, gender as well as between sampling sites (Table 1). At Riba Roja PGA significantly increased for approx. 30-40% in both sexes when reaching maximum size (stage 2 and 3) in comparison to stages 0, 1 and 4. At the same time PHA significantly decreased during stage 2 and 3 for approx. 10% in both sexes while maintaining a relatively constant size (21-27%) during the other stages. No significant differences in variations of annual PGA and PHA within same maturation stages were seen between both sexes. Similar to Riba Roja even though to a lesser extent, at Flix PGA significantly increased during stages 2 and 3 in both sexes for approx. 20-30% in comparison to stages 0, 1 and 4. However, gonad increase in stage two was considerably lower in comparison to Riba Roja indicating a delayed gonadal maturation process as well as a lower maximum gonad size. PHA remained relatively stable between 20-25% with the exception of a significant increase between stage 1 and 2 in males and a significant decrease in stage 3 in females. In contrast to Riba Roja, significant differences between sexes were seen at Flix for PGA at stage 0 which was significantly higher in females and PHA at stage 2 and 3 which was significantly higher in males. Thus, annual PGA was generally greater in females while annual PHA was greater in males.

Table 1. Changes in percentage of gonad and hepatopancreas area at different gonad maturation stages of zebra mussels from Riba Roja and Flix site. Data is presented as mean ± SEM (n=6-12).

		Males		Females	
	Gonadal Stage	% Gonad area	% Hepatopancreas area	% Gonad area	% Hepatopancreas area
Riba	0	18.39 ± 1.72	21.32 ± 1.78	18.71 ± 1.67	25.65 ± 1.60
Roja	1	24.12 ± 2.69	27.66 ± 2.58	25.29 ± 2.98	25.33 ± 2.17
	2	60.49 ± 3.84	17.01 ± 2.00	57.86 ± 5.61	13.09 ± 2.40
	3	63.11 ± 3.33	12.49 ± 1.89	63.44 ± 3.11	14.99 ± 1.98
	4	27.57 ± 3.51	24.32 ± 3.64	28.84 ± 1.92	21.39 ± 2.61
Flix	0	10.32 ± 1.43	24.68 ± 2.10	16.39 ± 1.77	22.18 ± 1.43
	1	17.36 ± 1.33	20.25 ± 1.61	19.79 ± 2.07	21.87 ± 2.50
	2	29.07 ± 1.98	30.37 ± 1.97	28.87 ± 3.16	22.74 ± 2.87
	3	43.96 ± 3.29	24.17 ± 1.79	49.77 ± 3.97	14.42 ± 2.20
	4	8.07 ± 0.62	22.91 ± 2.40	14.33 ± 3.12	25.96 ± 3.62

When comparing both sites, significant differences were seen in PGA for all maturation stages in males and for stages 2, 3 and 4 in females. In comparison to Riba Roja, PGA at Flix was 44%, 28%, 52%, 30% and 71% lower in males and 12%, 22%, 50%, 22% and 50% lower in females for stages 0, 1, 2, 3 and 4, respectively. PHA was significantly different at stage 2 and 3 in males and at stage 2 in females. Thus, in comparison to Flix, PHA at Riba Roja was 40%-48% lower in stage 2 and 3 in males and 42% lower in stage 2 in females.

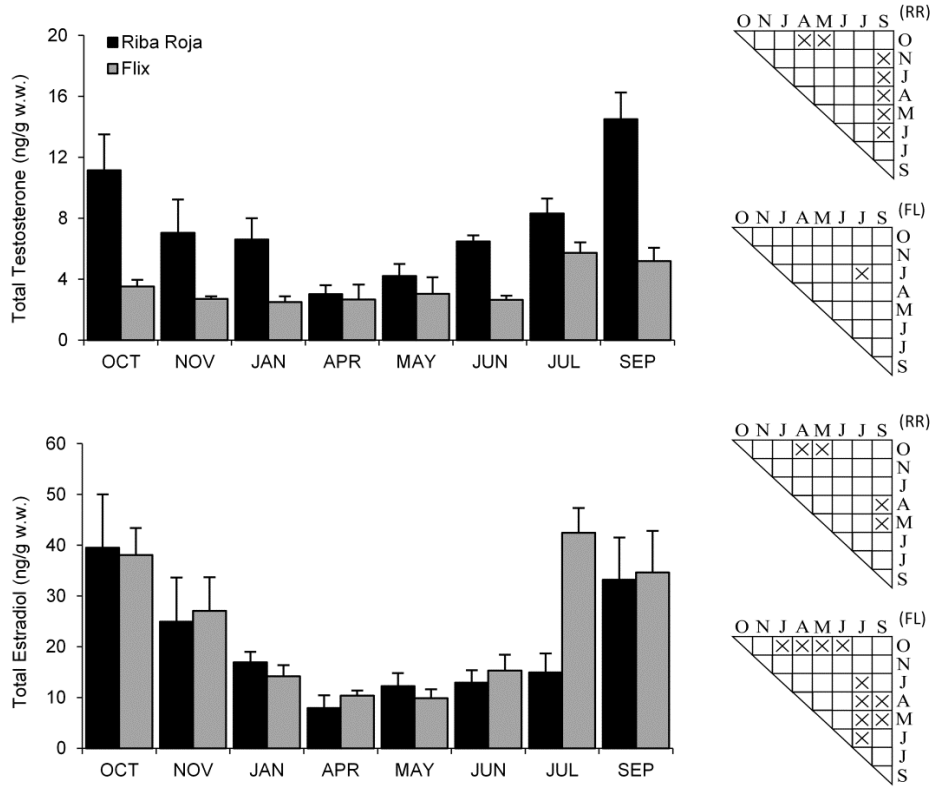

Figure 3. Seasonal variations in levels of total testosterone and estradiol in tissue extracts of zebra mussels. Data is presented as mean ± SEM (n=5). ^{x} denotes significant difference in annual variation levels using Tukey's test ($p<0.05$); Riba Roja (RR), Flix (FL).

Endogenous levels of total testosterone and estradiol showed significant differences in annual concentrations as well as differences among sampling sites (Figure 3). Total testosterone was in the range between 3.0 and 14.5 ng/g w.w. at Riba Roja, the highest concentrations were detected in September and October and the lowest in April. In contrast, at Flix total testosterone was in the range between 2.5 and 5.7 ng/g w.w, demonstrating highest values in July and lowest in January. Annual testosterone levels were significantly different between both sites ($p<0.01$) and in average 2-3 times higher at Riba Roja than at Flix. Highest differences were observed for the period between

October to January and June to September whereas levels in April and May were within the same range. Annual variations at Riba Roja followed a clear sigmoidal profile showing a constant decrease from October until April from where levels incrementally increased until reaching highest values in September. At Flix on the other hand, annual levels were constant between October and June which were followed by a strong significant increase in the month of July. Total estradiol levels were between 7.9 and 39.5 ng/g w.w at Riba Roja and between 9.9 and 42.5 ng/g w.w at Flix. At Riba Roja, highest levels appeared in October and lowest in April whereas at Flix, highest levels were measured in July and lowest in May. In contrast to total testosterone, annual estradiol concentrations were strikingly similar between both sites with the exception of July where levels were almost 3 times higher at Flix. Likewise, annual variations showed a similar profile for both sites following a sigmoidal progression with lowest levels between January and June and significant increases between July and October at Flix and September and October at Riba Roja. Due to significant differences in total testosterone, annual estradiol/testosterone ratios differed significantly for both sites, with an average of 2-3 at Riba Roja and 3-6 at Flix where months like October and November even showed ratios of 10.

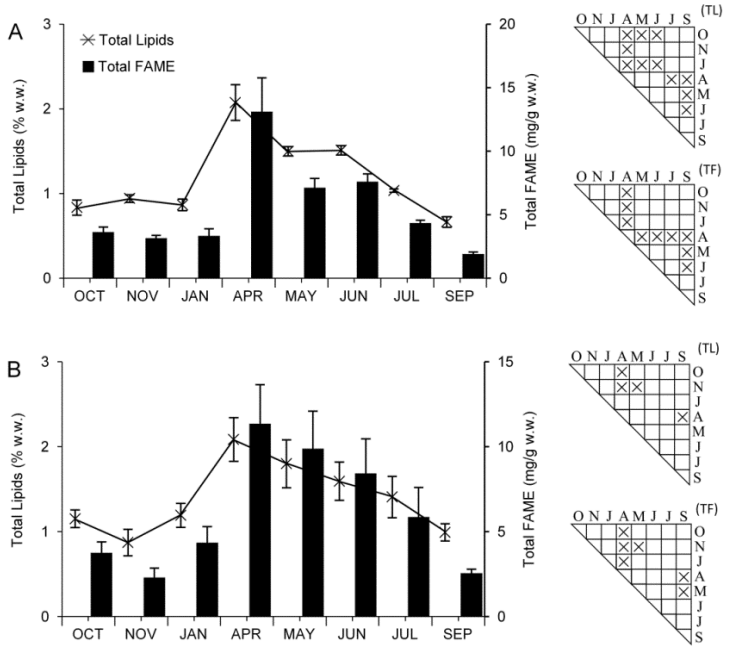

Figure 4. Annual variations of total lipid and total FAME levels in zebra mussels collected from (A) Riba Roja and (B) Flix. Data is presented as mean ± SEM (n=3). ^{x} denotes significant differences in annual variation levels using Tukey's test ($p<0.05$); Total lipids (TL), Total FAME (TF).

Measurement of total lipids showed a range between 0.66-2.07% w.w. at Riba Roja and between 0.87-2.09% w.w. at Flix. Although variations occurred within a narrow range, annual changes between different months were of statistical significance ($P<0.05$) for both sites (Figure 4). A clear seasonal trend was observed that was very similar at both sampling sites. Levels were relatively low between October and January and then significantly increased to peak in April from where they then gradually decreased to reach lowest levels in September at Riba Roja and in November at Flix. No significant differences of annual lipid levels between both sites were detected. Total FAME levels were between 1.9 – 13.2 mg/g w.w. at Riba Roja and between 2.3–11.3 mg/g w.w. at Flix (Figure 4). As expected, annual variations of total FAME levels were strongly related to total lipids (Riba Roja, $r^2=0.974$; Flix, $r^2=0.982$; $P<0.001$), hence, highest levels during April and lowest during September and November. However, coefficients of variation showed differences between total lipids and total FAME which were 40.3% and 65.9% at Riba Roja, and 30.0% and 56.8% at Flix, respectively. Differences were most pronounced in the months between April and June where average levels of total lipids increased 1.5-2 times whereas total FAME increased 3-4 times in comparison to preceding months. This may indicate a change in lipid composition during those months caused by a shift towards a higher abundance of fatty acids.

Fatty acid composition in both populations of zebra mussels showed a profile typically found in mussels feeding mainly on phytoplankton with polyunsaturated fatty acids as dominating fatty acid group and palmitic acid (16:0), palmitoleic acid (16:1n-7), 20:4n-6 (arachidonic acid), 20:5n-3 (eicosapentaenoic acid) and 22:6n-3 (docosahexaenoic acid) as most abundant fatty acids as shown in table 2. Among the most abundant fatty acids were 20:4n-6, 20:5n-3 and 22:6n-3 with relative abundances of respectively 3-10%, 6-12% and 8-12% at Riba Roja, and 3-9%, 7-12% and 7-11% at Flix. These fatty acids are considered essential and therefore were of special interest because of their high physiological importance (Xu et al., 1994). Annual progression of 20:4n-6 was very similar at both sites in contrast to 20:5n-3 and 22:6n-3 fatty acids which showed distinct differences in their patterns (Figure 5). At Riba Roja, 20:5n-3 showed a peak during the onset of spawning and then gradually decreased until reaching lowest values in September. Interestingly, at Flix the same peak was observed during April but an additional peak appeared in July which resulted in higher levels of 20:5n-3

during late-summer. Furthermore, 22:6n-3 demonstrated relative constant levels during November and June which then decreased during July and October at Riba Roja. At Flix, 22:6n-3 levels decreased already between November and May and then plateaued until September. In addition, significant differences in expression levels between the two populations were also seen for several other fatty acids as shown in table 2. Amongst those the 16:1n-7 and 18:1n-9 fatty acids, which are involved in energy-type functions, were significantly more abundant at Flix (10-20%) as well as the 22NMIT fatty acid which showed higher levels at Riba Roja (30%).

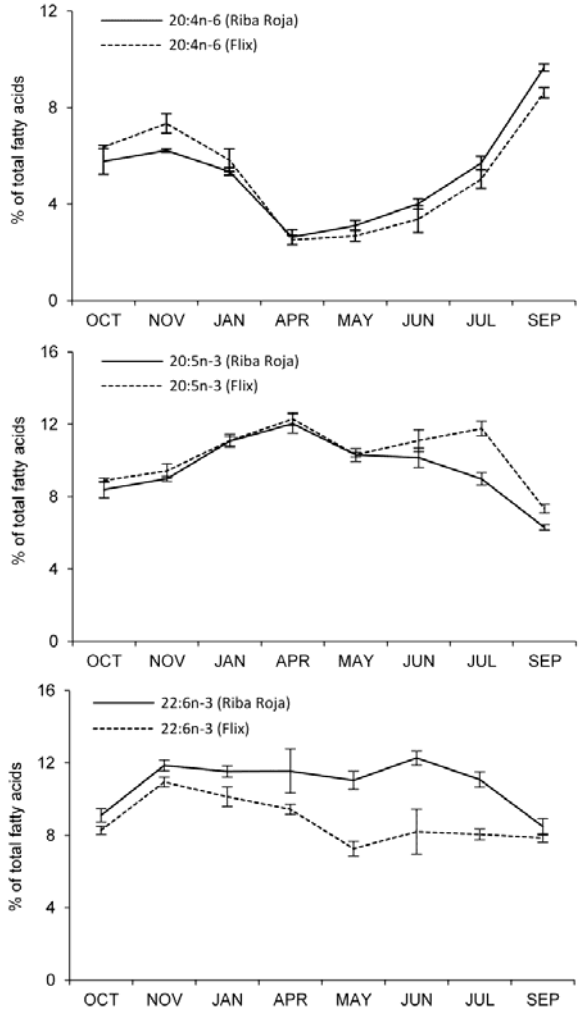

Figure 5. Annual variations of the essential fatty acids 20:4n-6, 20:5n-3 and 22:6n-3. Data is presented as mean ± SEM (n=3).

Discussion

Significant differences in the progression of key reproductive events such as gametogenesis and spawning were observed between populations collected from Riba Roja and Flix. Organisms from Riba Roja showed a peak in gametogenesis (90% of animals in stages 1+2) in January which coincided with the period of lowest water temperatures, while only 60% of the population at Flix was in the same development stage at that time. In April spawning began at Riba Roja where 90% of the animals started to release gametes whereas 100% of the animals at Flix were still in gametogenesis which lasted until May where a minor fraction of the population began to spawn. The main spawning event (100% of animals) occurred during the months of June and July at Flix, and between April and July at Riba Roja. In September both populations had entered the resting period which lasted until November at Riba Roja and until January at Flix. Hence, the main spawning period comprised 4 months at Riba Roja and only 2 at Flix indicating a significant reduction. In addition, for the major part of the population at Flix gametogenesis started approx. 2-3 months later and the resting period was significantly prolonged. Besides being in disagreement with the reference population, the reproductive cycle of the population from Flix also showed an annual progression which did not follow the typical variations in water temperature as established in other works with zebra mussels. Hence, main gamete development progressed during a period with significant higher water temperatures (~14 °C) as mentioned for most European and North American populations (<10 °C) (Borcherding, 1991; Mackie, 1991; Haag and Garton, 1992). Consequently, onset of spawning occurred at a temperature range (20-22 °C) which was much higher as described for several German populations (12 °C) or for most European populations (11-17 °C) in general (Borcherding, 1991; Jantz and Neumann, 1998; Sprung, 1992). Therefore, our work may show a considerable aberration from the natural progression of the reproductive cycle which in zebra mussels closely follows water temperature (Ram et al., 1996; Juhel et al., 2003; Wacker and von Elert, 2003).

Image analysis of histological cross-sections allowed the determination of the annual PGA and PHA development. According to the Delesse principle (1848; as cited by Coward and Bromage, 2002), the ratio of areas of the individual components equals the ratio of their composition by volume. Thus, the observed changes in gonadal development in both populations would coincide with other works on zebra mussels by

Borcheding (1991) who established a gonad index (volume of gonad divided by total volume of the visceral sack, consisting of most internal organs) to determine annual percentage changes of gonad volume, which ranged from 25% to as high as 70%. The gonad in bivalves normally shows a characteristic annual progression where the size gradually increases during gametogenesis until reaching a peak at time of spawning from where gonad size strongly decreases to reach minimum extension in the post-spawning period. The same cycle of annual variations in gonad mass with a peak during late gametogenesis and spawning have also been reported for scallops (Barber and Blake, 1991). Therefore, our study showed a gonadal development for both populations that followed the typical annual pattern reported in bivalves however with significant site specific differences. The most important changes in the Flix population were delay in gonad size development, significant lower total gonad size and significant differences between genders. Differences with the reference population were especially obvious in stage 2 where population from Riba Roja had almost reached full gonad extension (91-95% of maximum size) whereas population from Flix showed a significant delayed gonadal development (58-66% of maximum size). In addition, at Flix maximum gonad size was only 70-78% of that observed at Riba Roja for both sexes whereas annual size differences between populations were greater in males than in females for all stages. Thus, gonad development at Flix was not only significantly delayed but also maximum gonad size and total annual gonad development was strongly reduced with effects being more pronounced in males than in females. Furthermore, annual hepatopancreas development at Riba Roja varied inversely with that of the gonad; an effect which was not observed in males and only to a minor degree seen in females from Flix. Concomitant changes in both organs have already been reported in scallops where a reciprocal relationship between digestive gland and gonad weight was seen (Sastry, 1966; Fuji and Hashizume, 1974). Similar observations have been made in the blue mussels *Mytilus edulis* and *Mytilus trossulus* where, under the influence of the gametogenic cycle, digestive gland index decreased before spawning due to the utilization of the lipids and glycogen reserves stored in the digestive gland (Cartier et al., 2004). Due to the high nutritional requirement during gamete development, energy storage cycle of glycogen and lipids is a vital factor during gametogenesis (Gabbott, 1976). As a result, lipid levels in digestive gland strongly increase in several species prior to gametogenesis and decrease during gamete maturation (Pollero et al., 1979; Barber and Blake, 1981; Robinson et al., 1981). The transfer of material between

different body compartments especially during gonad growth and maturation has already been confirmed through the existence of increased plasma lipid transport via the hemolymph (Thompson, 1977). At Flix, male organisms showed an annual hepatopancreas development very different from what would normally be expected. Even though gonad size increased significantly during stage 2 and 3, the inverse development of the hepatopancreas did not occur but rather a further increase was noted in stage 2. The reason for the aberrant development of the hepatopancreas can only be speculated at the moment but may be strongly associated with the reduced development of the gonad. It would be obvious that a reduction in overall gonad development should significantly reduce energy demand which would result in a lower consumption of reserves stored in the hepatopancreas. The fact that the observed effects were more pronounced in males than in females (which showed a significant decrease in stage 3) may be explained through the increased demand of lipids during oogenesis and especially vitellogenesis which are mostly stored in the hepatopancreas and transferred to the gonad during gametogenesis (Barber and Blake, 1985; Pazos et al., 1996, 1997).

Determination of total steroids showed that testosterone levels were generally 2-3 times higher at Riba Roja than those found at Flix. Surprisingly, estradiol levels from both populations showed similar seasonal variations with exception of July where levels were significantly higher at Flix. As a consequence of the significant reduction in total testosterone, the ratio between estradiol and testosterone (E/T) was significantly altered. Hence, the population at Riba Roja showed a stable annual average E/T ratio between 2-3, where in comparison, the Flix population showed a more unsteady ratio between 3-6 and between the months of October and November even a ratio of 10. Several studies indicated that the estrogen to androgen ratio is vital for proper sexual differentiation in developing animals and alterations may result in incomplete or improper gonadal development like demonstrated in fish (Orlando et al., 2004) and gastropods (Bettin et al., 1996). Indeed, previous studies on wild carps (*Cyprinus carpio*) from the Flix area demonstrated an increased E2/T ratio in males (2-4 times higher) when compared to carps from the reference site (downstream of city of Lérida) which resulted from depressed plasma testosterone levels (up to 5-fold lower) (Lavado et al., 2004). The study also demonstrated arrested gametogenesis in males and delayed maturation in females. Moreover, works on *Dreissena polymorpha* showed that endocrine disrupters with estrogenic potential induced a significant increase in the levels of vitellin-like

proteins resulting in deleterious effect on gonadal development especially in male organisms, *viz*. increased proliferation of interstitial tissue (Quinn et al., 2004). In more recent studies Yan et al. (2011) postulated the importance of testosterone and estradiol as endogenous modulators for sex determination, development and sexual maturation in the razor clam *Sinonovacula constricta*. The study showed peaks of testosterone in males and of estradiol in females during spawning, resulting in significant changes in androgen/estrogen ratio which would suggest its importance in the regulation of gamete release. In addition, Sakr et al. (1992) reported that testosterone accelerated gamete development in males of the land snail *Theba pisana* demonstrating its importance in gamete development. Hence, misbalanced androgen/estrogen ratio may have caused alterations in key reproductive stages as well as the detected delay in gonad maturation, while depressed testosterone levels would have contributed to the more pronounced decrease in annual gonad volume in males from the Flix population.

Total lipids extracted from the whole body tissue of zebra mussels showed an equal seasonal progression at both sites with annual levels between 0.6-2% wet weight which are in agreement with other studies on bivalve molluscs (De Moreno et al., 1980; McLean and Bulling, 2005). Lipid levels are a common marker for the nutritional status of an organism which normally follow seasonal trends that clearly coincide with food abundance but also the reproductive cycle. In mussels, lipid levels tend to increase during the pre-spawning phase concomitantly with the onset of the bloom in phytoplankton (late-winter/early spring) to reach a peak shortly before spawning and are then gradually lost with the release of gametes (Nalepa et al., 1993). This phenomenon is explained by the fact that energy expenditure during gametogenesis is generally high and sufficient resources are needed for proper gamete development (Napolitano et al., 1992). Consistency of lipid levels between both sites would indicate a comparable access to food for both mussel populations. However, only for the population from Riba Roja onset of spawning would coincide, as expected, with the peak in total lipids in contrast to Flix where spawning occurred with a delay of approx. 2 months in June where lipid levels were already declining.

Analysis of fatty acid composition was focused on the essential 20:4n-6, 20:5n-3 and 22:6n-3 fatty acids because of their important role in bivalve growth, metabolism and reproduction (Ben-Mlih et al., 1992; Soudant et al., 1996). The seasonal progression of 20:4n-6 was very similar at both sites but distinct differences were seen for the

20:5n-3 and 22:6n-3 fatty acids. In case of 20:5n-3, major differences were seen within the months of June and July where a sudden increase in abundance occurred at Flix which did not appear at Riba Roja. Furthermore, at Riba Roja 22:6n-3 levels were relatively constant during most of the year and only decreased significantly during September. The population at Flix showed differing results for 22:6n-3 where levels were highest during November and then gradually decreased to reach their lowest values in September. It has been suggested that 22:6n-3 may play a major role at the structural and functional levels of cellular membranes and may also be involved in oogenesis and embryogenesis in bivalves (Soudant, et al., 1996). Furthermore, 22:6n-3 is among the most abundant fatty acids found in mussels and a major energy source (Smith et al., 2003). Interestingly, significantly higher annual levels of 16:1n-7 and 18:1n-9 fatty acids were seen at Flix as indicated in table 2. These fatty acids are also known to be important substrates for energy production (Freites et al., 2002; Smith et al., 2003) and their increase may be related to the lower levels of 22:6n-3. In addition, seasonal variations of the 20:5n-3 and 22:6n-3 fatty acids have been shown to be clearly influenced by the gametogenic cycle in females of *Pecten maximus* (Pazos et al., 1997). In populations studied by Besnard et al. (1989) both fatty acids followed cycles related to oocyte maturation and spawning. Our study may present evidence that suggests that changes in 22:6n-3 and especially 20:5n-3 may be a direct response to alterations in the reproductive cycle.

Environmental parameters like temperature and mainly availability of food are decisive factors for gonadal development in bivalve molluscs and seem to control the duration of the different phases of gametogenesis and spawning (Pazos et al., 1997, Delgado and Camacho, 2005; Bayne, 2004; Suárez et al., 2005). In our study water temperature and tissue levels of the major fatty acid groups like saturated fatty acids, monounsaturated fatty acids and polyunsaturated fatty acids as well as total FAME levels in addition to the n-6/n-3 ratio, which is an indicator for optimal nutrition (Sajjadi et al., 2009), were strikingly similar between Flix and Riba Roja as shown in figure 4 and table 2. Thus, taking into consideration the results of previous comparison studies on the Flix region from other groups which showed changes in reproduction, endocrine system and several biomarkers (Lavado et al., 2004; Navarro et al., 2009; Faria et al., 2010) we may conclude that environmental pollutants most likely are the main cause of the observed alterations presented in this study. Indeed, water and sediment analysis of

this area have already demonstrated high levels of organic and inorganic pollutants like polychlorinated biphenyls (PCB) (Fernández et al., 1999), hexachlorobenzenes (HCB), pentachlorobenzenes (PeCB) (Lacorte et al., 2006; Bosch et al., 2009), DDT and its breakdown products DDE and DDD (Lavado et al., 2004; Bosch et al., 2009) in addition to high amounts of heavy metals especially mercury (Ramos et al., 1999; Bosch et al., 2009). Furthermore, zebra mussels collected a few hundred meters downstream of the Flix chemical plant showed high tissue levels of heavy metals, DDT, PCBs and HCBs (Faria et al., 2010). Concurring, Carrasco et al. (2008) measured the highest total Hg (THg) and methylmercury (MeHg) concentrations in zebra mussels collected from the Flix area ever reported for this species (THg: 0.02 to 0.81 μg/g w.w.; MeHg: 0.22 to 0.60 μg/g w.w.). Successive studies demonstrated a high amount of dioxin-like PCBs in sediments and fish samples which also correlated with an increased ethoxyresorufin O-deethylase (EROD) and Cytochrome P450 1A (CYP1) activity (Eljarrat et al., 2008; Olivares et al., 2010). Furthermore, feral carp specimens sampled downstream the Flix plant showed significant higher mercury concentrations in liver, kidney and muscle than those collected upstream of the production facility (Navarro et al., 2009).

In conclusion, significant changes in reproductive cycle, gonad and hepatopancreas development, annual testosterone levels and fatty acid composition were observed in the zebra mussel population collected from a highly polluted area of the Ebro River. A clear temporal displacement of the gametogenic cycle was noted accompanied by a significant reduction of the spawning period and a prolongation of the resting period. Gonad development was significantly delayed and total gonad size was strongly reduced with effects being more pronounced in males than in females. Testosterone levels were considerably lower which led to an alteration of the estradiol/testosterone ratio. Our results would corroborate previous studies on this region which reported changes in reproductive cycle and a skewed sex steroid ratio. In addition, significant changes among important fatty acids have been detected which are involved in physiological functions especially energetic processes but also reproduction. We may hypothesize that those changes may be a reflection of alterations of the reproductive cycle which merit further investigation.

Table 2. Seasonal variations of the fatty acid profile, in the soft tissue of *Dreissena polymorpha* collected at Riba Roja (A) and Flix (B). Data expressed as % of total fatty acid methyl esters as mean ± SEM (n=3). * indicates fatty acids showing significant differences between sampling sites ($p<0.05$).

(A)

Fatty acid	OCT	NOV	JAN	APR	MAY	JUN	JUL	SEP
14:0	1.41 ± 0.12	1.70 ± 0.05	2.37 ± 0.28	0.95 ± 0.08	0.71 ± 0.05	0.59 ± 0.01	1.00 ± 0.08	1.08 ± 0.08
15:0 *	0.26 ± 0.02	0.37 ± 0.05	0.30 ± 0.04	0.14 ± 0.02	0.17 ± 0.02	0.14 ± 0.02	0.22 ± 0.02	0.34 ± 0.03
16:0	17.54 ± 1.17	15.27 ± 0.37	14.77 ± 0.70	23.05 ± 0.83	24.39 ± 0.29	24.13 ± 0.63	20.12 ± 0.40	13.27 ± 1.29
16:1n-7 *	7.05 ± 0.42	8.17 ± 0.30	10.09 ± 0.13	10.18 ± 0.93	8.14 ± 0.10	6.52 ± 0.07	6.28 ± 0.11	5.92 ± 0.72
16:1n-5	0.41 ± 0.06	0.52 ± 0.01	0.93 ± 0.03	0.73 ± 0.09	0.54 ± 0.00	0.40 ± 0.01	0.41 ± 0.02	0.33 ± 0.03
17 iso	1.75 ± 0.15	1.40 ± 0.06	1.94 ± 0.26	1.57 ± 0.13	1.50 ± 0.03	1.48 ± 0.02	1.82 ± 0.20	2.84 ± 0.16
16:2n-4 *	0.34 ± 0.02	0.30 ± 0.01	0.32 ± 0.02	0.59 ± 0.07	0.38 ± 0.03	0.30 ± 0.04	0.26 ± 0.01	0.08 ± 0.01
17:0 *	1.30 ± 0.02	1.12 ± 0.01	1.34 ± 0.17	1.16 ± 0.12	1.27 ± 0.04	1.31 ± 0.06	1.35 ± 0.04	1.40 ± 0.11
16:3n-4 *	0.16 ± 0.04	0.18 ± 0.02	0.16 ± 0.01	0.56 ± 0.01	0.22 ± 0.00	0.10 ± 0.01	0.10 ± 0.01	0.03 ± 0.01
18:0	3.51 ± 0.17	3.06 ± 0.20	2.99 ± 0.29	3.03 ± 0.53	3.55 ± 0.31	4.00 ± 0.32	4.09 ± 0.30	4.84 ± 0.25
18:1n-9 *	4.55 ± 0.05	4.03 ± 0.10	3.59 ± 0.05	3.19 ± 0.11	3.10 ± 0.09	3.01 ± 0.17	3.64 ± 0.12	4.25 ± 0.26
18:1n-7	2.90 ± 0.19	3.15 ± 0.10	4.19 ± 0.23	4.86 ± 0.30	4.28 ± 0.29	3.49 ± 0.27	3.35 ± 0.17	2.22 ± 0.18
18:1n-5	0.24 ± 0.02	0.25 ± 0.01	0.32 ± 0.02	0.17 ± 0.01	0.14 ± 0.00	0.10 ± 0.00	0.17 ± 0.02	0.12 ± 0.05
18:2n-6	2.31 ± 0.12	1.84 ± 0.05	2.01 ± 0.09	2.34 ± 0.06	2.16 ± 0.05	2.01 ± 0.11	2.11 ± 0.10	1.60 ± 0.08
18:2n-4	0.12 ± 0.01	0.12 ± 0.01	0.10 ± 0.01	0.30 ± 0.03	0.27 ± 0.03	0.19 ± 0.02	0.15 ± 0.01	0.10 ± 0.01
18:3n-4 *	0.23 ± 0.01	0.30 ± 0.01	0.24 ± 0.00	0.37 ± 0.03	0.30 ± 0.03	0.21 ± 0.02	0.26 ± 0.01	0.16 ± 0.07
18:3n-3	4.13 ± 0.43	2.25 ± 0.15	2.13 ± 0.12	3.33 ± 0.20	3.54 ± 0.18	3.35 ± 0.13	2.92 ± 0.21	1.88 ± 0.13
18:4n-3	2.22 ± 0.28	1.68 ± 0.08	2.89 ± 0.09	4.17 ± 0.48	3.32 ± 0.12	2.16 ± 0.23	0.80 ± 0.38	0.47 ± 0.06
20:1n-11	7.66 ± 0.62	7.13 ± 0.06	4.17 ± 0.17	2.61 ± 0.22	4.05 ± 0.16	4.62 ± 0.28	7.16 ± 0.60	11.36 ± 0.47
20:1n-9	1.71 ± 0.06	1.57 ± 0.04	1.16 ± 0.05	1.06 ± 0.19	1.48 ± 0.30	1.27 ± 0.08	1.78 ± 0.04	1.94 ± 0.08
20:1n-7	0.77 ± 0.08	0.87 ± 0.02	0.70 ± 0.05	0.34 ± 0.06	0.45 ± 0.01	0.53 ± 0.03	0.85 ± 0.09	1.18 ± 0.01
20:2NMID	0.37 ± 0.03	0.42 ± 0.01	0.46 ± 0.03	0.25 ± 0.02	0.29 ± 0.03	0.29 ± 0.02	0.39 ± 0.01	0.62 ± 0.05
20:2n-6	0.54 ± 0.03	0.43 ± 0.01	0.32 ± 0.03	0.37 ± 0.03	0.44 ± 0.05	0.40 ± 0.02	0.51 ± 0.01	0.39 ± 0.05
20:3n-6	0.23 ± 0.01	0.25 ± 0.01	0.24 ± 0.00	0.19 ± 0.04	0.20 ± 0.02	0.21 ± 0.01	0.26 ± 0.01	0.40 ± 0.01
20:4n-6	5.77 ± 0.54	6.22 ± 0.07	5.36 ± 0.16	2.63 ± 0.30	3.11 ± 0.21	4.02 ± 0.21	5.70 ± 0.28	9.66 ± 0.15
20:4n-3	0.66 ± 0.03	0.60 ± 0.03	0.59 ± 0.01	0.75 ± 0.04	0.86 ± 0.07	0.80 ± 0.01	0.65 ± 0.03	0.34 ± 0.03
20:5n-3 *	8.40 ± 0.46	8.99 ± 0.13	11.07 ± 0.26	12.04 ± 0.52	10.31 ± 0.36	10.15 ± 0.54	8.99 ± 0.34	6.29 ± 0.15
22:2NMID	2.57 ± 0.28	2.67 ± 0.08	1.92 ± 0.07	0.70 ± 0.05	1.23 ± 0.14	1.42 ± 0.05	2.05 ± 0.04	3.07 ± 0.26
22:3NMIT	0.61 ± 0.06	0.76 ± 0.01	0.92 ± 0.03	1.18 ± 0.05	1.22 ± 0.05	1.17 ± 0.01	0.52 ± 0.02	0.85 ± 0.31
22:4n-6	1.07 ± 0.14	1.18 ± 0.04	1.07 ± 0.03	0.27 ± 0.06	0.38 ± 0.03	0.57 ± 0.02	0.92 ± 0.06	2.06 ± 0.12
22:5n-6	5.18 ± 0.49	5.53 ± 0.10	5.08 ± 0.27	2.21 ± 0.28	2.85 ± 0.18	3.35 ± 0.17	4.34 ± 0.37	7.27 ± 0.42
22:5n-3	4.91 ± 0.41	5.76 ± 0.07	4.74 ± 0.20	3.15 ± 0.76	4.11 ± 0.12	5.42 ± 0.13	5.74 ± 0.41	5.14 ± 0.35
22:6n-3 *	9.10 ± 0.37	11.86 ± 0.30	11.53 ± 0.32	11.55 ± 1.22	11.05 ± 0.50	12.27 ± 0.39	11.09 ± 0.42	8.48 ± 0.46
SFA	25.77 ± 1.08	22.93 ± 0.53	23.71 ± 1.13	29.90 ± 1.30	31.58 ± 0.20	31.65 ± 0.91	28.59 ± 0.73	23.78 ± 1.22
MUFA	25.29 ± 0.37	25.70 ± 0.31	25.15 ± 0.14	23.15 ± 1.42	22.17 ± 0.72	19.94 ± 0.52	23.65 ± 0.90	27.31 ± 0.60
PUFA	13.00 ± 0.53	10.21 ± 0.25	10.55 ± 0.08	12.98 ± 0.74	12.16 ± 0.45	10.44 ± 0.55	9.56 ± 0.66	8.41 ± 0.12
n-3	6.35 ± 0.72	3.93 ± 0.23	5.02 ± 0.11	7.50 ± 0.68	6.87 ± 0.28	5.52 ± 0.35	3.72 ± 0.55	2.35 ± 0.19
n-6	2.85 ± 0.10	2.28 ± 0.06	2.33 ± 0.06	2.72 ± 0.04	2.61 ± 0.08	2.40 ± 0.13	2.62 ± 0.11	1.99 ± 0.07
HUFA	35.94 ± 1.72	41.16 ± 0.60	40.59 ± 1.20	33.98 ± 3.23	34.09 ± 1.19	37.97 ± 1.45	38.20 ± 1.80	40.50 ± 1.45
HUFA n-3	23.08 ± 0.81	27.21 ± 0.45	27.93 ± 0.78	27.50 ± 2.50	26.32 ± 0.79	28.65 ± 1.04	26.46 ± 1.14	20.25 ± 0.66
HUFA n-6	12.86 ± 1.12	13.95 ± 0.20	12.66 ± 0.43	6.48 ± 0.73	7.77 ± 0.41	9.32 ± 0.43	11.74 ± 0.74	20.25 ± 0.83

(B)

Fatty acid	OCT	NOV	JAN	APR	MAY	JUN	JUL	SEP
14:0	1.58 ± 0.37	1.36 ± 0.35	2.74 ± 0.11	1.34 ± 0.04	0.66 ± 0.23	0.54 ± 0.22	0.93 ± 0.09	1.32 ± 0.05
15:0	0.16 ± 0.01	0.18 ± 0.02	0.12 ± 0.04	0.10 ± 0.02	0.08 ± 0.03	0.09 ± 0.01	0.15 ± 0.03	0.22 ± 0.04
16:0	16.06 ± 0.54	14.26 ± 1.11	14.67 ± 0.76	21.71 ± 0.49	24.73 ± 0.09	26.49 ± 1.58	22.73 ± 1.41	14.23 ± 0.22
16:1n-7	8.69 ± 0.32	8.67 ± 0.16	10.19 ± 0.42	13.64 ± 0.39	11.72 ± 0.50	9.67 ± 1.01	8.06 ± 0.50	8.09 ± 0.20
16:1n-5	0.43 ± 0.01	0.49 ± 0.03	0.80 ± 0.06	0.60 ± 0.01	0.53 ± 0.02	0.54 ± 0.02	0.50 ± 0.03	0.44 ± 0.02
17 iso	1.51 ± 0.01	1.33 ± 0.12	1.43 ± 0.11	0.91 ± 0.07	1.04 ± 0.04	1.26 ± 0.10	1.43 ± 0.06	2.13 ± 0.07
16:2n-4	0.07 ± 0.02	0.04 ± 0.00	0.04 ± 0.01	0.01 ± 0.00	0.01 ± 0.01	0.01 ± 0.01	0.02 ± 0.00	0.08 ± 0.01
17:0	1.06 ± 0.01	1.06 ± 0.01	1.06 ± 0.01	1.07 ± 0.21	0.89 ± 0.01	1.05 ± 0.07	1.15 ± 0.05	1.34 ± 0.02
16:3n-4	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	0.00 ± 0.00	0.06 ± 0.06	0.00 ± 0.00	0.00 ± 0.00	0.01 ± 0.00
18:0	3.65 ± 0.14	3.74 ± 0.08	3.20 ± 0.03	3.24 ± 0.13	3.77 ± 0.17	4.05 ± 0.32	3.69 ± 0.17	4.13 ± 0.20
18:1n-9	5.00 ± 0.34	4.18 ± 0.25	4.29 ± 0.42	4.95 ± 0.24	4.46 ± 0.18	3.56 ± 0.18	3.67 ± 0.05	4.02 ± 0.15
18:1n-7	2.76 ± 0.15	2.67 ± 0.24	4.07 ± 0.41	5.29 ± 0.13	5.78 ± 0.20	4.94 ± 0.51	4.18 ± 0.26	2.82 ± 0.19
18:1n-5	0.15 ± 0.03	0.17 ± 0.05	0.21 ± 0.02	0.19 ± 0.00	0.15 ± 0.01	0.14 ± 0.01	0.14 ± 0.00	0.19 ± 0.01
18:2n-6	2.21 ± 0.10	1.88 ± 0.23	2.40 ± 0.16	2.93 ± 0.03	2.98 ± 0.08	2.46 ± 0.10	2.61 ± 0.09	1.80 ± 0.05
18:2n-4	0.12 ± 0.00	0.10 ± 0.01	0.10 ± 0.01	0.32 ± 0.03	0.33 ± 0.02	0.26 ± 0.03	0.19 ± 0.02	0.10 ± 0.00
18:3n-4	0.38 ± 0.01	0.32 ± 0.01	0.27 ± 0.01	0.46 ± 0.04	0.45 ± 0.01	0.40 ± 0.01	0.36 ± 0.01	0.44 ± 0.02
18:3n-3	3.50 ± 0.15	2.35 ± 0.18	2.44 ± 0.30	3.23 ± 0.16	4.14 ± 0.19	3.53 ± 0.14	3.36 ± 0.24	3.24 ± 0.14
18:4n-3	1.29 ± 0.06	1.45 ± 0.08	2.98 ± 0.15	3.49 ± 0.17	3.22 ± 0.11	2.11 ± 0.17	1.21 ± 0.19	0.99 ± 0.06
20:1n-11	8.24 ± 0.27	7.17 ± 0.30	4.08 ± 0.30	2.64 ± 0.12	3.90 ± 0.11	4.35 ± 0.14	5.47 ± 0.53	9.92 ± 0.17
20:1n-9	2.24 ± 0.07	1.76 ± 0.13	1.39 ± 0.05	1.00 ± 0.02	1.09 ± 0.06	1.21 ± 0.03	1.55 ± 0.12	1.98 ± 0.03
20:1n-7	0.96 ± 0.07	1.19 ± 0.13	0.98 ± 0.08	0.41 ± 0.05	0.52 ± 0.09	0.60 ± 0.09	0.74 ± 0.07	1.34 ± 0.03
20:2NMID	0.35 ± 0.02	0.44 ± 0.02	0.41 ± 0.03	0.18 ± 0.01	0.24 ± 0.03	0.23 ± 0.01	0.29 ± 0.03	0.54 ± 0.02
20:2n-6	0.57 ± 0.02	0.37 ± 0.03	0.26 ± 0.00	0.31 ± 0.00	0.37 ± 0.01	0.33 ± 0.02	0.35 ± 0.11	0.52 ± 0.04
20:3n-6	0.35 ± 0.01	0.35 ± 0.01	0.30 ± 0.01	0.18 ± 0.01	0.18 ± 0.01	0.24 ± 0.03	0.32 ± 0.02	0.53 ± 0.01
20:4n-6	6.37 ± 0.06	7.34 ± 0.40	5.83 ± 0.47	2.52 ± 0.19	2.69 ± 0.24	3.37 ± 0.55	5.04 ± 0.39	8.61 ± 0.21
20:4n-3	0.60 ± 0.02	0.52 ± 0.02	0.62 ± 0.03	0.64 ± 0.01	0.69 ± 0.01	0.71 ± 0.03	0.63 ± 0.05	0.45 ± 0.03
20:5n-3	8.91 ± 0.12	9.42 ± 0.40	11.10 ± 0.36	12.29 ± 0.35	10.33 ± 0.13	11.10 ± 0.60	11.77 ± 0.40	7.33 ± 0.24
22:2NMID	2.51 ± 0.13	3.00 ± 0.34	2.10 ± 0.19	0.83 ± 0.06	1.13 ± 0.13	1.12 ± 0.04	1.47 ± 0.21	2.86 ± 0.08
22:3NMIT	0.54 ± 0.02	0.65 ± 0.05	0.86 ± 0.03	0.94 ± 0.03	0.77 ± 0.01	0.65 ± 0.04	0.56 ± 0.04	0.31 ± 0.01
22:4n-6	1.31 ± 0.05	1.47 ± 0.11	1.21 ± 0.16	0.30 ± 0.06	0.31 ± 0.04	0.41 ± 0.09	0.80 ± 0.11	1.67 ± 0.06
22:5n-6	3.89 ± 0.10	4.72 ± 0.31	4.22 ± 0.41	1.65 ± 0.13	1.52 ± 0.08	1.86 ± 0.36	2.42 ± 0.21	5.06 ± 0.17
22:5n-3	6.29 ± 0.13	6.40 ± 0.19	5.52 ± 0.35	3.18 ± 0.47	3.97 ± 0.52	4.55 ± 0.85	6.15 ± 0.56	5.43 ± 0.21
22:6n-3	8.28 ± 0.22	10.95 ± 0.27	10.14 ± 0.54	9.44 ± 0.29	7.27 ± 0.40	8.20 ± 1.24	8.05 ± 0.29	7.86 ± 0.24
SFA	24.01 ± 0.24	21.93 ± 0.67	23.22 ± 0.86	28.38 ± 0.62	31.18 ± 0.48	33.47 ± 1.75	30.08 ± 1.19	23.36 ± 0.47
MUFA	28.48 ± 0.31	26.31 ± 0.31	25.99 ± 0.96	28.72 ± 0.53	28.16 ± 0.69	24.99 ± 1.71	24.31 ± 0.20	28.80 ± 0.48
PUFA	10.99 ± 0.15	9.95 ± 0.21	10.99 ± 0.37	11.76 ± 0.29	12.93 ± 0.26	10.45 ± 0.43	9.86 ± 0.27	10.59 ± 0.24
n-3	4.78 ± 0.21	3.80 ± 0.25	5.42 ± 0.44	6.72 ± 0.24	7.36 ± 0.31	5.64 ± 0.31	4.57 ± 0.42	4.23 ± 0.20
n-6	2.77 ± 0.09	2.24 ± 0.23	2.66 ± 0.16	3.24 ± 0.04	3.35 ± 0.07	2.79 ± 0.11	2.95 ± 0.20	2.33 ± 0.08
HUFA	36.52 ± 0.65	41.82 ± 0.85	39.79 ± 2.18	31.14 ± 0.67	27.73 ± 1.37	31.09 ± 3.78	35.75 ± 1.48	37.26 ± 0.88
HUFA n-3	24.07 ± 0.46	27.30 ± 0.44	27.38 ± 1.12	25.55 ± 0.36	22.26 ± 1.01	24.56 ± 2.72	26.61 ± 0.79	21.06 ± 0.56
HUFA n-6	12.45 ± 0.21	14.52 ± 0.76	12.41 ± 1.06	5.59 ± 0.36	5.48 ± 0.37	6.53 ± 1.06	9.14 ± 0.71	16.19 ± 0.43

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