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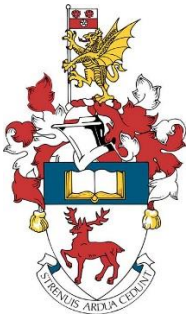

FACULTY OF ENVIRONMENT AND LIFE SCIENCES

School of Geography and Environmental Science

The influence of environmental factors, sex steroids and tributyltin on growth, survival and reproductive parameters in the European flat oyster (*Ostrea edulis* Linnaeus, 1758)

By

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Thesis for the degree of Doctor of Philosophy (PhD)

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ABSTRACT

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The influence of environmental factors, sex steroids and tributyltin on growth, survival and reproductive parameters in the European flat oyster (*Ostrea edulis* Linnaeus, 1758)

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The flat oyster *Ostrea edulis* (Linnaeus, 1758), a protandrous sequential hermaphrodite, is a commercially valuable species in Europe but its populations have been collapsing since the mid-1800s until recent years. In the Solent, Southern England, one such population of oysters has seen a significant reduction in the number of brooding female-phase oysters and skewed sex ratio towards male-phase oysters in recent years. Due to their ecological and economic importance, and numerous reintroduction schemes coming forward, understanding the many uncertainties still existing regarding their biology and reproduction are of critical importance. The aim of this thesis was to investigate some of the environmental factors (abiotic and biotic factors, and environmental pollutants) affecting physiological, biochemical and reproductive attributes in *O. edulis*.

In order to understand the effect of temperature on sex determination and the timing of gametogenesis, two experiments were carried out. In the first experiment animals (n=49 per treatment) were treated at different temperatures (10, 14 and 18°C) under laboratory conditions for four months. To understand the changes occurring during different seasons of the year and the interaction with other environmental factors such as food availability, a second experiment was completed using 120 oysters kept under semi-enclosed conditions at the National Oceanography Centre Southampton from May 2016 to June 2017. For these two experiments the variables response included biometric parameters, gametogenic stage and sex determination. Gonadal tissue from these oysters was analysed by Enzyme Linked Immunosorbent Assay (ELISA) to determine sex steroid concentrations. The results suggested a significant influence of temperature on reproductive parameters during both experiments. High temperatures were related to accelerated gametogenesis in all the experiments. Sex ratios changed throughout both experiments with lower temperatures causing a female-biased sex ratio whereas more males were found with the increase of temperature. Food availability also showed a direct relationship with gonadal maturation,

suggesting that this factor could play an important role in terms of energy allocation for sexual maturation. This study found a lack of relation between sex steroids in gonadal tissue and reproductive parameters, suggesting a different molecular pathway involved in reproduction in *O. edulis* and independent of vertebrate-related sex steroids.

In order to understand the effect and mechanism of action of exogenous sex steroids *O. edulis* was exposed to different concentrations of testosterone (20 ng/L (n=20) and 200 ng/L (n=20)), estradiol (5 ng/L (n=20) and 50 ng/L (n=20)), and a negative control (n=30) for 10 weeks. At the end of the exposure, gametogenic stage, hormones concentrations, energy reserves content and metabolomic profiling analysis were conducted to elucidate the metabolic alterations that occur in individuals exposed to those compounds. Results showed that oysters exposed to sex steroids presented an increase in mortality. It was also evidenced that *O. edulis* can uptake and accumulate estradiol and testosterone from the water. Although testosterone and estradiol did not cause masculinising or feminising effects other processes such as glycogenolysis and the synthesis of proteins and lipids were affected. In the same manner a reduction in metabolism evidenced by a lower content of energy reserves, down-regulation of molecules involved in the Tricarboxylic Acid (TCA) cycle and energy molecules was observed indicating an effect on homeostasis in *O. edulis* exposed to sex steroids.

Additionally, an experiment was carried out to evaluate the effect of a well-known endocrine disrupting environmental pollutant -tributyl tin (TBT)- to establish effects on sex determination, the gametogenic cycle, biochemical composition and the metabolome of *O. edulis.* Oysters were exposed to TBT chloride (20 ng/L (n=30), 200 ng/L (n=30) and 2000 ng/L (n=30) for 9 weeks. At the end of the exposure, gametogenic stage, hormones concentrations, energy reserves content and metabolomic profiling analysis were conducted to elucidate the metabolic alterations that occur in individuals exposed to those compounds. Oysters exposed to TBTCl presented an increase in mortality and changes in the gametogenic cycle with arrest in stages G0 and G1. Sex determination was affected by TBT causing a masculinization effect in *O. edulis* treated with low and environmentally relevant concentrations and an increase of inactive stages in oysters treated with high concentrations of this pollutant. The analysis of metabolites showed significant changes in the global biochemistry of oysters exposed to exogenous TBTCl by affecting ions involved in different and important molecular pathways such as TCA cycle and creating an imbalance between molecules involved in oxidative stress and the antioxidant system. A TBT mechanism of actions is proposed based on the results obtained in this study and the literature review.

Overall this study showed that a rise in sea temperatures and food quality through the year could influence reproductive parameters in *O. edulis*, potentially affecting the long term health of populations and contributing to recent declines. The presence and persistence of environmental

pollutants, such as sex steroids and TBT, could cause an additional threat to the declining *O. edulis* populations and related taxa around the world, by increasing mortality, changing reproductive maturation and skewing sex-ratios in natural populations. Furthermore, the presence of some intermediate molecules in the steroidogenesis pathway was detected in this study but their role in reproduction in invertebrates needs confirmation.

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Research Thesis: Declaration of Authorship

Print name:	Lina Maria Zapata Restrepo

Title of thesis:	The influence of environmental factors, sex steroids and tributyltin on growth, survival and reproductive parameters in the European flat oyster (*Ostrea edulis* Linnaeus, 1758)

I declare that this thesis and the work presented in it are my own and has been generated by me as the result of my own original research.

I confirm that:

1. This work was done wholly or mainly while in candidature for a research degree at this University;
2. Where any part of this thesis has previously been submitted for a degree or any other qualification at this University or any other institution, this has been clearly stated;
3. Where I have consulted the published work of others, this is always clearly attributed;
4. Where I have quoted from the work of others, the source is always given. With the exception of such quotations, this thesis is entirely my own work;
5. I have acknowledged all main sources of help;
6. Where the thesis is based on work done by myself jointly with others, I have made clear exactly what was done by others and what I have contributed myself;
7. Parts of this work have been published (see below):

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A version of Chapter 2 has been published in a scientific journal:

Zapata-Restrepo L.M., Hauton C., Williams I.D., Jensen A.C., & Hudson M.D. 2019. Effects of the interaction between temperature and steroid hormones on gametogenesis and sex ratio in the European flat oyster (*Ostrea edulis*). *Comparative Biochemistry and Physiology, Part A* 236:110523. DOI: https://doi.org/10.1016/j.cbpa.2019.06.023

Part of the results of Chapter 2 was also presented as oral presentation at the Society of Experimental Biology SEB 2019 Annual Meeting:

Lina M. Zapata-Restrepo, Malcolm D. Hudson, Ian D. Williams and Chris Hauton. (2019). Elevated temperature affects sex ratio and disrupts the timing of gametogenesis in the European flat oyster (*Ostrea edulis*). *Proceedings of the Society of Experimental Biology Annual Meeting*, FIBES II Conference and Exhibition Centre, Seville, Spain, 2-5 July 2019. Paper A13.11.

Part of the results of Chapter 5 was presented as a research poster at the SETAC Europe 29th Annual Meeting:

Lina M. Zapata-Restrepo, Malcolm Hudson, Chris Hauton**,** Ian D. Williams, David Hauton and Antony Jensen. (2019). Effects of tributyltin (TBT) exposure on gametogenesis and sex ratio in the European flat oyster (*Ostrea edulis). Proceedings of the SETAC Europe 29th Annual Meeting,* Helsinki, Finland, 26 - 30 May, 2019. Poster MO052.

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Finally. This thesis is dedicated to the memory of my parents. I greatly appreciate the support from my family and special thanks to my friends for their love, encouragement and support. Thank you for *being there* for me through all the *good* times and *bad times*. Thank you very much!

Definitions and Abbreviations

ΔΨm	Mitochondrial Membrane Potential
ALP	Alkali-Labile Phosphate
ANSA	Aminonaphthol sulfonic acid
ADP	Adenosine Diphosphate
ATP	Adenosine 5-triphosphate
BPA	Bisphenol A
CA	Comet assay
cAMP	Cyclic Adenosine Monophosphate
CEFAS	Centre for Environment, Fisheries and Aquaculture Science
Chl α	Chlorophyll α
CI	Condition Index
CV	Coeficient variation
CYP450	Cytochrome P450
DCM	Dichloromethane
DHA	Docosahexaenoic Acid
DHEA	Deshidroepiandrosterona
DNA	Deoxyribonucleic Acid
dNTPs	Deoxynucleotidesrna
E_2	Oestradiol-17β
EDCs	Endocrine Disruptors Compounds
EE_2	17α-ethinyloestradiol
EEC	European Economic Community
ELISA	Enzyme Linked Immunosorbent Assay
EPA	Eicosapentaenoic Acid
EQS	Environmental Quality Standards
ER	Estrogen Receptor

FAO	Food and Agriculture Organisation of the United Nations
F	Females
FW	Fresh tissue weight
GC/MS	Gas Chromatography Mass Spectrometry
GDP	Guanosine Diphosphate
GMP	Guanosine Monophosphate
GSH	Glutathione
GST	Glutathione S-transferase
GTP	Guanosine-5'-triphosphate
H_20	Water
H_20_2	Hydrogen peroxide
H	Height
HBS	Hermaphrodite with both sexes equally represented
HPF	Hermaphrodite predominantly female
HPM	Hermaphrodite predominantly male
HCl	Hydrochloric acid
HPLC	High-performance liquid chromatography
I	Inactive
IC-MS/MS	Ion chromatography tandem mass spectrometry
IFCA	Inshore Fisheries and Conservation Authority
IMO	International Maritime Organization
KOH	Potassium Hydroxide
L	Length
LC-MS	Liquid chromatography–mass spectrometry
LC-MS/MS	Liquid Chromatography with tandem mass spectrometry
M	Males
ml	Mililiter
MN	Micronucleus
mRNA	Messenger RNA

NA	Not applicable
NADH	Nicotinamide Adenine Dinucleotide Hydride
NADPH	Nicotinamide Adenine Dinucleotide Phosphate
Na_2HPO_4	Di-sodium hydrogen orthophosphate
NaCl	Sodium Chloride
ng	Nanogram
NaI	Sodium Iodide
NIEHS	National Institute of Environmental Health Sciences
NOCS	National Oceanography Centre Southampton
NP	Nonylphenol
OP	Octylphenol
OSPAR	Oslo/Paris convention
P	Progesterone
PAHs	Polycyclic Aromatic Hydrocarbons
PCA	Principal Component Analysis
PCBs	Polychlorinated Biphenyl
pg	Picogram
PLS-DA	Partial Least Squares Discriminant Analysis
PUFAs	Polyunsaturated Fatty Acids
PVC	Polyvinyl Chloride
RDA	Redundancy Anlaysis
ROS	Reactive Oxygen Species
SCGE	Single Cell Gel Electrophoresis
SRs	Steroid Receptors
SVol	Shell Cavity volume
T	Testosterone
TBT	Tributyltin
TBTCl	Tributyltin Chloride
TCA	Tricarboxylic Acid

TPhTCl	Triphenyltin chloride
TPT	Triphenyltin
UK	United Kingdom
UK BAP	United Kingdom Biodiversity Action Plan
ul	Microliter (1/1000 ml)
US-EPA	United States Environmental Protection Agency
Vtg	Vitellogenin
W	Weight
Wi	Width

Chapter 1 Introduction

1.1 Molluscs in the Marine Environment

Aquatic habitats, including marine ecosystems, are under threat from human impacts, and fragmented marine habitats are more common (Chatterjee, 2017). Marine ecosystems represent the most biologically diverse ecosystems on Earth but are very vulnerable to changes in sea temperatures and salinity (McCauley *et al.*, 2015; Chatterjee, 2017). The loss of species in marine environments has been increasing rapidly, but our understanding of these habitats is still limited in comparison to terrestrial systems (Broderick, 2015; McCauley *et al.*, 2015).

After insects, molluscs are probably the most diverse invertebrate animals with considerable economic and ecological importance (Broderick, 2015; Wanninger and Wollesen, 2019). Oysters are one of the most valued species by humans for food and subsistence but wild oysters are now extinct in many places around the world (Beck *et al.*, 2011). Due to the particular life characteristics of this species, such as relatively long-lived and sporadic reproduction, oyster beds are very vulnerable to commercial exploitation and overfishing (Orton, 1927c; Jackson *et al.*, 2001).

The demand for oysters stimulated commercial exploitation of several species, including *O. edulis,* around the world. This has led to a considerable reduction in its production around Europe (FAO, 2019). In response, the Oslo-Paris Convention included *Ostrea edulis* on the OSPAR List of Threatened Species and/or Declining Species and Habitats (OSPAR, 2008a). The *O. edulis* population from the Solent is one of these declining populations with several collapses during the last century (Key and Davidson, 1981; Tubbs, 1999; Gravestock, James and Goulden, 2014; Southern IFCA, 2018).

Studies have analysed the recent situation of the *O. edulis* population in the Solent, finding a significant reduction in the number of brooding female-phase oysters (Eagling, 2012), a reduction in fecundity of brooding oysters and skewed sex ratio towards male-phase oysters (Kamphausen, Jensen and Hawkins, 2011; Eagling, 2012). Some hypotheses point to trend changes in abiotic factors, habitat degradation, pollution as additional factors involved in population decline (OSPAR, 2008a; Kamphausen, Jensen and Hawkins, 2011; Harding, Nelson and Glover, 2016; Chatterjee, 2017). Recent studies have confirmed the imminent ecological collapse within the Solent harbours (Helmer *et al.*, 2019) so an effective intervention and adequate management are required. With numerous reintroduction schemes coming forward even though the complex reasons for previous declines are not fully understood, it is necessary to elucidate some of the factors affecting development, growth and reproductive parameters in *O. edulis*.

Abiotic factors, such as temperature, have been shown to be important in terms of influence on physiological, biochemical and reproductive attributes of oysters (Korringa, 1952; Loosanoff and Davis, 1952; Newell, Johson and Kofoed, 1977; Mann, 1979; Newell and Branch, 1980). With the increase in water temperatures in the north east Atlantic and UK coastal waters (Holliday *et al.*, 2008) and ongoing rises predicted (Marine Climate Change Impacts Partnership, 2015) a more refined understanding of the influence of this abiotic factor on gametogenesis and sex determination remains essential (Joyce *et al.*, 2013). Temperature has shown an effect on reproductive parameters in other mollusc species under controlled conditions, but different species display different responses making it difficult to extrapolate or predict effects on reproductive parameters in *O. edulis* (Chávez-Villalba *et al.*, 2003; Fabioux *et al.*, 2005; Joyce *et al.*, 2013; Teaniniuraitemoana *et al.*, 2016).

Reproductive activities, such as sex differentiation and sexual maturation, are well known to be regulated by sex steroids (e.g., estrogens, testosterone) in vertebrates. It has been shown that steroid hormones and other related molecules could be involved in reproduction, development, and maturation, especially in early life stages (i.e., gametes, larvae, and juveniles) in bivalves (LaFont, 2000; Lafont and Mathieu, 2007). Although the bivalve endocrine system does not appear to be as complex as in vertebrates, the mechanisms of the action of sex steroid hormones, if any, in those process are still poorly understood and evidence is inconclusive. Additional evidence suggests that environmental factors, especially temperature, when combined with hormonal control are involved in the gender determination in other species of bivalve (Mori, Muramatsu and Nakamura, 1972; Teaniniuraitemoana *et al*., 2016). However, the exact mechanism by which the temperature triggers these processes and the relation with steroid hormones for gametogenesis, sex change and sex ratio in *O. edulis* has not yet been resolved satisfactorily (Ketata *et al.*, 2007; Morishita *et al.*, 2010).

Evidence indicates that bivalves could be especially sensitive to environmental exposure to chemical substances interfering with the synthesis, metabolism, transport, secretion, and mode of action of natural hormones involved in the maintenance of homeostasis and regulation of developmental processes (Oehlmann and Schulte-Oehlmann, 2003). These chemical pollutans, known as Endocrine Disruptors Compounds (EDCs), can impact at early stages of maturation affecting sex determination, fecundity, and population dynamics. Further research on this topic is needed to clarify if these compounds could have some responsibility for male bias and the most recent *O. edulis* population decline.

Despite the fact that *O. edulis* has been studied as a species since the middle of last century, and it is currently the main species in projects guided to improve coastal and transitional water

ecosystems, such as in the BLUE Marine Foundation's Solent Oyster Restoration Project (Harding, Nelson and Glover, 2016) there are still gaps in the basic information relating to this species. The ecological and economic importance of this species, and the declining situation of some UK populations, requires research to address the many uncertainties that exist regarding their biology and reproduction. Rising ocean temperatures and anthropogenic pollution could have impacts in natural populations influencing the behavior, growth, reproduction, and survival of marine species including *O. edulis*.

The current chapter (Chapter 1) includes background information related to the ecological role of *O. edulis* in the ecosystem and the species' biological characteristics with special emphasis on reproductive parameters affected by environmental factors (Fig 1.1). As evidence suggests a disturbance of the reproductive processes as one of the main causes of the most recent collapse, this chapter will focus on parameters involved in reproduction in molluscs. The knowledge about the endocrinology of this species is scarce so this chapter also explains the role of sex steroids reported in vertebrates and discusses the evidence about its presence and role in invertebrates. Finally, this chapter will discuss pollution as one of the main possible factors affecting reproductive parameters in bivalves and will go through some of the most common methods used to analyse its effect on organisms.

1.2 The biological and ecological role of *Ostrea edulis*

O. edulis, the largest oyster native to Europe commonly known as the flat or native oyster, is a bivalve species of family Ostreidae found from the low intertidal down to the sublittoral zone throughout the Atlantic and Mediterranean coasts of Europe (Fig 1.2) (Perry and Jackson, 2017; CABI, 2019). It is recorded at higher abundance on the Mediterranean coasts of Europe especially in association with highly productive estuarine areas (Laing, Walker and Areal, 2005; Gibson *et al.*, 2007; Harding, Nelson and Glover, 2016).

Since the 1800s, the demand of oysters for human consumption stimulated commercial exploitation of several species of oysters around UK waters, including the Solent fishery on the central South coast of England which has been active since then (Cole, 1951; Smith, Low and Moore, 2006; Southern IFCA, 2015). *O. edulis* is still a commercially important marine resource in these waters but its populations have suffered several collapses during the last century (Smith, Low and Moore, 2006; Kamphausen, Jensen and Hawkins, 2011). According to the Food and Agriculture Organisation of the United Nations (FAO), *O. edulis* production has suffered a reduction in Europe

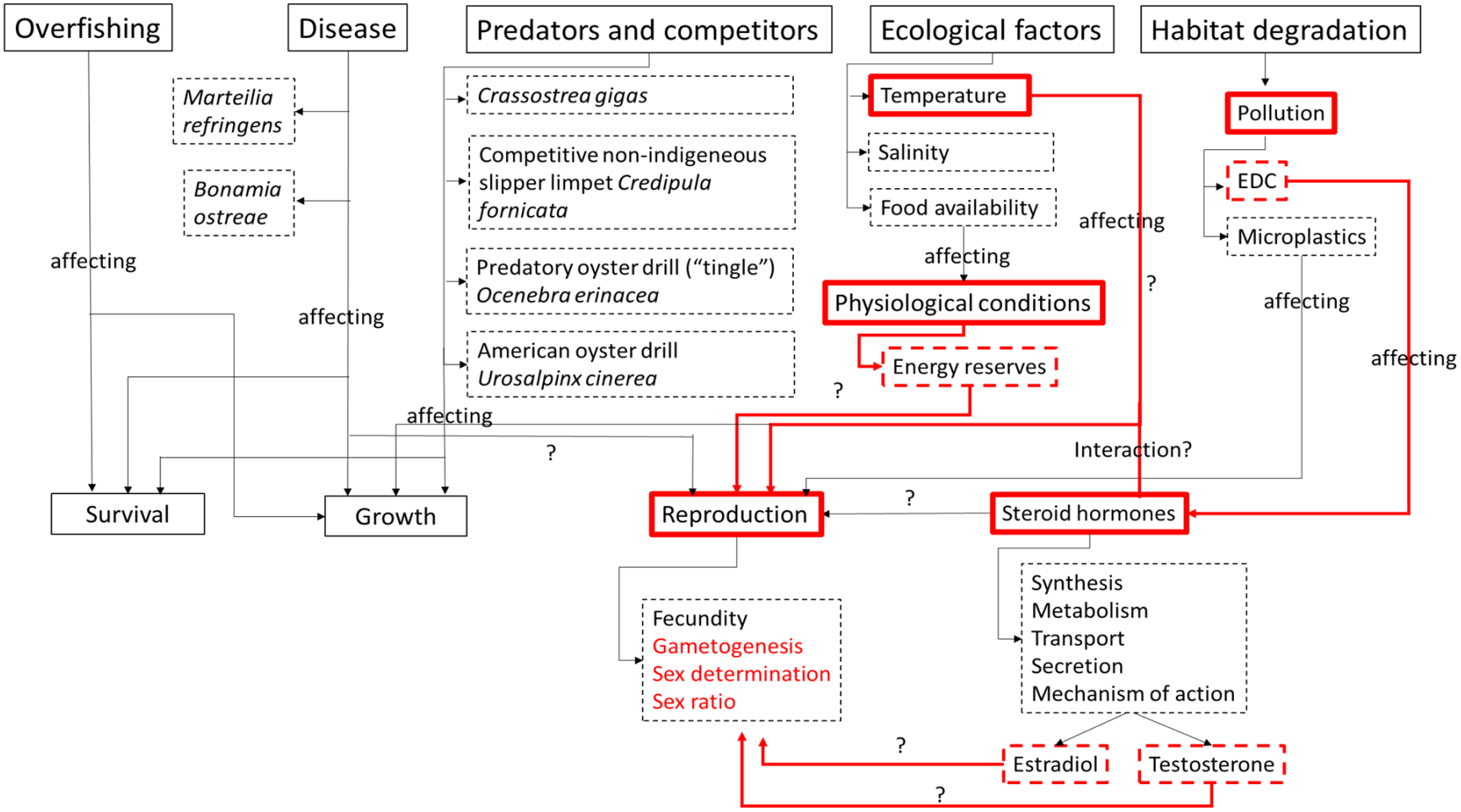

Figure 1.1 A summary showing some of the factors and biological effects reported as the main reasons causing *Ostrea edulis* declining populations around the world. Red–marked sections show the aspects evaluated and discussed in this thesis.

from nearly 30,000 tonnes in 1,961 to approximately 2,000 tonnes in 2016 (FAO, 2019). Due to the available supply has decreased and the bulk of production of all farmed oysters has been focused mainly in the Pacific oyster *Magallanas gigas* (formerly *Crassostrea gigas* (Bayne *et al.*, 2017)), average prices for *O. edulis* have increased reaching records as high as USD 13/Kg making its culture an important activity in the areas where it is still reared (FAO, 2019).

Oysters, and many of the invertebrates associated with oyster beds, are active suspension feeders of bacteria, phytoplankton, particulate detritus and dissolved organic matter making them important organisms in the filtration of water (Korringa, 1952; Gosling, 2004; Dame, 2016) with a single native oyster filtering up to 200 litres of water a day (Harding, Nelson and Glover, 2016). They are important organisms for pelagic-benthic coupling because, through the production of faeces and pseudofaeces; they increase turnover rates of nutrients and organic carbon, increase the overall productivity of the ecosystem and enrich the underlying sediment, providing a food source for benthic organisms (infaunal detritivores), deposit feeders, meiofauna and bacteria (Korringa, 1952; Gosling, 2004; Dame, 2016).

Another important ecological role played by oysters is the stabilization of sediments they inhabit so decreasing the turbidity of the water column allowing higher levels of light penetration causing a direct positive effect on primary production (Gosling, 2004; Dame, 2016). Their important role in

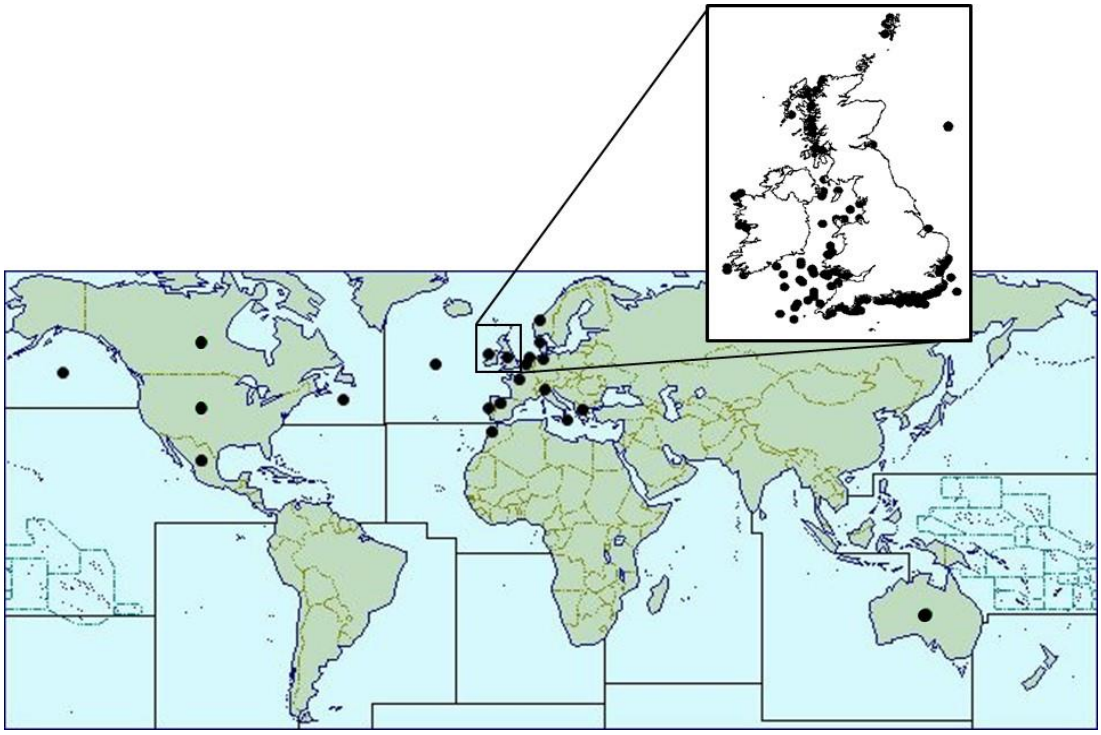

Figure 1.2 World distribution of *Ostrea edulis* (modified from CABI (2019) and Perry and Jackson (2017)).

stabilizing and regulating ecosystem conditions has been reported on several occasions, e.g. the decline in the size and abundance of *Crassostrea* spp. reefs in Chesapeake Bay caused an increased in nutrient levels and stronger algal blooms, increasing the risk of anoxic events (Dame, 2016).

1.3 Reproduction in *Ostrea edulis*

O. edulis has an unusual reproductive biology that is well documented (Orton, 1927c, 1927a, 1927b, 1933); it is a larviparous and a protandrous alternating hermaphrodite species, maturing at around 3 years of age as a male and then exhibiting an alternating sexuality between female and male sexual phases (Sparck, 1925; Orton, 1927a, 1927b, 1933; Cole, 1941; Korringa, 1952; Mann, 1979).

In brief, mature ripe male oysters release sperm in a constant flow into their exhalant siphon and into the water column and until their testes are depleted. In females, mature oocytes are transported from the exhalant to the inhalant chamber where they are kept (Korringa, 1952). Mature sperm is detected in the water by ripe females, it is taken up with the inhalant current, their eggs are consequently released into the suprabranchial chamber and branchial chamber by a series of contractions before internal fertilization takes place using the stored sperm. Fertilized eggs and larvae are brooded within the mantle cavity for a further 6 - 15 days until the veliger larvae have a fully formed shell of about 0.170 mm in length (Orton, 1927b; Newkirk and Haley, 1982). Once the veliger larvae are released into the water column, they spend between 10 - 16 days before settling and metamorphosing to juvenile oysters (Korringa, 1952).

The mechanisms and conditions which trigger gametogenesis, spawning after ripeness and sex determination are still unclear (Korringa, 1952; Wilson and Simons, 1985), but there are several factors that could be involved in this process. Some of the reproductive parameters of *O. edulis* and factors affecting those processes will be discussed in the next sections.

1.3.1 Sex cycle, phases, and gametogenesis

Ostreids have simple reproductive organs which do not differ between sexes, so in adults the genders can only be determined during the reproductive season from the presence of gametes (Orton, 1927c, 1927a). A thick matrix of connective tissue surrounding the digestive system provides support, communications and substrate for the differentiation of gonadal tissue, and annually it is transformed from storage tissue to gonads and back (Fig. 1.3). In winter with cold temperatures, the oysters are in a resting phase so the remaining sex products are resorbed for glycogen storage in the next reproductive cycle, and blood cells invade the gonadal tissues

(Andrews, 1979). Then, in spring, the connective tissue fills with gonadal tubules again, preparing the individual for another gametogenesis process and a new reproductive cycle (Andrews, 1979).

Gametogenesis of both sexes in a single follicle is a common phenomenon in flat oysters, resulting from the changing of sex (Andrews, 1979). Sometimes a few remaining gametocytes are retained over the winter, creating confusion about the presence of hermaphroditism in this species (Orton, 1927b). However, the majority of researchers have argued that most bivalves are unisexual and that they do not demonstrate true functional hermaphroditism, but instead transitional sexual phase (Loosanoff, 1962). *O. edulis* usually first undergo gametogenesis as a male and, when older, the oyster can alternate between female and male functions (Orton, 1927a, 1927b; Cole, 1942b; Loosanoff and Davis, 1952; Loosanoff, 1962; Laing, Walker and Areal, 2005). Once oysters are mature, it has been reported that they can spawn as both males and females during the same reproductive period (Orton, 1927c, 1927b, 1933) under optimal conditions, although subsequent to these early citations this has rarely been reported. The alternating female and male sexual phases do not occur at the same time and the production of eggs starts after the sperm has been completely released or vice versa (Coe, 1943). This release can follow immediately or be delayed until the next reproductive season. The lack of synchronicity in wild populations results in a mixed-sex population and makes it possible to find individuals of both sexual types during the spawning season (Coe, 1943; Loosanoff, 1962).

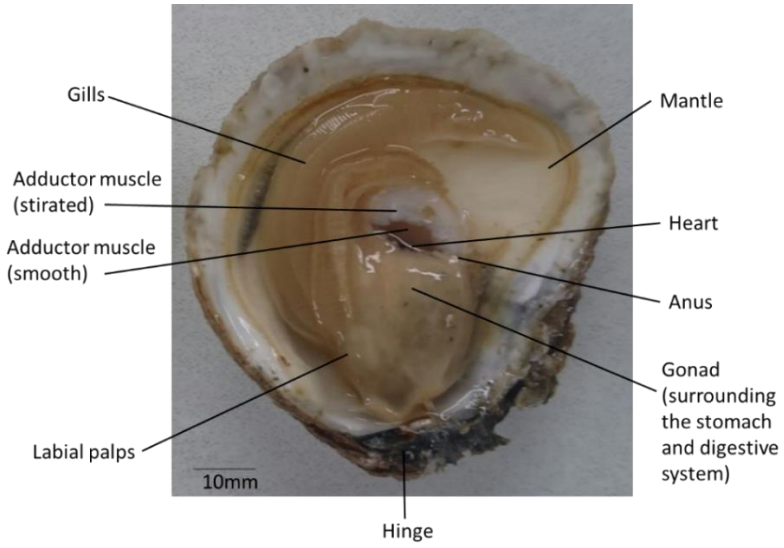

Figure 1.3 Gross anatomy of *Ostrea edulis*.

It has been observed that generally UK oysters spawn just once every summer (Orton, 1927c; Korringa, 1957). Under exceptionally favourable conditions, *O. edullis* has the potential to reach maturity and spawn several times during the same season because even just a few hours after

releasing eggs or sperm the gonads can begin to change into the opposite gender (Korringa, 1957). However, it has been reported that this species needs a large quantity of energy to produce ovaries and the ability to become a functional mature female occurs just after an exceptional summer period (Dodd *et al.*, 1937).

Histological analysis of reproductive tissue has been a useful strategy to determine the reproductive cycle, gonad development and sex determination in bivalves (Howard *et al.*, 2004; Kim, Ashton-Alcox and Powell, 2006). Six categories can be considered when assessing the gonad development of oysters at a particular moment: undifferentiated, male, female, predominantly male, predominantly female, and oysters with both sexes represented equally (Mann, 1979; Siddiqui and Ahmed, 2002). The gonadal stage (Table 1.1) is an important parameter that provides a useful indication about the reproductive status and activity at individual and population levels (Howard *et al.*, 2004; Kim, Ashton-Alcox and Powell, 2006).

Table 1.1 Criteria used in this study to define gonad development in bivalves, particularly in *Ostrea edulis* according to da Silva *et al*. (2009).

Gonad stage	Description	Gonad properties
GO	Inactive or resting gonad	No evidence of developing or ripe gametes. The gonad contained dilated and unfilled follicles. The follicles locate between the mantle and the digestive gland walled by abundant connective tissue.
G1	Early gametogenesis	Gonad follicles are more spread into the connective tissue. Oogonia and spermatogonia were mostly attached to the follicle cells. In addition, for male oysters, primary and secondary spermatocytes are observed and developing oocytes of female oysters are obviously attached to developing lines.
G2	Advanced gametogenesis	Gonad follicles are bigger than in the previous stage while connective tissue is reduced. Male oysters, development of few spermatogonia still occurred, but spermatocytes and spermatid balls are dominant; in females, oocytes in vitellogenesis are dominant but oocytes in post-vitellogenesis are thin.
G3	Ripe gonad	Large follicles can be observed in the whole area between the mantle and digestive gland. Both functional male and female developing lines, follicles contained gametes, plentiful spermatozoa balls and advanced oocytes.
G4	Partially spawned gonad	Gonad follicles are smaller than in the ripe stage and separate by some connective tissue. Residual mature gametes remain in the follicle lumen. More than 70% of gametes have been released. Residual spermatozoa/ oocytes appear in largely empty follicles and gonoducts. Presence of some phagocytes in the follicle lumen.
G5	Reabsoring gonad	Few gonad follicles appeared mostly located in the proximity of the mantle. Abundant connective tissue. Residual mature oocytes and sperm balls could be observed in the follicle lumen. A thin layer of primary germ cells appears in the follicle wall. Phagocytes were often observed in the follicle lumen.

The proportion of gonadal to somatic tissue and the timing of spawns can impact the physiology, and behaviour and response to environmental conditions (e.g. contaminants) in target species of oysters and mussels (Kim, Ashton-Alcox and Powell, 2006). This makes the stage of gonadal development an essential assessment to determine the physiological state of bivalve populations.

1.3.2 Sex determination and sex ratio

Sex ratio is a fundamental indicator for population reproductive success but the wide diversity of sexual systems generates natural populations displaying sex ratios different to the proportion 1:1 (Fisher, 1930; Charnov and Bull, 1989). This allows them to maximize reproductive potential in a given environment through the increase of reproductive success in populations with low densities with a biased sex ratio generally towards females rather than males, to create a more productive group (Cole, 1954; Morton, 1991).

Although sex parity in *O. edulis* inhabiting the waters of Britain and Ireland was reported in the last century (Orton, 1927b; Cole, 1941; Mann, 1979), early field studies reported a male-skewed ratio for *O. edulis* in natural populations (Cole, 1942b; Millar, 1964). More recent investigations have demonstrated extreme sex ratios towards male-phase oysters in members of the family Ostreidae, including the *O. edulis* population inhabiting the Solent, with ratios as high as 7:1 M:F being reported (da Silva, Fuentes and Villalba, 2009; Kamphausen, Jensen and Hawkins, 2011; Eagling, 2012; Acarli *et al.*, 2015; Hassan, Qin and Li, 2018). A cyclically skewed sex ratio, especially a male-biased sex ratio, could decrease the effective breeding population size (Baeza *et al.*, 2010) making the populations susceptible to other external factors that precipitate population declines. It is thus important to identify and understand the factors that may trigger or affect sex changes in this species.

Gender can be also affected by environmental factors such as temperature and food availability but only a few studies have covered the influence of temperature on sex ratio in marine bivalves (Loosanoff and Davis, 1952; Loosanoff, 1962; Fabioux *et al.*, 2005; Joyce *et al.*, 2013). The next sections will discuss the evidence for the effect of temperature and food availability on gametogenesis and sex determination in molluscs (Fig 1.4).

1.3.3 Temperature as a factor influencing reproductive attributes in bivalves

Temperature has been recognized as an important factor with great influence on ripening and spawning of oysters (Orton, 1927b; Nelson, 1928; Korringa, 1957; Mann, 1979; Wilson and Simons, 1985). Indeed, temperature has been shown to be an important requirement for gametogenesis in

many species of bivalves (Fig 1.4) (Korringa, 1952; Loosanoff and Davis, 1952; Mann, 1979). A direct effect of this abiotic parameter on sex ratio and sex determination in bivalves has also been shown (Chávez-Villalba *et al*., 2011; Fabioux *et al*., 2005; Joyce *et al*., 2013; Loosanoff, 1952, 1962; Orton, 1927a, 1927b; Teaniniuraitemoana *et al*., 2015).

The effect of temperature on reproductive parameters has been widely studied in the pacific oyster (Shpigel, Barber and Mann, 1992; Chávez-Villalba *et al.*, 2003; Fabioux *et al.*, 2005; Enríquez-Díaz *et al.*, 2009). *M. gigas*, a marine broadcast spawner with an alternative and irregular protandrous hermaphroditism, a male-biased sex ratio was obtained in adults by conditioning at low temperature (8°C) (Fabioux *et al*., 2005). In the same species, it has been shown that the temperature regulates the speed and thus the duration of gametogenesis presenting accelerated gametogenesis at high temperatures (Shpigel, Barber and Mann, 1992; Enríquez-Díaz *et al.*, 2009).

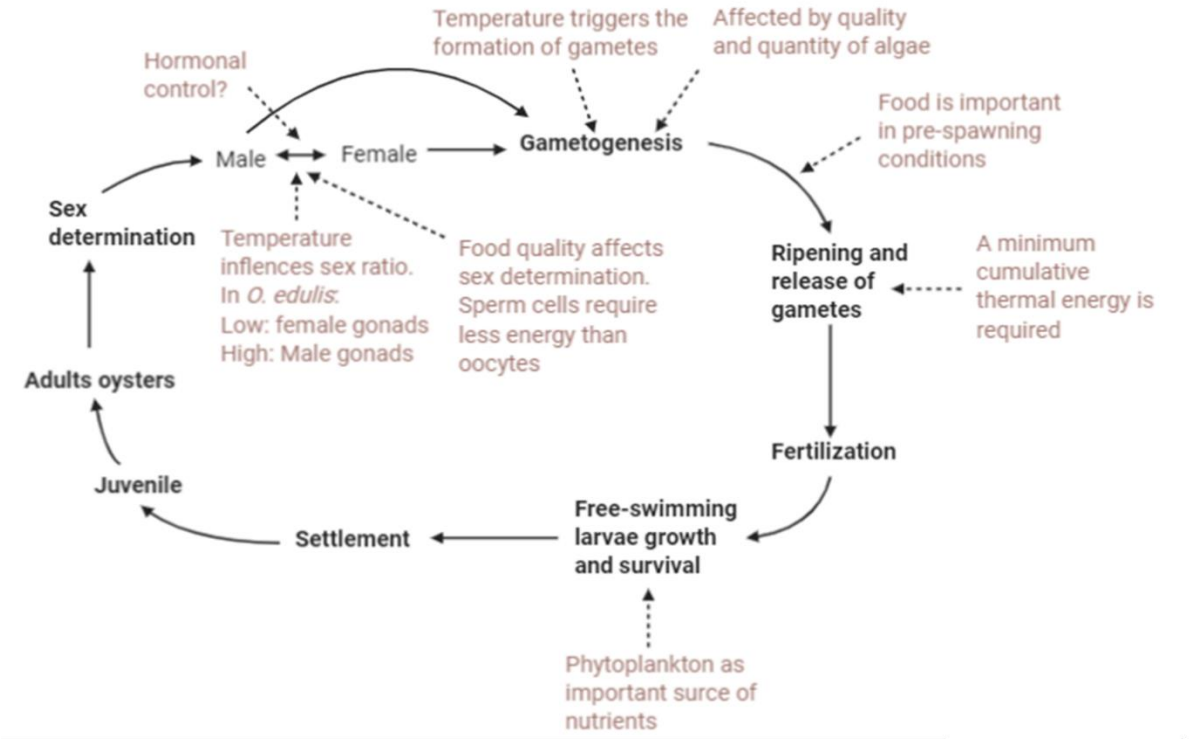

Figure 1.4 Scheme summarising the effect of temperature and food as regulators of reproduction in oysters (Image created with Biorender (https://biorender.com/).

As reviewed by Korringa (1957), *O. edulis* populations react differently to temperature, and specific temperature requirements for gametogenesis vary between populations with local adaptations. Nevertheless, there appears to be a minimum cumulative amount of thermal energy that is required for the ripening and release of gametes to the population (Fig 1.4) (Korringa, 1957; Mann, 1979; Wilson & Simons, 1985). This slightly contradicts Orton's suspicion of the existence of a trigger

temperature above which gametogenesis would automatically go ahead and continue until the temperature fell below that critical temperature (Orton, 1920). Populations of *O. edulis* in England, France, and the Netherlands undergo rapid gametogenesis once the water begins to warm in spring, and spawn once the water temperature has reached at least 16°C (Korringa, 1952, 1957; Loosanoff, 1962; Mann, 1979).

Lower temperatures appear to be implicated in the development of the female germinal cell lines in *O. edulis* (Loosanoff, 1952, 1962). Joyce *et al* (2013) tested the effect of photoperiod and temperature on gametogenesis and sex ratio in *O. edulis*, finding a predominantly female-bias at the beginning of the breeding season at the coldest water temperatures, whereas male gonads appeared when temperatures were warmer.

The exact mechanism by which the temperature triggers gametogenesis and affects sex determination in these species is still unknown and differences between species with different life histories can be expected (Breton *et al.*, 2018). The interaction with other biotic factors (e.g. food availability) can affect reproductive parameters in bivalves (Teaniniuraitemoana *et al.*, 2016) and need to be considered to understand the reproductive strategy of *O. edulis*.

1.3.4 Food availability influences on reproductive parameters in bivalves

Food quality is known to be an important factor in survival, development, and reproduction in bivalves affecting broodstock energy reserves, fecundity, quality and quantity of eggs, and larval development (Fig 1.4). The nutritional value of algae in bivalves is determined by the fatty acids content so the pre-spawning conditions of adult bivalves and larvae quality are influenced by quantity and quality of available food (Hendriks, Van Duren and Herman, 2003; Willer and Aldridge, 2019). Thereby the measure of certain type of fatty acids, such as the essential polyunsaturated fatty acids (PUFAs) eicosapentaenoic acid (EPA) 20:5(n-3) and docosahexaenoic acid (DHA) 22:6(n-3), are presumed to determine the nutritional value of algae for bivalves (Hendriks, Van Duren and Herman, 2003).

Despite some studies showing an absence of correlation between fatty acid concentration and gonad development in *O. edulis* (González-Araya *et al.*, 2011), it has been shown that gametogenesis can be affected by different types of algae used under controlled conditions (González-Araya *et al*., 2012; González-Araya, Quillien and Robert, 2013). It has been reported that an algal diet rich in carbohydrates produces the best gonadal development in this bivalve (González-Araya *et al*., 2012), and a mixed-algal species diets support generally better growth and competence than single-species diets in *O. edulis* (Fig 1.4) (Helm, Holland and Stephenson, 1973). In the same

manner, Willer and Aldridge (2019) showed that diets containing live microalgae in combination with microcapsules designed to carry high levels of nutrients, such as EPA and DHA, reduced mortality in spat and larvar in this species and improved the growth in juvenile oysters.

There is also an interaction between food availability and temperature that affects gametogenesis and sex ratio in marine bivalves. For instance, *Pinctada margaritifera* showed a significant sex ratio change, determined histologically, for oysters conditioned at high temperature (28°C) and low trophic level (40,000 cells/mL), with 50% of the initial females having changed sex after 60 days under these environmental conditions (Teaniniuraitemoana *et al.*, 2016). Conversely, in the same study a high concentration (40,000 cells/mL) of microalgae promoted female gonadal cell proliferation and gamete maturation.

It has been suggested that the production of female gametes is supposed to be more energetically costly than the production of male gametes. Sperm cells require less energy than oocytes to complete maturation, so it could be expected that mature females allocate more energy per unit organ to mantle-gonads than mature males showing differences in energy allocation between sexes during gonadal maturation (Pérez *et al.*, 2013). This has also been observed in *Aulacomya atra* and *Scrobicularia plana* males and females showing that even when they can reach a similar energy content of the mantle-gonad, they use this energy in a different way: males have gonads of larger size but with lower energy per unit of mass than females (Mouneyrac *et al.*, 2008; Pérez *et al.*, 2013). In an environment with poorly fed oysters and a high energy demand they do not gain enough energy from the diet and reserves to initiate gametogenesis (Mouneyrac *et al.*, 2008; Pérez *et al.*, 2013). In such a case, sex reversion to the male phase would be a strategy for maintaining sufficient energy for survival (Chávez-Villalba *et al.*, 2003).

1.4 Role and mechanism of action of sex steroids

Sex steroids are complex four-ringed organic molecules with many roles and functions in multicellular organisms (Norris and Carr, 2013; Cole, Short and Hooper, 2019). According to their structure, they can be grouped into four categories including polypeptides, steroids, amines, and fatty acid derivatives (Ruiz-Cortes, 2012). In vertebrates, all steroid hormones are synthesized from cholesterol through a common precursor steroid, pregnenolone (Fig 1.5) (Ruiz-Cortes, 2012). The cholesterol can be obtained from different sources: synthetized *de novo* from acetate, obtained from plasma low-density lipoproteins (LDL) and high-density lipoprotein (HDL), derived from the hydrolysis of stored cholesterol esters in the form of lipid droplets, and interiorize from plasma membrane (Medvei, 1982; Ruiz-Cortes, 2012; Cole, Short and Hooper, 2019).

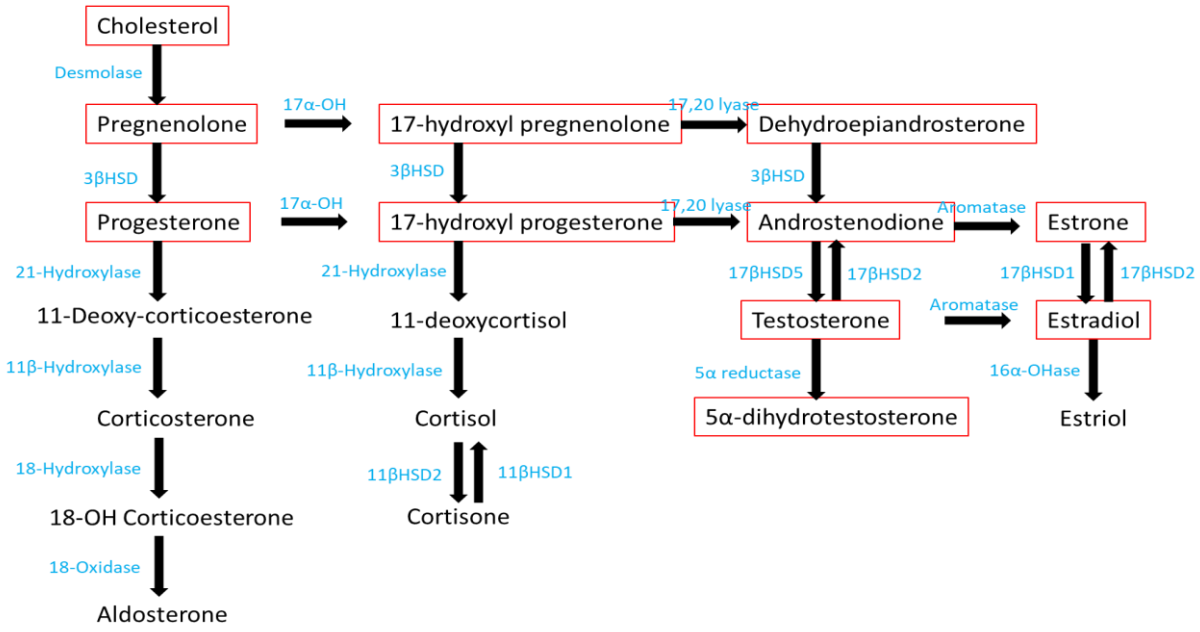

Figure 1.5 Steroidogenic and metabolic pathways described in vertebrates (adapted from Cole *et al*., 2019; Medvei, 1982; Norris and Carr, 2013). Key enzymes (in blue) involved in steroid hormone synthesis reported in vertebrates. Metabolites that have been reported in molluscs (in red squares) according to Janer and Porte (2007). HSD=hydroxysteroid dehydrogenase

In all organisms, hormones *in vivo* play key regulatory roles in mediating communication and regulation of important functions and processes within and between cells, and across tissues, to connect all organs of the body (Medvei, 1982; Norris and Carr, 2013; Cole, Short and Hooper, 2019). Due to their lipophilic nature, they can readily diffuse through or incorporate as structural components into the phospholipid bilayer of the cell membranes (Medvei, 1982; Cole, Short and Hooper, 2019). Sex steroids are secreted and transported to other parts of that body to evoke physiological responses (Medvei, 1982; Cole, Short and Hooper, 2019). In vertebrates, sex steroids are produced mainly in the gonads and regulate not only gonadal development and reproduction by the hypothalamic-pituitary-gonadal axis, but also a wide variety of other processes in the body such as growth, development, metabolism (fat, muscle, bone mass), immune function, blood salt balance, response to stress, and neuronal function (Ruiz-Cortes, 2012). They act in target cells through binding and activating specific receptors, including steroid receptors (SRs) (type II in Fig 1.6) that act as transcription factors and elicit the regulation of extensive molecular pathways, or receptors located at the cell membrane, generating rapid and non-genomic responses following hormone stimulation (type I in Fig 1.6) (Medvei, 1982; Ruiz-Cortes, 2012).

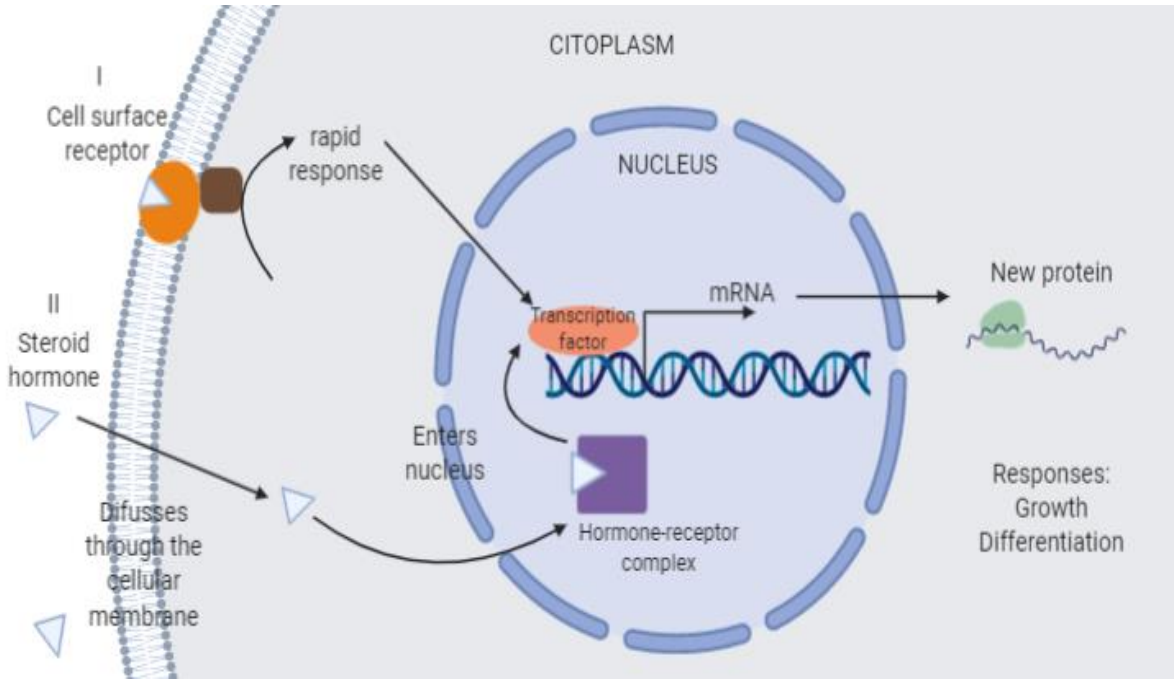

Figure 1.6 Schematic illustration of the mechanism of action of the sex steroid hormones using cell membrane receptor (I) and nuclear signaling (II) pathways (adapted from Medvei, 1982; Ruiz-Cortes, 2012; Wierman, 2007). Image created with Biorender (https://biorender.com/).

The most commonly occurring steroids in vertebrates are the corticosteroids (cortisol/corticosterone and aldosterone), progestogens, androgens, and estrogens (Ruiz-Cortes, 2012; Norris and Carr, 2013). Their common names and functions are giving in Table 1.2. In this thesis, interest will be focused on testosterone and estradiol due to their role in reproduction.

The principal estrogens found in vertebrate females are estradiol (E_2, estradiol-17β, or oestradiol), estrone and estriol. The former is the major estrogen produced by the ovary and is about 10 times as potent as estrone and 80 times as potent as estriol in its estrogenic effect (Ruiz-Cortes, 2012). It is mainly involved in oocyte maturation and the development of secondary sexual characteristics (Table 1.2).

Androgens are considered the male hormones because they confer masculinity by triggering and controlling male sexual development and behaviour in vertebrates (Ruiz-Cortes, 2012). In females, the effects of androgens are more subtle (Table 1.2). Testosterone (T) is metabolized by 5α-reductase in the potent androgen 5α-dihydrotestosterone and like androstenedione to estrone, followed by conversion of estrone to estradiol by 17β-HSD (Norris and Carr, 2013).

Table 1.2 Common vertebrate hormones and associated functions (Cole *et al.*, 2019; Medvei, 1982; Norris and Carr, 2013; Ruiz-Cortes, 2012)

Category	Commonly occurring steroids	Function
Glucocorticoids	Cortisol Costicosterone Cortison	Carbohydrate metabolism Stress response Immune response Protein catabolism Regulation of inflammation
Mineralocorticoids	Aldosterone	
Progestogens	Pregnenolone Progesterone	Maintenance of pregnancy Follicular growth and ovulation Induction of sexual receptivity
Androgens	Testosterone (T) Androstenedione	Sex steroids Development of secondary male characteristics (masculinizing agents) T is the substrate for E_2 synthesis (females)
Estrogens	Estradiol-17β (E_2) Estrone Estriol	Sex steroids Development of secondary female characteristics (feminizing agents) Cardiovascular physiology

Regarding steroidogenesis, a number of excellent studies were performed in molluscs in the 1970s using different radio-labeled steroid precursors combined with the tracing of their metabolism through the formation of different metabolites (Gottfried and Dorfman, 1970; Lehoux and Sandor, 1970; De Longcamp, Lubet and Drosdowsky, 1974; Lupo di Prisco and Dessi' Fulgheri, 1975; Krusch *et al.*, 1979). Nevertheless, since 1970s there has been a gap of research and information about the endocrinology in these animals. The study of the function and occurrence of hormones, with the exception of gastropods and cephalopods, has been limited (LaFont, 2000; Janer and Porte, 2007; Ketata *et al.*, 2007; Fernandes, Loi and Porte, 2011), but the economic importance of several species of bivalves has caused an increased interest of the study of its endocrinology.

1.4.1 Sex steroids in invertebrates

Invertebrates exhibit both asexual and sexual reproduction, with 99% of species reproducing sexually at some point in their life cycle (Giese and Pearse, 1974; Gosling, 2004). The endocrine system differs between the various classes of molluscs, and even among the major group of gastropods, whereby it may be considered as the most diverse hormonal system of the invertebrate phyla (Lafont and Mathieu, 2007). A number of studies have been carried out to better understand

the endocrine functions of steroids in molluscs, but the knowledge is still fragmentary. The presence of steroid hormones (e.g., estrogens, testosterone) or other molecules involved in reproduction, development and maturation, especially in early life stages (i.e., gametes, larvae, and juveniles) in bivalves is poorly understood (deFur *et al*. 1999) and evidence is inconclusive.

As already established, E_2 has been identified as an important primary female sex hormone and T as a key hormone in the development of male reproductive tissues in vertebrates (Table 1.2) (Ruiz-Cortes, 2012; Norris and Carr, 2013; Cole, Short and Hooper, 2019). However, the available information in invertebrates remains equivocal and information is contradictory about the effects of these sex steroids in bivalves (Table 1.3).

Some laboratory-based studies have reported the change in sex ratios in bivalves after exposure to sex steroids and the presence and role of neuropeptides and peptide hormones with physiological functions in some taxa has been proposed (Ketata *et al.*, 2007; Lafont and Mathieu, 2007). Early investigations revealed that injections of E_2 induced sex reversal from male to female in *M. gigas* (Mori, 1969). In the same way, the direct injection of E_2 into male gonads of *P. margaritifera* showed a significantly lower proportion of males and a significantly higher proportion of undetermined oysters with lower proportions of intermediate and mature gonads indicating a potential feminizing effect of E_2 (Teaniniuraitemoana *et al*., 2015). On the other hand, injections of testosterone, estradiol, progesterone, and DHEA stimulate male reproductive activities and spermatogenesis in other marine bivalves (Wang and Croll, 2004) and increase the male/female ratio in *Placopecten magellanicus* (Wang and Croll, 2006) showing a masculinizing effect caused by estrogenic and progestatic steroids. In the same manner, the mussel *Choromytilus chorus* treated with dihydrotestosterone (DHT) and E_2 produced more males and females, respectively (Ruiz-Velásquez *et al.*, 2018). Thus, steroidal involvement in gender determination has been suggested, but the exact mechanism and the effect of each steroid remains unclear and apparently differs between species.

Fluctuations in the concentrations of sex steroids have been found to be correlated with the sexual maturation cycle in a number of bivalves (Table 1.5), thus suggesting that sex steroids may play important stimulatory roles in their reproductive regulation (Reis-Henriques *et al.*, 1990; Osada, Tawarayama and Mori, 2004; Gauthier-Clerc, Pellerin and Amiard, 2006; Liu, Li and Kong, 2008; Yan *et al.*, 2011; Liu *et al.*, 2014). In the soft clam *Mya arenaria*, Gauthier-Clerc *et al*. (2006) suggested that estradiol-17β (E_2) and testosterone (T) act as endogenous regulators of gametogenesis. In the mussel *Mytilus edulis*, the levels of progesterone was correlated with the development of gonads (Reis-Henriques *et al.*, 1990) adding more evidence to the role of these steroids on gametogenesis.

Additionally to the reported presence and role of sex steroid hormones in bivalves (Ketata *et al.*, 2007; Lafont and Mathieu, 2007) it has been also shown that some groups of invertebrates are able to synthesize sex steroids from precursors such as cholesterol or pregnenolone (Fig. 1.5) (reviewed in Janer and Porte, 2007). For instance, some authors have demonstrated the conversion of androstenedione to E_2 by the action of the CYP450 aromatase in the ovary and testis of the Japanese scallop, *Patinopecten yessoensis* (Osada, Tawarayama and Mori, 2004). The activation of this enzyme by E_2 was also demonstrated in *Mytilus galloprovincialis* showing a concentration-dependant response (Janer, Lavado, *et al.*, 2004). The presence of other enzymes involved in the steroidogenic pathways has also been evaluated revealing its presence and action in bivalves. The androgen precursor androstenedione which is converted to testosterone in vertebrates by the action of 17β-HSDs (Fig. 1.5), could be metabolized mostly to 5α-reduced metabolites (5α-dihydroandrostenedione (5α-DHA) and 5α-dihydrotestosterone (5α-DHT)) in bivalves instead (Janer *et al.*, 2005; Lavado, Janer and Porte, 2006). These results together suggest that the presence of vertebrate-related steroid pathways are present in invertebrates, and particularly in molluscs.

However some authors argue that to be able to measure vertebrate steroids (such as E_2, T and progesterone) in the tissues of invertebrates or detect enzymes that are highly conserved between species do not mean that they are endogenously derived or that they play an important role acting as hormones in other species (Scott, 2013). In fact, a complete scheme of invertebrate-related steroid biosynthesis (enzymatic pathways and steroidogenic cells and tissues), transport, target tissues, and further catabolism is not convincing in bivalves (Scott, 2012, 2018). Some authors have reported a complete lack of the physiological role of steroids in mussels (Zabrzańska *et al.*, 2015). This adds more evidence to the open question about the origin of steroids and its actual role in marine molluscs' reproduction.

In fact, sex steroids have been shown to stimulate morphological differentiation in bivalves but not in a steroid-specific way (Wang and Croll, 2004). A number of endogenous and exogenous chemicals have shown an ability to regulate or affect growth and physiology (Lafont and Mathieu, 2007) (Krajniak, 2000; Lafont & Mathieu, 2007; Cheek *et al*., 1998). The evidence suggests a possible action of sex steroids in the regulation of the metabolism of glycogen, protein, and lipids in bivalves rather than a direct effect of sex steroids in morphological changes (Mori, 1969; Mori, Muramatsu and Nakamura, 1972).

There is not enough information about the role of sex steroids in *O. edulis* and the exact control for alternating sex change in this species has not yet been resolved satisfactorily (Ketata *et al*., 2008; Morishita *et al*., 2010). Further studies are needed to elucidate the real function and mechanism of action of E_2 and other hormones on reproduction.

Table 1.3 Reproductive effects reported in bivalves after injection or exposure (in water or sediments) to sex steroids (estradiol, testosterone and other molecules involved in reproduction) in laboratory-based studies

Species	Estradiol	Testosterone	Other molecules	Main findings	Reference
Eastern oyster (*Crassostrea virginica*)	Injection of 37.5 and 75 ng of E_2 per g of wet weight			Positive effect on maturation of gonads and spawning condition.	Quintana, 2005
Pacific oyster (*Crassostrea gigas*)	Injection of 50µg/100µL of E_2			E_2 treatment *in vivo* causes significant increases in oocyte diameter and vitellin content in the female oyster	Li *et al.*, 1998
	Injection of 0.1 mg/time/oyster of estradiol-3-benzoate in water			Estradiol-17B accelerated the sexual maturation.	Mori, 1969
	Injection of 0.10 mg/time/oyster of estradiol-17B			Sex reversal from male to female.	Mori, Muramatsu & Nakamura, 1972
	In water: 0.01, 0.1, 1, 10, 100 and 1000 ug/oyster/time of estradiol-3-benzoate			No indication of sex reversal.	Mori, Muramatsu & Nakamura, 1972
Sea scallop (*Placopecten magellanicus*)	Injections of 200 uL (1000 ug/ml)	Injections of 200 uL (1000 ug/ml)	Injections of 200 uL (1000 ug/ml) of progesterone	Estradiol induced spawning in both sexes. Testosterone induced spawning in males only. Progesterone blocked spawning in both sexes.	Wang & Croll, 2006

	Injections of 30 uL (1000 ug/ml)	Injections of 30 uL (1000 ug/ml)	Injections of 30 uL (1000 ug/ml) of progesterone and DHEA	Accelerated gonadal differentiation and shifted sex ratios toward more males with all the treatments. Estradiol appeared to stimulate oocyte growth. Testosterone appeared to induce degeneration of oocytes. Hermaphroditic animals were found in the progesterone and by DHEA injected groups.	Wang & Croll, 2004
Peppery furrow shell (*Scrobicularia plana*)	Sediments spiked with 100 µg/kg of E_2 (WW) and 100 µg/L kg EE_2 (WW)			Intersex condition had been induced. 44% of recovered males were found to have the ovotestis condition.	Langston, Burt & Chesman, 2007
Blue mussel (*Mytilus edulis*)	In water: 5 and 50 ng/L			5-HT receptor and COX mRNA expression levels were altered with estrogenic exposure (depending on the stages of the mussel reproductive cycle). 5-HT receptor mRNA expression levels in control samples did not vary significantly between sexes or stages of development, yet COX mRNA expression levels showed	Cubero-Leon *et al*., 2010

				sex-specific differences, male values higher than females at both gonadal stages.	
	In water: 5 ng/l, 50 ng/l, 200 ng/l of E_2, and 5 ng/l, 50 ng/l EE_2			A significant increase in VTG and ER2 mRNA expression in mussels exposed to estrogens at the early stage of gametogenesis. In contrast, mature mussels displayed no change in the VTG or ER2 mRNA expression.	Ciocan *et al*., 2010
Mediterranean mussel (*Mytilus galloprovincialis*)	In water: 20, 200, and 2000 ng/L			Total E_2 levels (free + esterified) increased in a dose-dependent manner.Testosterone levels, either free or esterified, did not significantly differ between control and exposure groups.	Janer, Lavado, *et al*., 2004
	In water: 20, 200, and 2000 ng/L			Free-estradiol was only significantly elevated at the highest exposure dose (up to 10-fold). Synthesis and esterification of testosterone were not altered by estradiol exposure but significantly increased the formation of 5α-DHA and	Janer *et al*., 2005

				5α-DHT. Exposure of mussels to low estradiol doses induced gametogenesis.	
	In water: 200 ng/L			No significant change in the expression of estrogen receptor (*ER*) or vitellogenin (VTG) genes.	Puinean *et al*., 2006
		In water: 20 ng/L, 200 ng/L and 2000 ng/L		Decreased CYP3A-like activity as a consequence of testosterone exposure. Neither the synthesis nor the esterification of estradiol was altered by T-exposure.	Fernandes *et al*., 2010
Magellan mussel or ribbed mussel (*Aulacomya ater*)	Exp 1: 0, 1, 100 µg/L of E_2 in water. Exp 2: injection of 0,5 y 1 mmol of E_2			A time-dependent increment in ALP levels for males exposed to the lowest E_2 levels. No response in Vg levels was detected in females after exposure to E_2.	Saavedra *et al*., 2012
Chorus mussel (*Choromytilus chorus*)	In water: 3,000 ng/L	3,000 ng/L of dihydrotestosterone (DHT)		Production of more females casued by exposure to E_2 and more males by DHT.	Ruiz-Velásquez *et al*., 2018

Table 1.4 Relationship between changes in sex steroid hormones and reproductive effects reported in bivalves as a response to seasonal variation

Species	Conclusion	Reference
Soft shell clam (*Mya arenaria*)	Increase of E_2 and T at the onset of vitellogenesis in females and during the spawning stage in both sexes.	Gauthier-Clerc, Pellerin & Amiard, 2006
Blue mussel (*Mytilus edulis*)	The levels of progesterone, androstenidione, testosterone, estradiol-17β and estrone were estimated at two different months of the year showing a different pattern between sampling times.	Reis-Henriques *et al*., 1990
Bay mussel or foolish mussel (*Mytilus edulis trossulus*)	No disturbances in the sex ratio and gametogenesis process were observed. High concentrations of T were more abundant for females and E_2 for males.	Zabrzańska *et al*., 2015
Japanese scallop (*Patinopecten yessoensis*)	Ovarian and testicular E_2 increased toward the mature stage and declined after the spawning stage. It was also observed an increase in aromatase activities of the ovary and testis preceded the onset of the ovarian and testicular development.	Osada, Tawarayama & Mori, 2004
Chinese razor clam or Agemaki clam (*Sinonovacula constricta*)	Oestradiol-17b and testosterone increased during sexual maturation in females and males, respectively, and then decreased markedly after spawning.	Yan *et al*., 2011
Egg cockle (*Fulvia mutica*)	Both steroids exhibited a similar annual pattern that correlated with the reproductive cycle. The two peaks of E_2 and T concentrations occurred at critical moments of the *F. mutica* reproductive cycle gametogenesis and of the spawning period). Steroid concentrations were low during the non-reproductive period.	Liu, Li & Kong, 2008
Chinse scallop (*Chlamys farreri*)	Concentrations of E_2 in females and T in males increased with development and maturation of gonad, attained the highest value before spawning, and decreased rapidly after spawning.	Liu *et al*., 2014

1.5 Chemical pollutants as environmental stressors affecting marine bivalves

The marine environment is exposed to different anthropogenic pollutants generated by industrial, domestic and agricultural activities. The loss and degradation of habitat associated with environmental pollution by anthropogenic activities have been identified as some of the main factors affecting declining natural populations worldwide (Lewis and Santos, 2016). Anthropogenic pollution can alter life parameters, such as fertility, fecundity, gametogenesis and sex determination, leading to serious disruptions such as reduction of the population size and alterations in reproductive activity (DeFur, 1999).

Pollutants are any substance capable of causing a damaging biological or ecological response (Freedman, 2015). They act as environmental stressors influencing and limiting the performance or fitness of organisms or populations (Schulte, 2014; Freedman, 2015). Thus the "performance" of an individual, considered as its productivity and reproductive fitness relative to its genetic potential, will be less under stress than it would be possible under optimal environmental conditions (Freedman, 2015). Depending on the intensity of a stressor regime, the growth and fitness of organisms may be diminished or even be made non-viable.

It is necessary to distinguish between the terms stressors and stress (Schulte, 2014; Freedman, 2015; Lewis and Santos, 2016). The term stressor refers to any environmental factor that can cause any stress response. They include biotic factors such as food availability, the presence of predators, infection with pathogenic organisms or interactions with conspecifics, as well as abiotic factors such as temperature, water availability and toxicants. Stress, on the other hand, is the way how the organisms respond to a stimuli (Kültz, 2005). The organisms respond to stressors through a complex set of physiological changes that caused different effects at multiple levels of biological organization and the measure of those changes informs about the stress response (Schulte, 2014).

According to Freedman (2015) "chemical stressors involve situations where the availability of certain substances is intense enough to cause toxicity or another kind of physiological detriment to organisms". The pollutants can cause damage via short or long term exposure, and their effects will depend on their properties, such as solubility, toxicity, bioavailability, and persistence (Fent, 2004; Walker, 2009). Chemical properties of the pollutants determine the uptake routes, transport, metabolism within organisms and, ultimately, the biological effects (Lewis and Santos, 2016).

Exposure of organisms, including invertebrates, to pollutants can lead to serious disruptions such as reduction of populations and changes to reproductive functions (DeFur, 1999). Understanding the effect of chemical pollutants on bivalve reproduction has important implications for establishing if populations of *O. edulis* in the Solent could be at additional risk caused by pollution, with adverse effects on survival and reproduction in a declining population.

1.5.1 Physiological impacts of chemical pollutants in marine animals

The presence of natural and anthropogenic derived chemicals is a key stressor affecting many marine ecosystems (Lewis and Santos, 2016). In marine organisms, many pollutants can interact with and impact physiological processes such as growth and reproduction (Depledge, 1998; Fent, 2004; Walker, 2009; Lewis and Santos, 2016). Due to their sedentary life characteristics and filter-feeding habits, bivalves are widely used as bioindicators to assess the concentrations and effects of contamination in marine ecosystems.

In order to assess the health of aquatic organisms and the biological effects of environmental pollutants, biomarkers that measure changes at the biochemical, cellular and physiological levels have been used as effective early warning tools in ecological risk assessment and marine environment monitoring (Lagadic, Caquet and Ramade, 1994; Hamza-Chaffai, 2014). The use of biomarkers in environmental assessment is an important tool to understand the stress response ranging from the biomolecular/biochemical to the population and community levels, analysing molecules and pathways regulating not only reproduction, but also growth, reproduction and physiology (Lagadic, Caquet and Ramade, 1994; Lafont and Mathieu, 2007).

A number of biomarkers of pollutant exposure have been identified in invertebrate species including detoxification enzymes (CYPs) (Peters and Livingstone, 2007), oxidative DNA damage (Gielazyn *et al.*, 2003; Rigonato, Mantovani and Jordão, 2005; de Lapuente *et al.*, 2015), DNA breaks (Baršienė *et al.*, 2008), among others that can be included among the most widely used parameters as biomarkers in invertebrates (Lagadic, Caquet and Ramade, 1994)**.** However, sometimes the long period between exposure and the expression of an adverse effect and the biological variability among organisms make hard to evaluate the effects of contaminant exposure in field conditions and its repercussions at the population/community level (Lagadic, Caquet and Ramade, 1994). Other methods widely used to better-understand the complexity of biological systems and organisms' responses to environmental disturbances are the omics-based approaches including genomic, transcriptomic, proteomic and metabolomics (Pinu *et al.*, 2019). These methods are more sensitive tools used in order to understand endogenous regulatory mechanisms involved in growth, survival and reproduction to external perturbations.

The use of “omics”, specifically metabolomics in this thesis, evidenced some mechanisms and biological aspects affected in oysters exposed to different pollutants, predominantly pathways involved in oxidative stress, genotoxicity and endocrine disruption. Most of the effects have been studied on fish and molluscs, mainly gastropods, so this literature review includes examples in these groups of animals as models evaluating these responses to chemical pollutants.

1.5.1.1 Oxidative stress

Many of the pollutants found in marine environments, such as metals, nanoparticles, polychlorinated biphenyls (PCBs) and polycyclic aromatic hydrocarbons (PAHs), cause damage to exposed aquatic organisms via oxidative stress damage (Lewis and Santos, 2016). Oxidative stress is caused by the imbalance between the production of reactive oxygen species (ROS) and the cells' ability to efficiently detoxify and remove the reactive intermediates or easily repair the resulting oxidative damage (Simon, Haj-Yehia and Levi-Schaffer, 2000; Elmore, 2007; Hongmei, 2012). ROS are mitochondrion-derived molecules generated through cellular oxidative metabolism and at the same time, they can target the mitochondrial membrane, increasing the mitochondrial membrane gating potential and promoting cytochrome c release as well as programmed cell death (Simon, Haj-Yehia and Levi-Schaffer, 2000). The most significant consequence of oxidative damage is the damage of molecules such as lipids, proteins and DNA, as well as disrupting cellular signalling pathways (Depledge, 1998; Lewis and Santos, 2016).

Cellular defences to protect against and repair oxidative damage include low molecular weight antioxidants, antioxidant enzymes, and DNA repair enzymes (Livingstone *et al.*, 2000; Valavanidis *et al.*, 2006). The best studied cellular antioxidants are the enzymes superoxide dismutase (SOD), catalase, and glutathione peroxidase. Their production and activity are often measured as biomarkers of oxidative stress (Arojojoye and Adeosun, 2016; Lewis and Santos, 2016). Glutathione (GSH) also plays a key role in the detoxification of a large number of xenobiotics, and it has been reported alongside to other antioxidant enzymes in marine invertebrates as part of the antioxidant defences (Lee, 1988; Gamble *et al.*, 1995; Belcheva and Chelomin, 2011). Conjugation with GSH is one biotransformation process that generally results in less toxic products (Lee, 1996).

Due to their involvement in the detoxification processes of xenobiotics, GST activity was proposed as a biomarker for several aquatic species such as fishes, crustaceans or mollusks (Hoarau *et al.*, 2001). Nevertheless, levels of GSTs can be modified by a large range of xenobiotics and also by abiotic factors, like salinity, temperature, pH, conductivity and dissolved oxygen (Davies, 1995) so a careful analysis should be done when interpreting its activity.

1.5.1.2 Genotoxicity

DNA damage occurs naturally in all cells as a result of normal oxidative stress from metabolism and exposure to environmental factors such as UV light (Jha, 2004; Lewis and Santos, 2016). A significant proportion of the chemicals entering the marine environments have the potential to induce DNA damage or interfere with the process involved in cell division (Depledge, 1998; Livingstone *et al.*, 2000; Choi, Yoo and Lee, 2004).

Some contaminants (PAHs, PCBs, heavy metals, herbicides and solvents) can act as genotoxicants, inducing genetic alterations in the integrity and functioning of the DNA of wildlife species (Hartwig, 1995). One of the mechanisms occurs via the ROS production which generates DNA strand breaks (Simon, Haj-Yehia and Levi-Schaffer, 2000; Cadet and Wagner, 2013).

The most common biomarkers employed to determine this type of damage in wildlife species are the micronucleus (MN) test and the comet assay (CA; or Single Cell Gel Electrophoresis, SCGE). They are two sensitive, rapid and extensively used methods that allow the characterization of DNA damage induced by physical and chemical agents due to their ability to detect chromosomal and DNA damage at an early stage (Al-Sabti and Metcalfe, 1995; Udroiu, 2006; Jha, 2008).

The MN test is based on the quantification of whole chromosomes or fragmented chromosomes that are not incorporated into the main nucleus during the cell division due to aneugenic or clastonegic effects (Al-Sabti, 1994, 1995; Al-Sabti and Metcalfe, 1995; Udroiu, 2006). The CA is also an indicator of DNA strand breaks, cross-links and alkali-labile sites in aquatic organisms (Jha, 2008; de Lapuente *et al.*, 2015). The advantages of the CA include the relative ease of application to most eukaryotic cell tissue types, the requirement for a small number of cells, the detection of multiple classes of DNA damage, its sensitivity for detecting low levels of DNA damage (1 break per 10^{10}Da of DNA), and the generation of single-cell data (Jha, 2008).

There is growing evidence that indicates that a range of pollutants can not only have endocrine disruptive effects interfering with the actions of endogenous hormones, but also possess mutagenic and carcinogenic activity (Choi, Yoo and Lee, 2004). The evidence has shown that genotoxic and clastogenic effects can be observed in molluscs after exposure to environmental contaminants indicating a sensitivity and potential use of these species as bioindicators of DNA damage caused by pollution (Jha *et al.*, 2000; Hagger *et al.*, 2002, 2006; Mičić *et al.*, 2002; Gielazyn *et al.*, 2003; Ferraro *et al.*, 2004; Rigonato, Mantovani and Jordão, 2005; Baršienė *et al.*, 2008; de Lapuente *et al.*, 2015)

1.5.1.3 Endocrine disruption

The endocrine system regulates many of the functions essential for life, such as development, growth and reproduction. The presence of chemical substances of natural or anthropogenic origin in the aquatic environment adds to increasing environmental stress (Gravestock *et al*., 2014) because many of these substances are suspected or known to interfere with the synthesis, metabolism, transport, secretion and mechanism of action of natural hormones involved in maintenance of homeostasis and regulation of developmental processes (Crisp *et al.*, 1997; DeFur, 1999). These chemicals are thus defined as Endocrine Disrupting Compounds (EDCs) and a list of more than 500 known or suspected EDCs has been established by the European Community (EEC, 1999). The increase of pollutants in the aquatic environment and its effects related to humans and wildlife has been growing over the last decades (Crisp *et al.*, 1997, 1998; Schug *et al.*, 2016).

EDCs can influence the endocrine system and the reproductive process of exposed organisms through diverse mechanisms: by mimicking or antagonizing the hormone effects, altering the synthesis and metabolism of hormones and modifying hormone receptor levels (Crisp *et al.*, 1997).

A brief history of EDCs

In the 1940s, ecologists noticed adverse health effects related to reproduction in wild populations but it was only until years later that these effects were linked to environmental chemicals (Fry, 1995). In 1958 the endocrinologist Roy Herts proposed that certain chemicals found in feedlots could find their way into the human body and mimic hormone activity (Gassner *et al.*, 1958). Then the publication of the book “Silent Spring” in 1962 introduced for the very first time evidence to the scientific and the public about the effects of chemical manufactured, used and discarded into the ecosystems (Carson, 1962). Since then, and after the creation of the National Institute of Environmental Health Sciences (NIEHS) in 1969 and the Environmental Protection Agency (US-EPA) in 1970, EDCs became a research priority on a global scale (Fry, 1995). Increasing evidence about the similarities of endocrine systems across species, effects on early development and evident effect until later in life have made it a priority to identify and use tools to understand the effect of these compounds on different species (Fry, 1995).

Effects of EDCs in molluscs

Some species of molluscs have been widely used in pollution biomonitoring programs; however, information regarding their endocrinology and enzymatic pathways involved in steroid synthesis and further catabolism of those steroids is still fragmentary and poorly known (Fernandes, Loi and Porte, 2011).

The first reports of EDC in molluscs in the late 1980s (Gibbs and Bryan, 1986; Bryan *et al.*, 1987; Gibbs, Pascoe and Burt, 1988; Ruiz, Bryan and Gibbs, 1994) showed that they could be especially sensitive to environmental exposure to EDCs and they may strongly affect sex determination, fecundity and population dynamics in different species. Nevertheless the gap in information about the endocrinology of molluscs since 1970s (Gottfried and Dorfman, 1970; Lehoux and Sandor, 1970; De Longcamp, Lubet and Drosdowsky, 1974; Lupo di Prisco and Dessi' Fulgheri, 1975; Krusch *et al.*, 1979) and the limited understanding of normal endocrine processes in invertebrates makes it difficult to assess the endocrine disruption effects in the field (DeFur, 1999). This has raised the need for a better knowledge of molluscs (and invertebrate) endocrinology in order to assess alterations caused by pollutants. A number of EDCs regulate or affect growth, reproduction, and physiology of bivalves molluscs (Porte *et al.*, 2006). One of the key elements in the investigations of EDCs' effects in natural populations of invertebrates includes biomarkers of exposure and effects, and indicators of responses to EDCs, such as sex determination, gametogenesis and gonadal development, vitellogenesis, sperm motility, larval growth, survival, and alteration of secondary sexual characters (Gibbs and Bryan, 1986; Thain and Waldock, 1986; Roberts *et al.*, 1987; Gibbs, Pascoe and Burt, 1988; Spooner *et al.*, 1991; Gibbs, 1993; Huet, Paulet and Le Pennec, 1996; Matthiessen and Gibbs, 1998; Ketata *et al.*, 2007; Eagling, 2012).

TBT as an endocrine disruptor compound in invertebrates

Among molluscs, the most widely studied effects of EDCs have been those caused by tributyltin (TBT) (Matthiesen & Gibbs, 1998 and literature cited there). This is the most well-known organotin and it was used widely in marine anti-foulant paints to prevent the growth of organisms such as barnacles on the hull of ships (Gadd, 2000; Pynaert and Speleers, 2000; Matthiessen, 2013). Several studies have shown the ability of TBT to cause many and diverse effects on gastropods (Gibbs and Bryan, 1986; Bryan *et al.*, 1987; Gibbs, Pascoe and Burt, 1988; Spooner *et al.*, 1991; Oehlmann *et al.*, 1996; Matthiessen and Gibbs, 1998; Morgan, Murphy and Lyons, 1998; Oberdörster, Rittschof and McClellan-Green, 1998; Terlizzi, Geraci and Gibbs, 1999; Oberdörster and McClellan-Green, 2002; Gooding *et al.*, 2003; Horiguchi, 2006). Imposex and female virilisation caused by TBT are the most well-known and extensively endocrine effect studied (Matthiessen and Gibbs, 1998). The evidence about the effects of TBT on oysters is scarce and the underlying mechanism remains unclear (Thain and Waldock, 1986; Roberts *et al.*, 1987; Matthiessen and Gibbs, 1998) (see more details about TBT and some related effect on gastropods in Chapter 5).

The effects observed for TBT exposure have also been found for other pollutants. An increase in apparent endocrine disruptors' effects (increased in the number of eggs, altered gamete development, increase in prevalence of oocyte atresia, changes in vitellogenin-like protein levels

and composition, skewed sex ratios and delayed gametogenesis) have been reported from mussels and bivalves exposed to another EDCs such as bisphenol A (BPA), octylphenol (OP), PAHs, PCBs and metals (Oehlmann *et al.*, 2000; Gagné *et al.*, 2002; Gauthier-Clerc *et al.*, 2002; Aarab *et al.*, 2004; Ortiz-Zarragoitia and Cajaraville, 2006).

Sex steroids in the environment

Sex steroids in the environment can act as EDCs causing adverse effects on reproduction in invertebrates. The (xeno)estrogens have been capable to induce intersex in *S. plana* under experimental exposure to sediment spiked with mixtures of E_2, 17α-ethinyloestradiol (EE_2), OP and nonylphenol (NP) (Langston, Burt and Chesman, 2007). Intersex is a phenomenon where animals remain in their original sex, but develop some tissues associated to the opposite sex within their sexual organs. It can occur in both sexes, being more frequently found in males. However, intersex is less catastrophic (compared with imposex) because does not mean complete sterility and is not typically fatal. Intersex is widely reported in gastropods and bivalves and it has been reported in several species including *Littoraria angulifera* from Brazil (Costa *et al.*, 2013), *Ruditapes sp*. from Spain (Delgado and Perez-Camacho, 2002) and Korea (Lee *et al.*, 2010), *M. gigas* from Korea (Lee *et al.*, 2010), *M. galloprovincialis* from Spain (Ortiz-Zarragoitia and Cajaraville, 2010) and *S. plana* from south coast of the UK (Chesman and Langston, 2006; Langston, Burt and Chesman, 2007), Portugal (Gomes, Gonzalez-Rey and Bebianno, 2009) and France (Tankoua *et al.*, 2012).

The exact biological and reproductive effects of exposure to sex steroids is not clear (Table 1.2), and the evidence about the feminizing or masculinizing effects of these hormones is inconclusive and contradictory in some studies (Mori, 1969; Wang and Croll, 2006; Teaniniuraitemoana *et al.*, 2016). A major difficulty understanding the effect of endocrine disruptors has been the variability in effects of the same compound (e.g. hormones or pollutants) in different species. Exposure to estradiol caused sex reversal from males to females in the Pacific oyster, *M. gigas* (Mori, 1969), but the opposite effect has been observed in the scallop *P. magellanicus* (Wang and Croll, 2004). Thain & Waldock (1986) reported that exposure to TBT reduced the proportion of a population which developed as females in European flat oysters, *Ostrea edulis,* and reduced larval production. However, no such effects were observed for the Eastern oyster, *Crassostrea virginica,* exposed to TBT (Roberts *et al.*, 1987). A significant shift towards females has been observed after oestrogenic exposure of the Sydney rock oysters (*Saccostrea glomerata*) (Andrew *et al.*, 2010). Also, a feminization of male clams (*S. plana*) from estuaries in Southwest UK, including Southampton Water, was seen as a result of exposure to endocrine-disrupting chemicals (Langston, Burt and Chesman, 2007). The difference in responses and effects caused by exposure to sex steroids in different bivalve families could suggest that even when these species present the steroidogenosis,

the final response will depend on the evolution and current role of the enzymes involved in this molecular pathway (discussed in detail in section 4.4.4).

Other indicators of reproductive disruption in invertebrates

Vitellogenin (Vtg) in female bivalves has been used as an indicator of reproductive status, especially in the spawning period. Vtg are glycolipophosphoproteins produced in the liver or equivalent organs in response to endogenous estrogens, and are released into the bloodstream, or stored in developing oocytes (Clayton, 1996). Vtg provide energy reserves for embryo development in oviparous organisms (Wallace, 1985; Suzuki *et al.*, 1992) and a similar response can be observed in invertebrates, particularly molluscs (Blaise *et al.*, 1999). Thus, the lipid content of Vtg has been used as an indirect method to measure oocyte proteins (Gagné *et al.*, 2002). Most studies concerning Vtg induction in aquatic invertebrates have been conducted using the alkali-labile phosphate (ALP) method (Blaise *et al.*, 1999) that detects inorganic phosphate liberated from phosphorylated proteins, including Vtg-like proteins in molluscs, e.g. *M. arenaria* (Gagné *et al.*, 2002). Depending on the species Vtg induction has been either successful or unsuccessful after estradiol exposure (Li *et al*., 1998; Gagné *et al*., 2002; Osada *et al*., 2003; Won *et al*., 2005).

In many commercially-exploited and coastal bivalve species, local population densities have been reduced by overfishing and habitat degradation to a point where reproductive success is compromised (Gravestock, James and Goulden, 2014; Southern IFCA, 2015, 2018). In such cases, even modest effects of EDCs may substantially reduce reproductive success. Recent studies have analysed the current situation on reproductive parameters of *O. edulis* population founding a significant reduction in the number of brooding female-phase oysters (Eagling, 2012), low values of fecundity of brooding oysters and skewed sex ratio towards male-phase oyster of 3:1 and 6:1 (Kamphausen, Jensen and Hawkins, 2011; Eagling, 2012). Understanding the effect of environmental factors on *O. edulis* is important to define the main factors affecting a declining population in the Solent where efforts are being made to restore populations (Gravestock, James and Goulden, 2014; Harding, Nelson and Glover, 2016).

1.5.1.4 Metabolomes to understand homoeostasis

The omics-based approaches including genomic, transcriptomic, proteomic and metabolomics are sensitive tools used in order to understand endogenous regulatory mechanisms involved in growth, survival and reproduction to external perturbations (Pinu *et al.*, 2019).

Metabolomics (also known as metabolic profiling) is the non-targeted analysis that characterizes endogenous and exogenous low molecular mass metabolites within a cell, tissue, or biofluid of an

organism in response to external stressors (Lankadurai, Nagato and Simpson, 2013). The ion regulation is essential to maintain homeostasis and cellular osmotic potential to keep the cell membrane integrity so this method allows the determination of changes in individual ions or metabolites (Pinu *et al.*, 2019). These metabolites are end products of gene and protein expression and are exceptionally sensitive to genetic and environmental perturbations (Johnson, Ivanisevic and Siuzdak, 2016; Young, 2016). Metabolites' profiles are used to identify biomarkers indicating changes in physiological responses of organisms exposed to different conditions (Lankadurai, Nagato and Simpson, 2013; Johnson, Ivanisevic and Siuzdak, 2016). For instance, changes in the expression of enzymes that are involved in the regulation of different pathways in living organisms reflect responses to cellular and environmental conditions in real-time (Johnson, Ivanisevic and Siuzdak, 2016). In this manner, a metabolomic profile provides evidence about what occurs in the cellular and physiological processes presenting a general overview of an organism's phenotype (Lankadurai, Nagato and Simpson, 2013). This way each phenotype will reflect specific activities of particular pathways (e.g. glycolysis, TCA cycle, steroidogenesis, fatty acid biosynthesis, amino acid metabolism) under environmental changes or perturbations (Lankadurai, Nagato and Simpson, 2013). For this reason, the use of this technique has shown a good response in environmental pollution assessment using different species as bioindicators (Bundy *et al.*, 2007; Gonzalez-Fernandez *et al.*, 2013). Due to its wide applicability, the interest in metabolomics-based approaches for studying marine invertebrate has increased (Young, 2016; Clark *et al.*, 2017).

In terms of advantages compared with genomic, proteomic or transcriptomic analysis, metabolomics involves less sample preparation, lower costs, can be performed using non-invasive body fluids/solid (e.g. plasma or faeces) and its interpretation is more direct in term of effects (Young, 2016). Another important advantage is that metabolites are not species-specific, compared with genes and proteins, and can be easily applied to the study of non-model organisms (Young, 2016). These characteristics make this method useful in terms of understanding the effect of exposure to different pollutants in molluscs.

1.6 Current situation of a declining *Ostrea edulis* population in the Solent

O. edulis, naturally distributed around the UK and Ireland (Fig. 1.2) has been an attractive traditional product for human consumption (Cole, 1951). This created an interest for formal commercial exploitation of this species and since the 1800s the Solent fishery has undergone substantial fluctuations of prosperity and decline. In addition to exploitations and overfishing, this species has been endangered by cold winters, predation pressure and diseases, and is today in the OSPAR (Oslo

and Paris Conventions for the protection of the marine environment of the North-East Atlantic) and UK BAP lists as threatened and/or declining species and habitats (OSPAR, 2008a, 2008b).

Three population crashes have been reported for the Solent *Ostrea edulis* population during the last century: in 1920-21 triggered by disease; in 1962-63 caused by a harsh winter and the most recent decline (since 2006) where the cause or causes remain unknown (Key and Davidson, 1981; Tubbs, 1999; Southern IFCA 2014 in Gravestock, James & Goulden, 2014). Before the recent decline, 60 boats were licenced to fish in the Solent oyster fishery, providing an important source of income during the winter months (Davidson, 1976; Harding, Nelson and Glover, 2016). In 1978, 450 vessels and 700 fishermen relied on oysters for a substantial part of their income fishing between Weymouth and Chichester (Key and Davidson, 1981; Harding, Nelson and Glover, 2016). In the 1979-1980 season, landings of native oysters peaked at 840 tonnes or 15 million oysters (Key and Davidson, 1981). However, in 2013, the Southern IFCA reported the need to severely restrict the fishery after a drop in the annual harvest from 200 to 20 tonnes of oysters over five years (Harding, Nelson and Glover, 2016).

Recruitment in *O. edulis* beds is a variable and irregular process because they experience periods of natural contraction and expansion making the frequency of these events dependant on a variety of environmental and physiological factors (Spärck, 1949). This behaviour makes these populations vulnerable to overexploitation and a long series of good recruitment events is required for populations to recover after depletion, which can take in the region about 20 to 25 years (Spärck, 1949; Laing, Walker and Areal, 2005). Natural populations inhabiting areas under high fishing pressure can suffer the effects of low population densities that result in disproportionately low recruitment (e.g. Gascoigne and Lipcius (2004)). Hence, according to the Allee effect, when population size becomes smaller its gene pool is reduced and individual fitness decreases, leading the population to a decline in growth rate. A population suffering an Allee effect may decline rapidly due to demographic stochasticity (Stephens, Sutherland and Freckleton, 1999) which added to poor habitat quality and delayed functional maturity can increase a selection pressure acting on this species.

O. edulis population is valuable both ecologically and economically, and the decline in its populations has been well documented, but the exact reason for the decline in the Solent oyster population from 2006 onwards is still unknown (Gravestock, James and Goulden, 2014). A number of factors have been attributed to these events including commercial exploitation, overfishing, habitat degradation, increasing sea temperatures, changes in food availability, the occurrence of diseases caused mainly by parasites, and the presence of indigenous predators and competitors (Fig 1.1) (Orton, 1927c; Laing, Walker and Areal, 2005; OSPAR, 2008a; Gravestock, James and

Goulden, 2014; Harding, Nelson and Glover, 2016). The presence of chemical and physical pollutants of natural or anthropogenic origin in the aquatic environment adds an additional environmental stress to this species influencing growth, development and reproductive process of exposed organisms (Crisp *et al.*, 1997; DeFur, 1999; Porte *et al.*, 2006; Ketata *et al.*, 2007). This chapter has reviewed some of these factors with special emphasis on those affecting reproduction and creating gender imbalance in *O. edulis.* It is important to consider that this species has been through a history of overfishing, diseases and competitors in the Solent, and additional factors such as climate change with rising temperatures and pollution could create additional pressure for this species.

1.6.1 A history of overfishing, diseases and competitors in the UK

Although this study will not evaluate the effect of overfishing, diseases, predators and competitors on the native oyster, it is important to mention briefly these aspects as possible reasons involved in the declining of *O. edulis* populations. Overexploitation combined with a lack of incentive to preserve and protect openly-accessible resources is causing local extinctions (Jennings, Kaiser and Reynolds, 2001). This idea was first established in 1968 by the ecologist Garrett Hardin in his economic theory named "The tragedy of the Commons" (Hardin, 1968). In this text, the author proposed a set of solutions to avoid this situation through strategies such as promoting an incentive to manage exploitation sustainably by fisheries and promoting stronger laws to avoid unlawful exploitation. However, even today, there appears to be limited success in preventing the problem of overfishing.

Culture and exploitation of native oysters beds have been common activities since Roman times (Günther, 1987) and oyster fisheries turned into one of the main economic activitites for Britain in the mid-19th century (Perry and Jackson, 2017). This led to rapid overexploitation causing a population decline by the 19th century in most UK areas, including the Solent, with many beds being depleted (Davidson, 1976; Key and Davidson, 1981). In response, efforts were made to restore the population by re-laying oysters from other areas into the estuaries and harbours, but by the mid-20th century native oyster beds were declared as scarce (Korringa, 1952).

In an attempt to improve the status of oyster fisheries the South of England Oyster Company established oyster parks protected from dredgers and seeded in the 1860s and 70s, importing species as *Crassostrea angulata* from Iberia, *Crassostrea virginica* from the eastern United States, and *M. gigas* from the Pacific (Utting and Spencer, 1992a). These species would not normally reproduce in the temperate UK climate and were mostly used for ongrowing, but they were

successfully implanted in the new environment and became into competitors for *O. edulis* causing a serious long term impact on its populations.

Some actions were taken and under the Solent Oyster Fishery Order 1980 the maximum number of boats exploiting the fishery was limited, licences were taken out from the Southern Sea Fisheries Committee (now Southern Inshore Fisheries and Conservation Authority, IFCA) and agreements were established to shorten the fishing season each year (Gravestock, James and Goulden, 2014; Harding, Nelson and Glover, 2016). In 2010, prior to the closure of the Solent fishery, fishing efforts reduced markedly due to decreasing economic viability. CEFAS surveys of the Solent stock documented declining numbers from 1990-2011, and CEFAS stopped monitoring the stock after extremely poor catch rates (Palmer and Firmin, 2011). Due to a failure of stocks to recover from the population crash in 2006-2007, the Solent oyster fishery was closed between 2013 and 2015 (Southern IFCA, 2015). By 2015, only large (>50mm) oysters in small numbers were caught in the Solent, typically numbering 0-3 per site, implying that a natural recovery in the next few years was highly improbable (Southern IFCA, 2015, 2018).

In addition, since the 1960s the production of the flat oyster *Ostrea edulis* in Europe dropped as a result of the epizootics of two protozoans, *Marteilia refringens* (Grizel *et al.*, 1974) and *Bonamia ostreae* (Pichot *et al.*, 1980). The former causes the "digestive gland disease'' (Grizel *et al.*, 1974) and the latter is responsible for bonamiosis in *O. edulis* (Grizel *et al.*, 1988). This disease affects the viability and quality of oysters. Oyster age has been reported as critical in relation to disease development mainly affecting oysters that are more than 2 years old (Chávez-Villalba *et al.*, 2003; Montes *et al.*, 2003; Lallias *et al.*, 2008).

Something similar occurred when North American imports in the 1880s introduced the slipper limpet *Crepidula fornicata* to Essex, which now colonizes the same habitat as oysters and competes with them for space and food (Loosanoff, 1955; Davidson, 1976), while depositing mud on them (Utting and Spencer, 1992b). It was showed that this settlement of mud converts the substratum and makes it unsuitable for the settlement of spat (Barnes, Coughlan and Holmes, 1973). The presence of *C. fornicata* across Europe has been a matter of concern mainly due to the disruption caused by this species in oyster harvesting and farming (Blanchard, 1997). In recent years the presence of *C. fornicata* has been reported as significantly higher than *O. edulis* within the Solent harbours (Helmer *et al.*, 2019).

Minimum winter temperatures may be important in limiting the ability to develop extensive *C. fornicata* populations in the UK (Minchin, Mcgrath and Duggan, 1995), but with increasing sea

temperatures around the world, including UK waters, this species could become more widely dispersed, and so become more problematic.

The tingle or oyster drill, *Ocenebra erinacea*, and the introduced American oyster drill *Urosalpinx cinerea,* are gastropods inhabiting the same habitat as *O. edulis*. These gastropods prey on small oysters and oyster spat. In 1953, it was reported that 55% of oyster spat were predated by *Urosalpinx cinerea* in Essex (Hancock, 1954). It has long been believed that *O. erinacea* is one of the greatest dangers to newly-settled spat in the Solent. However, there is a lack of evidence of drilled oyster spat shells on the seabed in the Solent and a direct relationship between mortality in adult oysters and the presence of *O. erinacea* has not (yet) been demonstrated (Kamphausen, 2012). At the current time it is difficult to establish a causal relationship between recruitment failures of the native oyster in the Solent and the presence and activity of *O. erinacea*.

1.7 Project framework

An important antecedent for this study was the skewed sex ratio towards male phase oysters in *O. edulis* from The Solent for at least two consecutive years reported by Kamphausen (2012). The data collected in that study showed that these oysters were in a good conditions in terms of growth, survival, and immunology. Temperature is the most important abiotic factor triggering gametogenesis and affecting sex determination in some bivalve species; however, understanding of its ability to affect the reproductive parameters in *O. edulis* is still fragmentary.

On the other hand, the presence of pollutants in the aquatic environment has led to an increasing potential for endocrine disruption affecting physiological and reproductive processes in invertebrates. However, there is a growing amount of evidence regarding contradictory information about the endocrinology in oysters and the presence and role of vertebrate-related steroids are controversial. This makes an assessment of chemical endocrine disruption in this species extremely difficult. More studies need to be undertaken in this research area to clarify what physiological/reproductive role(s) of sex steroid hormones, such as estradiol and testosterone, may play in *O. edulis*.

Therefore, this study will increase the information about biological and reproductive processes of the European flat oyster *O. edulis* recognizing some of the main factors that can affect reproductive parameters in *O. edulis*. Understanding which, if any, steroid hormonal effects may have in *O. edulis* and the relation with change in abiotic factors, such as temperature, have important implications for fisheries' managers and restoration activities. Furthermore, this study will determine whether chemical pollutants, specifically TBT -a well-known endocrine disruptor in molluscs-, have a

significant impact on the ability of *O. edulis* to survive and reproduce, identifying if this pollutant plays an important factor affecting a declining population.

1.8 Research aims and objectives

Although populations have declined in recent years, *O. edulis* remains economically important in the areas where wild or cultivated stocks are present (e.g. regions of France, UK, Spain, Italy, the Netherlands, Ireland). The aim of this project is to clarify the effect and interaction of temperature, steroid hormones and pollutants on *O. edulis* reproduction in laboratory settings and under seminatural conditions. The outcome of this will contribute to the identification and understanding of factors affecting the reproduction in *O. edulis*.

1.8.1 Chapter 2: Effect of temperature and endogenous steroid hormones on gametogenesis and sex ratio in *Ostrea edulis*

Temperature could have an effect on survival and reproductive parameters in *O. edulis*. In this chapter, the role of temperature driving gametogenesis and sex determination in *O. edulis* under controlled laboratory experiment was established. The determination of endogenous steroids hormones homologues was carried out in order to provide evidence related to its presence and possible role in reproduction in this species.

By investigating the effects of temperature increases alongside hormonal factors, it may be possible to identify interactions between two factors which might be strongly implicated in reproductive changes in *O. edulis*. Consequently, the following aims of this chapter are:

- To determine the effects of temperature on mortality, gametogenesis and sex ratio in *O. edulis* under laboratory conditions.
- To evaluate the presence and concentrations of endogenous steroid hormones (estradiol and testosterone) on gametogenesis and sex ratio in *O. edulis* in a laboratory setting.
- To establish the relation between temperature and hormonal expression patterns affecting gametogenesis and sex ratio in *O. edulis* under different temperature treatments.

From these objectives the effect of temperature on gametogenesis and sex determination in *O. edulis* was determined. These findings have been published (Zapata-Restrepo *et al*., 2019), and employed as reference to the other design experiments in this project.

1.8.2 Chapter 3: Annual changes in biochemical composition, gametogenesis, sex determination and endogenous steroid hormones in *Ostrea edulis*

In bivalves, many functional parameters show seasonal changes in relation to abiotic (such as temperature and salinity) and biotic factors (such as food availability). Biochemical and reproductive attributes in *O. edulis* could be affected by seasonal variations that need to take into account to analyse the reproductive changes observed in this species. However, there are no studies on the annual reproductive cycle and biochemical composition of the flat oyster in the Solent.

Understanding of the effects that the Solent conditions have upon storage metabolism in relation to the gametogenic cycle in this species could be considered essential for planning restoration projects in this region. This chapter aimed to examine the seasonal changes in biochemical composition of *O. edulis* in relation to the gametogenic cycle. This allows to determine periods of low energetic condition which increase the fragility of oysters and its sensitivity to confront the different environmental stresses.

This chapter aims to determine if seasonal changes in abiotic and biotic factors in the Solent are important in relation to changes in the biochemical composition (lipids, proteins, carbohydrates, sex steroid hormones) of *O. edulis* triggering gametogenesis and affecting sexual determination. The objectives to achieve this were:

- To evaluate changes on mortality and growth in *O. edulis* kept under semi-enclosed natural conditions in the Solent during an annual cycle.
- To identify changes in biochemical parameters (lipids, carbohydrates, proteins, sex steroids, Vtg-like proteins) of *O. edulis* during an annual reproductive cycle.
- To describe the gonadal maturation and sex determination in *O. edulis* during an annual reproductive cycle under semi-enclosed natural conditions.
- To elucidate the interaction between environmental factors (temperature, salinity, dissolved oxygen, conductivity) and biological variables in *O. edulis* during an annual reproductive cycle kept under semi-enclosed natural conditions.

1.8.3 Chapter 4: Effect of exogenous steroids on survival, homoeostasis and reproduction of *Ostrea edulis*

Information about the role of hormones in normal endocrine system function in bivalves is still contradictory. However, evidence shows that they can uptake these steroids from the environment

creating an endocrine misbalance. This can lead to changes in the normal development and performance of organisms. The aim of this chapter was to understand the effect of the exposure to environmentally relevant concentrations of sex steroid hormones (estradiol and testosterone) in *O. edulis*. The objectives were:

- To evaluate changes on mortality and growth in *O. edulis* treated with different concentrations of estradiol and testosterone for 10 weeks.
- To determine changes in biochemical parameters (lipids, carbohydrates, proteins and sex steroids) of *O. edulis* exposed to different concentrations of estradiol and testosterone for 10 weeks.
- To identify gonadal maturation and sex determination changes in *O. edulis* exposed to different concentrations of estradiol and testosterone for 10 weeks.
- To establish changes in the metabolome profile in *O. edulis* treated with different concentrations of estradiol and testosterone for 10 weeks.
- To identify changes in some of the main pathways involved in survival and reproduction in *O. edulis* after the exposure to different concentrations of estradiol and testosterone for 10 weeks.

1.8.4 Chapter 5: TBT as a model endocrine disruptor pollutant and its effects on reproductive parameters in *Ostrea edulis*

Chemical pollutants are able to modify the physiological, biochemical and reproductive attributes of organisms exposed. TBT, as a masculinizing model EDC pollutant, has shown adverse effects on reproductive parameters in molluscs, mainly gastropods. This pollutant is a widespread global contaminant and still persists in some UK estuaries and harbours. The ecological and population impacts of TBT, and its derivatives, are well studied. But the mechanism through which they induce and promote adverse effects in physiology and reproduction remain unclear.

Understanding the alterations resulting from TBT can help to clarify if pollution by this organotin could be one of the causes for the declining populations of *O. edulis* declining populations around the UK. Therefore, this chapter aimed to determine the effect of the exposure to TBT on survival, growth, and reproductive parameters of *O. edulis* after exposure to TBT under laboratory conditions.

The objectives proposed to achieve this were:

- To evaluate changes on mortality and growth in *O. edulis* after exposure to TBT under laboratory conditions for 9 weeks.

- To determine changes in biochemical parameters (lipids, carbohydrates, proteins and sex steroids) of *O. edulis* exposed to different concentrations of TBT for 9 weeks.
- To determine the effect on gametogenesis and sex determination in TBT-treated oysters exposed to different concentrations of TBT for 9 weeks.
- To establish changes in the metabolome profile in *O. edulis* treated with different concentrations of TBT for 9 weeks.
- To identify changes in some of the main pathways involved in survival and reproduction in *O. edulis* after the exposure to different concentrations of TBT for 9 weeks.

1.8.5 Chapter 6: General discussion

The aim of this chapter was to discuss the most relevant findings of the main factors affecting biological attributes in *O. edulis.* This thesis contributes important evidence about the effect of environmental factors on the growth, physiology and reproduction of *O. edulis*. The main outcomes and limitations of this project are discussed in this chapter. It has also been included the future work proposed to understand the mechanism involved in the reproduction process in *O. edulis*.

1.8.6 Chapter 7: Conclusions

This thesis provides important evidence about biological and physiological attributes in *O. edulis* affected by environmental factors, sex steroids and TBT. The chapter includes the main conclusions of this thesis regarding the biology and endocrinology of this species and some of the molecules related to reproduction that are present in this species and can be affected by environmental pollutants.

Chapter 2 Effect of temperature and its relationship with steroid hormones on gametogenesis and sex ratio in *Ostrea edulis*

2.1 Introduction

This chapter was published as Zapata-Restrepo *et al*. (2019), see Appendix A.

Temperature has been recognized as an important factor influencing reproductive parameters in many species of bivalves, including *Ostrea edulis* (Orton, 1927b, 1933; Dodd *et al.*, 1937; Korringa, 1957; Mann, 1979; Wilson and Simons, 1985). Sea-surface temperatures in the north east Atlantic and UK coastal waters have been rising since the 1980s by around 0.2-0.9^{o}C per decade, with further rises predicted (Holliday *et al*., 2008; Marine Climate Change Impacts Partnership, 2015). This could have impacts in natural populations influencing the behaviour, growth, reproduction and survival of marine species.

A direct effect of temperature has been shown on sex determination and gametogenesis in marine bivalves (Chávez-Villalba *et al.*, 2003; Fabioux *et al.*, 2005; Joyce *et al.*, 2013; Teaniniuraitemoana *et al.*, 2016). Only a few studies have shown the effect of temperature on gametogenesis and sex ratio in *O. edulis*, reporting that lower temperatures could be implicated in the development of female characteristics while warmer temperatures can be related to development of male gonads in this species (Sparck, 1925; Loosanoff and Davis, 1952; Loosanoff, 1962; Joyce *et al.*, 2013).

Sex ratio is an important parameter for population reproductive success (Fisher, 1930; Charnov and Bull, 1989). A male-biased sex ratio could decrease the effective breeding population size (Baeza *et al.*, 2010) making the populations susceptible to other external factors that precipitate population declines. Recent investigations reported a shift in sex ratios towards the male phase in *O. edulis* populations in UK waters (da Silva, Fuentes and Villalba, 2009; Kamphausen, Jensen and Hawkins, 2011; Eagling, 2012; Acarli *et al.*, 2015; Hassan, Qin and Li, 2018) but the relationship with the rise in sea temperatures has not been established. Moreover, in the case of commercially exploited bivalves, including ostreid and crassostreid oysters, the temperature is the most readily modulated environmental factor in hatcheries, so it is useful to understand if this factor may trigger or affect sex changes in this species.

Although the role of hormones in development, maturation, gametogenesis and sex determination is not well understood in bivalves, it has been suggested that an interaction with environmental factors, such as temperature, could be responsible for some of the responses observed in other

species (Mori, Muramatsu and Nakamura, 1972; Ketata *et al*., 2007; Morishita *et al*., 2010; Teaniniuraitemoana *et al*., 2016). Evidence of the presence and role of steroid hormones is scarce and contradictory. Some authors have presented evidence about the presence of these hormones in bivalves as well as some of the enzymes involved in the steroidogenic pathway (Reis-Henriques *et al.*, 1990; Osada, Tawarayama and Mori, 2004; Fine, Johnson and Matt, 2006; Gauthier-Clerc, Pellerin and Amiard, 2006; Janer and Porte, 2007; Ketata *et al.*, 2007; Lafont and Mathieu, 2007; Liu, Li and Kong, 2008; Yan *et al.*, 2011; Liu *et al.*, 2014). On the other hand, for some authors the supporting evidence remains equivocal and more rigorous studies need to be conducted in order to resolve current uncertainties (Scott, 2012, 2013). There is not enough information about the role of sex steroids in *O. edulis* and the exact controls and regulators for alternating sex change in this species have not yet been resolved satisfactorily (Ketata *et al.*, 2007; Morishita *et al.*, 2010). At this time, additional experimental studies are required to understand the potential function and mode of action of sex steroids on sex ratio and gametogenesis.

In spite of the ecological and economic importance of *O. edulis* and the influence of temperature in reproductive parameters in other bivalve species, the role of this abiotic factor in driving gametogenesis and sex determination in this species is not well understood. Similarly, there are not studies about the endocrinology, presence and role of sex steroids in *O. edulis*. By investigating the effects of temperature and the interaction, if any, with hormonal factors, it may be possible to identify a relationship between two factors which might be strongly implicated in reproductive changes in *O. edulis*. Consequently, the aim of this study was to provide evidence of the effects of temperature on gametogenesis and sex ratio in *O. edulis* under laboratory conditions. This chapter also aimed to determine the presence, role of steroid hormones (E_2 and T) and relationship with temperature on reproductive parameters in this species.

2.2 Methods

The Ethics and Research Governance Office (ERGO) run by the University of Southampton reviewed and approved the ethical implications in terms of conditions during transportation, manipulation, acclimation and dissection of the animals throughout this project (Project ID: 20658) and ethics guidelines were followed during the experiment.

2.2.1 Biological material

Oysters (*Ostrea edulis*) were provided by the Loch Ryan Oyster Company, a Centre for Environment, Fisheries and Aquaculture Science (CEFAS)-certified *Bonamia* sp. free location in Scotland. Four-hundred and fifty oysters were transferred to the aquarium of the National Oceanography Centre

Southampton at the beginning of March 2016 and acclimated to 8°C and salinity 33.1 for four weeks (Fig. 2.1) to restrict early gonadal development. The oysters used in this experiment were 5-7 cm at their maximum diameter. Oysters of this size are considered adults as they are reported to be more than two years old at this size in local natural populations (Walne, 1964).

Figure 2.1 Acclimation of oysters to aquarium conditions at National Oceanography Centre Southampton.

2.2.2 Feeding of oysters

Oysters were fed *ad libitum* daily with 40000 cells/ml of a mixed algae diet (40% *Tetraselmis suecica*, 40% *Pavlova lutheri* and 20% *Phaedactylum tricornutum*) following a standard protocol at National Oceanography Centre Southampton (Appendix B). These algal species typically show adequate characteristics as food for several bivalve species and complementary profiles in essential fatty acids (Pernet *et al.*, 2003). Cultures of every algae species were produced in 20 L bottles containing filtered seawater enriched with nutrient solution at 20°C and each bottle was supplied with 0.2 µm-filtered air under continuous light. Microalgae were harvested during the exponential growth phase

(6–8 days). Every day the cell concentration in every culture was counted using a 0.1mm deep Improved Nebauer™ hemocytometer.

2.2.3 Temperature treatments

Oysters were divided randomly among three temperature treatments (10, 14 and 18°C) with 49 oysters per tank and three replicates per treatment. The 75L tanks were filled with filtered natural seawater to get a final concentration of 50 oysters/L. Using different platforms distributed at different levels in each tank the density of animals was kept at 3 oysters/m^2 throughout the experiment (Fig. 2.2). Aquariums were kept under static conditions with aerated filtered seawater being circulated through particulate filters and protein skimmers and with an 80% water change twice per week. Dead animals were counted and removed every day. After each sampling point the total volume of water and algae concentration was re-calculated per tank to keep the same conditions than at the beginning of the experiment.

Temperature was raised at a rate of 1°C per day until the target temperature was reached for all the treatments. The temperatures were chosen so as to remain within the range of temperatures recently reported for the Solent during a year (www.seatemperature.org) whilst accounting for the observation that the maximum scope for growth, optimal filtration and reproduction in *Ostrea* has been reported at 20°C (Newell, Johson and Kofoed, 1977). During the experimental period, mean salinity was 34.5, pH range was 7.8-8.0 and dissolved oxygen was always more than 98%. Water temperatures were controlled throughout the experiments using either a free-standing chiller unit (TECO, model TR60) or using a constant temperature room.

Ten oysters were sampled at the beginning of the experiment (t_0) before randomly divide the animals between the treatment tanks. At each of four months (t_1-t_4), 10 oysters across tanks were dissected from each temperature treatment into discrete tissues. Oysters were opened and dissected in natural saltwater to reduce the stress and damage of tissues. Samples of the visceral mass were immediately fixed in Bouin's solution for histological examination and the other tissues were stored at -20°C until further analysis.

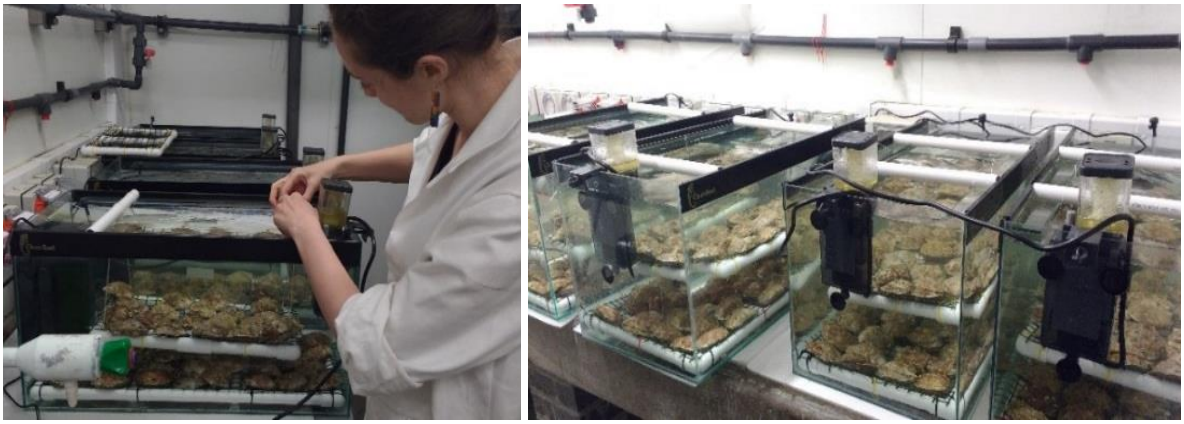

Figure 2.2 Preparation and assembly of aquaria for temperature treatments.

2.2.4 Biological indices

Measurements of height (H), length (L), width (Wi; all to 0.01mm) for every animal were taken using a digital caliper. Shell cavity volume (SVol), Fresh tissue weight (FW) and total weight (W, to the nearest 0.1g) were measured using a Denver instrument SI-603 balance. Maximum antero-posterior length was measured as length, maximum length in the dorso-ventral axis from umbo as depth (height) and maximum thickness of clam when both valves closed as width (Gaspar *et al.*, 2002). After removal of the shells, the Condition Index (CI) was calculated for each bivalve: ([total fresh tissue weight/total weight] * 100) (Walne, 1976; Lawrence and Scott, 1982). This index is a standard method widely used as a health and fitness state used to evaluate the condition of oysters (Crosby and Gale, 1990).

2.2.5 Histological analysis

Sections of the visceral mass were sampled for histological examination following a standard protocol (Howard *et al.*, 2004; Kim, Ashton-Alcox and Powell, 2006). After dissection, a 5-mm thick section was cut along the sagittal plane containing gill, gonad, digestive gland, and mantle lobes and was fixed in Bouin's solution (Sigma-Aldrich™, Dorset, UK) for 24h (Fig. 2.3). The samples were dehydrated through an ethanol series (70%, 80%, 90% and dehydrated ethanol) overnight for each concentration. The samples were embedded in paraffin, and the wax blocks were sectioned at 6-µm using a rotary microtome (*Leitz Wetzler, model 1212*), and stained with hematoxlyn/eosin (Cellpath Ltd) (Howard *et al.*, 2004; Kim, Ashton-Alcox and Powell, 2006). Because maturation is not a homogenous process and female and male gametes can be present in different follicles at different maturation stages at the same time (Sparck, 1925; Coe, 1932; Korringa, 1952; Loosanoff, 1962), three slides per animal were prepared from three different sections separated by 500 µm to determine sex and developmental stage of the gonad. All microscope analysis was carried out using

an Olympus BH-2-RFCA microscope fitted with a Nikon Coolpix E4500 microscope camera. Sex was recorded as indeterminate (I), female solely (F), male solely (M), hermaphrodite with both sexes equally represented (HBS), hermaphrodite predominantly male (HPM) and hermaphrodite predominantly male (HPF) according to (da Silva, Fuentes and Villalba, 2009). The gametogenic stage of the gonad was identified as inactive (G0), early gametogenesis (G1), advanced gametogenesis (G2), ripe gonad (G3), partially spawned gonad (G4) and reabsorbing gonad (G5) adopted by da Silva, Fuentes and Villalba (2009).

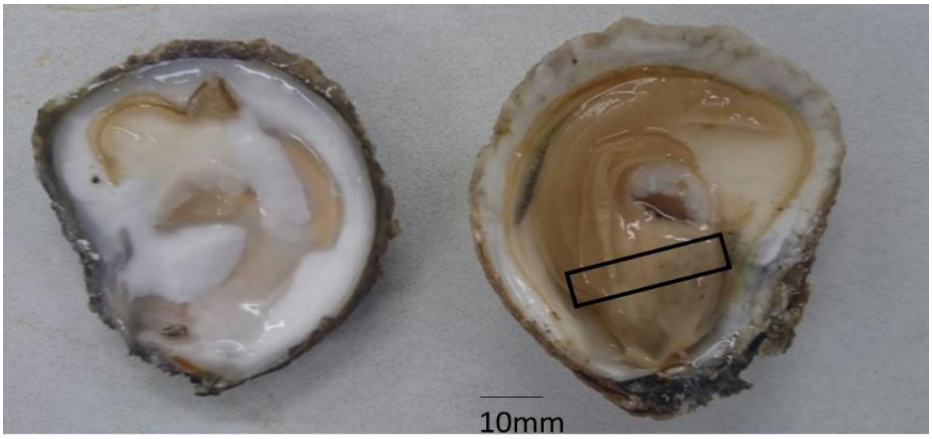

Figure 2.3 Dissection of *Ostrea edulis* for histological examination and biochemical analysis. Black square: section of the visceral mass sampled for histological examination.

2.2.6 Steroid Hormone Homologue Analysis

Extraction and analysis of homologues of the sex hormones E_2 and T concentrations were quantified in the gonads of each oyster using enzyme-linked immunosorbent assay (ELISA) assays (Cayman Chemical Co.; Ann Arbor, MI, USA) as described by Gauthier-Clerc *et al*. (2006). Gonad tissue (0.1 g) from each animal was homogenized in water (1:5 w:w) and sonicated twice for 30s. 400µl of 25mM of hydrochloridic acid (HCl) was added to 500µl homogenate and allowed to stand for 15 min at 40°C. Then, 1.25ml 0.07 M of sodium phosphate dibasic (Na_2HPO_4) (pH 7.4) was added before organic extraction. Homogenates were extracted twice with 14ml dichloromethane (DCM) and organic extracts were evaporated to dryness under a nitrogen stream at room temperature (20-25°C). The resulting pellet was dissolved in 250-µl enzyme immuno-assay buffer. E_2 and T concentrations were determined by competitive ELISA kits according to the manufacturer's instructions. This assay is based on competition between free E_2 (T) and an E_2 (T) tracer (E_2 or T linked to an acetylcholinesterase) for a limited amount of E_2 (T) antiserum. The acetylcholinesterase

bound to the antibody is catalysed into a distinct yellow colour, the absorbance of which was measured at 405 nm. The intensity of the colour, determined spectrophotometrically, is proportional to the amount of E_2 (T) tracer bound to the well, which is inversely proportional to the amount of free E_2 (T) present in the well during the incubation. E_2 and T standards were prepared and determinations carried out in duplicate. Standard curves were carried out with E_2 between 6.6 and 4000 pg/ml and T between 3.9 and 500 pg/ml. *A priori* criteria for intra-assay CVs reported by the manufacturer using a reference standard curve for E_2 and T were 7.8-18.8% and 2.8-14.2%, respectively. The mean intra-assay CVs for standards and samples in this study were ≤ 9.6% for E_2 and ≤ 8.27% for T. Mean inter-assay CVs were ≤ 5.6% and ≤ 6.3% for E_2 and T, respectively.

2.2.7 Vitellogenin-like (Vtg-like) protein assays

Vtg-like levels were determined using an alkali-labile phosphate (ALP) assay (Blaise *et al.*, 1999). In brief, immediately after dissection oyster haemolymph (1.2 ml) was extracted from the adductor muscle sinus of each oyster sample using a hypodermic syringe and 21G needle. Haemolymph was centrifuged at 4°C and 10,000 *g* for 15 minutes to pellet the haemocytes. After centrifugation, the supernatant was retained and the haemocyte pellet was discarded. The free-cell haemolymph (1000 µl) was extracted with 500 µl of 100% methyl-t-butyl ether, vortexed and centrifuged at 1000 *g* for 5 minutes and left at room temperature for 15 minutes. The ether phase of each sample (top phase) was removed from the phase extraction and mixed with 100 µl of 1M sodium hydroxide to release the labile phosphates, stirred and left in dark at room temperature for 60 minutes. After one hour, the alkali-digested phosphate emulsion was centrifuged for 2.5 minutes at 10000 *g* and total inorganic phosphate in the aqueous phase was determined. 1 ml of the aqueous phase was removed and mixed with 1 ml of the acid molybdate solution and 0.4 ml of aminonaphthol sulfonic acid (ANSA) reagent. The reaction was diluted to 10 ml with deionised water and reactions were incubated for 40 minutes in the dark at room temperature. The absorbance of each sample was measured using a spectrophotometer (Jenway 7315, Bibby Scientific Ltd, Staffordshire, UK) at 815 nm and compared to a standard curve to determine the total phosphate concentration in the alkali extraction.

2.2.8 Statistical analysis

Normality of data and homogeneity of variances were evaluated using the Shapiro Wilk and Levene's tests, respectively. Differences between biometric parameters (W, H, Wi, Vol, FW), CI, each steroid hormone concentration, and Vtg-like proteins between temperature and time were tested using the non-parametric Kruskal-Wallis H test at $p<0.05$. In the same manner, the comparison for hormonal concentration and Vtg-like proteins between stages of gonadal

development (G0-G5) was undertaken. When non-parametric Kruskal and Wallis test was significant, differences were then evaluated using a non-parametric the Mann and Whitney test. Spearman correlation was performed to evaluate the correlation between biological variables (biometric parameters, hormone concentrations and Vtg-like proteins). Chi-square statistics were used to test sex ratios against a 1:1 ratio. For the statistical analysis, the Windows 24.0 SPSS was used. Statistical significance was assigned at $p \leq 0.05$.

2.3 Results

2.3.1 Temperature treatments

A record of temperature was taken during the whole treatment. The temperature for each treatment at 10, 14 and 18 °C was kept constant along every treatment with a final average of 10.13±0.70, 14.94±0.57 and 18.20±1.08, respectively.

The monthly mortality across the tanks per treatment is reported in Table 2.1. The observed mortality was 2% during the acclimation at 8°C but it increased during the experiment in all temperatures. In the first two months mortality was less than 5% in every treatment. In the third month, mortality was 5%, 7% and 10% for oysters kept at 10, 14 and 18°C, respectively. But in the fourth month mortality presented the higher values with 7%, 10% and 21% at 10, 14 and 18°C, respectively.

Table 2.1 Observed mortality across tanks per treatment in *Ostrea edulis* kept at different temperatures for four months.

Temperature (°C)	Time (months)	Cumulative Mortality (%)
8	0	2
10	1	4.08
	2	4.96
	3	5.22
	4	7.09
14	1	4.08
	2	4.96
	3	7.46
	4	10.48
18	1	4.76
	2	5.00
	3	10.53
	4	21.01

2.3.2 Biometric measurements after temperature treatment

There was a significant effect of temperature on some of the biometric parameters analysed in oysters under the different treatments (Fig. 2.4, Appendix C). Total weight showed differences that were statistically significant between temperatures (Kruskal-Wallis test value = 8.585, p = 0.035, N = 120) during the experiment but not between different months for each temperature (Fig. 2.4A). Oysters reared at 18°C had the lowest overall weight during the majority of the experiment showing an evident weight loss at this temperature by the second month (Fig. 2.4A, Appendix C). Other parameters such as height, length and width did not show significant difference ($p > 0.05$) between temperatures and time (Fig. 2.4B, 2.4C and 2.4D, Appendix C).

Shell cavity volume showed significant changes during the study (Kruskal-Wallis test value = 9.8314, p = 0.02, N = 120). During the third and fourth months of treatment this parameter increased for oysters kept at 10°C and 18°C compared with the beginning of the experiment (t0) (Fig. 2.4E). Shell volume was lower for oysters kept at 18°C during the first three months, but at the end of fourth month the volume was larger for these oysters (Fig. 2.4E, Appendix C).

There was a significant interaction of temperature and time on fresh tissue weight study (Kruskal-Wallis test value = 11.6551, p = 0.008, N = 120). There was a significant difference (p = 0.04) between fresh tissue weight at the second and fourth months of treatments. This parameter was always lowest for oysters kept at 18°C, and this tendency was even more pronounced at the end of the experiment (Fig. 2.4F, Appendix C).

Condition Index (CI) showed a significant effect (Kruskal-Wallis test value = 6.8393, p = 0.077, N = 120) of temperature throughout the experiment (Fig. 2.4G). Just at the end of the experiment, significant differences between temperatures were found for this parameter. Compared to the beginning of the experiment, CI was lower for all the treatments at months three and four. This was even more evident for oysters kept at 18°C that showed the lowest mean for this parameter.

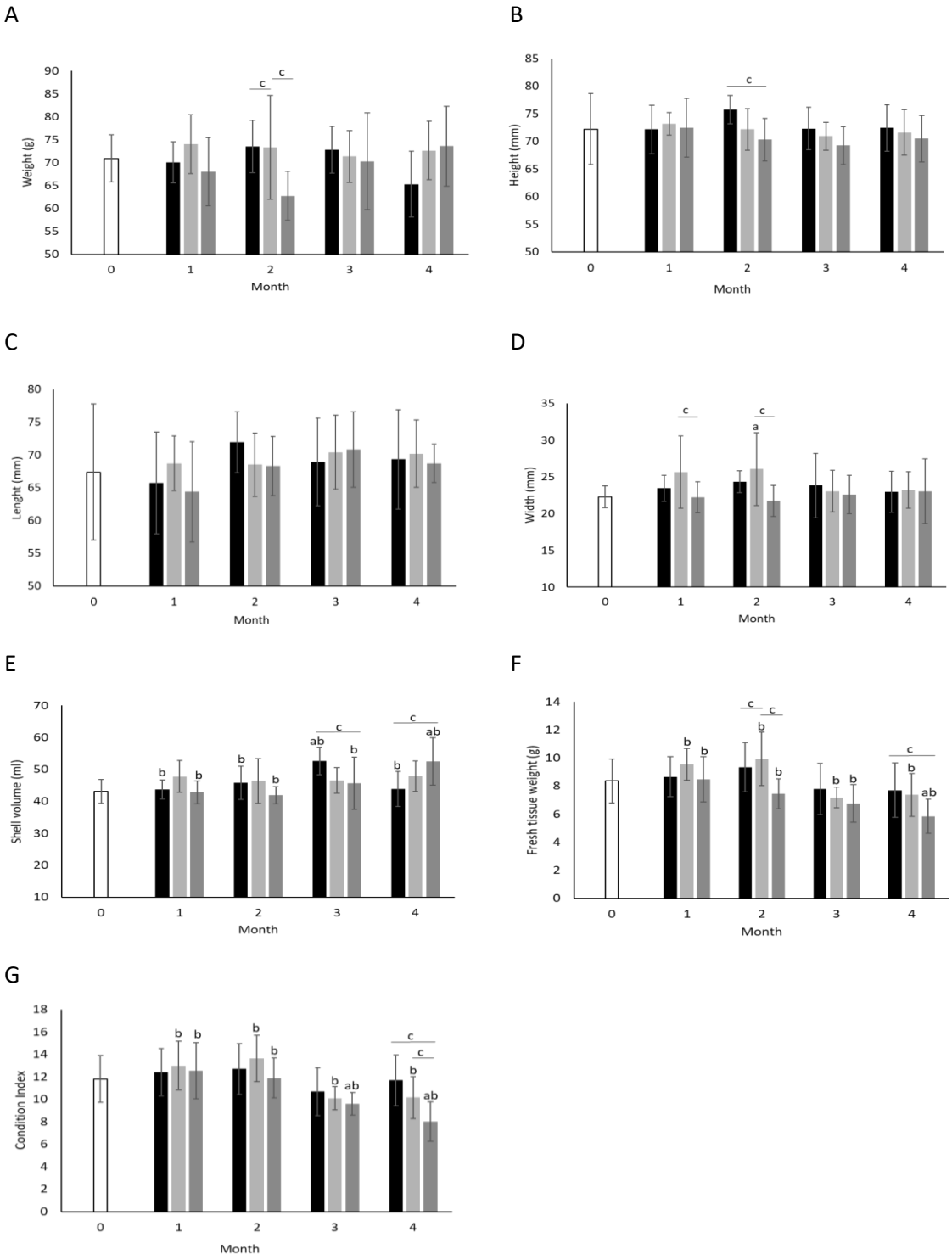

Figure 2.4 Biometric parameters including total weight (A), height (B), length (C), width (D), shell volume (E), flesh weight (F) and condition index (G) measured in *Ostrea edulis* during temperature treatments under laboratory conditions (mean±SD; n=10 per treatment per month). Different letters bars are significantly different ($p < 0.05$) (a) Significant differences compared to the beginning of the experiment (t0), (b) Significant differences between time of treatment at the same temperature, and (c) Significant differences between temperatures at the same time. White bar: beginning of the experiment (t0), black bar: 10°C, light grey: 14°C, and dark grey: 18°C.

2.3.3 Effect of temperature treatments on gonadal development and sex ratio

According to results obtained through histological examination of gonadal tissue (Fig. 2.5), sexes changed throughout the experiment suggesting that it was significantly (Kruskal-Wallis test value = 25.745.585, $p < 0.001$, N = 120) influenced by temperature during the treatments. At the beginning of the experiment 90% of oysters were hermaphrodites and 10% males (Fig. 2.6). Thereafter different sex proportions and different stages of gonad development were identified depending on treatment temperature (Fig. 2.6).

At 10°C the ratio of females increased by the end of the experiment. The sex ratio (males:females) was not significantly different from 1:1 during the first three months (Fig. 2.6). At the end of the treatment at 10°C, 80% of oysters had developed as females and just 20% as males presenting significant differences from the proportion 1:1 (Chi-square test: $\chi^2 = 28.16$, df = 1, $p = 0.035$). The percentage of hermaphrodites decreased over time with HPF occurring in a similar proportion during the exposure and HPM only found at the initial time and first month.

At 14°C the percentage of females and males increased during the first two months as the proportion of hermaphrodites decreased. Throughout the exposure there was a smaller proportion of females compared with males. Incubation at 14°C had the greatest effect on stimulating male gonad development, with sex ratios (M:F) significantly different from 1:1 at the second (2:1) (Chi-square test: $\chi^2 = 13.5$, df = 1, $p = 0.05$) and third (2:1) (Chi-square test: $\chi^2 = 4.17$, df = 1, $p = 0.04$) (Fig. 2.6). At the end of the fourth month at 14 °C, just 10% of oysters were identified as females, 30% as males, 10% as HPF and 50% were in an inactive or undifferentiated state of gonadal development (Fig. 2.6 and Fig 2.7).

At 18°C the percentage of females increased until the end of the second month and then decreased at the end of the third month. The proportion of males was similar during the treatment with a slight reduction at the end of the third month (Fig. 2.6). The sex ratios (M:F) at this temperature showed significant differences from 1:1 at the second (0.43:1) (Chi-square test: $\chi^2 = 20.17$, df = 1, $p < 0.05$) and third (0.2:1) (Chi-square test: $\chi^2 = 8.17$, df = 1, $p < 0.05$) months. Hermaphrodites decreased at the end of first month and they disappeared at the second and third months of treatment (Fig. 2.6). The first undifferentiated animals were observed again at the third month showing an inactive state/spent of gonadal development. At the end of the treatment all the oysters showed inactive gonads.

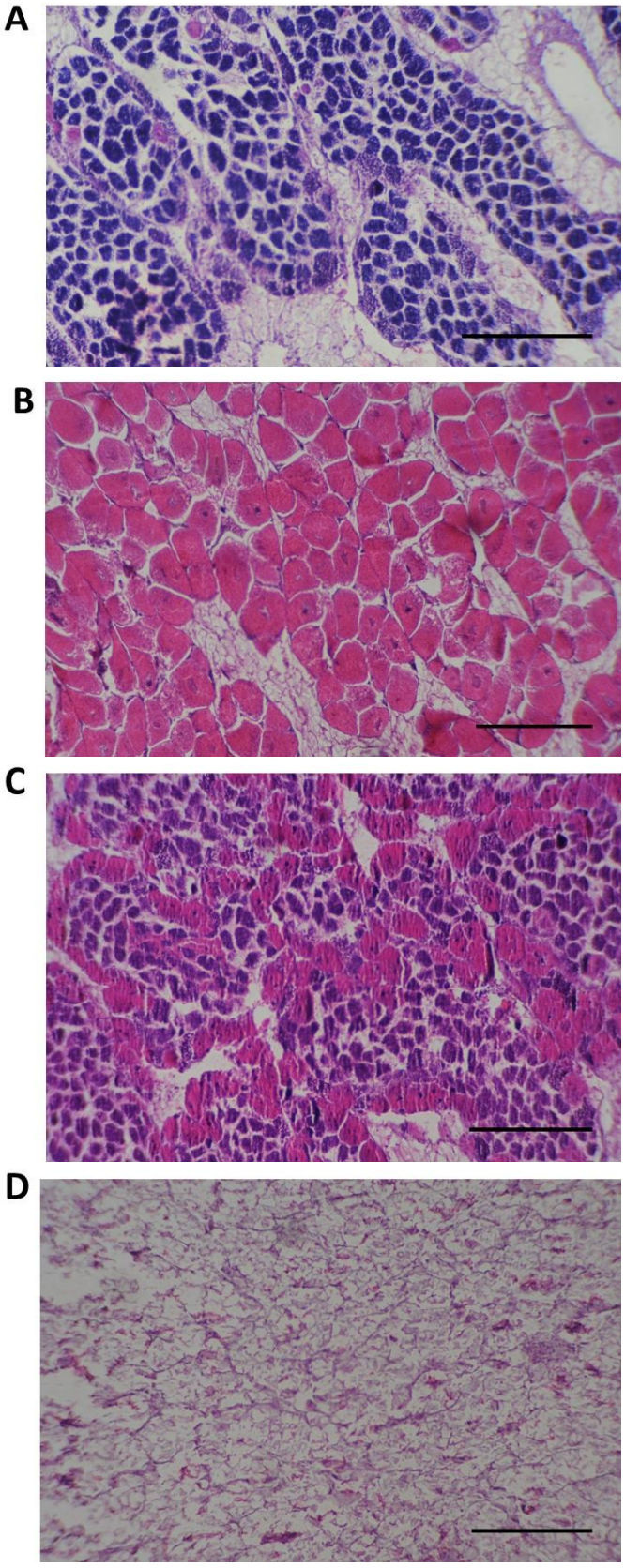

Figure 2.5 Micrographs at 20X magnifications of histological sections of *Ostrea edulis*, showing the gonad area of different sex categories. A: Male, ripe gonad. B: female, ripe gonad. C: hermaphrodite, ripe gonad. E: inactive. Bar = 100µm.

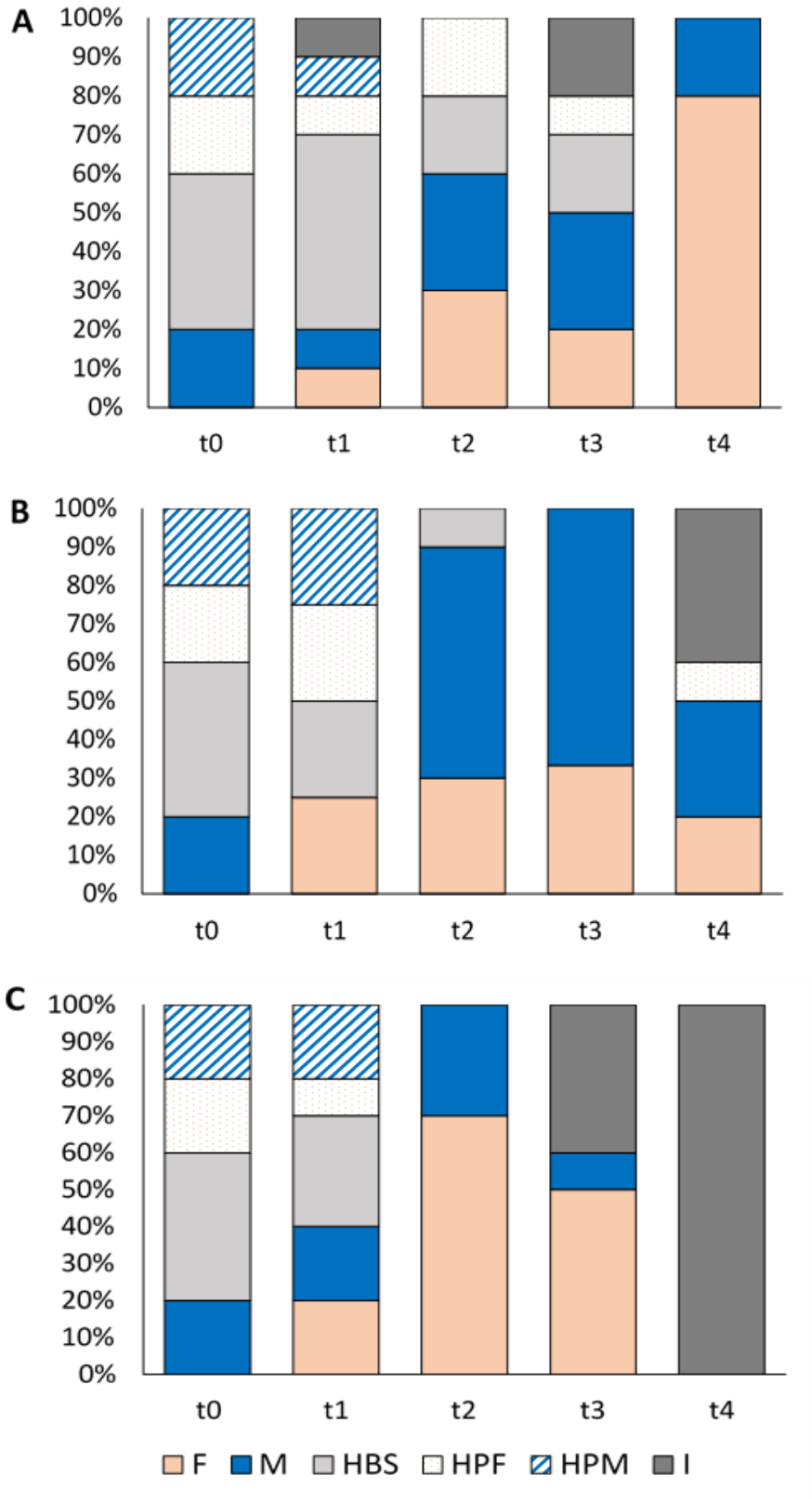

Figure 2.6 Sex ratio in *Ostrea edulis* exposed to (A) 10°C, (B) 14°C and (C) 18°C during four months. Specimens samples (n=10 per treatment per month) were identified by histological examination as females (F), males (M), hermaphrodite with both sexes equally represented (HBS), hermaphrodite predominantly male (HPM), hermaphrodite predominantly male (HPF) an indeterminate (I).

Gametogenic changes showed an effect of temperature on the gonadal maturation in *O. edulis* (Fig. 2.7). At the beginning of the experiment, 40% of the oysters analysed were classified in gonadal stage G1 and 60% in stage G2. At 10°C, a slow progress in gonadal maturation was observed during the first month, presenting mainly stages determined as G1 and G2 and around 30% of the animals were classified as G3. Then the gonad follicles were filled mainly by a few oogonia and spermatogonia, and the follicles gradually became larger with more developed cells. By the third month of treatment at 10°C, the proportion of animals with gonads in G2 decreased and 20% of the animals were classified as G4. The proportion of oysters in G1 and G2 increased by the fourth month at this temperature suggesting the beginning of a new gametogenic cycle.

Accelerated gametogenesis was more evident at 14°C. The proportion classified as G1, G2 and G3 were similar to those at 10°C during the first month (Fig. 2.7). By the second month the percent classified as G3 increased to 50% and stage G4 was observed for first time in this treatment. The third month was characterized by animals in the later maturation stages (G3, G4 and G5) showing well developed gonads. Oysters with gonads in G0 were found at the end of the treatment at 14°C reflecting the preparation of the oysters to start a new cycle again.

No gamete release, brooding or spawning events were observed during this experiment.

2.3.4 Effect of temperature treatments on sex steroids levels

The standardization of the protocol for ELISA assays for hormonal analysis showed a good response (see example in Appendix D), producing linear standard curves of R value of 0.999 and 0.988 for E_2 and T standards, respectively.

E_2 concentrations significantly increased (Kruskal-Wallis test value = 43.9524, $p < 0.001$, N = 120) at all temperatures (Fig. 2.8A). The maximum peak concentration for this hormone was reached at the fourth, third and second month at 10, 14 and 18°C, respectively (Fig. 2.8B, 2.8D and 2.8F). Significant differences (Mann Whitney U test, $p = 0.003$) were found between all the temperatures analysed in the same month except at the fourth month when significant differences were found only between 14 and 18°C (Fig 2. 8B, 2.8D and 2.8F). Testosterone concentration showed an increase at the beginning of the experiment reaching the maximum peaks at the second moth for 10 and 14°C treatments (Fig. 2.8C and 2.8E). At 18°C the maximum peak concentration for this sex steroid was detected at the end of the first month of treatment and thereafter it decreased until the end of the experiment (Fig. 2.8G).

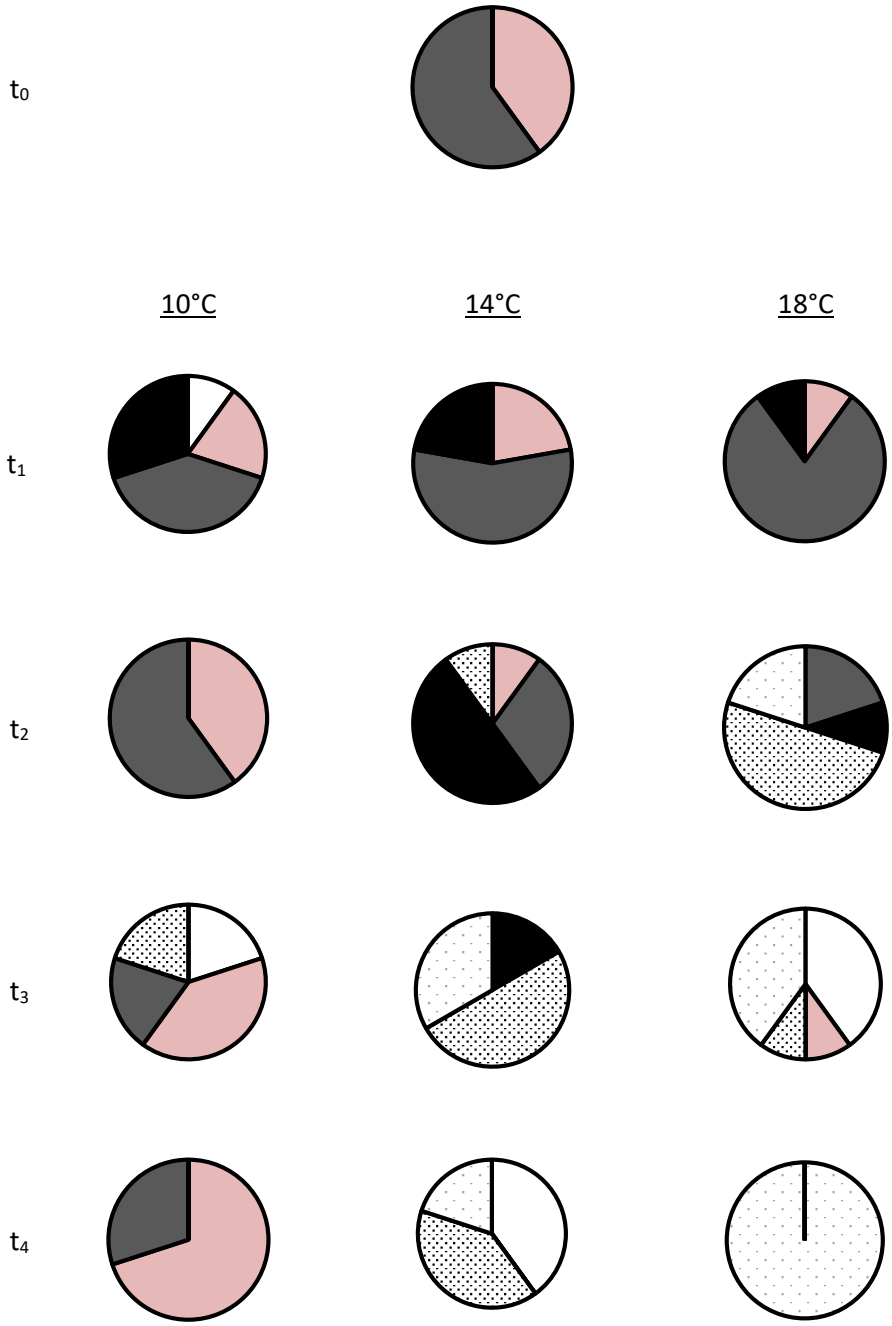

Figure 2.7 Proportion of *Ostrea edulis* at different stages of gonad development (n=10 per treatment per month) under different temperature treatments (10, 14 and 18°C) during four months. According to da Silva *et al*. (2009) developmental stage was classified by the gametogenic stage of the gonad as inactive (G0) ☐ , early gametogenesis (G1) ☐ , advanced gametogenesis (G2) ■ , ripe gonad (G3) ■ , partially spawned gonad (G4) ⬚ and reabsorbing gonad (G5) ☐ .

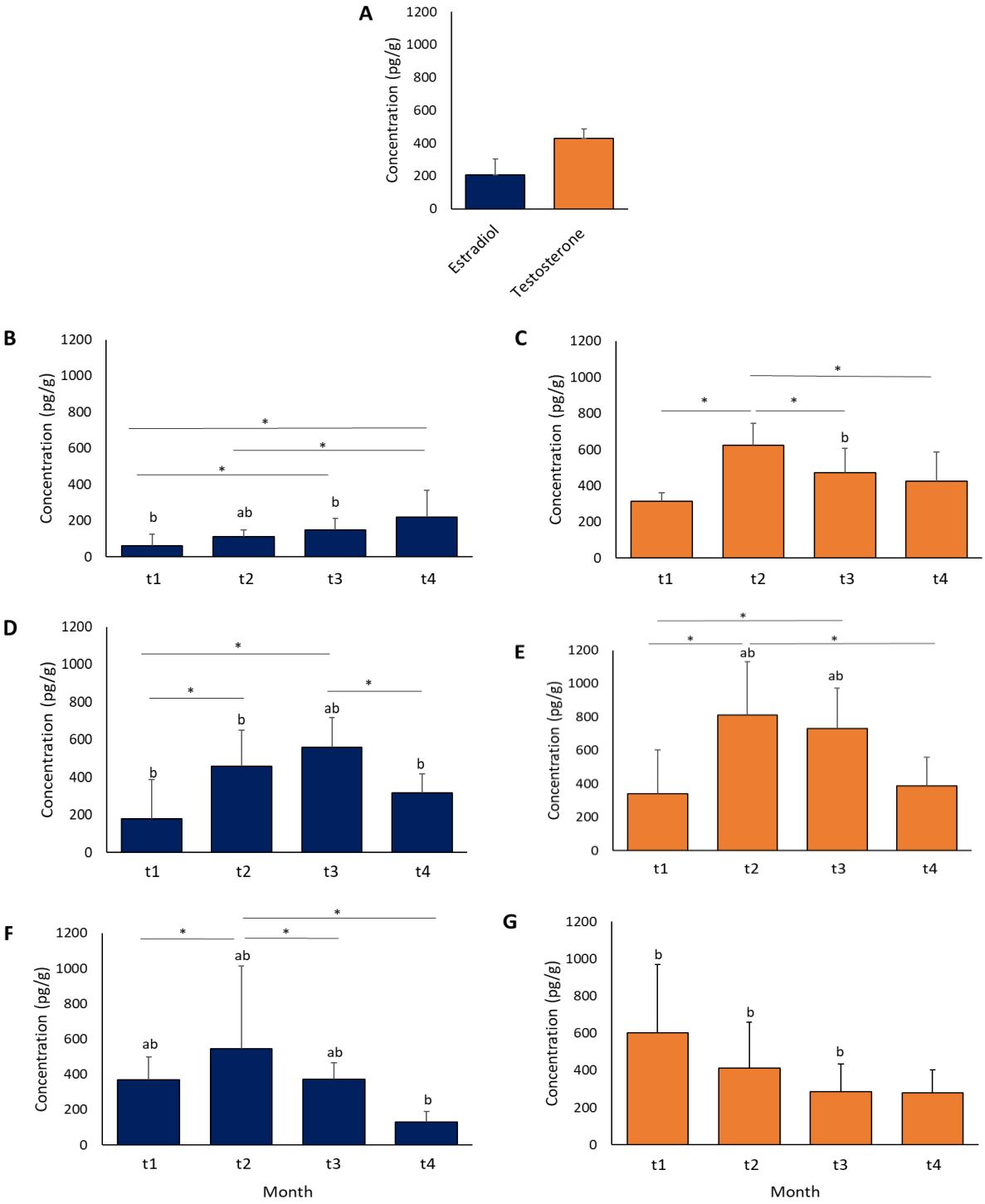

Figure 2.8 Hormone concentrations (mean±SD; n=10 per treatment per month) (A) in *Ostrea edulis* at the beginning of the experiment (t0) and during four months of treatment at 10°C (B, C), 14°C (D, E) and 18°C (F, G). Estradiol concentration (dark blue bars, panels B, D, F) and testosterone concentration (orange bars, panels C, E, G). (a) significant differences compared to the beginning of the experiment (t0), (b) significant differences between temperatures at the same time, and (*) significant differences between times at the same temperature.

Some biometric parameters seemed to be affected by hormone concentrations. A significant weak negative relation between E_2 concentrations and weight (Spearman correlation r_s = -0.198; p = 0.023) was found. Significant weak positive relations between T concentration and width (r_s = 0.179; p < 0.04), fresh tissue weight (r_s = 0.174; p = 0.046) and CI (r_s = 0.204; p = 0.019) were found.

Hormone homologue concentrations in gonadal tissue of *O. edulis* showed a weak but highly significant correlation (r_s = 0.311; p = 0.0003) between E_2 and T concentrations during the temperature treatments. A direct relationship between sex determination results through histology and hormone concentrations for all the temperatures was not found.

No significant differences between hormone concentrations were found between hermaphrodites (HBS, HPM and HPF) at any temperature so they were treated in the same group (as hermaphrodites) for comparison with the other sex categories. The behaviour of both hormones at 10°C was very similar without any significant difference between sexes (Fig. 2.9A). At 14°C the T concentration was always higher than E_2 for all the sex categories, with the highest values presented by individuals classified through histology as males (Fig. 2.9B). However, no significant differences were found for T concentration between sexes at this temperature. There was a significant difference for E_2 concentration for males compared with hermaphrodites, but not between males and female oysters kept at 14°C.

Estradiol concentrations at 18°C were significantly higher than testosterone for females (Fig. 2.9C) Males showed the same tendency, although no significant difference was found. Animals classified through histology as females exhibited the highest values for E_2 (Fig. 2.9C). A significant difference for both hormones between males, females and hermaphrodites was found at 18°C.

The analysis of hormones concentration in gonadal tissue of *O. edulis* after four months of temperature treatments showed a significant correlation (r_s = -0.3720; p = 0.035) between temperature and T concentrations, but there was no correlation between temperature and E_2 levels. At the same time, female oysters presented T concentrations as high as males, and E_2 concentrations were less than T in most of the samples. A direct relation between sex determination and hormone concentrations for these oysters at the end of the treatments was not found (Fig. 2.9).

The comparison of hormone concentrations between stages of gonadal maturations showed an increase from G0 to G2, then showed a slight decrease for both hormones at G3 and finally a second increase was observed at G4 and G5 (Fig. 2.10). However no significant differences for hormone concentrations were found between stages.

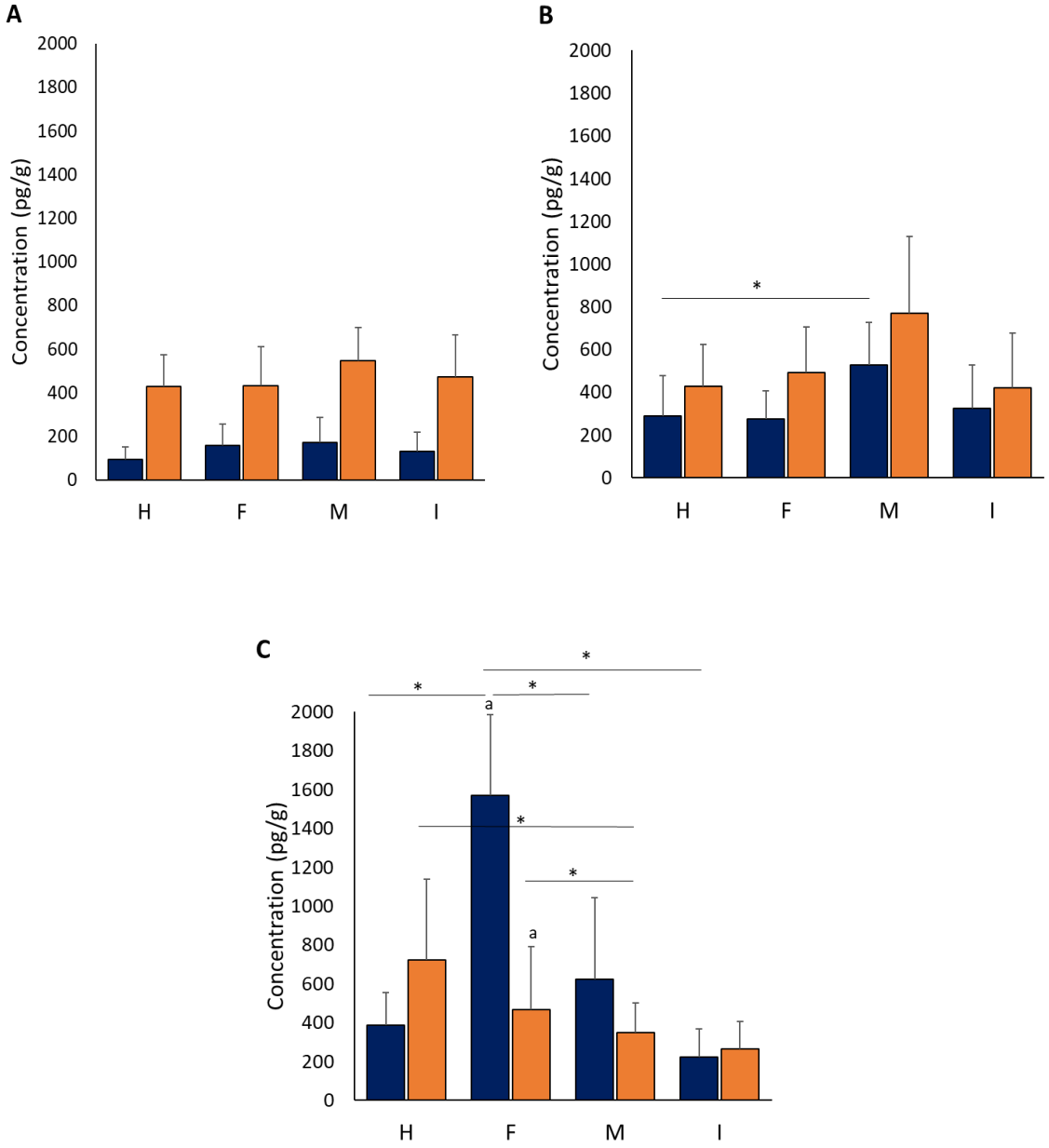

Figure 2.9 Hormone concentrations (mean±SD) for *Ostrea edulis* classified through histology as hermaphrodites (H), females (F), males (M) or in an inactive stage (I) under a treatment of (A) 10°C, (B) 14°C and (C) 18°C. Dark blue bars: estradiol. Orange bars: testosterone. (*) Significant differences for the same hormone between sex categories at the same temperature. (a) Significant differences between hormones for the same sex category. Error bars denote standard deviation.

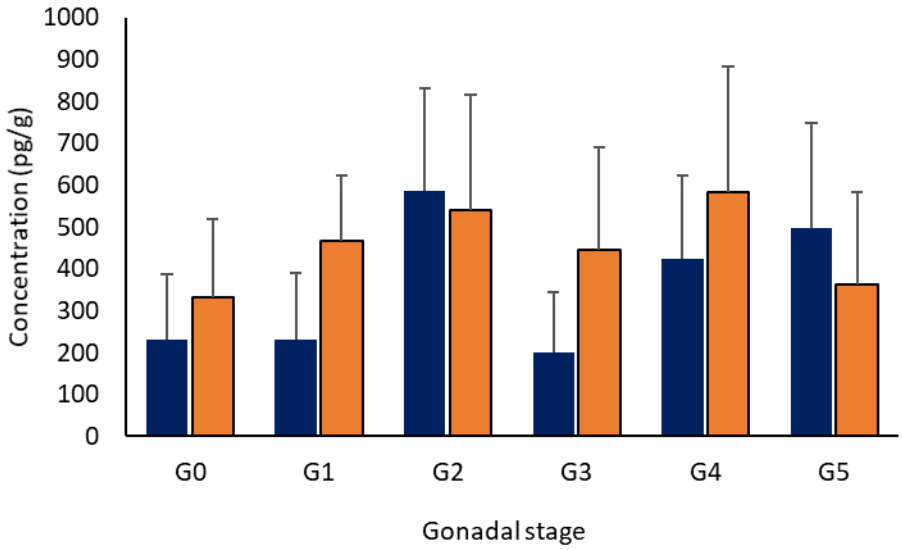

Figure 2.10 Hormone concentration (mean±SD) according to the stage of gonadal maturation in *Ostrea edulis* classified as inactive (G0), early gametogenesis (G1), advanced gametogenesis (G2), ripe gonad (G3), partially spawned gonad (G4) and reabsorbing gonad (G5) according to da Silva *et al*. (2009). Dark blue bars: estradiol. Orange bars: testosterone. Error bars denote standard deviation.

2.3.5 Effect of temperature treatments on Vtg-like protein

The maximum peak for Vtg-like protein concentrations was found at the second month at 10 and 14°C, but the maximum values were found at the month 4 for the highest temperature (Fig. 2.11). Significant differences in Vtg-like protein concentrations at different times and temperatures were not found (Mann Whitney U test, $p > 0.05$). In the same manner, significant differences for Vtg-like protein concentrations between different sex categories were not found (Mann Whitney U test, $p > 0.05$) (Fig 2.12). Furthermore a direct relationship between oysters classified as females through histology and Vtg-like protein levels for these oysters at the end of the treatments was not found. Female oysters presented Vtg-like protein concentrations as high as males, and some females presented lower concentrations of this protein than males for the same temperature and time (Fig 2.12).

The analysis of Vtg-like protein concentrations in gonadal tissue of *O. edulis* did not show a correlation with estradiol or testosterone concentrations during the temperature treatments.

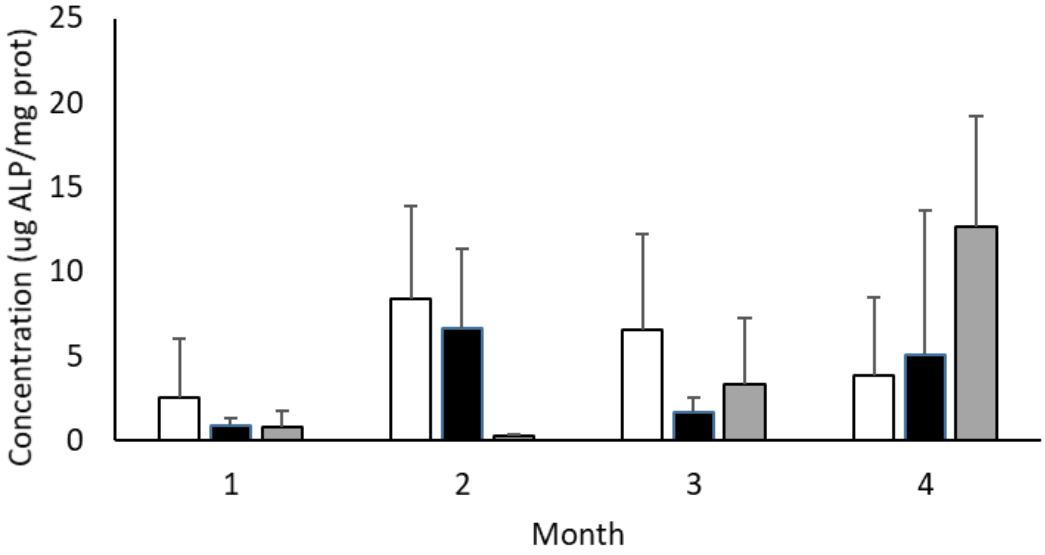

Figure 2.11 Vtg-like protein concentrations (mean±SD; n=10 per treatment per month) in *Ostrea edulis* kept at different temperatures during four months. White bars: 10°C, black bars: 14°C and grey bars: 18°C. Error bars denote standard deviation.

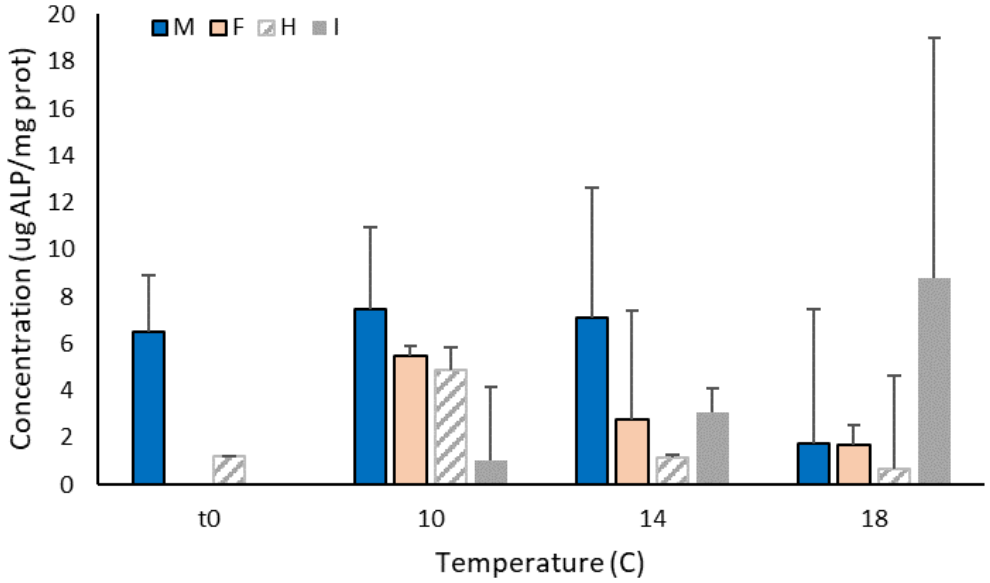

Figure 2.12 Vtg-like protein concentrations (mean±SD; n=10 per treatment per month) for *Ostrea edulis* classified through histology as males (M), females (F), hermaphrodites (H), or in an inactive stage (I) under different treatments of temperature. Error bars denote standard deviation.

2.4 Discussion

2.4.1 Effect of temperature treatments on gametogenesis in *Ostrea edulis*

A positive effect of temperature on the gonadal development of *O. edulis* has been shown in this study. Accelerated gametogenesis was more evident at the highest temperature compared with the other treatments and by the fourth month of treatment all the oysters were in an inactive state of gonadal development. It is known that elevated temperatures increase metabolism, accelerate rates of oxygen consumption and ammonia excretion but also accelerate gametogenesis and gonadal development in bivalves (Shpigel *et al*., 1992;Chávez-Villalba *et al*., 2003; Pérez *et al*., 2013; Santerre *et al*., 2013; Teaniniuraitemoana *et al*., 2016). Usually the production of gametes results in a reduction in condition index (CI) due to the demand for energy reserves obtained from carbohydrates, lipids and protein stored in tissues (Shpigel, Barber and Mann, 1992). Shpigel *et al*. (1992) also showed that diploid and triploid Pacific oysters, *Magallanas gigas*, were metabolically stressed at high temperatures showing accelerated rates of oxygen consumption and ammonia excretion. Accordingly, *Ostrea edulis* in this study kept at 18°C showed the lowest values for most of the biometric measurements. Thus the relation between temperature and the energy allocation to initiate gametogenesis in bivalves could explain the rapid maturation and the accelerated gametogenesis process observed for oysters kept at 18°C in this study.

A thick matrix of connective tissue surrounding the digestive system provides support and substrate for the differentiation of gonadal tissue, and annually during autumn and winter any remaining sex products are resorbed for glycogen storage in the next reproductive cycle as haemocytes invade the gonadal tissues (Giese and Pearse, 1974). With the rise of water temperatures during spring, it has been observed that connective tissue filled with gonadal tubules prepares the animals to start a new gametogenic cycle (Giese and Pearse, 1974). It has been showed that gonadal development in bivalves normally takes place using reserves of carbohydrate and lipids stored prior to the initiation of gametogenesis (Shpigel, Barber and Mann, 1992). This potential makes the process of gametogenesis dependent on temperature and the availability of stored nutrient reserves.

An asynchrony of gonadal development between animals was found in the present study. Since gonadal maturation is not a homogeneous process and spawning is not a synchronous event, gametogenesis of both sexes in a single follicle is a common phenomenon in flat oysters resulting from the changing of sex (Sparck, 1925; Coe, 1932; Korringa, 1952; Loosanoff, 1962). It is common to find males and female cells at a different grade of maturation stage at the same time (Maneiro *et al*., 2016, 2017).

During this study no larvae or settlement was observed. Systems for broodstock conditioning use flow-through tanks containing a mesh-based sieve used to retain oyster larvae (Utting and Spencer, 1991; Helm and Bourne, 2004). The tanks in this study did not present a flow-through system so particulate filters, protein skimmers and frequent water change were necessary to keep the conditions in the aquariums. This might cause the loss of gamets and larvae liberated by the adults.

2.4.2 Effect of temperature treatments on sex ratio in *Ostrea edulis*

Factors controlling the sex ratio in natural populations of oysters remain unclear. Some field studies on oysters in the family Ostreidae have reported the effects of environmental factors, such as temperature, salinity and food availability, amongst others, on sex ratio (Acarli *et al.*, 2015; Eagling *et al.*, 2018; Hassan, Qin and Li, 2018). In natural populations biased sex ratios are expected and frequently observed for strict sequential hermaphrodites (sex-changing species) and in species where sex is determined by environmental conditions experienced during pre-adult development (Charnov and Bull, 1989). Modelling has demonstrated a trend to present a skewed sex ratio towards the first sex in some sequential hermaphrodite species (Charnov and Bull, 1989; Baeza *et al.*, 2010); however, the sex ratio and other sex allocation parameters differ between species. For instance, not all the protandric species featured a male-skewed sex ratio in the adult life showing a large range in sex ratio variation (Allsop and West, 2004; Collin, 2006).

O. edulis can alternate between female and male functions (Orton, 1927c; Cole, 1942a; Loosanoff and Davis, 1952; Loosanoff, 1962) but the influence of temperature on this process and the determination of sex ratios in natural populations remains unclear in this species. Few studies have experimentally evaluated the effect of temperature in *O. edulis,* and the results have indicated that lower temperatures are implicated in the development of female germinal cell lines causing a female-bias at the beginning of the breeding season with coldest water temperatures, whereas male gonads appeared when temperatures were warmer (Loosanoff and Davis, 1952; Loosanoff, 1962; Joyce *et al.*, 2013).

These earlier reports are supported by this study, which demonstrated a higher proportion of females found at the lowest temperature (10°C) and a higher proportion of males at 14°C. It has been mentioned that an energetic cost related to the production of female gametes has been proposed (Wright, 1988; Pérez *et al.*, 2013). Thus, oysters experiencing faster metabolism in environments with higher temperatures will need more energy to initiate gametogenesis that cannot be gain from the diet and reserves (Santerre *et al.*, 2013). If more energy is required to produce eggs than sperm, then a protandric species will save energy producing the low-cost male gonads and allocating the energy reserves into survival or growth (Mouneyrac *et al.*, 2008; Pérez *et*

al., 2013), and later when the environmental conditions become more favourable, they would be able to change to female.

In this study, the sex ratio was biased towards early developing females at 18°C. This behaviour contradicts the expected response that at high temperatures protandric species will save energy through the production of the low-cost male gonads (Wright, 1988; Pérez *et al.*, 2013). However, it has been shown that food availability can affect reproductive parameters in *Pinctada margaritifera* (Teaniniuraitemoana *et al.*, 2016). In that study, the animals kept at high temperature (28°C) showed females transitioning into males after exposure to low food availability and females presenting male and female gametes together under a high food treatment. The same behaviour has been shown in other bivalves. In a similar manner, *Aequipecten irradians concentricus* showed that the oogonia differentiation started when a minimum water temperature was reached but the fecundity and gonadal size were determined mainly by food availability (Sastryz, 1965).

The oysters used in this study were fed *ad libitum* to meet the energy demands needed to go through gametogenesis. This could suggest that under favourable conditions and in an environment with enough food to supply a high energy demand *O. edulis* could be expected to go through a faster gametogenesis which favours female gonadal production. It has been reported that under exceptionally favourable conditions, *O. edulis* have the potential to reach maturity and spawn several times during the same season because, even just a few hours after releasing eggs or sperm the gonads can begin to change into the opposite sex (Korringa, 1957). Furthermore, sex ratios in oysters could favour females when food availability is high (Chávez-Villalba *et al.*, 2003). It has been reported that *O. edulis* only has the ability to become a functionally mature female following an exceptional summer period because it needs a large quantity of energy to produce ovaries (Dodd *et al.*, 1937). This evidence supports the female-biased sex ratio observed in this study at the highest temperature with an excess of food, but further studies with larger sample sizes would help in understanding natural sex ratios in this species.

Sea-surface temperatures in the north east Atlantic and UK coastal waters have been rising since the 1980s by around 0.2-0.9^{o}C per decade with the most rapid rises occurring in the southern North Sea and the English Channel (Holliday *et al.*, 2008), with ongoing rises predicted (Marine Climate Change Impacts Partnership, 2015). The year 2006 was the second-warmest year in UK coastal waters since records began in 1870 and seven of the 10 warmest years have occurred in the last decade (Marine Climate Change Impacts Partnership, 2015). This also could be implicated in the skewed sex ratio towards male-phase oyster found in oyster populations in the Solent (Eagling, 2012; Kamphausen, 2012). This study showed an increase in the proportion of males under a large

and longterm exposure to 14 °C suggesting that a rise in seawater temperatures could intensify this skewed sex ratio towards males observed in field studies in this area.

The reason why different species show a different effect in gametogenesis and gender determination in response to changes in environmental conditions is not clear and more studies are needed. Some species have a fixed size at sex change and others have plastic responses (Hamilton *et al.*, 2007; Benvenuto *et al.*, 2017). Fisheries exploiting hermaphroditic species may affect sex ratios by skewing these towards the sex that matures first, producing a population with smaller and younger individuals (Hamilton *et al.*, 2007). Thus, finding *O. edulis* populations in the Solent with a considerable skew towards males (Kamphausen, Jensen and Hawkins, 2011; Eagling, 2012) raises the question of whether fishery practice is responsible for the removal of larger individuals leaving only smaller animals with the first sex (males). Considering that this species can alternate between sexes, changes in local environmental temperatures can modify the reproductive behaviour of this species.

Effective restoration programs should also understand the effect of distribution and demographic rates of oysters inhabiting natural environments with the population density acting as an important attribute that can affect the success in reproduction (Peters *et al.*, 2017; Theuerkauf *et al.*, 2017; Lipcius and Burke, 2018). Beds of *O. edulis* were reported occurring at densities of 5 or more per m^2 in the UK (OSPAR, 2008b). Analysis of densities of *O. edulis* in Chichester Harbour in 1998 indicated an overall value of 8 oysters per m^2 (Helmer *et al.*, 2019). However a study carried out in the Solent population in 2012 revealed that the density of *O. edulis* on Ryde Middle, considered as one of the "denser" oysters beds, was as low as 1 oyster per 20.9 m^2 (Kamphausen, 2012). By 2017 the density of *O. edulis* was 0.1 oyster per m^2 in Chichester Harbour or absent in Portsmouth Harbour (Helmer *et al.*, 2019). It has been reported that *O. edulis* with a nearest neighbour ≤1.5 m were found to brood significantly more larvae than individuals with nearest neighbours ≥1.5 m (Guy, Smyth and Roberts, 2019) indicating that the low densities reported in the South coast harbours in the UK could be an important factor limiting the recruitment in *O. edulis* populations and affecting reproduction in this species.

The density of animals at the beginning of this experiment was 3 oysters per m^2 and it was reduced over time until the end of the experiment. This way, even when an effect of removing animals over time cannot be ignored in this study, these densities reflect the values reported during the last years for *O. edulis* population inhabiting the Solent and could predict the behaviour of this species facing changes in temperatures alongside low density numbers in the wild.

2.4.3 Concentration of hormone levels in *Ostrea edulis* under different temperature treatments

Most research on steroid concentrations in invertebrates, including this study, has used immunoassays as the detection system, relying on a cross-reactivity of these assay antibodies with other steroids (Porte *et al.*, 2006; Lafont and Mathieu, 2007). In spite of the manufacturer (Cayman Chemical Co.; Ann Arbor, MI, USA) claiming that these kits have 100% specificity and the percentage of detection for hormone homologues is low, it is strongly recommended to include the detection and characterization of sex steroids as an important step in this type of studies.

It has been reported that environmental factors, especially temperature, combining with hormonal control are involved in the gender determination of adult pearl oysters *P. margaritifera* (Teaniniuraitemoana *et al.*, 2016). However, the lack of a direct relation between sex determination results (from histology) and hormone concentrations for the oysters at the end of the current study indicates that other biochemical pathways are involved in the gonadal development and that maturation in *O. edulis* appears independent of steroid hormones measured in this study.

This evidence, along with our results showing similar hormone concentrations for female and male gonads in most of the treatments and sex categories, demonstrates a lack of a direct relation between the different stages of gonadal maturation, sex determination through histology and hormone concentrations could indicate that these steroids may not be actively involved as endogenous modulators in gonadal maturation and sex determination in this species. A lack of differences in T and E_2 content has been reported in other bivalve species such as *Mya arenaria* (Gauthier-Clerc, Pellerin and Amiard, 2006), *Mytilus edulis* (Reis-Henriques *et al.*, 1990), and *Patinopecten yessoensis* (Osada, Tawarayama and Mori, 2004).

It has been argued that the main classes of molluscs are able to synthesize sex steroids from precursors such as cholesterol or pregnenolone (Fernandes *et al*., 2011; Lafont and Mathieu, 2007). Several reviews have reported evidence about the presence, metabolism and enzymatic pathways of sex steroids, e.g., testosterone, androstenedione, and estradiol occurring in several invertebrate species (Le Curieux-Belfond *et al.*, 2001; Porte *et al.*, 2006; Lafont and Mathieu, 2007; Fernandes, Loi and Porte, 2011). Fluctuations in sex steroids have been found to be correlated with the sexual maturation cycle in a number of bivalves, thus suggesting that sex steroids may play important stimulatory roles in their reproductive regulation (Le Curieux-Belfond *et al.*, 2001; Gauthier-Clerc, Pellerin and Amiard, 2006; Porte *et al.*, 2006; Lafont and Mathieu, 2007). In this context, some studies have concluded the central role of estrogens in the natural gametogenic cycle in oysters, scallops, and clams (Mori, Muramatsu and Nakamura, 1972; Gauthier-Clerc, Pellerin and Amiard,

2006). In the soft clam *M. arenaria*, Gauthier-Clerc *et al*. (2006) suggest that estradiol-17β and testosterone act as endogenous regulators of gametogenesis, and other studies suggest similar, though species-specific, roles in oyster *M. gigas* (Mori, 1969; Mori, Muramatsu and Nakamura, 1972) and scallops *Placopecten magellanicus* (Wang and Croll, 2006).

However, others have questioned the role of endogenous origin of vertebrate-type steroids, their regulation and synthesis in molluscs (Lafont and Mathieu, 2007; Fernandes, Loi and Porte, 2011; Scott, 2012, 2013). Scott (2012) argued that the seasonal changes in hormone concentrations reported in some studies could be more related to an increase in fatty acids, lipids and proteins during reproductive maturation, with hormones taken up from the environment or through a dietary source and stored in the form of fatty acid esters for days or even months. In fact, various steroids are always present in the animal's food and in the environment as a product of the physiological process in other animals or anthropogenic activities (Lafont and Mathieu, 2007) and this could be an external source for these hormones identified in the current study. This is supported by other studies showing a fast bioaccumulation of hormones, e.g. in less than 48h estradiol-17b from seawater was concentrated up to 31 times in the soft tissues of oysters *M. gigas* during in vivo experiments (Le Curieux-Belfond *et al.*, 2001). It was also reported in adult blue mussels, *Mytilus* spp. an optimum uptake of E_2 from water in just 24h (Schwarz *et al.*, 2016). It may well be that the presence of E_2 and T in the gonad tissues of these oysters was a function of the accumulation of these steroid homologues from the phytoplankton food source. The potential role for exogenous steroid hormones and the presence of steroid pathways in oysters represent the focus of chapter 4 in this thesis.

2.4.4 Concentration of Vtg-like protein in *Ostrea edulis* under different temperature treatments

The Vtg concentration measured as ALP levels has been used to indicate reproductive status in female bivalves (Suzuki *et al.*, 1992; Blaise *et al.*, 1999; Matozzo and Marin, 2008; Arcos *et al.*, 2009). The lack of relationship between oysters classified as females through histology and Vtg-like protein levels in this study and the similar concentrations presented by males and females at the same temperature and time could indicate that this technique is not the most sensitive method to distinguish between sex or stages of gonad development in this species. This is supported by Sánchez-Marín, Fernández-González, Mantilla-Aldana, Diz, & Beiras (2017) who found that ALP analysis could detect similar amounts of phosphorylated proteins regardless of sex or gonad development stage in marine mussel gonads.

The use of Vtg as a biomarker for feminization in fish has been widely used (Sumpter and Jobling, 1995; Kirby *et al.*, 2004; Scott *et al.*, 2006; Hamilton *et al.*, 2007). However, its use in other species is still controversial and has recently been brought into question (Ford, 2012). Other studies have also suggested that Vtg expression should not be considered as an appropriate biomarker of feminisation/de-masculinisation in crustaceans (Short *et al.*, 2014; Boulangé-Lecomte *et al.*, 2017). However, this method is still widely used in bivalves as an indicator of reproductive status (Suzuki *et al.*, no date; Blaise *et al.*, 1999; Gagné *et al.*, 2002; Won *et al.*, 2005; Matozzo and Marin, 2008) and it is included to measure sexual maturation, especially in females (see examples in Table 1.3). In *O. edulis* has been observed a relationship between the gametogenic stage and ALP concentrations suggesting the use of this method as a potential indicator of female reproductive investment (Sawusdee, 2015). In opposition to the results reported by Sawusdee (2015) the present study did not find a relation between ALP concentrations and gametogenetic stage or sex in *O. edulis* but more research is needed to establish the effectivity of this method to indicate the reproductive status of this species.

2.5 Conclusions

Temperature accelerates the gonadal development of *O. edulis*. At the highest temperature treatment, the oysters went through a faster gametogenesis process and all of them were in an inactive state of gonadal development at the end of the treatment.

Furthermore, in this study, the sex ratios (males:females) changed throughout the experiment suggesting that the ratio was significantly influenced by temperature and time during the treatments. The lowest and highest temperatures analysed in this study caused a female-biased sex ratio in adults, but at 14°C a higher proportion of males than females was found. The results from this study also suggest that sex determination could be affected by other parameters such as food availability, indicating a complex relationship in terms of energy allocation for sexual maturation.

It could, therefore, be expected that a rise in sea temperatures and warmer conditions in European waters through the year, potentially combined with differences in phytoplankton food supply (species assemblage and concentration), could influence the processes of gametogenesis, sex determination and sex ratios, affecting the long term health of populations.

Although it has been reported that environmental factors, especially temperature, combined with hormonal control are involved in sex determination and the maturation of other bivalves, our results showed a lack of a direct strong relationship between sex determination results through histology and hormone concentrations at three different temperatures. These results together

could indicate that other biochemical pathways are involved in the gonadal development and maturation in *O. edulis* independent of steroid hormones.

The Vtg-like protein has been a widely used method to indicate reproductive status in female bivalves. However, our results showed a lack of relationship between these proteins and sex determination suggesting that careful verification is needed before assuming this technique is a good proxy to assess reproductive status in other species.

To conclude, this chapter has shown that gametogenesis and sex determination in *O. edulis* are influenced by temperature and these processes are independent of E_2 and T concentrations in gonadal tissues under long-term and controlled laboratory exposure. Chapter three will next look at the annual variations in these parameters under semi-enclosed natural conditions to establish if seasonality and changes in environmental factors could have an impact in reproductive parameters in this species.

Chapter 3 Seasonal changes in biochemical composition, gametogenesis, sex determination and sex steroids in *Ostrea edulis*

3.1 Introduction

Changes in some reproductive parameters such as gametogenesis and sex ratio in response to seasonal variations in the family Ostreidae have been studied (Ruiz *et al.*, 1992; Acarli *et al.*, 2015; Eagling *et al.*, 2018; Hassan, Qin and Li, 2018). However, the knowledge about changes in physiological and biochemical attributes and their relationship with the reproductive cycle of *O. edulis* remain poorly understood. Abiotic factors have been argued to be important in terms of influence on the physiological, biochemical and reproductive attributes of oysters (Newell, Johson and Kofoed, 1977; Mann, 1979; Newell and Branch, 1980). Early evidence suggested that temperature plays an important role in *Ostrea edulis* by keeping this species in a resting stage under winter temperatures with a post-spawning period characterized by reduced metabolism, an increase of glycogen reserves, and reset of the gametogenic cycle (Loosanoff and Davis, 1952). Then, an increase in this factor has been associated with the formation of eggs or sperm and adult spawning when higher temperatures are reached in summer (Korringa, 1952; Loosanoff and Davis, 1952; Loosanoff, 1962; Mann, 1979; Ruiz *et al.*, 1992; Kennedy and Roberts, 1999; Hedgecock *et al.*, 2007; Eagling *et al.*, 2018). The absence of gonadal development at a temperature below 7°C in *O. edulis* (Korringa, 1952; Mann, 1979; Wilson and Simons, 1985), the accelerated gametogenesis observed at higher temperatures (Joyce *et al.*, 2013) and the results obtained in the second chapter of this thesis confirm that this process is directly affected by temperature in this species.

On the other hand, it has also been shown that food quantity and quality of food can also affect reproduction and food availability could be an important factor in controlling gonad growth once gametogenesis has been initiated (Newell, Johson and Kofoed, 1977; Newell and Branch, 1980; Ruiz *et al*., 1992; Chávez-Villalba *et al*., 2003; González-Araya, Quillien and Robert, 2013; Pogoda *et al*., 2013; Teaniniuraitemoana *et al*., 2016). It has been shown that oogonia differentiation starts when a minimum temperature in warmer waters is reached but the fecundity and gonadal size is determined mainly by food availability (Sastryz, 1965). This suggests that seasonality and frequency of the reproductive cycle in marine bivalves could depend on environmental parameters such as temperature and available phytoplankton.

It is known that biochemical components are used for growth and as energy reserves for reproductive activity in bivalves (Newell, Johson and Kofoed, 1977; Newell and Branch, 1980; Mouneyrac *et al.*, 2008; Pogoda *et al.*, 2013; Acarli *et al.*, 2015). Lipids, carbohydrates and proteins may act as energy reserves for gametogenesis and reproduction, and seasonal metabolic activities in molluscs result from complex interactions among food availability, environmental conditions, growth and the gametogenic cycle (Acarli *et al.*, 2015). How species use these substrates can vary among populations and species that have different strategies for energy storage and utilization (Giese and Pearse, 1974; Pogoda *et al.*, 2013). In *O. edulis,* an increase in energy reserves when food is abundant as preparation for the spawning period has been reported (Ruiz *et al.*, 1992). Moreover, spawning has been associated with a subsequent dramatic change in biochemical composition in this species (Ruiz *et al.*, 1992).

The role of estrogens in the natural gametogenic cycle in oysters, scallops, and clams has been proposed based on fluctuations in concentrations of sex steroids during the sexual maturation cycle in bivalves (Mori, 1969; Osada, Tawarayama and Mori, 2004; Gauthier-Clerc, Pellerin and Amiard, 2006; Ketata *et al.*, 2007). However, the results obtained in the second chapter suggest a lack of relationship between gametogenesis and sex determination and sex steroids in *O. edulis*. Some authors have argued that the available evidence about the formation and metabolism of vertebrate-type steroids is not solid enough and the seasonal changes in hormone concentrations reported by some authors could be more related to hormones taken up from the environment and stored in the form of lipids, carbohydrates and proteins during reproductive maturation (Scott, 2012).

In bivalves, many functional parameters show seasonal changes in relation to both abiotic (non-living influences such as light intensity, pH, temperature and salinity) and biotic factors (such as food availability). It has been shown that gametogenesis in *O. edulis* is correlated with water temperature so some variation in the exact temperature triggering gametogenesis and spawning between different populations can be expected (Cole, 1942a; Loosanoff, 1962; Mann, 1979; Wilson and Simons, 1985; Ruiz *et al.*, 1992; Shpigel, Barber and Mann, 1992). Some studies have shown seasonal variations in biochemical and reproductive attributes in *O. edulis* in Turkey, Spain, Italy, Germany and Scotland (Ruiz *et al.*, 1992; Cano, Rosique and Rocamora, 1997; Carlucci *et al.*, 2010; Pogoda *et al.*, 2013; Acarli *et al.*, 2015; Eagling *et al.*, 2018). However, there are no studies on the biochemical composition and energy reserves during an annual cycle of the flat oyster in the Solent and additional studies are required to understand the effect of environmental factors on sex ratio and gametogenesis in this species. The purpose of this study was therefore to examine the changes in the biochemical composition of *O. edulis* in relation to the sex determination and gametogenic

cycle during a year to determine periods of low energetic condition which increase the fragility of oysters and its sensitivity to confront different environmental stresses. Understanding of the effects that changes in abiotic and biotic factors in the Solent have upon storage metabolism in relation to the gametogenic cycle in this species could be considered essential for planning restoration projects in this region.

3.2 Methods

3.2.1 Animals and experiment set up

A low incidence of bonamiosis has been reported in oysters from the Solent (Kamphausen, 2012). This disease affects the viability and quality of oysters (Cáceres-Martínez, Robledo and Figueras, 1995; Montes *et al.*, 2003) and oyster age has been reported as critical in relation to disease development mainly affecting oysters which are more than 2 years old (Cáceres-Martínez, Robledo and Figueras, 1995; Culloty and Mulcahy, 1996). To avoid any effect caused by this disease, oysters (*O. edulis*) were provided by the Loch Ryan Oyster Company, a Centre for Environment, Fisheries and Aquaculture Science (Cefas) certified Bonamia sp. free location. Oyster were > 2y old, and were 5-7 cm at their maximum diameter.

In April 2016, 120 oysters were transferred to the aquarium of the National Oceanography Centre Southampton (NOCS). During the acclimation period they were placed in seawater tanks (about 1L/oyster) with continuous aeration at the same temperature (8°C) and salinity (33.1) as at the hatchery site. During this period animals were fed *ad libitum* daily with 40,000 cells/ml of a mixed algae diet (40% *Tetraselmis suecica*, 40% *Pavlova lutheri* and 20% *Phaedactylum tricornutum*). These algal species typically show adequate characteristics as food for several bivalve species and complementary profiles in essential fatty acids (Pernet *et al*., 2003). As the temperature in the Solent was higher at this time of the year, 1°C was increased every other day from the initial acclimation temperature (8°C), until the environmental temperature was reached, thus finalizing the acclimation period. Then the oysters were transferred to a tank (1.15 m (length) x 0.75 m (with) x 0.5 m (height)) kept at the Empress Dock at the NOCS. In order to keep the oysters under the same conditions than in the environment, they were kept under a constant water flow system directly from the dock (flow rate: 1000L/h), with no food (algae) supply and without temperature control over a period of 13 months. The density of animals at the beginning was 2 oysters/m^2 and it decreased until 0.5 oysters/m^2 at the end of the experiment.

3.2.2 Environmental variables

Water parameters (temperature (°C), salinity (PSU), luminescence dissolved oxygen (LDO; mg/L), percentage of oxygen saturation (%O_2)) were monitored at least twice per week using a HACH HQ 30d digital multimeter, with a conductivity, pH and dissolved oxygen meter.

For chlorophyll *a* analysis (Chl *a*; µg L-1), 200 mL aliquots were collected from the surface at intervals of two weeks and immediately filtered through a Whatman GF/F filter (47 mm) and Chl *a* was extracted with 90% acetone. The extracts were stored for one night in the dark at 4°C until analysis. Then ultrasonic treatment was performed twice for 30 seconds, centrifuged at 2500 rpm for 5 min and fluorometric measurement of Chl *a* in the supernatant was performed using a Turner fluorometer (10-AU) as described by Parsons *et al*. (1984).

3.2.3 Biological indices

Six specimens were processed monthly for determination of height (H), length (L), width (Wi; all to 0.01mm), shell cavity volume (SVol). Fresh tissue weight (FW) and total weight (W, to the nearest 0.1g) and condition index (CI) for every animal were taken following the same criteria as reported in Chapter 2 (see section 2.2.4 Biological indices). Additionally, a thick layer was observed around the shell so this was measured (mm) and reported as new growth (NG) of the shell.

3.2.4 Histological analysis

In order to investigate the reproductive cycle of the species in the Solent, sections of the visceral mass were sampled for histological examination following a standard protocol (Howard *et al.*, 2004; Kim, Ashton-Alcox and Powell, 2006). After dissection, a 5-mm thick section was cut along the sagittal plane containing gill, gonad, digestive gland, and mantle lobes. This was fixed in Bouin's solution (Sigma-Aldrich™, Dorset, UK) for 24h (see section 2.2.5). Sex was recorded as *indeterminate* (I), *female solely* (F), *male solely* (M), *hermaphrodite with both sexes equally represented* (HBS), *hermaphrodite predominantly male* (HPM) and *hermaphrodite predominantly male* (HPF) according to da Silva *et al* (2009). The developmental stage was classified by the gametogenic stage of the gonad as *inactive* (G0), *early gametogenesis* (G1), *advanced gametogenesis* (G2), *ripe gonad* (G3), *partially spawned gonad* (G4) and *reabsorbing gonad* (G5) according to da Silva *et al* (2009).

3.2.5 Energy reserves

Energy reserves (lipids, carbohydrates and proteins) were quantified in the gonad of each animal. The tissue was freeze-dried and ground by hand in liquid nitrogen with a porcelain mortar and pestle into a fine powder for biochemical analysis, and the samples were analysed in duplicate. 1mg of the dried tissue obtained was homogenized with 1000 uL of distilled, deionized water.

Total lipids content was determined using a gravimetric method suggested by Mann and Gallager (1985). From the homogenized 300 µL were mixed well with 100 µL of distilled water and a mixture of methanol/chloroform (2:1, v/v) (Bligh and Dyer, 1959). The homogenate was centrifuged at 1000 g for 10 minutes. A second extraction in methanol/chloroform (1:2, v/v) (Folch, Lees and Sloane Stanley, 1957) was undertaken and centrifuged at 1000 g for 10 minutes. Both supernatants were taken and 950 µl of 0.7% w/v sodium chloride (NaCl) solution was added and vigorously mixed. After overnight separation and additional centrifugation was carried out at 500 g for 10 min, the bottom layer was collected in pre-weighed 1.5 mL glass vials, dried in nitrogen stream and weighed. Cholesterol (95%, ACROS Organics^{TM}) was used for method calibration. Recoveries were reported at 90% for cholesterol every time the method was carried out.

Carbohydrate and protein assay began with extraction of the initial water homogenate (500 µL) with trichloracetic acid to give a final concentration of 5% w/v after mixing (Mann and Gallager, 1985).

The carbohydrate content of the supernatant was assayed by the phenol-sulphuric acid method of Raymont *et al* (1964) using glucose (D-glucose anhydrous, analytical grade, Fisher Scientific) as a standard. Briefly, 500 µL of supernatant was mixed with 500 µL of water and 500 µL % w/v phenol. Then 2.5 mL of H_2SO_4 was added, mixed and allowed to stand for at least 20 min. The absorbance value was measured at 490 nm against a water blank.

The total protein content in the precipitate was measured using a Bicinchoninic Acid Protein Assay Kit (BCA) (Sigma-Aldrich®) with Bovine Serum Albumin (BSA, Sigma-Aldrich®) as a standard. Briefly, a BCA working reagent was prepared by mixing 50 parts of bicinchoninic acid solution (Sigma-Aldrich™, B9643) with 1 part of copper (II) sulphate solution (Sigma-Aldrich™, USA, C2284). A standard curve was prepared as described in the manufacturer's instructions using 2mg/mL BCA protein standard diluted to the concentration range of 0 – 1000 µg/mL. 200 µl of BCA working reagent was added into a 96 well plate (Sterilin®, UK) and then left on ice. Duplicate 25 µl volumes of sample from each oyster were added into the 96 well plate. The plate was covered and incubated at 37 °C for 30 minutes. The absorbance value was measured at 815 nm (Labsystems Multiskan RC,

Finland) and then absorbance values were compared with the standard curve to determine the gonad protein concentration.

The calculation of total protein, lipid, and carbohydrate was based on the dry tissue weight (DW) of each individual and determined as a percentage (%).

3.2.6 Steroid Hormone Homologue Analysis

Extraction and analysis of homologues of the sex hormones E_2 and T concentrations were quantified in the gonads of each oyster using enzyme-linked immunosorbent assay (ELISA) kits (Cayman Chemical Co.; Ann Arbor, MI, USA) as described by Gauthier-Clerc, Pellerin and Amiard (2006) as referenced in Chapter 2 (section 2.2.6). The mean intra-assay CVs for standards and samples were ≤ 9.1% for E_2 and ≤ 7.8% for T. Mean inter-assay CVs were ≤ 15.6% and ≤ 7.82% for E_2 and T, respectively.

3.2.7 Vitellogenin-like (Vtg-like) protein assays

Vtg-like levels were determined using an alkali-labile phosphate (ALP) assay (Blaise *et al.*, 1999) as referred in Chapter 2 (section 2.2.7).

3.2.8 Statistical analysis

The normality of data and homogeneity of variances were evaluated using the Shapiro Wilk and the Levene's tests, respectively. Mortality and growth were analysed using generalized linear models (GLMMs). When the assumptions of parametric tests were not met, non parametric tests were applied. All percentages of the data were transformed by arcsine transformation prior to the analysis and reversed afterwards. Kruskal-Wallis was used to analyse changes of biometric (W, H, NG, L, Wi, Vol, FW, CI) and biochemical variables (lipids, carbohydrates, proteins, sex steroid hormones, Vtg-like proteins) through the year. Spearman's correlation was applied to describe the relationship between environmental factors (temperature, salinity, conductivity, LDO, % O_2 and Chl α), biometric parameters and biochemical variables. Additionally lipids, carbohydrates, proteins and sex steroids were combined by seasons as follows: Summer (June 2016 to August 2016), Autumn (September 2016 to November 2017), Winter (December 2016 to February 2017) and Spring (March 2017 to May 2017) in order to understand the changes in biochemical composition of *O. edulis* according to sex. The Kruskal-Wallis H-test was also used for the comparison of biochemical variables between seasons and between males and females in the same season. In the same manner comparison for biochemical variables between stages of gonadal development (G0-G5) was undertaken. When non-parametric Kruskal and Wallis test was significant, differences were

then evaluated using a non-parametric the Mann and Whitney test. Chi-square statistics were used to test sex ratios against a 1:1 ratio.

After verifying correlations between environmental variables and some of the variables response, a redundancy analysis (RDA) was useded: (1) to provide a reduced description of the large data set, (2) to analyse relationships between variables, and (3) to extract and summarise the variation in biological variables that are "redundant" or explained by the environmental factors (Lepš and Šmilauer, 2003). This method is the direct extension of multiple regressiom to the modelling of multivariate response data and is used when the X variables display linear relationships with the Y raviables (Lepš and Šmilauer, 2003). The RDA generates one ordination in the space defined by the matrix of response variables and another in the space defined by the matrix of explanatory variables. Two similar variables are represented by lines close to each other, while two different or dissimilar variables are represented by two points apart from each other.

Statistical significance was assigned at $p<0.05$. Statistical analyses were performed using SPSS_v24.0. RDA anlaysis was performed using Canoco 4.5.

3.3 Results

3.3.1 Variation in environmental variables

The temperature data in the Solent during the period of study varied from a minimum of 7.1°C in February 2017 to a maximum of 19.9°C in August 2017 according to the Bramblemet (www.bramblemet.co.uk), which is a long-term weather information system weather reports located in the center of the Solent (50°47'. 41N, 001° 17'. 15W). Water temperature measured monthly at the dock at NOCS showed a similar seasonal pattern with the lowest value (7.9 °C) in January 2017, while the highest temperature (22.4 °C) was recorded in August 2016 (Fig 3.1, Table 3.1). Salinity average varied between 27.9 and 31.6 PSU during the period of study. Salinity values were higher during the summer periods of high temperature but then decreased during the autumn and winter (Fig 3.1, Table 3.1). The Chl α level displayed marked seasonal changes. Peaks in Chl α were observed in July 2016 (4.79 µg/l) and May 2017 (4.21 µg/l) (Fig. 3.1, Table 3.1). Other environmental factors such as dissolved oxygen, conductivity and LDO presented similar values during the sampling time (Table 3.1).

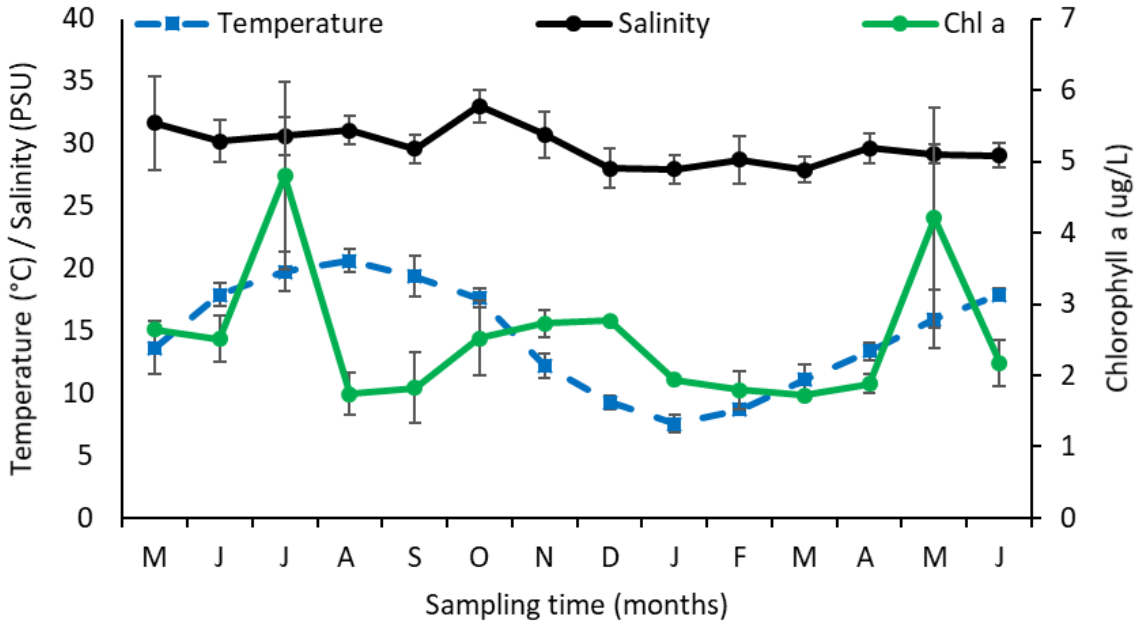

Figure 3.1 Mean monthly variation of temperature, salinity and Chl α from May 2016 to June 2017 in the dock side tank at NOCS. Error bars denote standard deviation.

Table 3.1 Monthly variation (mean± S.D.) of temperature, salinity, conductivity, LDO, % O_2 and Chl α measured monthly in the dockside tank at NOCS from May 2016 to June 2017.

Month	Temperature	Salinity (PSU)	Conductivity (mS/cm)	LDO (mg/L)	% O_2	Chl α (µg/L)	% Monthly mortality
May 2016	13.64±2.14	31.64±3.73	38.44±1.96	11.60±0.86	113.78±12.0	2.64±0.10	3.33
Jun 2016	17.89±0.94	30.20±1.70	39.37±1.40	9.08±0.90	95.15±7.65	2.51±0.32	5.45
Jul 2016	19.76±1.55	30.62±1.51	41.73±2.38	9.07±0.31	99.58±6.53	4.80±1.32	8.16
Aug 2016	20.60±0.92	31.06±1.16	43.74±1.59	8.66±0.45	95.51±4.10	1.74±0.29	5.95
Sep 2016	19.37±1.64	29.58±1.17	41.06±1.74	8.08±1.25	87.45±14.31	1.82±0.50	0.00
Oct 2016	17.63±0.73	33.00±1.26	41.03±0.98	9.49±0.17	94.26±1.42	2.52±0.53	5.97
Nov 2016	12.20±1.02	30.72±1.89	32.50±0.42	10.38±0.26	96.52±1.11	2.72±0.19	1.75
Dec 2016	9.25±0.58	28.03±1.63	30.70±1.39	10.97±0.74	92.50±3.70	2.76±0.11	0.00
Jan 2017	7.53±0.71	27.97±1.14	29.20±1.01	11.92±0.72	99.70±4.27	1.93±0.15	0.00
Feb 2017	8.66±0.26	28.70±1.87	30.66±1.97	11.75±0.19	99.12±1.82	1.79±0.27	0.00
Mar 2017	11.08±1.23	27.93±1.08	30.93±1.63	11.45±0.36	100.33±1.71	1.72±0.09	0.00
Apr 2017	13.34±0.72	29.64±1.20	35.12±1.71	10.96±0.22	102.78±1.73	1.88±0.13	0.00
May 2017	15.92±2.33	29.18±0.73	37.08±2.28	10.39±0.36	103.28±3.79	4.21±1.54	5.00
Jun 2017	17.87±0.50	29.07±1.01	38.53±1.55	10.12±0.46	107.10±5.60	2.17±0.33	0.00

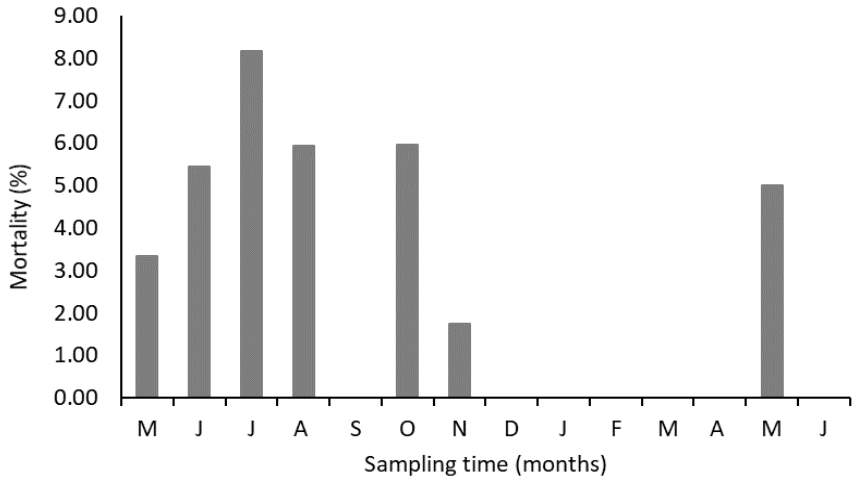

Figure 3.2 Percent of dead animals registered monthly from May 2016 to June 2017 in the dockside tank at NOCS.

3.3.2 *Ostrea edulis* mortality and growth

In July 2016, mortality showed the highest value (8.16%) reported during the period of study (Fig 3.2, Table 3.1). The mortality of oysters significantly changed during the period of study (Kruskal-Wallis; $p < 0.0001$). Temperature and mortatility presented a significant correlation ($r_s = 0.642$; $p < 0.0001$) with the highest mortality rates were observed in the summer period when the highest temperatures were recorded.

Changes in biometric parameters are illustrated in Fig. 3.3 and reported in Appendix E. Total weight, shell height, length and width did not show significant changes over the sampling period (Fig. 3.3A, B, D, and E). A new thick layer of tissue in the oysters' shells was observed showing a significant change (Kruskal-Wallis test value = 45.590, $p < 0.001$, N = 84) throughout the period of study. The major change was observed with an increase from June (1.40±1.97 mm) to July 2016 (9.94±5.38 mm), and then presenting the highest values from July to September 2016 (10.49±2.90 mm) (Fig. 3.3A). Shell volume showed large significant variations over the year (Kruskal-Wallis test value = 30.549, $p < 0.004$, N = 84), with the highest value in August (61.89±7.71 ml), December 2016 (61.77±12.87 ml) and February 2017 (58.91±11.27 ml) (Fig. 3.3F). Fresh dry weight showed significant changes during the year (Kruskal-Wallis test value = 40.926, $p < 0.001$, N = 84), increasing after summer from September to November 2016, and then decreased alongside with the decrease in the water temperature (Fig. 3.3G). The CI showed significant changes during the year (Kruskal-Wallis test value = 49.708, $p < 0.001$, N = 84) with an increase in CI from June to October 2016 (15.11±1.44), and then the highest values for this parameter were observed in January 2017 (13.86±2.02) (Fig. 3.3H). After January a CI decrease was observed over time.

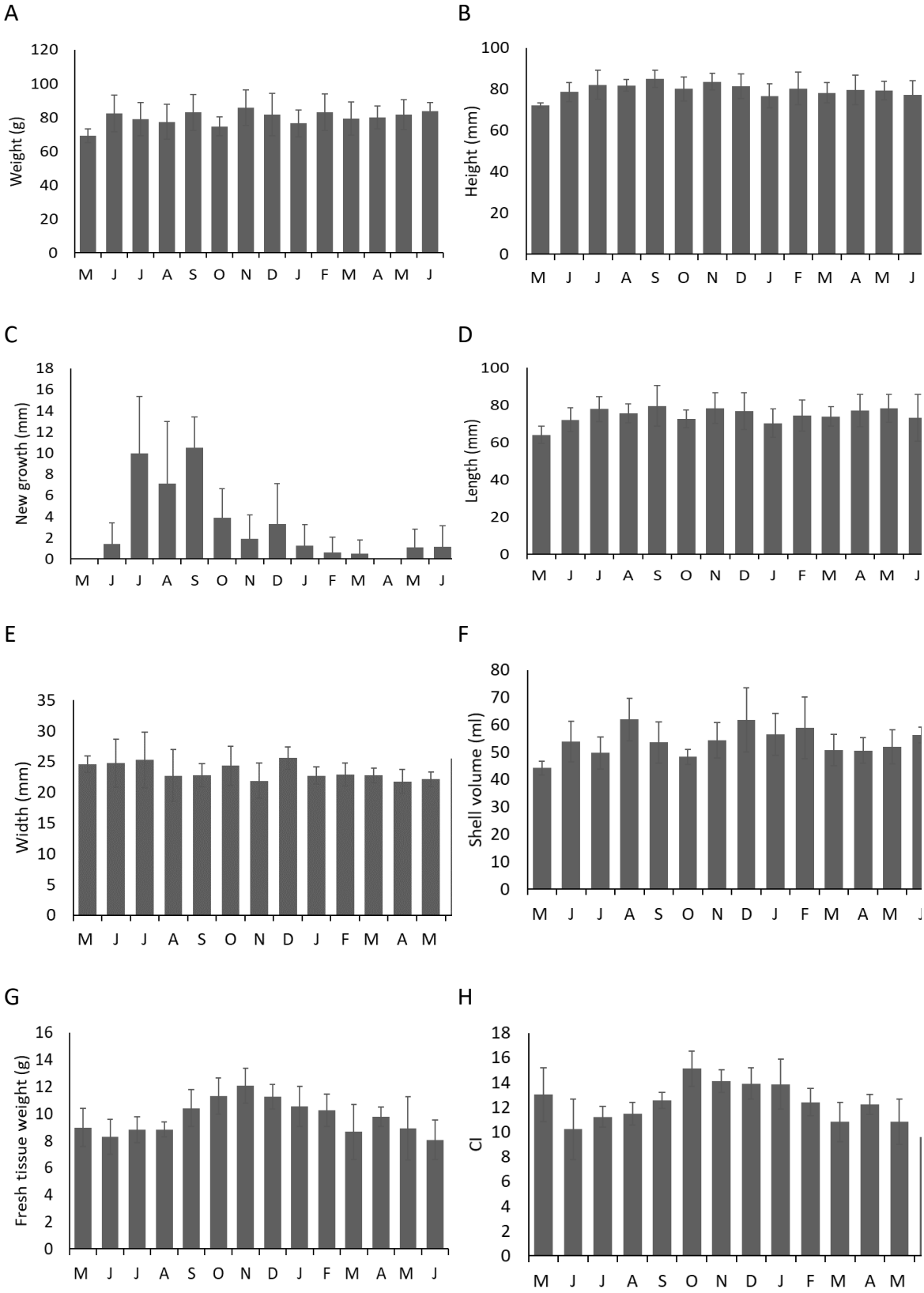

Figure 3.3 Biometric parameters including total weight (A), height (B), new growth (C), length (D), width (E), shell volume (F), flesh weight (G) and condition index (H) of *Ostrea edulis* (n=6 per month) kept in the dockside tank at NOCS from May 2016 to June 2017. Error bars denote standard deviation.

3.3.3 Gametogenic cycle

The gametogenic cycle of *O. edulis* was studied from May 2016 until June 2017. The percentage distribution of different gametogenic stages observed during the period of study is illustrated in Fig. 3.4. The asynchrony of gonadal development among individuals was evident during the study showing high variability in each month. That variability was expected since gonadal maturation is not a homogeneous process and spawning is not a synchronic event. In spite of this variability, it was possible to follow the gonadal maturation process during the year of study. At the beginning of the experiment, 20% of oysters were inactive and 40% were at a stage of advanced gametogenesis (stage G2) or with ripe gonads (stage G3). By August, the percentage of oysters in G0 increased (40%) and an increase in oysters in their resting phase reabsorbing gonads (stage G5) was found at this time. From this point until November 2016, an increase in oysters at G0 was observed. From December 2016 onwards, oysters at stage G0 decreased and some of the early and advanced gametogenic stages slightly increased until March 2017 when ripe gonads were found again until the end of the study. Although some individuals were at an early stage of gonad maturation (stages G1 and G2) from May to June, spawning activity (stage G4) was observed in July 2016 (35%), August 2016 (20%), April 2017 (10%), May 2017 (33%) and June 2017 (12%).

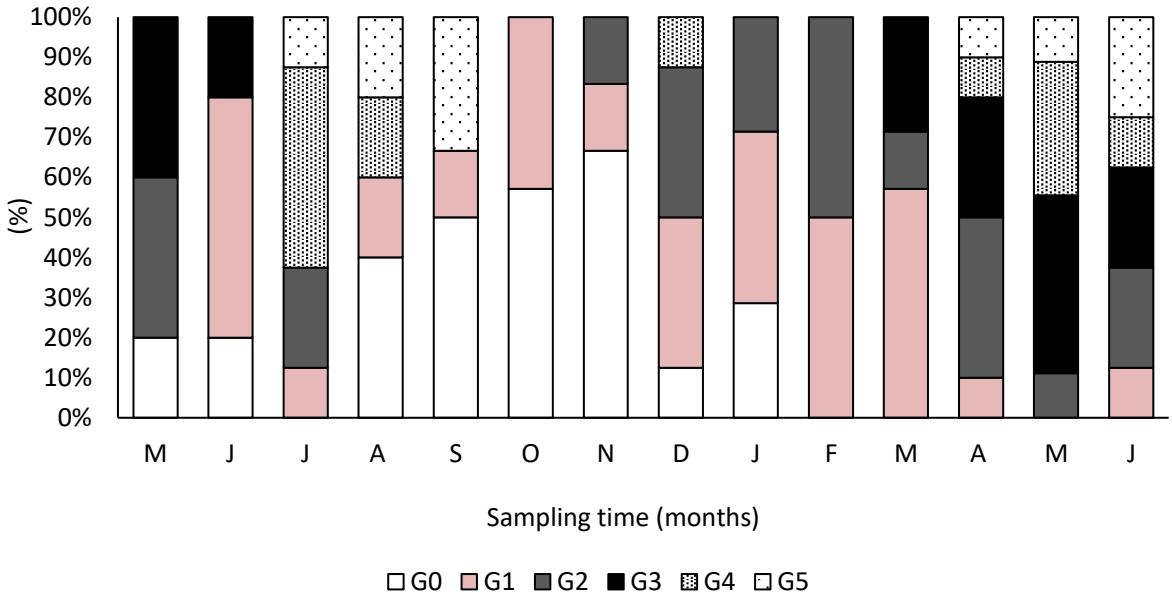

Figure 3.4 Seasonal distribution of *Ostrea edulis* at different stages of gonad development. Oysters (n=6 per month) were kept in the dockside tank at NOCS from May 2016 to June 2017. According to da Silva *et al*. (2009) developmental stage was classified by the gametogenic stage of the gonad as inactive (G0), early gametogenesis (G1), advanced gametogenesis (G2), ripe gonad (G3), partially spawned gonad (G4) and reabsorbing gonad (G5).

In this study, an annual reproductive cycle of *O. edulis* can be identified presenting a resting phase from August to November, then a slow increase in gonadal development when temperature starts increasing, and then gametogenesis ripeness or reabsorption of gonads during the summer (from May to July). Spawning events were not observed in this study but oysters classified in G4 (partially spawned gonad) fulfilled the characteristics described by da Sila *et al*., (2009) presenting small gonad follicles than in the previous stage (G3), residual mature gametes and presence of phagocytes in the follicle lumen (Table 1.1).

3.3.4 Sex ratio

A total of 78 individuals were studied and the sex of all individuals was determined during this study. Figure 3.5 illustrates the change in the sex category proportions per month of this protandrous alternate hermaphrodite sex ratio over the year. The proportion of sex categories varied during the study period but the changes were not significant (Kruskal-Wallis test value = 14.125, p = 0.36, N = 84) and the sex ratio was not statistically significantly different from a ratio of 1:1. For a few months each year (October 2016 and March 2017), the ratio was skewed towards females, whilst a male-skewed sex ratio was found in June for both years.

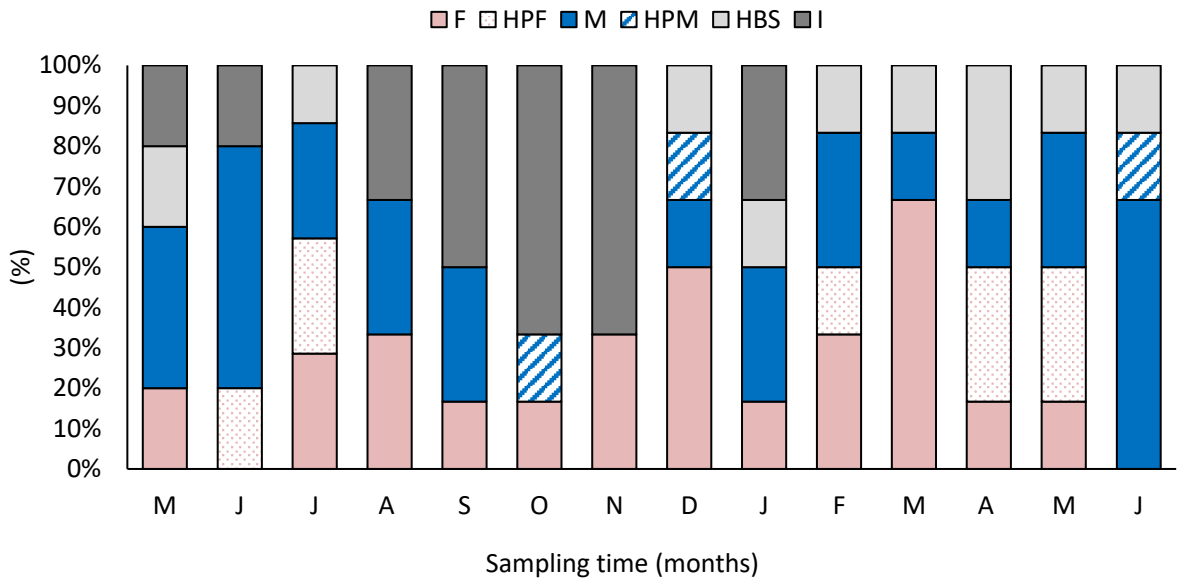

Figure 3.5 Proportion of sex categories in *Ostrea edulis* kept in the dock side tank at NOCS from May 2016 to June 2017. Specimen samples (n=6 per month) were identified by histological examination as females (F), males (M), hermaphrodite with both sexes equally represented (HBS), hermaphrodite predominantly male (HPM), hermaphrodite predominantly male (HPF) an indeterminate (I) according to da Silva *et al*. (2009).

Female individuals were observed throughout the study period but predominantly in December 2016 and March 2017. Males were present most of the time during the study but the proportion of males:females was higher in May and 2016, January and February 2017, and June and July 2017. In June 2016 and July 2017 no females were found, and the percentages of males was the highest throughout the year (60% in June 2016 and 65% in July 2017). The highest percentage of individuals in the inactive stage was recorded between September and November, and inactive individuals were scarce or absent in the other months. A comparison of different sex categories indicated that, in general, the population studied presented more females when the water temperature was less than 12°C. On the other hand, males were more frequently observed with an increase in water temperature. Hermaphrodites were variable throughout the year with the highest values found in April (70%) and May (50%) 2017.

3.3.5 Seasonal cycle of energy reserves

The changes in biochemical composition of gonad visceral mass of *O. edulis*, throughout the year, are shown in Fig. 3.6. In the present study, seasonal variation of total lipids (% DW) indicated that the highest content of total fatty acids is reached between November (8.55±0.97 %DW) and March (8.12±3.70 %DW), including the end of winter and the beginning of spring, although these changes were not statistically significant (Kruskal-Wallis test value = 12.568, $p = 0.48$, $N = 84$) (Fig 3.6A).

Carbohydrate content (% DW) changed significantly over the period of study (Kruskal-Wallis test value = 44.642, $p < 0.001$, $N = 84$). Oysters in May and June 2016 showed the highest values for carbohydrate content (% DW) with 12.00±6.06 %DW and 10.98±8.82 %DW, respectively. From June 2016 onwards seasonal fluctuation of the carbohydrate content stored in the gonad-digestive gland shows progressive declines (Fig 3.6B). Thereafter it increases slightly in September but decreased again throughout late autumn and winter, presenting the lowest values in January (1.72±0.94 %DW). Then a period of slight increase was observed until the end of the period of study.

Protein content also presented significant differences throughout this study (Kruskal-Wallis test value = 23.927, $p < 0.03$, $N = 84$). Protein content (% DW) represented around 30% of the dry weight from May to December (Fig. 3.6C). This percentage increased significantly reaching the maximum values in March 2017 (44.05±9.02 %DW) and June 2017 (41.03±10.50 %DW) (Fig 3.6C).

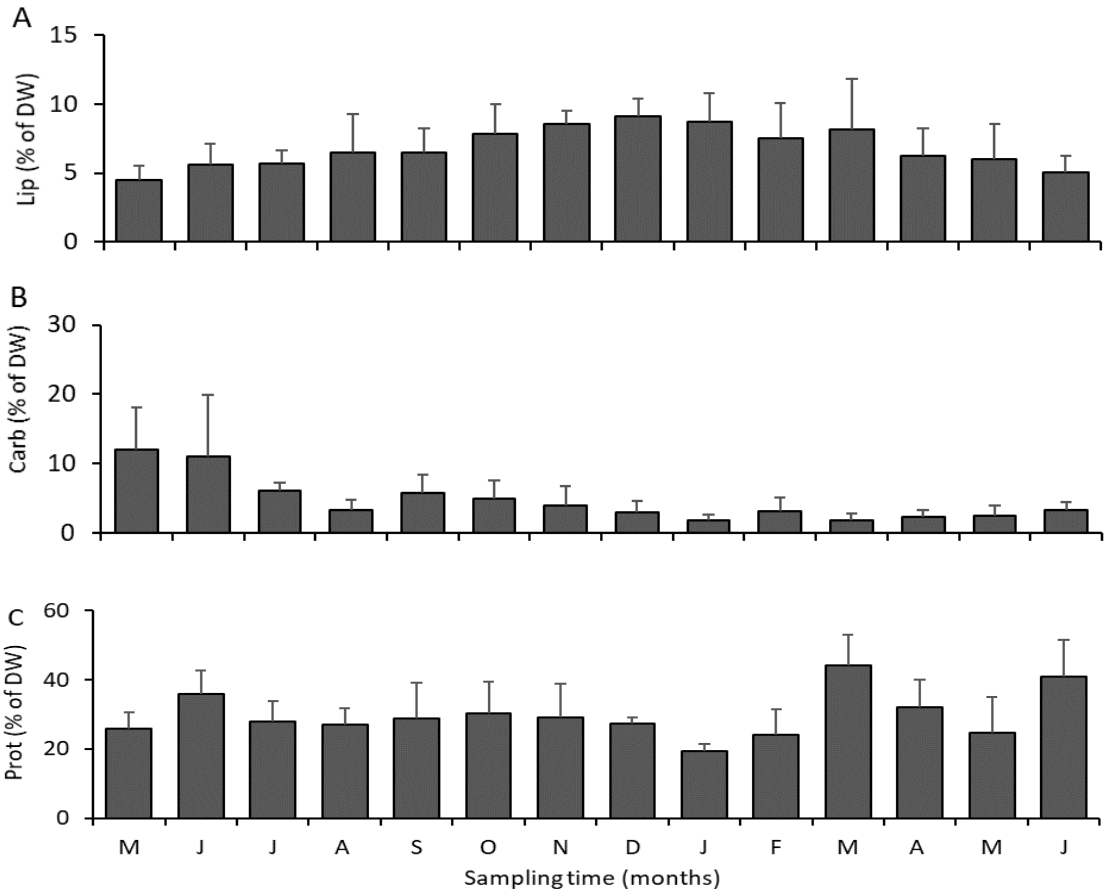

Figure 3.6 Monthly variations in (A) total lipids (% of dry mass, % DM), (B) total carbohydrates (% of dry mass, % DM) and (C) protein content (% of dry mass, % DM) of *Ostrea edulis* (n = 6 per sampling time) kept in the dockside tank at NOCS from May 2016 to June 2017. Error bars denote standard deviation.

3.3.6 Seasonal changes in sex steroid hormones

The standardization of the protocol for using ELISA kits for hormonal analysis showed a good response. The analysis of samples from this experiment was carried out including a standard curve for both hormones obtaining a good linear relationship with R^2 values of 0.9855 and 0.9865 for estradiol and testosterone, respectively.

The analysis of E_2 and T concentrations in gonadal tissue of *O. edulis* showed significant changes throughout the year of study (E_2: Kruskal-Wallis test value = 56.670, $p < 0.001$, N = 84; T: Kruskal-Wallis test value = 51.025, $p < 0.001$, N = 84). Concentrations of E_2 in gonads showed maximum values from October 2016 to January 2017, reaching a peak (1,647.41±772.6 pg/g) in November 2016. Testosterone showed a progressive increase throughout the year presenting the highest values between January (1,995.3±1.658.68 pg/g) and May 2017 (2,890.07±1126.57 pg/g).

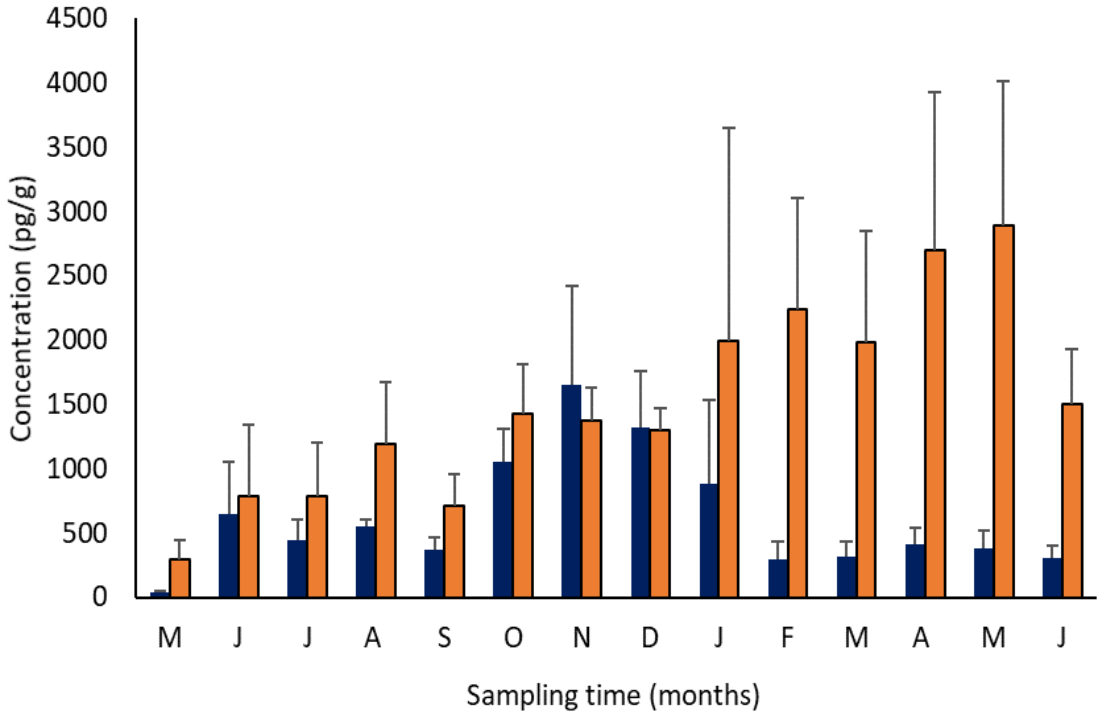

Figure 3.7 Mean sex steroid hormone concentrations in *Ostrea edulis* (n = 6 per sampling time) kept in the dockside tank at NOCS from May 2016 to June 2017. Error bars denote standard deviation. Dark blue bars: estradiol. Orange bars: testosterone.

3.3.7 Seasonal changes in Vtg-like protein

The analysis of Vtg-like protein concentrations in gonadal tissue of *O. edulis* showed significant changes throughout the year of study (Kruskal-Wallis test value = 36.546, $p < 0.001$, N = 84). Similar values were observed between September 2016 (17.97±14.73 ug ALP/mg prot) and December 2016 (15.44±9.39 ug ALP/mg prot), and then a slight increase was observed presenting the highest value in February 2017 (24.65±19.38 ug ALP/mg prot). After this point, a decrease in the concentrations of Vtg-like protein was observed with 19.94±12.35, 11.21±6.07 and 8.41±10.69 ug ALP/mg prot for March, April and May, respectively.

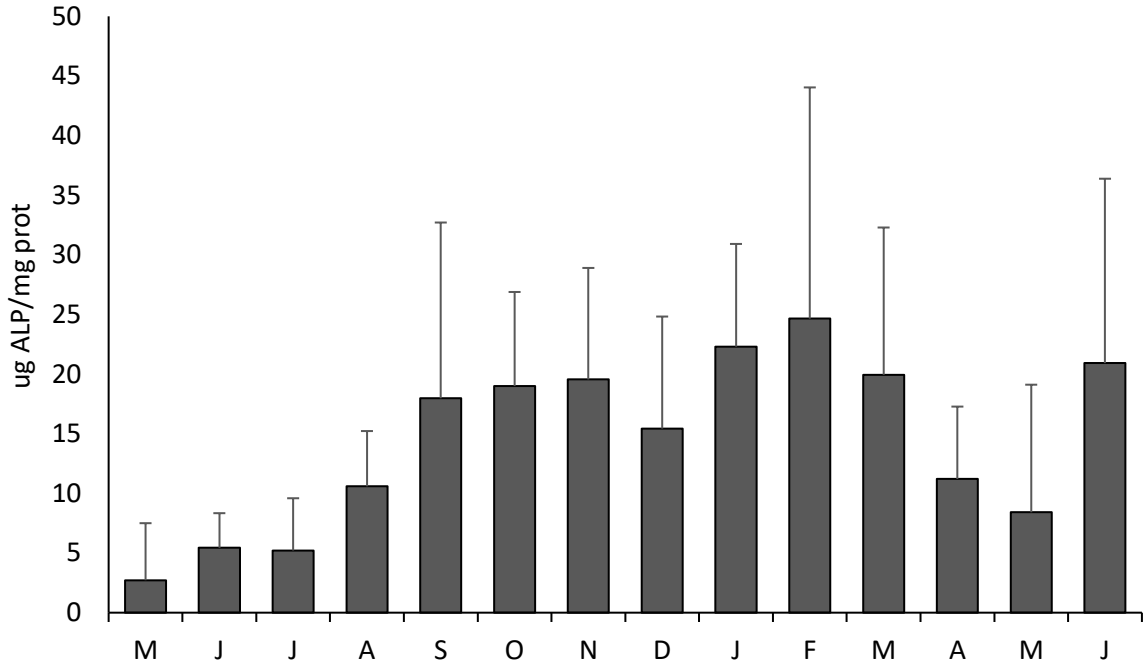

Figure 3.8 Mean Vtg-like protein concentrations in *Ostrea edulis* (n = 6 per sampling time) kept in the dockside tank at NOCS from May 2016 to June 2017. Error bars denote standard deviation.

3.3.8 Effect of sex on energy reserves and sexual steroids

The concentration of energy reserves in gonads quantified in males or females in each season is illustrated in Fig. 3.9A, B and C. In gonads, no significant intersex differences (Mann Whitney U test, $p > 0.05$) in carbohydrates levels were observed in *O. edulis*. Females showed higher lipid content than males in all seasons but autumn was the only season when significant differences ($p = 0.04$) between sexes were found. Concerning proteins, higher concentrations were observed in females in autumn (Mann Whitney U test, $p = 0.034$), winter (Mann Whitney U test, $p = 0.003$) and spring (Mann Whitney U test, $p > 0.05$) compared to males in the same season. In contrast, no intersex differences in sexual steroids in gonads (estradiol and testosterone) or Vtg-like proteins were found (Fig. 3.9D, E and F).

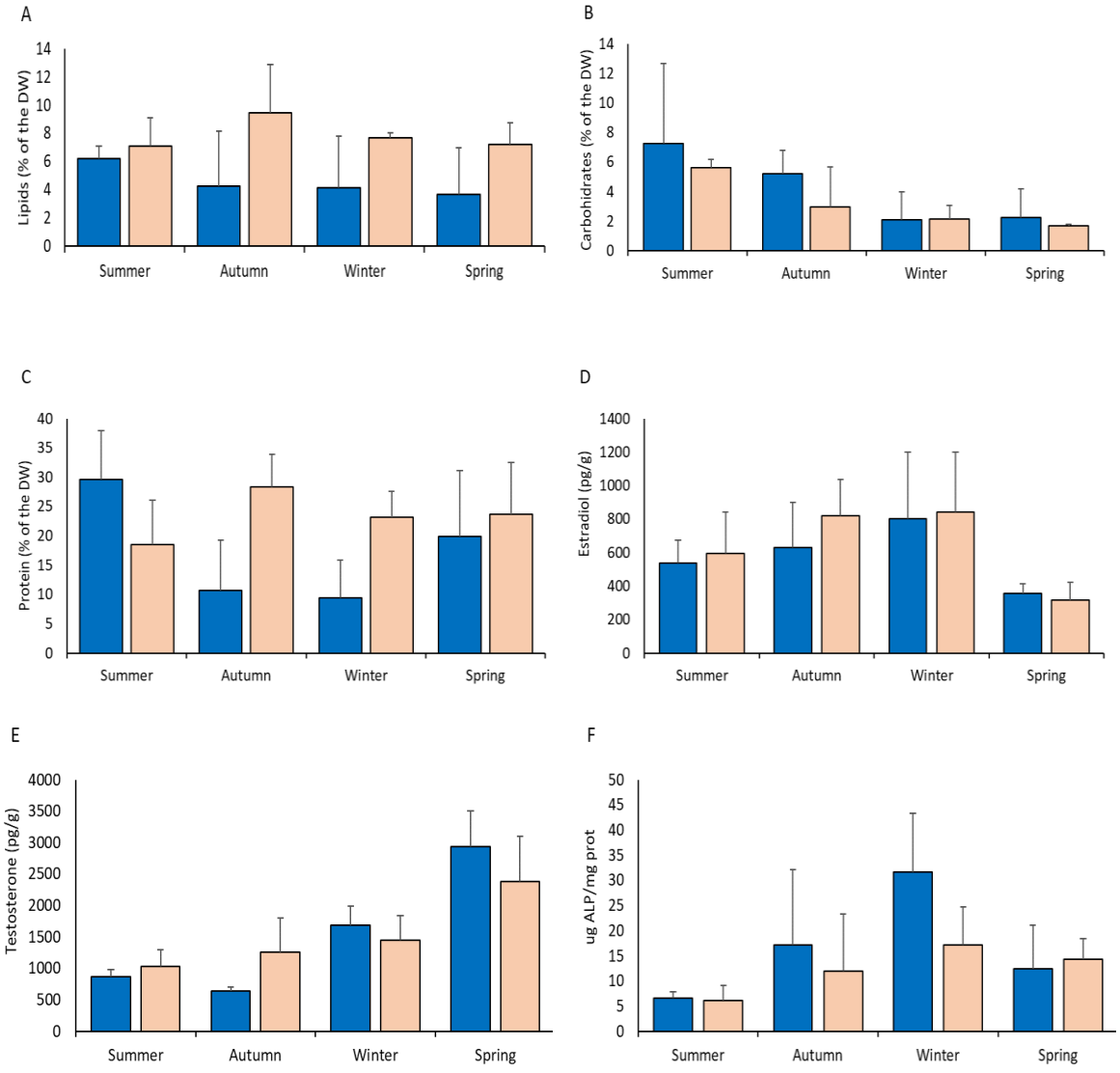

Figure 3.9 Influence of sex on energy reserves ((A)lipids, (B)carbohydrates, (C)proteins), sex steroid hormones ((D)estradiol and (E)testosterone) and (F)vtg-like proteins in *Ostrea edulis* (n=18 per season) in different seasons throughout a year kept in the dock side tank at NOCS. Blue bars: males + hermaphrodites predominately males. Beige bars: females + hermaphrodites predominately females. Error bars denote standard deviation.

3.3.9 Linking energy reserves, steroid hormones and gonadal stage

The influence of the sexual maturity stage of *O. edulis,* the concentration of steroid hormones and the energy reserves is illustrated in Fig. 3.10. In the oysters, the concentrations of E_2 in showed a gradual increase from inactive (G0) to ripe developed gonads (G3), and gradually decreased after that (Figure 3.9A). Concentrations of E_2 in oysters classified in stage G5 were significantly different

(Mann Whitney U test, p = 0.03) from the other stages. Overall the concentrations of T showed a gradual decrease with no significant differences (Mann Whitney U test, p > 0.05) among the concentrations of T at the different gonadal stages.

The concentrations of carbohydrates and proteins did not present significant changes between gonadal stages. However, lipid content showed a significant increase (Mann Whitney U test, p = 0.02) for oysters classified in stage G3 compared to the other stages.

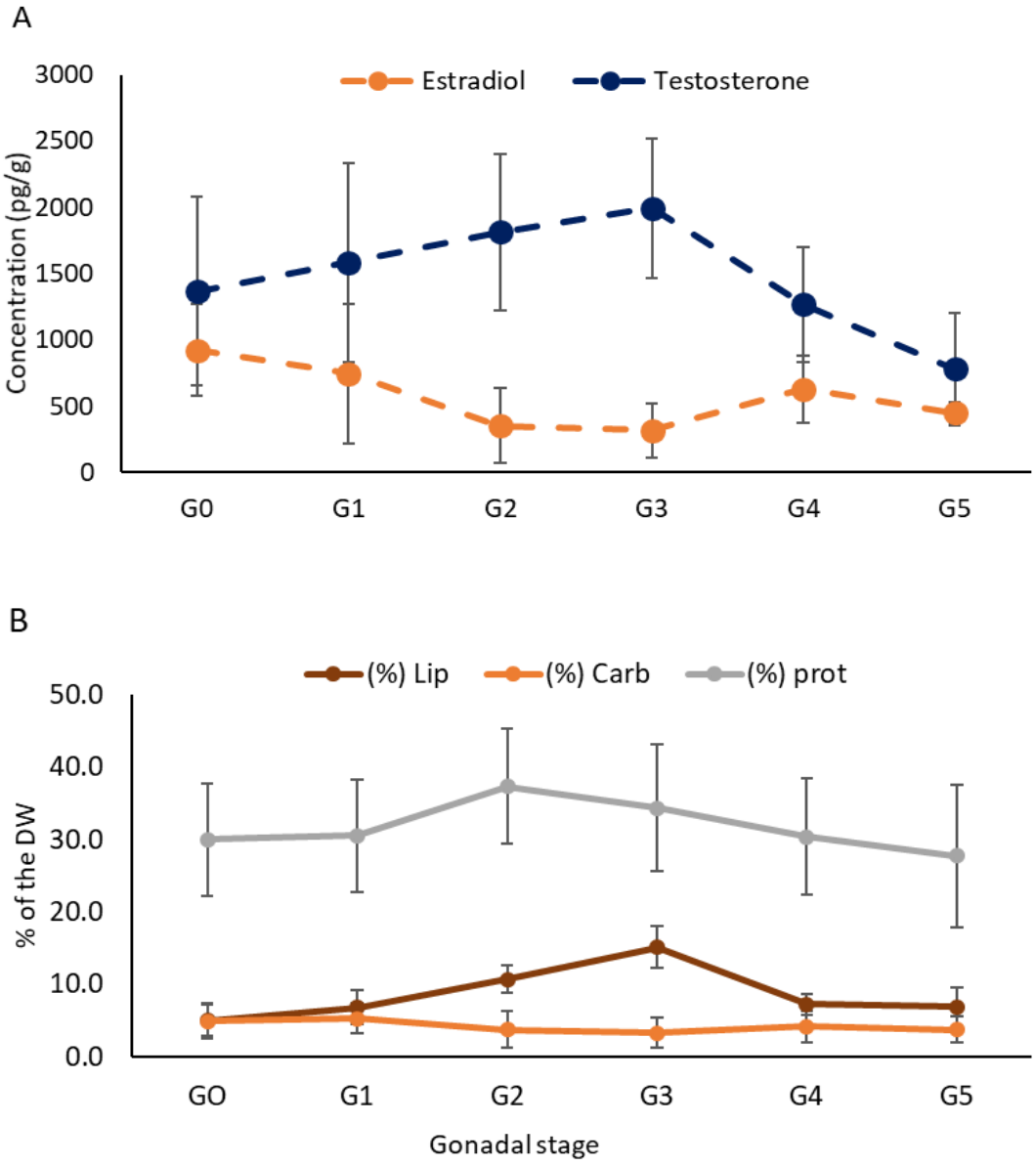

Figure 3.10 Changes in (A) sex steroids and (B) energy reserves (lipids, carbohydrates, proteins) (Mean±SD) at different stages of gonadal development (G0, n=9; G1, n=21; G2, n=20; G3, n=13; G4, n=12; G5, n=9) of *Ostrea edulis* kept in the dockside tank at NOCS from May 2016 to June 2017. Error bars denote standard deviation.

3.3.10 Relation between environmental variables and biological and reproductive parameters in *Ostrea edulis*

Mortality was significantly influenced by temperature (r_s = 0.6421; $p < 0.001$), salinity (r_s = 0.7622; $p < 0.001$) and LDO (r_s = 0.6421; $p < 0.001$). Chl α showed a weak significant effect on mortality (r_s = 0.4820; $p < 0.001$).

Some of the biometric parameters were significantly affected by environmental variables. Temperature showed a significant effect on NG (r_s = 0.4880; $p < 0.001$), FW (r_s = -0.3875; $p < 0.05$) and CI (r_s = -0.3682; $p < 0.001$). An inverse correlation between the CI and the mean temperature in the water was observed throughout the study period, with the CI showing a significant increase in October 2016, and then a significant decrease in March 2017 when temperatures were colder. Salinity had a weak significant effect on NG (r_s = 0.2429; $p = 0.03$) and SVol (r_s = -0.2471; $p = 0.03$).

Temperature, salinity, LDO and Chl α did not show a significant effect on sex determination. However temperature and Chl α showed a significant effect on gonadal maturation (temperature: r_s = 0.2527; $p = 0.02$ and Chl α: r_s = 0.3676; $p = 0.02$).

The CI was correlated with gonad maturation stage (r_s = -0.4133; $p < 0.001$). The significant CI increase coincided with increases in the frequency of inactive (G0) and early gametogenic (G1) stages. In the same manner, the CI decrease coincided with the increase in the frequency of gonads at stages G2 to G3.

The changes in biochemical composition of gonad visceral mass of *O. edulis* throughout the year showed a complex relationship between environmental parameters and biochemical attributes. Carbohydrate content was affected by temperature (r_s = 0.4389; $p < 0.001$), salinity (r_s = 0.4775; $p < 0.001$) and Chl α (r_s = 0.3331; $p < 0.05$). However, none of the environmental parameters studied significantly correlated lipid or protein content in gonads of *O. edulis*. Lipid content showed a progressive increase throughout the year reaching peaks at the end of winter and the beginning of spring. This corresponded with a slight increase in Chl α and a decrease in temperatures. At the same time, during this time a reduction of oysters at stage G0 and the increase in gametogenic stages (G1 and G2) were observed. A similar trend was observed for proteins in gonads of *O. edulis* showing an increase that coincided with the period of gametogenic maturity. The carbohydrate content showed a negative significant correlation with lipid content (r_s = 0.6785; $p < 0.05$). An increase in carbohydrate content was observed when lipid content decreased throughout late autumn and winter. The protein content did not show any correlation with the other biochemical

variables measured but an increase that coincided with the period of gametogenic maturity was observed.

An analysis of hormone concentrations showed a relationship with some environmental variables, biometric and biochemical parameters. Estradiol concentration in gonads was not significantly affected by any of the environmental variables, but testosterone was significantly correlated with temperature (r_s = -0.3665; $p < 0.05$) and salinity (r_s = 0.3538; $p < 0.05$). The CI correlated with estradiol concentration (r_s = 0.4258; $p < 0.001$), but not with testosterone concentrations in gonads. A positive significant relationship between lipid content and E_2 (r_s = 0.3980; p = 0.02) and T (r_s = 0.3413; p = 0.03) concentrations was found. Carbohydrate content correlated negatively with testosterone concentrations (r_s = -0.5376; $p < 0.001$) but did not show a correlation with estradiol. Proteins did not appear to be affected by hormone concentrations. The analysis of Vtg-like protein concentrations in gonadal tissue of *O. edulis* showed positive correlations with estradiol (r_s = 0.2343; p = 0.03) and testosterone (r_s = 0.2653; p = 0.03) concentrations.

Gonadal maturation correlated negatively with estradiol concentrations (r_s = -0.2900; $p < 0.05$). However, no correlation between gametogenic stage and T concentrations or Vtg-like proteins was found. Sex determination did not show any correlation with hormone concentrations or Vtg-like proteins in gonads in *O. edulis*.

3.3.11 A synthesis based on RDA analysis

The particular challenge in the case of environmental factors involved in biological response is the complexity associated with analyzing a large number of measured variables and their effects. Therefore, in this study a RDA was used to extract biological responses explained by environmental factors (ter Braak and Verdonschot, 1995; Lepš and Šmilauer, 2003) (Fig. 3.11). In this analysis the different variables taken into account were: biometric parameters (height, length, width, new growth, shell cavity volume, fresh tissue weight, total weight and condition index), energy reserves (lipids, carbohydrates and protein content), sex steroids concentrations (estradiol and testosterone), sex and sexual maturity stage. The matrix of scores provides information about the distribution of patterns in the data. In this study, the scores of the two retained principal axes (AXs) (Appendix F) showed AX1 and AX2 explaining 49.8% and 31.4% of the variance, respectively. Between both axes 81.2% of the variance is explained by this model.

The data are distributed in two main regions of space spanned by the two well-defined PC axes (Fig 3.11). The closer two-parameter lines lie together, the stronger the mutual correlation is. For example, temperature, conductivity and Chl *a* lines are very close which shows a high positive

correlation coefficient between these factors, indicating than they had similar trends during the period of study. In the same manner, mortality, the content of carbohydrates, NG and gonadal maturation seem to be the biological responses more affected by these environmental factors. These biological parameters are positively and significantly correlated with the first axis, whereas H, L, W, SVol and E_2 concentration are negatively correlated with AX1. On the other hand, the percent of proteins and lipids, Vtg-like proteins concentration, T levels, FW and CI could be more directly affected by LDO levels than any of the other environmental factors.

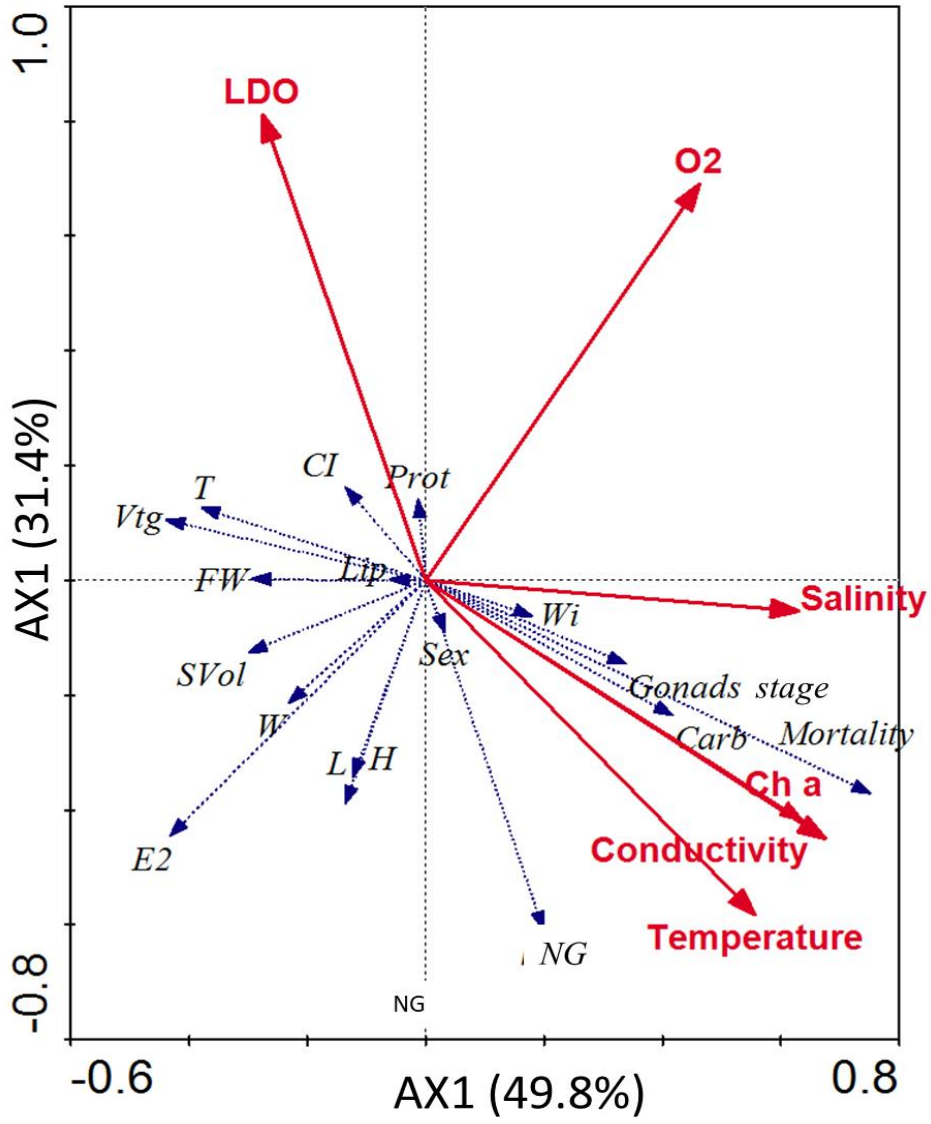

Figure 3.11 RDA biplot including environmental factors (temperature, conductivity, salinity, Chl *a*, LDO and O_2) (red lines) and biological responses in *Ostrea edulis* (biometric parameters: W, H, L, Wi, NG, Svol, FW, CI; hormones concentrations: E_2 and T; Vtg-like proteins; energy reserves: lipids, carbohydrates, proteins; sex and gonad maturation stage) (blue dotted lines). Variance explained by each principal component is shown in brackets.

3.4 Discussion

Biological indices, reproductive parameters and energy balance in *O. edulis* are likely to respond to numerous environmental conditions being especially affected by temperature, salinity and food availability (Diaz-Almela *et al*., 2004; González-Araya, Quillien and Robert, 2013). This study has found some environmental factors affecting biological responses in *O. edulis* during natural seasonal changes that could be relevant in the understanding of the main factors affecting declining oyster populations.

3.4.1 Environmental factors affecting growth in *Ostrea edulis*

It is generally assumed that CI is a good indicator of the health and ecophysiological state in commercially exploited bivalve and other mollusc species (Orban *et al.*, 2002; Filgueira *et al.*, 2013). Fluctuations in CI have important implications for cultivation and harvesting strategies. Carlucci *et al*. (2010) found a seasonal oscillation in the growth pattern of *O. edulis* with an increase during spring-summer. In this study, a different trend was found, with the highest CI values found during colder conditions from October 2016 to January 2017. The decrease of CI in February may be attributed to gonadal maturation and low food availability. This coincides with a non-significant drop in lipid content that could indicate a mobilization of somatic molecules as an energy reserve as shown in other clams (Mouneyrac *et al.*, 2008).

3.4.2 Effect of environmental variables on gametogenesis in *Ostrea edulis*

The gametogenic cycle of bivalve species is affected by exogenous factors such as food availability, temperature, photoperiod and salinity (Korringa, 1957; Newell, Johson and Kofoed, 1977; Mann, 1979; Newell and Branch, 1980; Shpigel, Barber and Mann, 1992; Pérez *et al.*, 2013; Santerre *et al.*, 2013; Acarli *et al.*, 2015; Eagling *et al.*, 2018; Hassan, Qin and Li, 2018) and endogenous rhythms such as energy reserves and hormonal cycles (Newell, Johson and Kofoed, 1977; Mouneyrac *et al.*, 2008; González-Araya *et al.*, 2011; Pérez *et al.*, 2013; Hassan, Qin and Li, 2018).

The effect of temperature in terms of affecting biochemical and physiological components involved in the maturation process of oysters is well-known (Chávez-Villalba *et al.*, 2003; da Silva, Fuentes and Villalba, 2009). Gametogenesis in *O. edulis* has been correlated with water temperature (Marteil 1960, Sastry 1975, Mann 1979, Wilson and Simons 1985), but the onset of gametogenesis and spawning in *O. edulis* occurs at different temperatures and depends on location (Cole, 1942a; Loosanoff, 1962; Mann, 1979; Wilson and Simons, 1985; Ruiz *et al.*, 1992; Shpigel, Barber and Mann, 1992). In general the connective tissue surrounding the digestive system provides substrate

for the differentiation of gonadal tissue, and with the rise of water temperatures during spring this tissue is filled with gonadal tubules preparing the animals for a new gametogenic cycle (Giese and Pearse, 1974).

In this study, an annual reproductive cycle of *O. edulis* was well defined showing a resting phase from August to November, then a slow increase in gonadal development when the temperature starts increasing and then gametogenesis ripeness or reabsorption of gonads during the summer were observed. Due to the conditions of this experiment and the constant water flow in the tank containing the oysters it was not possible to measure gamete release and fertilization rate. Evidence of settlement was found on the shell of some of the adults suggesting that the spawning events and fertilization was occurring but due to the space between samplings (a month between sampling times) the evidence of other reproductive and biological parameters (such as release of eggs and sperm into the water, fertilization, free-swimming larvae) could have been missed.

Small gonad follicles and residual mature gametes remaining in the follicle lumen were observed in July 2016 to August 2016 and April 2017 to June 2017. According to the criteria described by da Silva *et al*., (2009) (see table 1.1) these observations could suggest partially spawned gonads. These results are in agreement with literature data indicating that the highest percent of the spawning stage in this species could be found when temperatures start decreasing (Acarli *et al.*, 2015). However, spawning was not directly observed or recorded in this study. A decline in temperature also coincided with the resting phase characterized by the increase of oysters in resting stage (G5) and slow development of early gonad stages (G0) from August 2016 and increasing considerably by November 2016. Then, early gametogenic stages (G1 and G2) were more abundant between December 2016 to March 2017 indicating that *O. edulis* starts maturating under cold conditions and continues when temperatures slowly start increasing. Carlucci *et al*. (2010) found that under low temperatures (12–13°C) the metabolism of *O. edulis* was mainly oriented to the production of mature sperms and eggs rather than towards somatic growth. This is not in agreement with the results of this study reporting the increase of oysters in developed stages (G3 and G4) at the same time that the highest environmental temperatures were reached. This could indicate a shift in the reproductive cycle caused by a delay in the maturation process.

It has been shown that elevated temperatures increase metabolism and accelerate gametogenesis and gonadal development in bivalves (Shpigel, Barber and Mann, 1992; Pérez *et al.*, 2013; Santerre *et al.*, 2013). In Chapter 2 a rapid maturation and an accelerated gametogenesis process were reported for *O. edulis* kept at high temperatures (Zapata-Restrepo *et al*., 2019). However, the delay in oysters kept in the dockside tank at NOCS to reach G3 and G4 stages could reflect that the

conditions (such as temperature, food quality, etc.) for activation of the gametes growth phase were not enough.

The change in temperatures during spring could be an important factor to start gametogenesis and it has been demonstrated that some species require a minimum temperature for activation of the oocyte growth phase, and although oogonia can develop below this threshold, further differentiation only occurs at warmer temperatures (Sastryz, 1965; Maneiro *et al.*, 2017). Maneiro *et al*. (2017) reported that the percentages of the gonadal volume occupied by germinal cells were almost double for *O. edulis* conditioned under a gradient of temperature between 14 and 18°C compared to those oysters kept at 15°C, indicating that the change in temperature could trigger and accelerate this process. Whilst some variation in the exact temperature triggering these processes between different populations can be expected, 15–16°C has been identified as the minimum temperature required for maturation for UK populations (Korringa, 1957). Under exceptionally favourable conditions, *O. edullis* has the potential to reach maturity and spawn several times during the same season because even just a few hours after releasing eggs or sperm the gonads can begin to change into the opposite gender (Korringa, 1957). However, it has been observed that generally UK oysters spawn just once every summer (Orton, 1927b; Korringa, 1957). Dodd *et al*. (1937) reported that *O. edulis* only has the ability to become a functional mature female following an exceptional summer period because it needs a large quantity of energy to produce ovaries (Dodd *et al.*, 1937).

Sastryz (1965) suggested that, above the minimum threshold for gonadal maturation, the rate of gamete development is temperature dependent, whereas fecundity and size of the gonad are determined primarily by food availability. In this study, a significant effect of temperature and Ch*a* on gonadal maturation has been found. Besides temperature, food availability is also affecting broodstock energy reserves and gametogenesis in bivalves (Teaniniuraitemoana *et al.*, 2016). The reproductive cycle of a bivalve can be divided into two processes: i) storage accumulation (generally glycogen) and ii) gametogenesis (Pouvreau *et al*., 2006; Saucedo *et al*., 2002), which is achieved by using accumulated reserves and/or available food. It is difficult to differentiate the role and effect of adult reserves from that of additional food. Although some authors have not found a clear evidence of the utilization of carbohydrate from the *O. edulis* adductor muscle as source of energy (González-Araya *et al*., 2012), it is considered that bivalves can use mantle, digestive gland and adductor muscle tissues as important energy reserve sites for storage and translocation of protein and glycogen, lipids and proteins, respectively (Barber and Blake, 1985; Saucedo-Lastra *et al.*, 2002; Pérez *et al.*, 2013).

The relationship between gametogenesis and energy metabolism is of great interest because considering that the investment of resources in reproduction reduces somatic investment, the life cycle is a pattern of allocation of resources between reproductive and somatic functions with a constant trade-off between resources invested in reproduction and somatic growth (Barnes *et al*., 1988). Initial egg lipid content has been found to be positively correlated with larval survival (e.g., *Mercenaria mercenaria* and *Crassotrea virginica*: Gallager & Mann, 1986; *Pecten maximus*: Le Pennec *et al*., 1991), larval growth (*O. edulis*: Helm *et al*., 1973) and fecundity (e.g., *Magallanas gigas*: Chavez-Villalba *et al*., 2003; *Argopecten purpuratus*: Martinez *et al*., 2000a, 2000b). However, the influence of the relative food values of different phytoplankton species (nutritional quality) on mollusc gonadal development has been little explored, especially in *O. edulis*, and there is not enough evidence to demonstrate that these energy reserves can be kept and used during the reproductive season.

Despite some studies showing an absence of correlation between fatty acid concentration and gonad development in *O. edulis* (González-Araya *et al*., 2011), it has been shown that gametogenesis can be affected by different types of algae used under controlled conditions (González-Araya *et al*., 2013). Furthermore, the examination of nutrient biochemical allocation in the gonads and ecophysiological parameters of *O. edulis* consuming different types of monospecific microalgal diets has shown a difference between ingestion, digestion, assimilation and efficiency allocation in the reproductive compartment (González-Araya *et al*., 2012). It has been reported that an algal diet rich in carbohydrates produces the best gonadal development in this bivalve (González-Araya *et al*., 2012), and a mixed-algal species diets support generally better growth and competence than single-species diets in *O. edulis* (Helm *et al*., 2004) suggesting that this species is highly dependent on the food quality.

It can be expected that molluscs species with different life histories could respond in different manners to changes in temperature and food availability (Collin, 2006; Breton *et al.*, 2018). Broadcasting and brooding species are the reproductive strategies in molluscs. Broadcasting species, such as *M. gigas*, release their gametes in seawater where fertilization and larval development take place. On the other hand, brooding species such as *O. edulis* incubate their larvae in the inhalant chamber before their release into seawater (Bayne, 1976). Even when these species have different reproductive strategies seem to respond in a similar way to changes in temperature and food availability showing a positive effect on fertility, sex determination and speed of gametogenesis (Shpigel, Barber and Mann, 1992; Chávez-Villalba *et al*., 2003; Fabioux *et al*., 2005; González-Araya *et al*., 2012; González-Araya, Quillien and Robert, 2013; Joyce *et al*., 2013).

3.4.3 Effect of environmental variables on sex determination in *Ostrea edulis*

Factors controlling the sex ratios in natural populations of oysters remain unclear. Some field studies of this species have reported the effects of environmental factors, such as temperature, salinity and food availability, on sex ratio in oysters in the family Ostreidae (Acarli *et al.*, 2015; Eagling *et al.*, 2018; Hassan, Qin and Li, 2018). *O. edulis* alternate between sexes (Orton, 1927a, 1927b; Cole, 1942b; Loosanoff and Davis, 1952; Loosanoff, 1962) and the development of female gonads has been related with lower temperatures whereas male gonads are associated with the increase of the water temperature (Loosanoff and Davis, 1952; Loosanoff, 1962; Joyce *et al.*, 2013). These earlier reports are supported by the results in chapter 2, in which it was found a higher proportion of females at the lowest temperature (10°C) and a higher proportion of males at 14°C (Zapata-Restrepo *et al*., 2019). In this study, female individuals were observed throughout the study period but predominantly in December 2016 and March 2017 when the water temperature was under 12 °C. Males were more frequently observed with an increase in water temperature. Hermaphrodites were variable throughout the year. In marine bivalves quality and quantity of food have shown to influence the sex ratio (e.g. in *Pinctada margaritifera*: Teaniniuraitemoana *et al.,* (2016)) and gonadal size and fecundity (e.g. in *A. irradians*: Sastryz (1965)). This study did not report significant bias respect the expected ratio 1:1 so there is no evidence of changes in temperature or food availability affecting this proportion. Variation in sex ratios in natural populations could be affected by other factors such as changes in population density or size-frequency distribution (Collin, 2006; Baeza *et al.*, 2010).

As it was mentioned in section 2.4.2, the density dependent effect in a brooding species such as *O. edulis* is an important factor to consider and it has been shown that in presence of a nearest neighbour ≤1.5 m a higher larvae production is reached in this species (Guy, Smyth and Roberts, 2019). The low densities reported for *O. edulis* inhabiting the South coast harbours could be implicated in recruitment failures and the decrease in populations occurring in this area (Kamphausen, 2012; Helmer *et al.*, 2019). The density of animals at the beginning of this experiment was 2 oysters per m^2 and it was reduced until 0.5 oysters per m^2 at the end of the experiment. Even when these values represent a low density of animals and an effect of removing animals over time cannot be ignored throughout this study, the densities used in this experiment reflect values reported during the last years for *O. edulis* population inhabiting the Solent.

3.4.4 Seasonal cycle of energy reserves in *Ostrea edulis*

It is difficult to differentiate the role and effect of adult reserves from that of additional food. The main energy reserves for gametogenesis in bivalves are lipid and glycogen (Dridi, Romdhane and Elcafsi, 2007; Pogoda *et al.*, 2013). Although some authors have not found a clear evidence of the utilization of carbohydrate from the *O. edulis* adductor muscle as source of energy (González-Araya *et al*., 2011, 2012), it is considered that bivalves can use mantle, digestive gland and adductor muscle tissues as important energy reserve sites for storage and translocation of protein and glycogen, lipids and proteins, respectively (Barber & Blake, 1985; Pérez *et al*., 2013; Saucedo *et al*., 2002). In our study, carbohydrate content correlated with levels of Chl *a* during the year, but lipids and proteins were not affected by environmental variables.

It has been shown that gonadal development in bivalves normally takes place using reserves of carbohydrate and lipids stored prior to the initiation of gametogenesis (Shpigel, Barber and Mann, 1992). This potential makes the process of gametogenesis dependent on temperature and the availability of stored nutrient reserves. Elevated temperatures increase metabolism and accelerate gametogenesis and gonadal development in bivalves, but the production of gametes results in a reduction in CI due to the demand for energy reserves obtained from carbohydrates, lipids and protein stored in tissues (Shpigel, Barber and Mann, 1992). Lipids and carbohydrates presented a variable behaviour during this study whereas protein content was more constant showing an increase by the end of the study.

In bivalves the gonads consist mainly of protein and lipids provided by the diet and they are used as energy sources during gametogenesis (Pieters *et al.*, 1980; Mouneyrac *et al.*, 2008). These lipids play a major role as membrane constituents and reserve energy. In our study, *O. edulis* presented a progressive lipid content increase throughout the year. However, the maximum peaks were reached at the end of winter and the beginning of spring 2017 when a slight increase in Chl *a* and a decrease in temperatures were observed. At the same time, during this time a reduction in oysters at stage G0 and the increase in gametogenic stages (G1 and G2) were observed. During spring, a reduction in lipid content was observed which coincided with gametogenic progression and the appearance of developed stages (G3 and G4). This inverse relationship between total lipids and gonadal maturation has been observed in other species presenting a significant decrease in lipid concentrations coinciding with the progress in gametogenesis (Mouneyrac *et al.*, 2008). However, other authors have reported different behaviour for *O. edulis* and *M. gigas* with the highest levels of lipids in the periods of maximum ripeness (Dridi, Romdhane and Elcafsi, 2007; Yildiz *et al.*, 2011).

During gametogenesis, the transfer of nutrients from storage or digestive sites to the gonad has been shown in other bivalve species (Beninger *et al*., 2003 and literature cited therein; Mouneyrac *et al*., 2008). In this study, the carbohydrate content increases when lipid content decreased throughout late autumn and winter. This could be attributed to the achievement of gametogenesis whilst maximum values in spring could correspond to glycogen conversion to lipids at the onset of gametogenesis (Mouneyrac *et al.*, 2008).

Available food appeared to be a very important factor in controlling gonad growth once gametogenesis was initiated (Ruiz *et al.*, 1992). The behaviour between Ch*a* and energy reserves in *O. edulis* in this study suggests that the amount of energy used for gametogenesis activity in this species could depend more on food quality than on the nutrient storage. Bayne (1976) suggested that the stored energy of bivalves used for gametogenesis can be classified as a conservative or opportunistic species, with the former using the energy stored in various organs (mantle, digestive and adductor muscle) and the latter using the energy from recently ingested food (Bayne, 1976). But some authors consider that some of the bivalve species cannot be classified as conservative or opportunistic species because both methods can be used under different circumstances depending on the quality and quantity of food (Joaquim *et al.*, 2008). It is even possible that oysters of the same species use different strategies to provide the energy required for gametogenesis (Li *et al.*, 2006).

In this study, a progressive increase in lipids was associated with the first phase of gametogenesis. However, the lipid content decreased by spring time during ripeness and spawning. These dates correspond with an increase and the peak for Chl *a* in May and June 2017 suggesting a compensation with food availability in the environment. This suggests that the metabolic cost of this phase, coinciding with the low lipid content and highest food levels in the environment, was supported by the availability of food rather than energy reserves. It could be expected that abundant food (e.g. phytoplankton bloom) is used in bivalves to build up energy reserves and spawning produces a decrease in biochemical constituent levels (Ruiz *et al.*, 1992). Under this assumption and the categories suggested by Bayne (1976) *O. edulis* could be classified as an opportunistic species. Other authors have also classified this species as an opportunistic species which concentrates its reproductive effort during a short period of favourable conditions depending on the nutritive availability in the environment (Ruiz *et al.*, 1992). Yildiz *et al*. (2011) found that glycogen was not accumulated in this species during the study period but those oysters inhabited an area with sufficient food throughout the year letting them go through gametogenesis using food availability (Yildiz *et al.*, 2011). This study confirms the opportunistic strategy suggested by these authors for *O. edulis*, and changes in food quality and quantity need to be considered as an important environmental factor for fisheries' managers and restoration programmes.

Concerning the influence of sex on energy reserves, a sexual differentiation has been shown in *O. edulis*, with females presenting a greater quantity of lipids than males. Similar behaviour was observed in *S. plana* showing differential accumulation of certain types of lipids according to the sex (Mouneyrac *et al.*, 2008). Different lipid components do not accumulate in the same way, with some of them clearly influenced by sex whereas others did not show significant differences between males and females (Delgado *et al*., 2004; Napolitano *et al*., 1992). This could be related to the energy cost of producing eggs and sperm already mentioned before in this text. Considering that this species seems to be an opportunistic in terms of stored and use of energy reserves, female oysters in wild populations would depend on the food quality to go through gametogenesis rather than males. A delay in this process could create a male-skewed sex ratio affecting population dynamics and causing a slow decrease in reproduction in wild populations (Allsop and West, 2004; Collin, 2006). This could be implicated in the skewed sex ratio toward males for *O. edulis* reported in previous years in the Solent (Kamphausen, Jensen and Hawkins, 2011; Eagling, 2012).

Protein was the major organic component found in oysters but its content did not show any correlation with the other biochemical variables measured. However, an increase that coincided with the period of gametogenic maturity was observed. Some authors reported that variations in protein seem to be influenced by the reproductive cycle and that protein accumulated in the tissues at earlier stages of maturation could be used for the oocyte development and growth (Berthelin, Kellner and Mathieu, 2000; Marin *et al.*, 2003). It has also been reported that the use of protein as an energy source depends on the availability of carbohydrates and lipids, being used just when the other reserves are scarce (Mladineo *et al.*, 2007).

Southampton Water is a dynamic area presenting sustained phytoplankton blooms mainly during the spring and summer months, anoxic waters and toxic algal blooms (concentrations above 10 mg/m^3) (Xiong, 2000; Holley *et al.*, 2007). Chlorophyll concentrations ranging from 1 to 64 mg/m^3 have been reported in Southampton Waters (Holley *et al.*, 2007) making this area a hypernutrified system (Xiong, 2000). Considering this the Solent could be a favourable area for *O. edulis* providing enough food for reproduction and survival. However it has been shown that ingestion, digestion, and assimilation of different types of algae affect gonadal development and growth by in this species (González-Araya *et al*., 2011, 2012, 2013). Thus the sex ratio towards male-phase oysters of 3:1 and 6:1 reported by Eagling (2012) and Kampahusen (2012) in *O. edulis* populations in the Solent could be explained in terms of food quality instead of quantity.

3.4.5 Seasonal cycle of hormones and effect on gametogenesis and sex determination in *Ostrea edulis*

The lack of a direct relation between sex results from histology and sex steroids concentrations for *O. edulis* in the present chapter, and supported by results obtained in chapter 2, could indicate that other biochemical pathways are involved in this process. In the same manner, the negative correlation between gonadal maturation with E_2 concentrations and the lack of correlation with T could indicate that gametogenesis is independent of steroid hormones. This has been suggested by authors who consider the evidence of presence, synthesis and regulation of vertebrate-type steroids in molluscs is not convincing (Lafont and Mathieu, 2007; Scott, 2012, 2013).

Fluctuations in levels of sex steroids have been found to be correlated with the sexual maturation cycle in a number of bivalves, thus suggesting that sex steroids may play important stimulatory roles in their reproductive regulation (Le Curieux-Belfond *et al.*, 2001; Gauthier-Clerc, Pellerin and Amiard, 2006; Ketata *et al.*, 2007). In this context, some studies have concluded the central role of estrogens in the natural gametogenic cycle in oysters, scallops, and clams (Mori, Muramatsu and Nakamura, 1972; Gauthier-Clerc, Pellerin and Amiard, 2006; Wang and Croll, 2006). But it is difficult to correlate annual variations in steroid content and reproductive cycle stages and a lack of differences in T and E_2 concentrations has been shown in this study and in other bivalve species such as *Mya arenaria* (Gauthier-Clerc, Pellerin and Amiard, 2006), *M. edulis* (Reis-Henriques *et al.*, 1990), and *Patinopecten yessoensis* (Osada, Tawarayama and Mori, 2004).

Scott (2012) argued that these seasonal changes in hormones concentrations could be more related to an increase in fatty acids, lipids and proteins during reproductive maturation, and then those hormones taken up from the environment can be stored in the form of fatty acid esters during days or even months. Our results found a weak relationship between lipid content and E_2 concentrations in gonads of *O. edulis* between October and January. This supports the idea that an increase in hormones measured in tissues of bivalves could be related to an increase in energy reserves and a careful analysis needs to be done concerning the presence of hormones and their role in reproduction in this species.

Several reviews have reported evidence about the presence, metabolism and enzymatic pathways of sex steroids, e.g., testosterone, androstenedione, and E_2 occurring in several invertebrate species as a prove of endogen origin of those molecules (Le Curieux-Belfond *et al.*, 2001; Janer and Porte, 2007; Lafont and Mathieu, 2007; Fernandes, Loi and Porte, 2011). However, the lack of evidence indicating a role in reproduction found in this study supports that endogenous origin of sex steroids, its regulation and synthesis in molluscs cannot be argued just by the presence of these hormones

in bivalves tissues (Lafont and Mathieu, 2007; Scott, 2012, 2013). Sex steroids are a product of the physiological process and anthropogenic activities (Lafont and Mathieu, 2007) so the transfer from the food (algae) and the environment is an external source for sex steroids detected in bivalves tissue (Le Curieux-Belfond *et al.*, 2001; Schwarz *et al.*, 2016).

No sex differences in testosterone and estradiol content were observed in *O. edulis* during this study. Also, female gonads presented a similar level of testosterone and estradiol than males. This same behaviour has been reported in other species such as *Mytilus edulis trossulus* (Reis-Henriques *et al.*, 1990; Zabrzańska *et al.*, 2015), *M. arenaria* (Gauthier-Clerc, Pellerin and Amiard, 2006) and *P. yessoensis* (Osada, Tawarayama and Mori, 2004) which presented a lack of intersex differences in sexual steroids in gonads.

Most research on steroid concentrations in invertebrates, including this study, has used immunoassays as the detection system, with the possibility of having cross-reactivity of these assays with other steroids (Janer and Porte, 2007; Lafont and Mathieu, 2007). Thus it is strongly recommended to include the detection and characterization of homologues as an important step in this type of studies. There is not enough information about the role of sex steroids in *O. edulis* and the exact controls for alternating sex change in this species has not yet been resolved satisfactorily (Ketata *et al.*, 2007; Morishita *et al.*, 2010).

3.4.6 Seasonal changes in Vtg-like protein concentrations in *Ostrea edulis*

Vtg provides energy reserves for embryo development in oviparous organisms (Suzuki *et al*., 1992; Wallace, 1985) and a similar response can be observed in invertebrates, particularly molluscs (Blaise *et al*., 1999). Thus, the lipid content of Vtg has been used as an indirect method to measure oocyte proteins and as an indicator of reproductive status, especially in spawning period (Suzuki *et al.*, 1992; Blaise *et al.*, 1999; Matozzo and Marin, 2008; Arcos *et al.*, 2009). Most studies concerning Vtg induction in aquatic invertebrates have been conducted using the alkali-labile phosphate (ALP) method (Blaise *et al.*, 1999) that detects inorganic phosphate liberated from phosphorylated proteins, including Vtg-like proteins in molluscs, e.g. *Mya arenaria* (Gagne *et al*., 2002). In some studies, Vtg induction has been either successful or unsuccessful after estradiol exposure (Gagné *et al*., 2002; Won and Novillo, 2005). In other studies, a delay of variable duration has been observed before elevated Vtg was recorded (Li *et al*., 1998; Osada *et al*., 2003).

The analysis of Vtg-like protein concentrations in gonadal tissue of *O. edulis* showed a correlation with both hormones (estradiol and testosterone) concentrations during this study. Some authors have found an effect of 17B-estradiol on vitellin formation in *C. gigas* and *P. yessoensis* (Osada,

Unuma and Mori, 1992; Li *et al*., 1998) indicating that estradiol is one of the major factors which control the vitellogenesis in the oyster and that the ovary is the site of synthesis of vitellin. Additionally, it has been shown that estradiol promoted vitellin formation in the ovary of the oysters (Li *et al*., 1998).

However, our results showed a lack of relation between Vtg-like proteins and sex determination. In addition, the lack of differences between sexes suggests that careful verification is needed before assuming this technique is a good proxy to assess reproductive status in bivalve species. This is supported by Sanchez *et al*. (2017) who found that ALP analysis could detect similar amounts of phosphorylated proteins regardless of sex or gonad development stage in marine mussel gonads (Sánchez-Marín *et al.*, 2017). These results suggest that the ALP method may not provide reliable information about Vtg concentrations in molluscs and careful verification is needed before assuming its results as a good proxy to assess Vtg levels in other bivalves.

3.5 Conclusions

A clear reproductive cycle was observed in *O. edulis* kept in the dockside tank at NOCS with three phases: a resting phase from August to November, an increase in gonadal development between December and April, and gametogenesis ripeness and spawning during the summer. However, a delay in oysters reaching development stages (G3 and G4) was observed indicating that environmental conditions were not enough for activation of the gametes growth phase.

The ratio 1:1 (F:M) was not considerably affected during this study. More female individuals were observed throughout the study period but predominantly in December 2016 and March 2017 when the water temperature was under 12°C. Males were more frequently observed with an increase in water temperature. Hermaphrodites were variable throughout the year.

The effect of temperature and Chl *a* on gonadal maturation confirms the importance of these environmental factors in gametogenesis in this species. Moreover, this species shows a dependence on the food quality rather than energy reserves to go through this process. The biochemical composition of *O. edulis* indicates that proteins were very constant throughout the year whereas lipids and carbohydrates tended to vary in an opposite manner with lipids increasing continually parallel to gametogenic development and carbohydrates decreasing at the same time. Female oysters of this species presented a greater quantity of lipids than males suggesting an influence of sex in the storage of energy reserves. It could, therefore, be expected that female oysters could be more susceptible to gametogenesis failures in an environment with poor food quality. Eventually,

this could lead to a skewed male sex ratio in natural populations affecting the long term health and sex balance of populations.

The lack of a direct relationship between sex results from histology and sex steroid concentrations for *O. edulis* in the present study confirms that other biochemical pathways could be involved in the sex determination and gonadal development in *O. edulis* independent of steroid hormones.

To conclude, this chapter has shown that gametogenesis and sex determination in *O. edulis* are influenced by temperature, food availability and energy reserves. Also, these processes are independent of E_2 and T concentrations in *O. edulis* gonadal tissues suggesting that a different biochemical mechanism could be involved in the reproduction of this species. However, the effect of hormones acting as environmental pollutants cannot be discharged in the skewed sex ratios reported for this species. The evidence suggests that other species of bivalves are able to respond to external exposure to sex steroids presenting adverse effects on growth, reproduction and survival. Chapter four will study the potential effect on biological and reproductive parameters resulting from external exposure to estradiol and testosterone, in order to understand the role of these hormones as environmental pollutants that might affect natural populations.

Chapter 4 Effect of exogenous steroids on survival, homoeostasis and reproduction of *Ostrea edulis*

4.1 Introduction

It is well known that physiological, development and reproductive activities (sex determination, sexual maturation, reproductive cycle and sexual behavior) are regulated by sex steroids in vertebrates (Markov *et al.*, 2009; Cole, Short and Hooper, 2019). However as it has been pointed out in the previous chapters the existing evidence about the endogenous origin, metabolism, physiological/reproductive role and regulation of neuropeptides and peptide hormones in the Mollusca is still contradictory (LaFont, 2000; Janer and Porte, 2007; Ketata *et al.*, 2007; Scott, 2012, 2013). The results from the two previous experimental chapters showed a lack of a direct strong relationship between reproductive parameters (such as sex determination and gametogenesis) and hormone concentrations in *Ostrea edulis*. These results together could indicate that other biochemical pathways are involved in the gonadal development and maturation in *O. edulis,* independent of steroid hormones.

Nevertheless studies have not been conclusive about the endogenous metabolism of hormones or its role in normal endocrine system function in bivalves, the evidence showing the uptake from the environment suggest that environmental exposure to estradiol, testosterone, and potentially other mimicking compounds, might create an endocrine misbalance affecting the normal development and function of organisms. According to Scott (2012, 2018) the presence of hormones in invertebrates does not necessarily mean an endogenous metabolism as they could be taken up from the environment and they can be stored in the form of fatty acid esters for days or even months. The evidence has shown that a fast bioaccumulation of hormones in bivalves is possible, e.g. in less than 48h estradiol-17b was concentrated up to 31 times from seawater in the soft tissues of oysters *Magallanas gigas* during *in vivo* experiments (Le Curieux-Belfond *et al.*, 2001, 2005), meanwhile an optimum uptake of E_2 from water in just 24h in adult blue mussels was reported for *Mytilus* spp. (Schwarz *et al.*, 2016).

The presence of pollutants in the aquatic environment has led to an increasing number of endocrine disruption studies concerning both vertebrates and invertebrates and involving physiological processes controlled by steroid hormones. In this instance, a number of exogenous chemical compounds, including environmental pollution by estrogens and androgens, could play a role as EDCs affecting exposed organisms (Porte *et al.*, 2006; Shore and Shemesh, 2016). The exposure of bivalves to E_2 and T in water has suggested that these steroids can act as regulators of gametogenesis and sex determination showing feminizing or masculinizing effects in molluscs

exposed to those steroids (Mori, 1969; Gauthier-Clerc, Pellerin and Amiard, 2006; Wang and Croll, 2006; Teaniniuraitemoana *et al.*, 2016). However, the exact role of E_2 and T is not clear presenting contradicting effect in different species, which makes difficult to generalize about an effect for each hormone in the reproductive cycle of molluscs.

Tarrant *et al*., (2005) summarised environmental concentrations of EDCs, including sex steroids and related synthetic compounds, in the UK. Significant concentrations of E_2 and T were reported in soil, sediments and surface water in UK freshwater and marine systems (Tarrant *et al*., 2005). In the UK concentrations of E_2 ranged from 13.5 to 24 ng/L in sewage influent, 3-88 ng/L in sewage effluent and 0.73 ng/L in estuarine has been reported (reviewed in Tarrant *et al*., (2005)). There is no available information about T concentrations in the environment in the UK.

In order to assess the health of aquatic organisms and the biological effects of environmental pollutants, a wide range of methods measuring changes at the biochemical, cellular and physiological levels have been used in marine environmental monitoring (Lagadic, Caquet and Ramade, 1994; Hamza-Chaffai, 2014). The use of metabolomics methods has created a better-understand of the complexity of biological systems and organisms' responses to environmental disturbances (Young, 2016; Clark *et al.*, 2017; Pinu *et al.*, 2019). This method is a non-targeted analysis that characterizes changes in endogenous and exogenous low molecular mass metabolites within a cell, tissue, or biofluid of an organism in response to external stressors (Lankadurai, Nagato and Simpson, 2013). These metabolites are end products of gene and protein expression and are exceptionally sensitive to genetic and environmental perturbations (Johnson, Ivanisevic and Siuzdak, 2016; Young, 2016). For this reason, metabolome profiles could be determined in order to understand endogenous regulatory mechanisms involved in growth, survival and reproduction to external stressors, and therefore these approaches offer greater utility/insight that some of the conventional methods used regularly in environmental monitoring studies (Young, 2016; Clark *et al.*, 2017; Pinu *et al.*, 2019).

The limited understanding of normal endocrine processes in invertebrates makes an assessment of chemical endocrine disruption in this field extremely difficult (Scott, 2018). Additional experimental studies are required to understand the physiological/reproductive role of exogenous sex steroid hormones on growth, survival, performance and reproduction in *Ostrea edulis*. The aim of this chapter is to understand the effect of the exposure to environmentally relevant concentrations of sex steroid hormones (estradiol and testosterone) in these biological aspects of *O. edulis*. Advancing our knowledge about the mollusc physiology and endocrinology, and the alterations resulting from environmental chemical exposure can result in a better understanding of these processes as a critical point for assessing the impacts of exogenous chemical compounds in marine bivalves.

4.2 Methods

4.2.1 Biological Material

Oysters (*Ostrea edulis*) were obtained from Galway Bay in Ireland in January 2018 and transferred to the NOCS where they were acclimated for four weeks. During the acclimation period, they were placed in seawater tanks (about 1L/oyster) with continuous aeration at the same temperature (8°C) as at the grow-out site (https://www.seatemperature.org/europe/ireland/gaillimh.htm). Oysters were > 2y old, and were 5-7 cm at their maximum diameter.

4.2.2 Feeding of oysters

During this period animals were fed *ad libitum* daily with 40,000 cells/ml of a mixed algae diet (40% *Tetraselmis suecica*, 40% *Pavlova lutheri* and 20% *Phaedactylum tricornutum*) (see procedure in section 2.2.2 Feeding of oysters)

4.2.3 Sex steroid hormones treatments

After the acclimation process and before starting the exposures 6 oysters were taken as a control and those data are referred as initial time (t0). One-hundred and ten oysters were divided randomly among five treatment tanks: 20 ng/L T (n=20), 200 ng/L T (n=20), 5 ng/L E_2 (n=20), 50 ng/L E_2 (n=20), and a negative control with no hormones (n=30). All the treatments were kept at 10°C because in the previous experiment two months of exposure at that temperature resulted in a similar proportion of females and males developing (Chapter 2). Also at that temperature the gonadal development was slower, so any response in the current experiment was likely to be a direct effect of the treatments rather than a maturation process resulting from incubation temperature. Air and water temperatures were controlled throughout the experiments using a free-standing chiller unit (TECO, model TR60). The salinity, pH, temperature, conductivity and dissolved oxygen were measured in every aquarium at least twice per week.

Both hormones (E_2 and T) were obtained from Sigma-Aldrich®, dissolved in 100% ethanol, which was then dried under a nitrogen stream and diluted 1:100 with 1-µm filtered sterilized seawater to give a 0.5mg/mL stock solution. It has been reported that the concentration of E_2 decreased to about a third of the nominal concentration in 24 h (Puinean *et al.*, 2006). For this reason during exposure, water spiked with E_2 and T was renewed every 48h (renewal volume 80% of total aquarium water).

Oysters were opened and dissected in saltwater to reduce the stress and damage of tissues. Then, samples were immediately fixed in Bouin's solution for histological examination and the other tissues were immediately frozen in liquid nitrogen and kept in the freezer at -20°C until further analysis. After 10 weeks of exposure the individuals (n=13) were sacrificed, except for treatment 50 ng/L E_2 (n=11) in which case the high mortality during the experiment did not permit the same number of individuals to be sampled. Ten individuals from the control were used to standardize conditions for biochemical analysis.

4.2.4 Biological indices

Measurements of H, L, Wi, SVol, FW and W were taken following the same criteria referred in Chapter 2 (see section 2.2.4 Biological indices)

4.2.5 Histological analysis

For each oyster, gametogenic stages and sex were determined by histological examination of a 5-mm thick section of the visceral mass fixed in Bouin's solution (Howard *et al.*, 2004; Kim, Ashton-Alcox and Powell, 2006) (Sigma-Aldrich™, Dorset, UK) for 24h (see section 2.2.5 Histological analysis). Sex was recorded as indeterminate (I), female solely (F), male solely (M), hermaphrodite with both sexes equally represented (HBS), hermaphrodite predominantly male (HPM) and hermaphrodite predominantly male (HPF) according to (da Silva, Fuentes and Villalba, 2009). The gametogenic stage of the gonad was identified as inactive (G0), early gametogenesis (G1), advanced gametogenesis (G2), ripe gonad (G3), partially spawned gonad (G4) and reabsorbing gonad (G5) adopted by da Silva, Fuentes and Villalba (2009).

4.2.6 Energy reserves

Energy reserves were quantified in the gonad of each oyster following the same procedure explained in section 3.2.5.

4.2.7 Steroid Hormone Homologue Analysis

Extraction and analysis of homologues of the sex hormones E_2 and T concentrations were quantified in the gonads of each oyster using enzyme-linked immunosorbent assay (ELISA) kits (Cayman Chemical Co.; Ann Arbor, MI, USA) as described by Gauthier-Clerc, Pellerin and Amiard (2006) (See section 2.2.6). Standard curves were carried out with E_2 between 6.6 and 4,000 pg/ml and T between 3.9 and 500 pg/ml. Mean intra-assay CVs for standards and samples were ≤ 8.9% for E_2 and ≤ 7.2% for T. Mean inter-assay CVs were ≤ 9.4% and ≤ 7.67% for E_2 and T, respectively.

4.2.8 Metabolomic profile

Gonadal samples (0.1 g) were immediately placed into liquid nitrogen and stored at -80°C until further analysis. To characterize the metabolic changes that occurred in *O. edulis* exposed to different steroid types and concentrations, the metabolomic profiles of all the animals in each treatment was carried out by the McCullagh Metabolomics Laboratory for untargeted metabolomics, Department of Chemistry at the University of Oxford.

In brief, each sample was analysed using up to three separate LC-MS/MS methods using two different LC systems (Thermo Scientific ICS-5000+ ion chromatography system and a Thermo Ultimate 3000). Each was coupled directly to a Q-Exactive HF Hybrid Quadrupole-Orbitrap mass spectrometer with a HESI II electrospray ionisation source (Thermo Scientific, San Jose, CA).

IC-MS/MS: Ion exchange chromatography was performed using a ICS-5000+ HPLC system incorporating an electrolytic anion generator (KOH) which was programmed to produce a OH– gradient over 37 min. An inline electrolytic suppressor removed OH– ions and cations from the post-column eluent stream prior to MS analysis (Thermo Scientific Dionex AERS 500). A 10 µL partial loop injection was used for all analyses and the chromatographic separation was performed using a Thermo Scientific Dionex IonPac AS11-HC 2 × 250 mm, 4 µm particle size column with a Dionex Ionpac AG11-HC 4 µm 2x50 guard column inline. The IC flow rate was 0.250 mL/min. The total run time was 37 mins and the hydroxide ion gradient comprised as follows: 0mins, 0mM; 1min, 0mM; 15mins, 60mM; 25mins, 100mM; 30mins, 100mM; 30.1mins, 0mM; 37mins, 0mM. Analysis was performed in negative ion mode using a scan-range from m/z 60-900 and resolution set to 70,000. The tune file source parameters were set as follows: Sheath gas flow 60 mL/min; Aux gas flow 20 mL/min; Spray voltage 3.6v; Capillary temperature 320 oC; S-lens RF value 70; Heater temperature 350oC. AGC target was set to 1e6v ions and the Max IT value was 250ms. The column temperature was kept at 30°C throughout the experiment. Full scan data were acquired in continuum mode.

C18 Reversed Phase (underivatised): C18 reversed-phase analysis of underivatised samples was performed using a Thermo Utimate 3000 UHPLC system with a gradient elution program coupled directly to a Q-Exactive HF Hybrid Quadrupole-Orbitrap mass spectrometer. A 5 µL partial loop injection was used for all analyses with pre and post-injection wash program. A Waters CORTECS UPLC T3 1.6µm (2.1x100mm) column was used with a flow rate of 0.4mL/min. The total run time was 18 mins. Mobile phase A comprised milli-Q water with 0.1% formic acid and mobile phase B was 100% methanol with 0.1% formic acid. The gradient elution program was as follows: 0mins, 5%B; 4min, 50%B; 12min, 99%B; 15mins, 99%B; 15.1min, 5%B; 18min, 5%B. The column temperature was kept at 40°C throughout the experiment. Mass spectrometry analysis was

performed in positive and negative ion mode separately using a scan-range from m/z 60-900 and resolution set to 70,000. The tune file source parameters were set as follows: Sheath gas flow 60 mL/min; Aux gas flow 20 mL/min; Spray voltage 3.6v; Capillary temperature 320 oC; S-lens RF value 70; Heater temperature 350oC. Full MS setting were AGC target 5e6 ions and the Max IT value was 120ms. Full scan data were acquired in continuum mode. A data directed tandem mass spectrometry method was utilised (ddMS2) with no inclusion list. The orbitrap detector and HCD setting for ddMS2 were as follows: Microscans 2, resolution 17,500, AGC target 5e4 ions, maximum IT 80ms, loop count 10 and NCE 35.

C18 Reversed phase (derivatised): The third LC-MS method used a sample derivatisation protocol followed by analysis based on a modified version of the Waters AccQ-Tag method (Salazar *et al*., 2012). C18 reversed-phase analysis of derivatised samples was also performed using the Thermo Utimate 3000 UHPLC system coupled directly to a Q-Exactive HF Hybrid Quadrupole-Orbitrap mass spectrometer. A 5 µL partial loop injection was used for all analyses with pre and post injection wash program. A Waters AccQ-Tag column (2.1x100mm) was used with a flow rate of 0.5mL/min. The total run time was 9.5 mins. Mobile phase A and B comprised commercially available AccQ-Tag reagents prepared as recommended by Waters (Waters PLC, Elstree, UK). The gradient elution program was modified from the published AccQ-Tag method as follows: 0mins, 0.1%B; 0.54min, 9.1%B; 5.74min, 21.2%B; 7.74mins, 59.6%B; 8.04min, 90%B; 8.05min, 90%B; 8.64min, 0%B; 9.5min, 0.1%B. The column temperature was kept at 40°C throughout the experiment. Mass spectrometry analysis was performed in positive ion mode separately using a scan-range from m/z 70-1050 and resolution set to 70,000. The tune file source parameters were set as follows: Sheath gas flow 60 mL/min; Aux gas flow 20 mL/min; Spray voltage 3.6v; Capillary temperature 320 oC; S-lens RF value 70; Heater temperature 350oC. Full MS setting were AGC target 3e6 ions and the Max IT value was 200ms. Full scan data were acquired in continuum mode.

Data processing: Ion species were identified with reference to an 'in-house' database created from authenticated standards. Briefly, pure compounds were purchased from chemical suppliers (e.g. Sigma-Aldrich, UK; Tocris UK; Tokyo Chemicals industry, UK). These standards were then diluted in appropriate solvent (80% methanol) and separated chromatographically by different methods. Each compound was then examined using QExactive Mass Spectrometer (Thermo, UK). Each authenticated standard was identified by collection of discrete data: this included chromatographic retention time; accurate mass (5 decimal places), compound fragmentation hence allowing the identification if different structural isomers with reference to differing fragmentation and retention characteristics.

Raw data files were processed using ProgenesisQI (Waters, Elstree, UK). This process included alignment of retention times, peak picking by identification of the presence of natural abundance isotope peaks, characterising multiple adducts forms and identification of metabolites using our in house database. Retention times, accurate mass values, relative isotope abundances and fragmentation patterns were compared between authentic standards and the samples measured. Identifications were accepted only when the following criteria were met: <5ppm differences between measured and theoretical mass (based on chemical formula), <30 seconds differences between authentic standard and analyte retention times, isotope peak abundance measurements for analytes were >90% matched to the theoretical value generated from the chemical formula. Where measured, fragmentation patterns were matched to least the base peak and two additional peak matches in the MS/MS spectrum to within 12ppm. The top 10 data directed fragmentation method was not always able to provide fragment ions for all ions measured in the MS 1 spectrum.

Ions identified using the different methods was as follows:

ION EXCHANGE METHODS: Total ions identified= 5733, CV less than 30%= 1773, identified compounds= 157.

C18-Reverse Phase Chromatography: Total ions identified= 10103, CV less than 30%= 2577, identified compounds= 147.

Derivatised-C18-Reverse Phase Chromatography: Total ions identified= 16027, CV less than 30%= 10940, identified compounds= 58.

4.2.9 Statistical analysis

The normality of data and homogeneity of variances were evaluated using the Shapiro Wilk and the Levene's tests, respectively. The assumptions of parametric tests were not met, so non parametric tests were applied. The Kruskal-Wallis H-test was used to determine differences in mortality, biometric parameters (W, H, Wi, SVol, FW, CI), biochemical variables (lipids, carbohydrates, proteins, sex steroid hormones concentrations) and gonadal development. Spearman's correlation was used to test the relationship between biochemical variables (lipids, carbohydrates, proteins, sex steroid hormones, Vtg-like proteins). When non-parametric Kruskal and Wallis test was significant, differences were then evaluated using a non-parametric the Mann and Whitney test. Chi-square statistics were used to test sex ratios against a 1:1 ratio. For the statistical analysis, the Windows 24.0 SPSS statistical pack was used. Statistical significance was assigned at $p \leq 0.05$.

MetaboAnalyst is a free online tool that can be used to analyse a wide range of metabolomics datasets (Chong *et al*., 2018): Univariate statistical analysis includes determining fold-change and t-tests between experimental groups for compound features and combined in volcano plots (FDR-adjusted p-values should be reported). A fold-change threshold of 1.5 and a FDR adjusted p-value cut-off of 0.05 (occasionally an FDR adjusted p-value <0.1) when few significant changes is used to determine significance. Two analysis were carried out to visualise the behaviour of the data and to evaluate similarities and differences between variables: Principle Component Analysis (PCA) and Partial Least Squares Discriminant Analysis (PLS-DA). PCA is an unsupervised multivariate statistical analysis used to visualize global metabolic profiles and determine sample outliers. This method ignores the information regarding the groups or treatments showing similarities in the data colleted. On the other hand PLS-DA is a supervised multivariate statistical analysis used to model important compound feature when the number of samples per experimental group is sufficient (Ruiz-Perez and Narasimhan, 2017). For this method the information about each sample's group is supplied to show discrimination between variables. Feature loadings and variable importance in the projection (VIP) scores can be ranked for validated models to determine important metabolite features. Models are assessed by cross validation (using R^2, Q^2 and Accuracy; R^2 should be higher than Q^2 which should be >0.4 and within 0.3 if the R^2 values, the closer to 1 the R^2 and Q^2 the strong the model). To avoid overfitting of the model a permutation test is performed using 100 or more permutations with a separation distance (B/W) test statistic (this should always be reported). Hierarchical clustering can be visualised using heat-maps with a Euclidean distance measure and all compound features.

Pathways Analysis can be performed to aid functional interpretation of the dataset and interpret within a biological context. MetaboAnalyst is used for this analysis in addition to pathways heat-maps using an in-house Excel macro that mapped identified compound features onto KEGG metabolic pathway relevant to known metabolic pathways active in Homo sapiens (https://www.kegg.jp/kegg/atlas/?01100). The pathway Enrichment Analysis uses a global Test statistic and Relative Betweeness Centrality for topology analysis.

4.3 Results

4.3.1 *Ostrea edulis* mortality and growth

Mortality was significantly ($p < 0.05$) affected by the treatments compared to the control during this study. In particular, the highest mortality percent was observed for the highest doses of E_2 (Fig 4.1). Both T concentrations presented a similar increasing trend in mortality.

No significant differences in any of the biometric parameters between the experimental and control groups were found (Table 4.1). These results indicate that sex steroids had no detectable effects on the overall growth of the oysters or the soft body parts over 10 weeks examined in the present study.

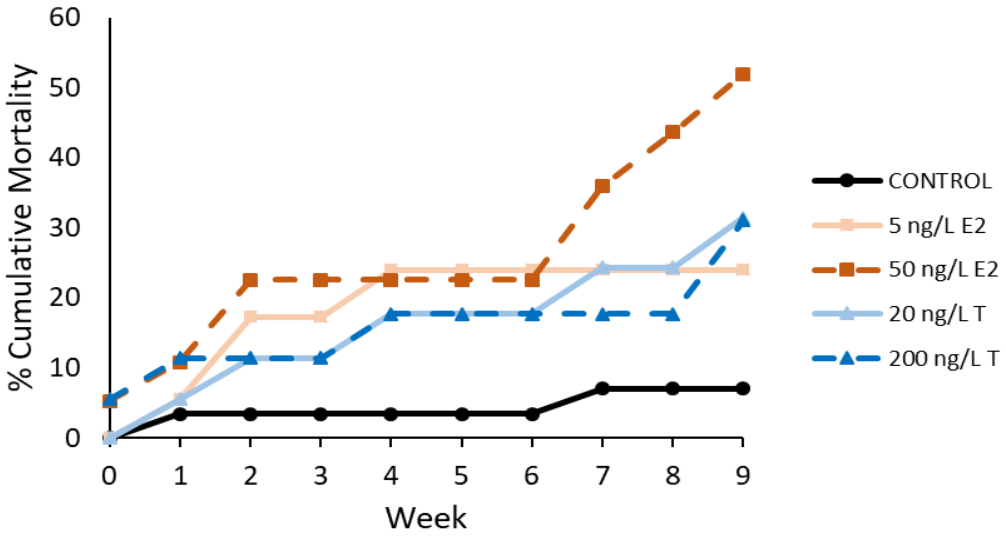

Figure 4.1 Cumulative mortality of oysters treated with 5 ng/L E_2 (n=20), 50 ng/L E_2 (n=20), 20 ng/L T (n=20), 200 ng/L T (n=20), and a negative control (n=30) during 10 weeks.

Table 4.1 *Ostrea edulis* biometric parameters (mean+SD) for total Weight (W), Height (H), length (L), width (Wi), Shell volume (SVol), flesh weight (FW) and Condition Index (CI) at the beginning of the experiment (t0), under different treatments (5 ng/L E_2, 50 ng/L E_2, 20 ng/L T and 200 ng/L T), and a negative control during 10 weeks.

Treatment	n	W (g)	H (mm)	L (mm)	Wi (mm)	SVol (ml)	FW (g)	CI
t0	6	71.66±10.99	77.20±3.00	72.02±7.05	19.33±4.22	48.37±9.71	8.29±2.03	11.49±1.83
20 ng/L T	13	72.8±10.30	74.92±3.61	72.48±10.79	20.63±2.79	44.51±7.06	8.11±1.41	11.14±1.25
200 ng/L T	13	67.42±7.74	76.27±4.17	71.39±6.01	19.14±2.80	48.18±6.06	7.23±1.21	10.70±1.85
5 ng/L E_2	13	67.93±6.70	71.81±3.94	71.74±5.09	21.51±1.45	40.60±3.80	6.82±1.13	10.04±1.49
50 ng/L E_2	11	68.05±9.02	72.03±5.64	69.20±4.21	20.19±2.55	44.41±6.86	7.44±1.75	10.85±1.54
Control	13	65.51±10.09	73.51±5.46	71.44±3.93	20.00±2.50	35.91±6.63	7.41±1.18	11.40±1.75

4.3.2 Effect of sex steroid hormones on gonadal development

Gonadal development between the experimental groups or compared with the control was not significantly different (Kruskal-Wallis test value = 25.646, $p > 0.05$, N = 63) (Fig 4.2). At the beginning of the experiment the oysters were classified in gonadal stage G1 or G2, at 66.67% and 33.33%, respectively. The proportion of oysters in stage G1 was very similar between treatments with the highest percentage (81.81%) found at 50 ng/L E_2. Slow progress in gonadal maturation was observed during the 10 weeks for the control group, presenting more oysters in stage G2 (46.15%) compared with oysters from t0 and treated groups. An increase in oysters in stage G0 was observed in most of the treatments including the control group, except for 200 ng/L T where no oysters in stage G0 were found. However this concentration was the only where stage G3 was found in 1 oyster. A few oysters at stage G4 were found at the lowest concentration for both hormones.

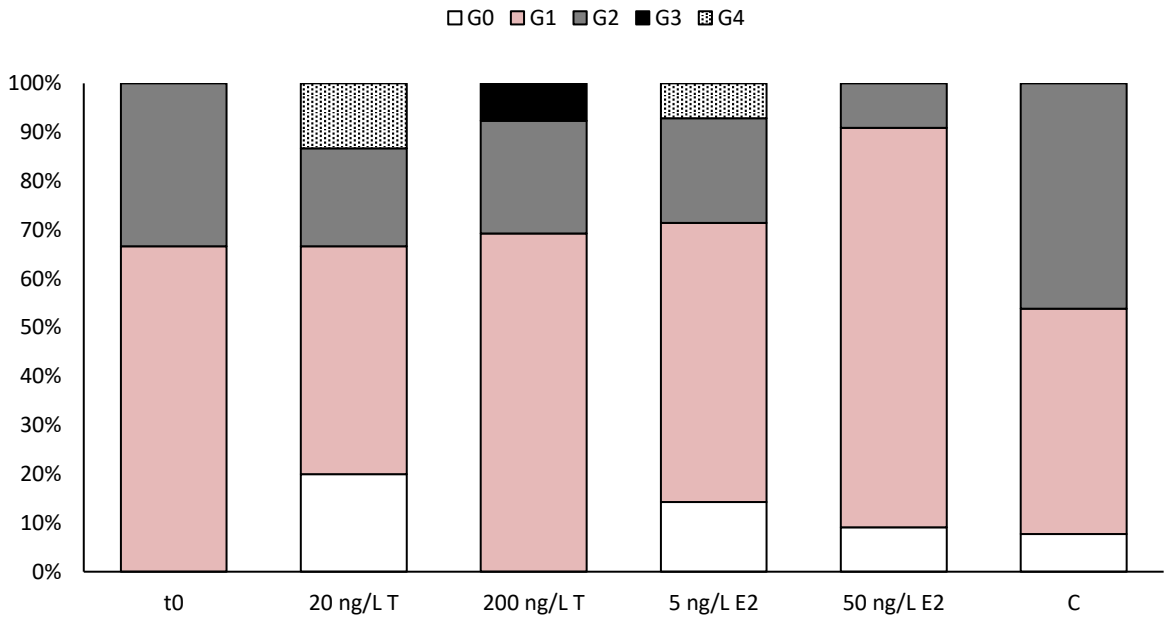

Figure 4.2 Distribution of *Ostrea edulis* at different stages of gonad development at the beginning of the experiment (t0; n=6) and exposed during 10 weeks to 20 ng/L T (n=13), 200 ng/L T (n=13), 5 ng/L E_2 (n=13), 50 ng/L E_2 (n=11), and a negative control (n=13). According to da Silva *et al*. (2009) developmental stage was classified by the gametogenic stage of the gonad as inactive (G0), early gametogenesis (G1), advanced gametogenesis (G2), ripe gonad (G3), partially spawned gonad (G4) and reabsorbing gonad (G5).

4.3.3 Effect of sex steroid hormones on sex ratio

Exposure to steroids caused significant changes in the sex ratio in the experimental groups (Fig 4.3). Sex ratios (male:female) were significantly shifted to 0:8 at the highest concentration of E_2 ($p < 0.05$) and to 0:6 by the lowest concentration of T ($p < 0.05$), compared to 1:1.5 in the control group (Chi-square test for all comparisons). The other treatments did not have a significant effect on the expected proportion 1:1.

In the present study, exposure of *O. edulis* to 200 ng/L of testosterone increased the number of males over two months of exposure. However, the proportion was lower compared to the control. Interestingly, after the exposure to the lower concentration of T no males were found. The exposure to the highest concentration of E_2 increased the proportion of females after two months of treatments. In contrast, exposure to the lowest concentration of E_2 caused an increase in males and just a few females.

Hermaphrodites were found in all the treatments. The percentage of hermaphrodites was higher at t0 with 66.67% of animals classified as HBS. During the treatments, the highest percentage of hermaphrodites (61.54% including HBS, HPM and HPF) was found in 200 ng/L T compared with 15.38% in the control group.

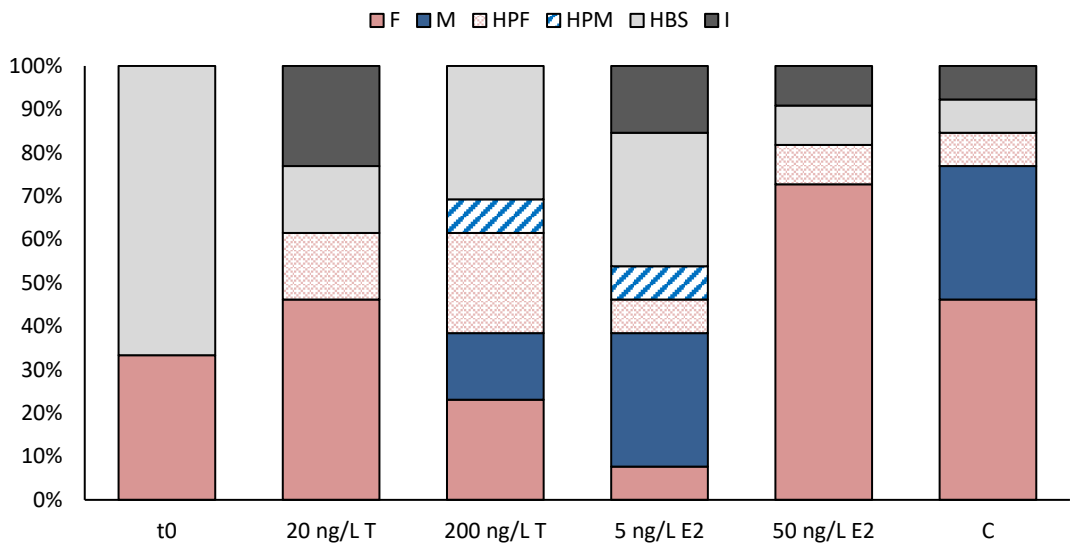

Figure 4.3 Proportion of sex categories in *Ostrea edulis* at the beginning of the experiment (t0; n=6) and exposed during 10 weeks to 20 ng/L T (n=13), 200 ng/L T (n=13), 5 ng/L E_2 (n=13), 50 ng/L E_2 (n=11), and a negative control (n=13). Specimen samples were identified by histological examination as females (F), males (M), hermaphrodite with both sexes equally represented (HBS), hermaphrodite predominantly male (HPM), hermaphrodite predominantly male (HPF) an indeterminate (I).

4.3.4 Changes in energy reserves and energy production in *Ostrea edulis* after exposure to sex steroid hormones

The changes in the biochemical composition of gonad visceral mass of *O. edulis* throughout the experiment are shown in Fig. 4.4. In the present study, total lipids (% DW) indicated that the highest average content of total fatty acids (53.04±33.01 % DW) was reached by oysters kept as part of the control group (Fig 4.4A). No significant differences in lipids content between hormone treatments were found presenting average values of 22.28±5.44, 28.40±6.14, 22.80±7.85 and 36.33±7.76 %DW for oysters treated with 5 ng/L E_2, 50 ng/L E_2, 20 ng/L T and 200 ng/L T, respectively. Treatments with both sex steroids showed a similar effect in lipid content in *O. edulis* with no significant differences between concentrations but significantly lower respect to the control group (Mann Whitney U test, $p < 0.001$) (Fig 4.4B). No significant differences were found in carbohydrates content between the treatments (5 ng/L E_2: 11.64±2.20 %DW, 50 ng/L E_2: 12.79±4.57 %DW, 20 ng/L T: 11.82±2.63 %DW and 200 ng/L T: 13.13±2.87 %DW), or between the treatments and the control group (13.67±6.60 %DW) (Fig 4.4B). The exposure to both concentrations of E_2 showed a similar effect in protein content in *O. edulis* with no significant differences between treatments (5 ng/L E_2: 23.51±5.89 %DW, 50 ng/L E_2: 24.71±12.87 %DW, 20 ng/L T: 27.13±6.81 %DW, 200 ng/L T: 19.57±6.92 %DW and negative control: 34.74±7.49 %DW) (Fig. 4.4C).

4.3.5 Biochemical changes in metabolites related to homestoasis in *Ostrea edulis*

The analysis of the global biochemistry to screen for metabolites significantly affected in oysters treated with both sex steroids showed an overall change in performance compared to the control group. PCA and PLS-DA were used to assess metabolomic data (Fig 4.5 and 4.6). PCA provides an overview of variation in datasets, but principal components may not identify variables driving maximum separation among treatments (Want and Masson, 2011). In contrast, PLS-DA is a supervised method that allows maximizing separation among known groups or treatments (e.g. different concentrations of the same sex steroid in the present study) (Want and Masson, 2011; Ruiz-Perez and Narasimhan, 2017). Thus, the combination of both PCA and PLS-DA provides a more comprehensive analysis of patterns in metabolomic profiles among treatments. The PLS-DA model was well-validated using a permutation test and presented a $Q^2 > 0.7$ in all cases. Combined, PCA components 1 and 2 explained 35.7% and 30.4% of the total variance among concentrations of testosterone and estradiol, respectively (Fig 4.5A and Fig 4.6A). PLS-DA revealed separate clustering of all treatments, and components 1 and 2 explained 25.7% and 29.2%of the total variance among concentrations of testosterone and estradiol, respectively (Fig 4.5B and Fig 4.6B).

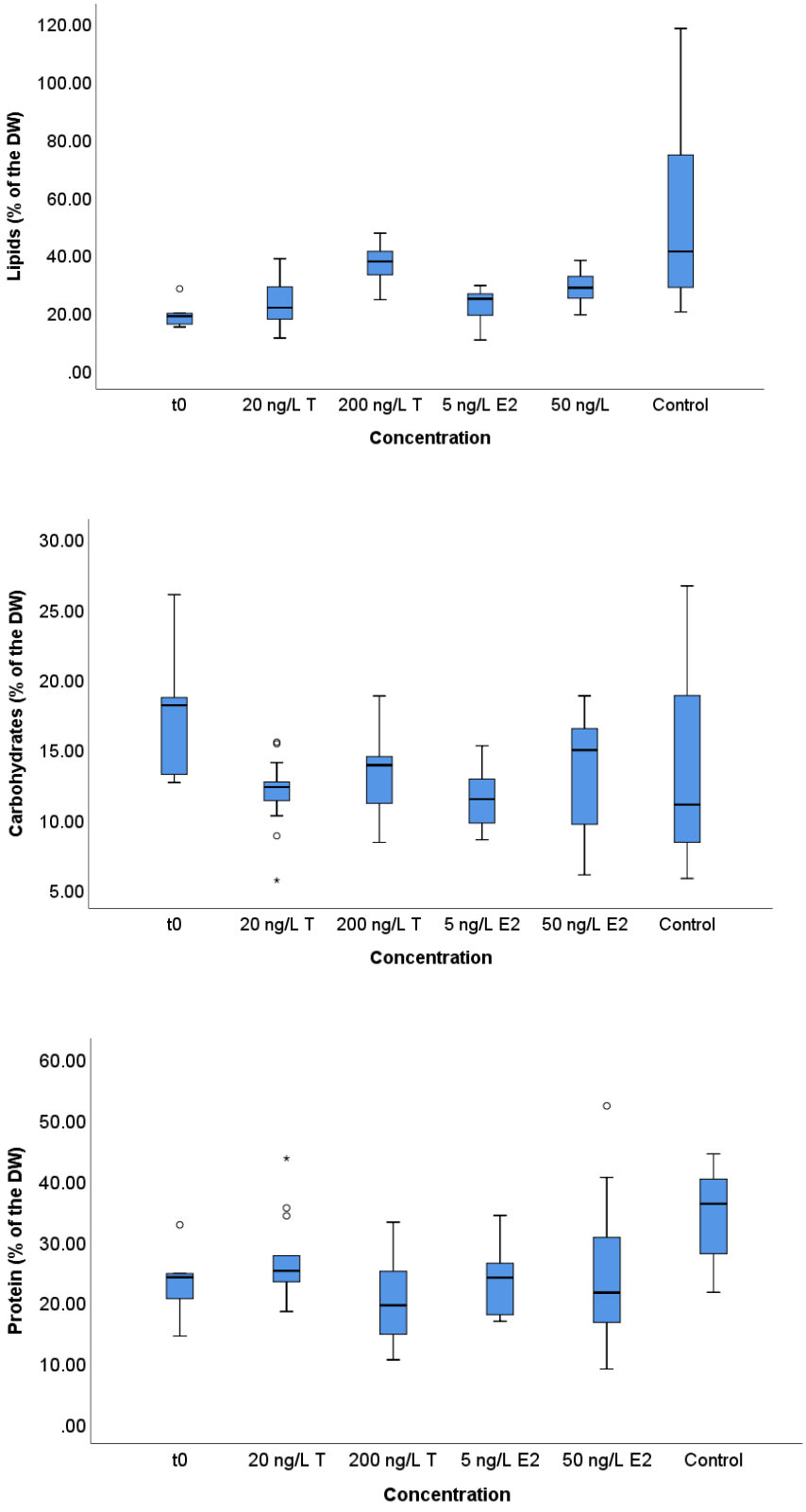

Figure 4.4 (A) Total lipids (% of dry mass, % DM), (B) total carbohydrates (% of dry mass, % DM) and (C) protein content (% of dry mass, % DM) of *Ostrea edulis* at the beginning of the experiment (t0) and exposed during 10 weeks to 20 ng/L T (n=13), 200 ng/L T (n=13), 5 ng/L E_2 (n=13), 50 ng/L E_2 (n=11), and a negative control (n=13).

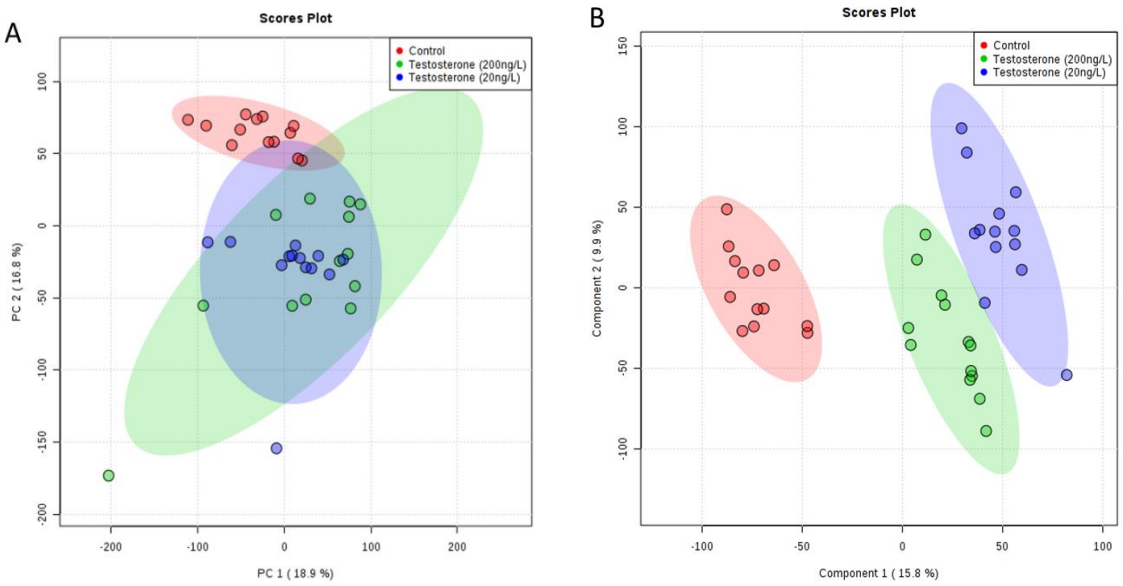

Figure 4.5 (A) Principal component analysis and (B) Partial least square discriminant analysis model comparing *Ostrea edulis* metabolomic profiles among testosterone treatments (20 ng/L T (n=13) and 200 ng/L T (n=13)) and a negative control (n=13) after 10 weeks of exposure.

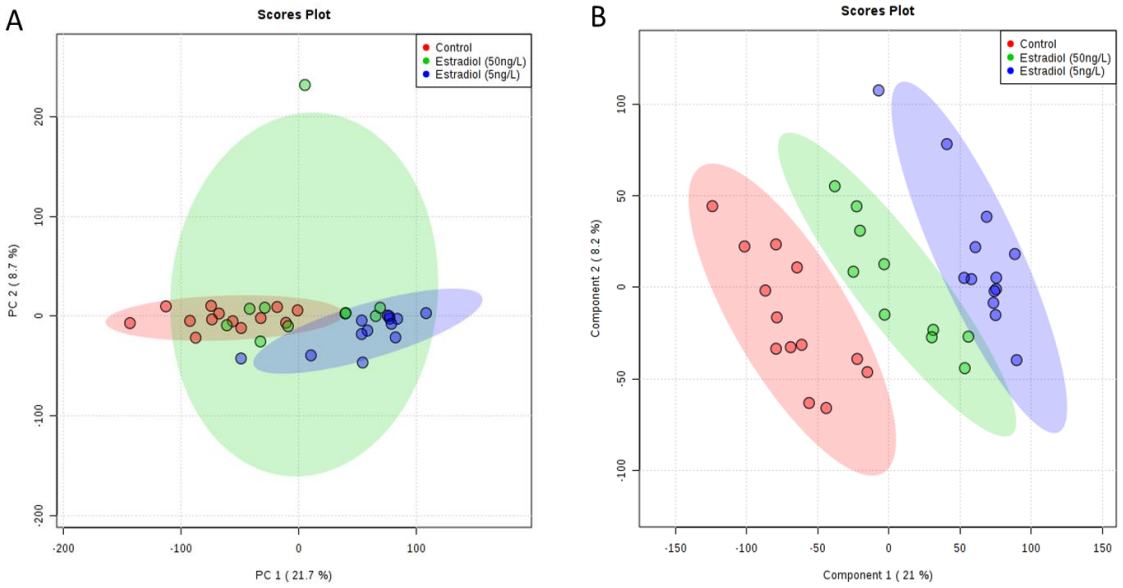

Figure 4.6 (A) Principal component analysis and (B) Partial least square discriminant analysis model comparing *Ostrea edulis* metabolomic profiles among estradiol treatments (5 ng/L E_2 (n=13), 50 ng/L E_2 (n=11)) and negative control (n=13) after 10 weeks of exposure.

It should be noticed that a considerable amount of ions have changed in oysters exposed to the different treatments changing the general metabolomic profile and affecting biochemical pathways involved in diverse functions such as gluconeogenesis, energy balance, mitochondrial electron transport chain, etc. (see an example in Appendix G). From the information obtained in this analysis, some of the main metabolites and the pathways affected were selected to understand the overall effect of exposure to sex steroids (see an example in Appendix H).

The analysis of metabolites showed that some of the main molecules affected by the treatments were those involved in the citric acid cycle pathway (also known as Krebs cycle) (Fig. 4.7). In summary, with most of the treatments a down-regulation of the TCA cycle intermediates such as citric acid, ketoglutaric acid, fumaric acid, malic acid and oxalacetic acid suggests decreased in TCA cycle metabolism. Furthermore, glucose which is the preferred energy source for most body cells decreased at all the treatments compared with the control group. These changes could be related to the down regulation of the principal molecules for storing and transferring energy in cells (ATP and GTP) (Fig 4.8).

The energy reserves are used in the cells in the production of glucose and energy molecules and carriers such as ATP, ADP, GDP and GTP. The metabolomics analysis of molecules involved in the production and transfer of energy in the cells showed a reduction in all those ions compared to the control group (Fig 4.8).

4.3.6 Sex steroid concentrations in gonadal tissue in *Ostrea edulis* after exposure to estradiol and testosterone

Sex steroid concentrations analysed by ELISA increased in all treatments respect to the beginning of the experiment (Fig. 4.9). Only oysters exposed to the highest concentration of testosterone showed a significant increase (Mann Whitney U test, $p < 0.001$) in the concentration of this hormone in tissue compared with the control group (290.99±314.23 pg/g). However, the concentration of both hormones did not show significant differences between T treatments (Fig. 4.9).

The treatment with estradiol showed that only the treatments with 50 ng/L E_2 showed a significant increase in the concentration of this hormone in tissue (838.91±609.10 pg/g) compared to the lowest concentration (400.28±258.17 pg/g; $p < 0.001$) and the control group (349.74±90.79 pg/g). However the concentration of testosterone in tissue was similar between treatments.

In this study, E_2 and T concentrations in tissue showed a significant correlation ($r_s = 0.8188$; $p < 0.001$).

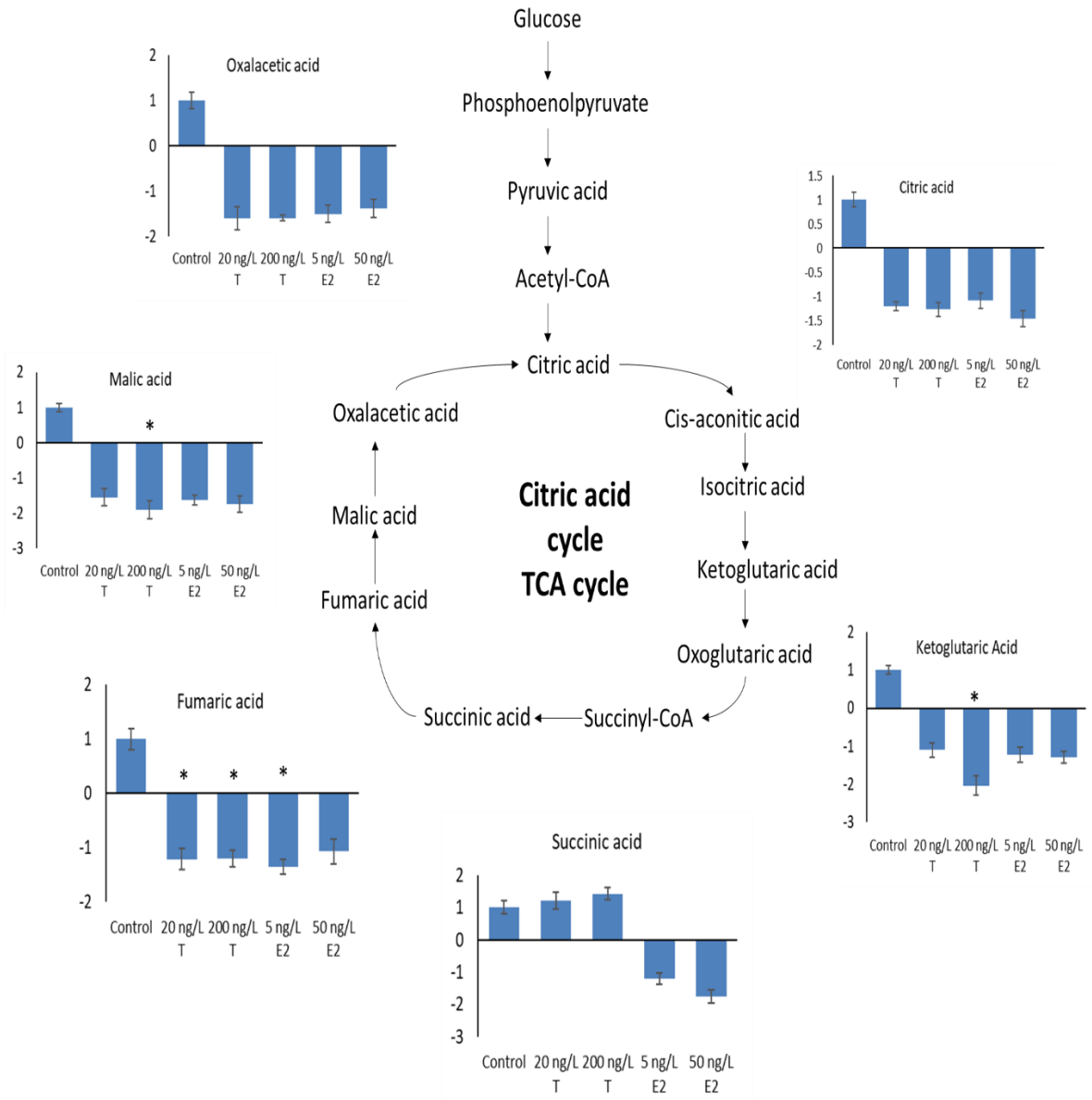

Figure 4.7 A summary of the biochemical pathway and fold changes in some of the molecules of TCA cycle metabolism in *Ostrea edulis* exposed to 20 ng/L T (n=13), 200 ng/L T (n=13), 5 ng/L E_2 (n=13), 50 ng/L E_2 (n=11), and a negative control (n=13).

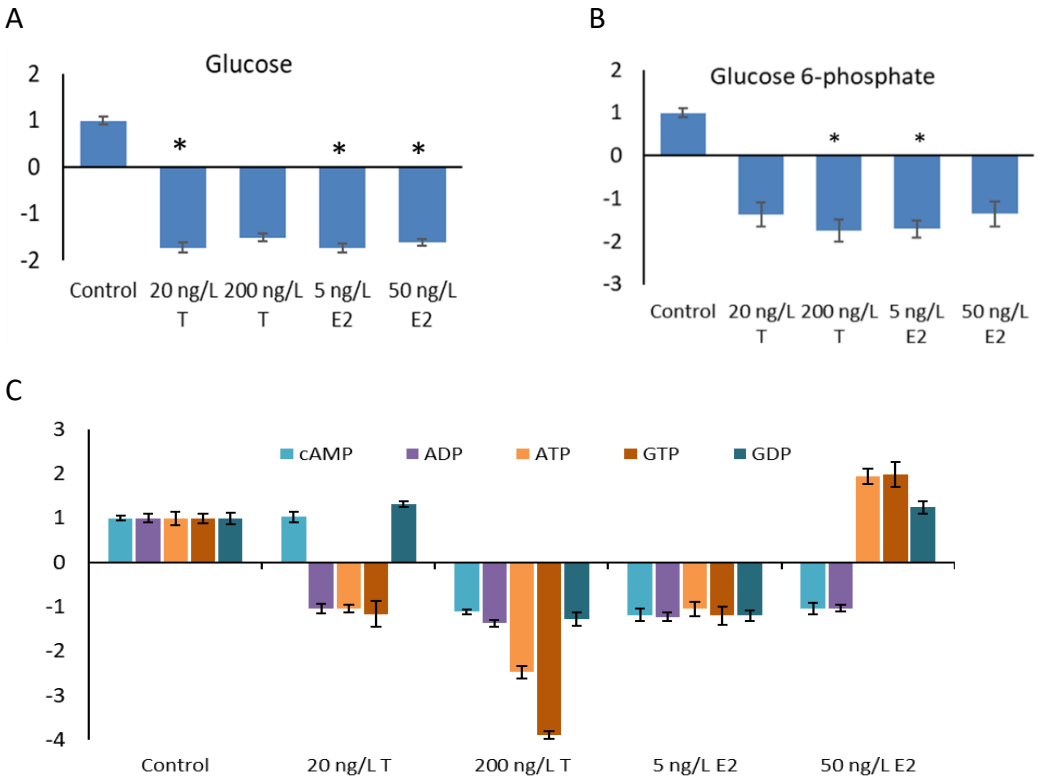

Figure 4.8 Fold changes for (A) glucose, (B) glucose 6-phosphate and the energy carrier molecules (C) cAMP, ADP, ATP, GDP, GTP in *Ostrea edulis* exposed to 20 ng/L T (n=13), 200 ng/L T (n=13), 5 ng/L E_2 (n=13), 50 ng/L E_2 (n=11), and a negative control (n=13).

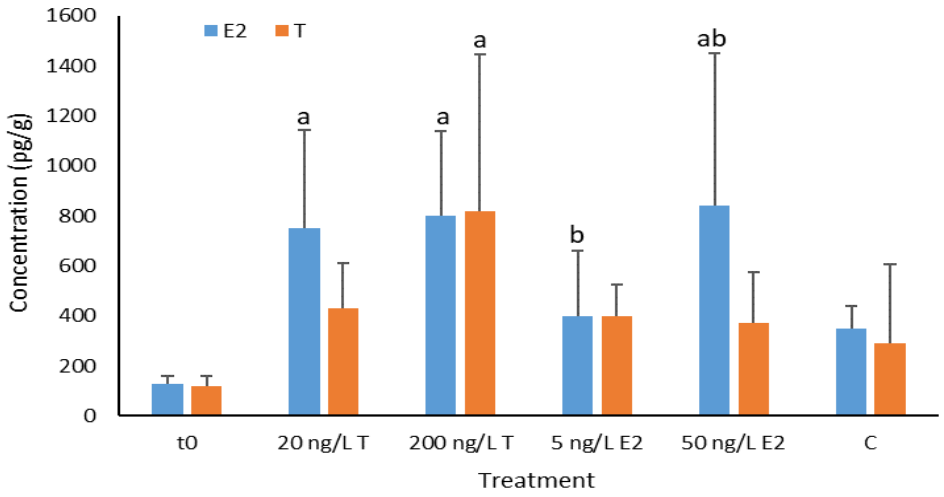

Figure 4.9 Hormones concentrations (mean±SD) in *Ostrea edulis* at the beginning of the experiment (t0; n=6) and exposed for 10 weeks to 20 ng/L T (n=13), 200 ng/L T (n=13), 5 ng/L E_2 (n=13), 50 ng/L E_2 (n=11), and a negative control (n=13). (a) Significant differences compared to the control group. (b) Significant differences between concentrations for the same hormone. Error bars denote standard deviation.

To analyse if the sex had any effect on the hormone concentration in tissue the females and males were analysed per treatment (Fig 4.10). Due to the low number of individuals with the same sex in some treatments, the individuals classified as females were counted together with hermaphrodites predominantly females. In the same manner, males were analysed in the same group with hermaphrodites predominantly females. The control group showed significant differences (Mann Whitney U test, $p < 0.05$) in the hormone concentrations between sexes. It was evident that F/HPF and M/HPM had similar hormones concentrations in all the treatments with no significant differences found between sexes. However due to the lack of males in some cases it was not possible to do the analysis for all the treatments.

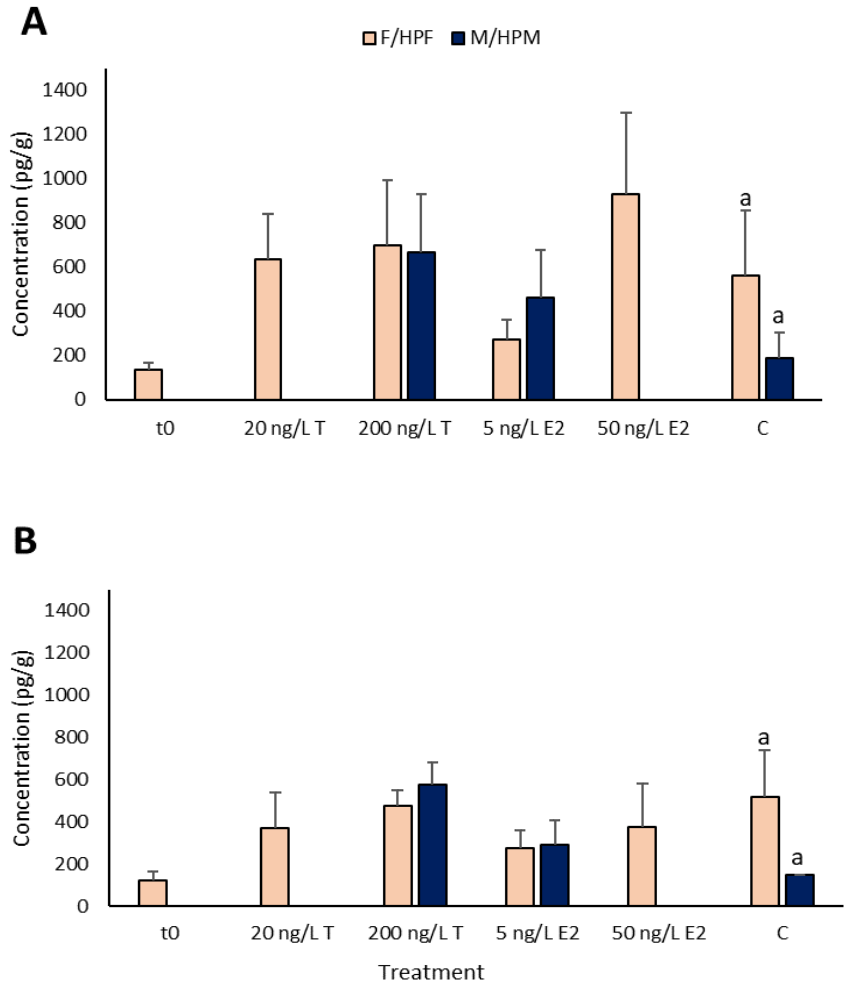

Figure 4.10 (A) Estradiol and (B) testosterone concentrations for *Ostrea edulis* classified through histology as females (F) + hermaphrodites predominantly females (HBF) (light-red bars) and males (M) + hermaphrodites predominantly males (HBM) (blue bars) at the beginning of the experiment (t0) and exposed to 5 ng/L E_2, 50 ng/L E_2, 20 ng/L T, 200 ng/L T, and a negative control. (a) Significant differences between sexes. Error bars denote standard deviation.

4.3.7 Presence and change of molecules related to vertebrate-sex steroid pathway in *Ostrea edulis*

Based upon the aims and objectives of this thesis, and the focussed pathway showed in Fig 1.4, this chapter will focus mostly on the molecules known to be relevant in the vertebrate-sex steroid pathway from Cholesterol (Fig 1.4). In summary, some of the molecules expected according to the vertebrate-sex steroid pathway were identified in all the groups (treatments and control) (Fig 4.11). Some of the initial precursors, such as pregnenolone and progesterone were down-regulated by both T concentrations and the lowest concentration of E_2.

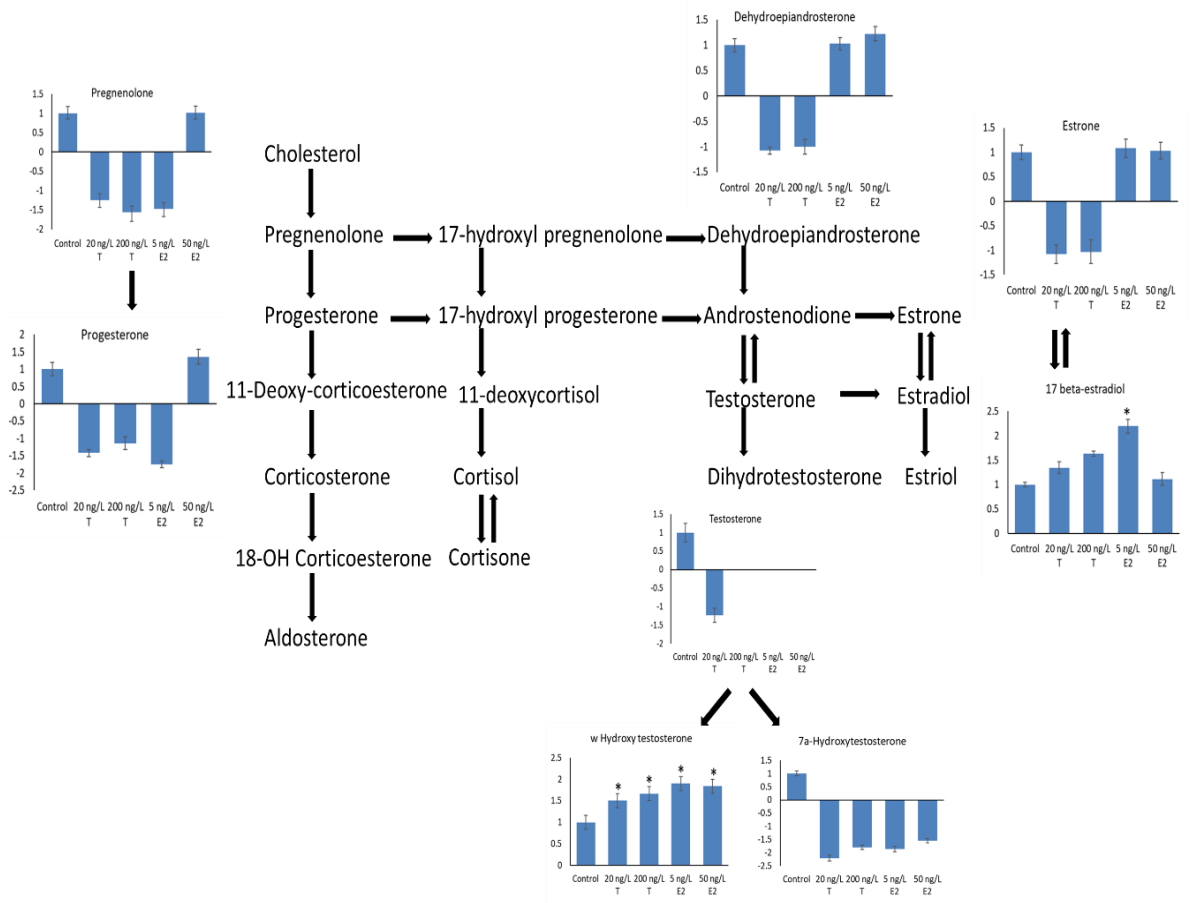

Figure 4.11 Presence and fold changes of some of the molecules expected according to the vertebrate-sex steroid pathway from cholesterol. *Ostrea edulis* exposed to 20 ng/L T (n=13), 200 ng/L T (n=13), 5 ng/L E_2 (n=13), 50 ng/L E_2 (n=11), and a negative control (n=13). (*) Significant differences ($p<0.05$) respect to the control group.

Of particular interest to the aims of this thesis, E_2 increase in all the treatments compared to the control group. The lowest concentration of E_2 was the only treatment with a significant increase in this molecule compared to the control group. The same trend found through the ELISA test was observed with T-treated oysters showing an increase in the levels of E_2 (Fig 4.9).

Testosterone was found for oysters exposed to the lowest concentration of T, but not for any of the other treatments. However, other androgen sex steroid metabolites such as hydroxytestosterone and 7a-hydroxytestosterone were found in all the treatments (Fig 4.11). The former was significantly up-regulated at 20 ng/L T, while the latter was down-regulated in all the treatments compared to the control.

4.4 Discussion

4.4.1 Effect of steroid treatments on survival and growth in *Ostrea edulis*

The lack of significant differences in biometric parameters in *O. edulis* between treatments matches that observed in scallops and mussels (Wang and Croll, 2004; Ruiz-Velásquez *et al.*, 2018). The weight fluctuations in bivalves could be attributed to the gonadal tissue weight as this is the main organ that varies throughout the reproductive cycle (Filgueira *et al.*, 2013). In this study the oyster gonads were immature, being classified mainly in stages G0, G1 and G2 of sexual maturity, thus a small change of weight of gonadal tissue occurred.

The mortality in *O. edulis* treated with different concentrations of E_2 and T were higher than the control. These results, alongside with the change in energy reserves and the production of energy in the cells (see more details in the section 4.3.4) indicate that the effect of these hormones could be more related to an imbalance in physiological function and homoestatic processes in this species in response to exposure to exogenous hormones for a period of 10 weeks. This is supported by the down-regulation of energy molecules, such as ATP, indicating that this exposure could be also affecting survival due to a lack of enough level of ATP needed to survive and the basic functions of the oysters.

4.4.2 Effect of steroid treatments on gonadal development and sex ratio in *Ostrea edulis*

In some species the injection of sex steroids has affected the speed of gonad maturation and shifted sex ratios. For instance, injection of E_2, T, progesterone, and dehydroepiandrosterone accelerated

gonad differentiation and induced masculinization increasing male/female ratio in the sea scallop *Placopecten magellanicus* (Wang, 2000; Wang and Croll, 2004).

In the present study, the exposure of *O. edulis* to 200 ng/L of T increased the number of males over two months of exposure. This is in accordance with other studies showing the masculinization effect of exposure to testosterone (Wang and Croll, 2004; Fernandes *et al.*, 2010; Ruiz-Velásquez *et al.*, 2018).

In this study, a low concentration of estradiol induced the differentiation of more males than females. This behaviour was also observed in *P. magellanicus* injected with estradiol (Wang and Croll, 2004). These results are contradictory to previous reports indicating the positive effect on ovarian maturation and feminizing effects of estradiol in bivalves (Mori, 1969; Quintana, 2005; Teaniniuraitemoana *et al.*, 2016; Ruiz-Velásquez *et al.*, 2018), but the current observations are consistent with other reports that estradiol can stimulate male reproductive activities and spermatogenesis in other marine bivalves (Wang and Croll, 2006).

Another possible effect of steroids on sexual differentiation may have been manifested in the present study by the increase in hermaphrodites of *O. edulis* exposed to E_2 and T, while fewer hermaphrodites were observed in the control groups. This effect was also observed in the sea scallop, *P. magellanicus* injected with progesterone and dehydroepiandrosterone (DHEA) (Wang and Croll, 2004). Due to the small sample numbers in the present study, it is unclear if the occurrence of hermaphrodites was caused by steroid treatments. That hypothesis has been proposed in simultaneous hermaphrodite bivalves (Kat, 2009) but there is no available information about the effect of hormones in hermaphroditism in members of the family Ostreidae.

It is important to mention that all the experiments evaluated in bivalves have been carried out for short periods. This study is the only one to my knowledge exposing oysters for more than two months to different concentrations of hormones to test the effects of chronic exposure to steroids in *O. edulis*. This makes difficult the comparison with other studies evaluating short-term exposures (hours or days) to hormones. It has been argued that the response to short-term exposures could predict the response in the same parameter after long-term exposures in bioassays (Giesy and Graney, 1989). However triggered mechanisms in response to chronic exposures could be related to toxicity and physiological effects (Crane, Watts and Boucard, 2006; Cope *et al.*, 2008).

4.4.3 Effect of steroid treatments on energy reserves and energy production in *Ostrea edulis*

When food is abundant, nutrients are stored in various tissues in the form of glycogen (mainly in the adductor muscle of scallops, and in the mantle and connective tissues of other bivalves such as oysters and mussels), lipid (mainly in the digestive gland and the adductor muscle) and protein (mainly in the adductor muscle or liver in bivalves) (Wang, 2000). This occurs mainly prior to periods of sex maturation so the preparation and storage of energy reserves prior to a reproductive period is crucial.

Lipid and protein content were significantly lower in exogenous steroid-exposed groups compared to the control group, indicating a decrease in these parameters after two months of exposure to steroid hormones. It has been shown that sex steroids could regulate the main metabolic pathways of glycogen, proteins and lipids (Matute and Kalkhoff, 1971; Wang and Croll, 2004; Durou and Mouneyrac, 2007). However there are relatively few studies on the direct effects of sex steroids on energy reserves in bivalve molluscs.

Mori (1969) demonstrated that estradiol stimulated gametogenesis in the Japanese oyster by accelerating glycogenolysis which provided the energy for the process of gametogenesis (Mori, 1969). The injection of estradiol accelerated gametogenesis and vitellogenesis and stimulated the transfer of energy reserves from the pyloric caeca to the gonad in the sea star *Asterias rubens* (Schoenmakers, Van Bohemen and Dieleman, 1981). Estradiol may stimulate glycogenolysis and lipogenesis through the regulation of the activity of the enzyme glucose-6-phosphate dehydrogenase and malate dehydrogenase in molluscs (Mori, 1969; Mori, Muramatsu and Nakamura, 1972. It seems like the mobilization of energy from storage tissues to gonad is a long-term process probably under the regulation of steroids, especially estrogens in female bivalves (Wang, 2000). This evidence suggests that even when sex steroids could not be synthesized and metabolized in *O. edulis*, and do not have a direct effect on sex determination, exogenous hormones might be influencing gametogenesis and other physiological processes, such as transfer of energy reserves, trough accumulation in lipid-rich gonad.

The major metabolic processes in the gonad during sexual maturation are the breakdown of glycogen, and the synthesis of proteins and lipids (Mori, Muramatsu and Nakamura, 1972; Mori, Muramatsu and Nakamura, 1972; Wang, 2000). In this study, the exposure of *O. edulis* for two months to lower concentrations of both hormones caused a decrease in carbohydrate content. However the lipid and protein content were always lower than the control (Fig. 4.4A). This could

suggest that after a long exposure with these hormones the glycogenolysis, and the synthesis of proteins and lipids could be altered, affecting the energy reserves in the organisms.

Lipid synthesis is essential for gametogenesis because lipids are needed not only for the cell membranes but also for the synthesis of lipoproteins including Vtg (Mori, 1969; Mori, Muramatsu and Nakamura, 1972; Wang, 2000). This means that reserves of energy are used for gonadal maturation in animals to go through gametogenesis (Wang, 2000). The lower energy reserves in the oysters exposed to the different treatments in this study, alongside the slow maturation process observed in these animals, suggest that the dynamic process including both anabolic and catabolic metabolism and the changes in contents are the affected by a long term exposure to these hormones.

During gametogenesis, glycogen stored in the gonad is broken down into glucose which is used as energy resource for the production of ATP, NADPH, and NADH which are necessary for the synthesis of other organic compounds including fatty acids and nucleic acids, etc. (Wang, 2000). The respiratory pathways of glycolysis, the tricarboxylic acid (TCA) cycle and the mitochondrial electron transport chain are ubiquitous throughout nature and essential for both energy provision in heterotrophic cells and a wide range of other physiological functions (Fernie, Carrari and Sweetlove, 2004). Enzymes and proteins that participate in these pathways are well known, but their regulation and control are much less well understood and just a few reports have shown the importance of those processes in invertebrates (Alp, Newsholme and Zammit, 1976).

For instance, in the process of sexual maturation, the glucose-6-phosphate dehydrogenase (G-6-PD) plays a central role in the interaction of glycogenolysis, Iipidogenesis and steroidogenesis (Wang, 2000). This is the major regulatory enzyme in the pentose phosphate pathway of glucose breakdown and it has shown to be induced by estrogen-treatment in mammals (Cummings and Barker, 1986; Murray, 2006). However the direct effects of steroids on glucose levels and its break down have not been studied in molluscs. In this study a reduction in the production of molecules, such as glucose, ATP and NADH, involved in the production and transfer of energy was observed for all the treatments. This indicates that exposure to steroids could cause an unbalance in the energy reserves and the production of ATP in the cells affecting not only reproduction but also other biological processes in the organisms exposed.

4.4.4 Sex steroids and their intermediate metabolites in tissue of *Ostrea edulis* after exposure to steroid treatments

In this study the hormone concentrations in gonadal tissue of *O. edulis* significantly increased in all the treatments (Fig 4.9 for ELISA and Fig 4.11 for metabolomics results), indicating that *O. edulis* can uptake and accumulate these hormones from the environment.

An increase in a concentration-dependent manner of steroid hormones could be expected in a study like this. However the concentration of both hormones was very similar between treatments. In some studies the increase of free steroids has been very low or even not significant compared with the control, but the response was different taking in consideration total steroids (free + esterified) with a fast increase in a concentration-dependent manner (Janer *et al.*, 2005; Fernandes *et al.*, 2010). This has led to the assumption that esterification of the excess of steroids with fatty acids might help to maintain stable endogenous levels of free steroid (Janer, Lavado, *et al.*, 2004; Janer, Mesia-Vela, *et al.*, 2004; Janer *et al.*, 2005; Fernandes *et al.*, 2010). Although esterified steroids do not bind steroid receptors, they are important because they could be considered as long-acting steroids, since they can be hydrolyzed by esterases (Hochberg *et al.*, 1991). This could imply that beyond a threshold the concentration of free steroids would not increase because they can diffuse throughout the organism and are available for a quicker excretion and metabolism, while esterified forms could increase because they become immobilized in the fat for very long periods (with half-lives measured in weeks rather than days) (Scott, 2018). However, the physiological role of those steroid esters in molluscs, and the mechanisms by which steroid esterification is regulated remain uncertain.

The slight increase in hormone concentration found in the control group was not expected as the experiment was carefully designed to avoid cross-contamination between tanks. Low concentrations of steroids have been detected in water from control tanks in similar experiments (Fernandes *et al.*, 2010). The presence of steroids in molluscs due to the transformation and synthesis of steroids by microalgae or bacteria has been reported (Pollio *et al.*, 1994; Panter *et al.*, 1999). It may well be that the presence of E_2 and T in the gonad tissues of the control group was a function of the accumulation of these steroid homologues from the phytoplankton food source or human manipulation during the experiment (Scott, 2012, 2018).

The evidence for the presence and activity of critical enzymes involved in the steroidogenic pathway such as 5α-reductase, 17β-HSD and Cytochrome P450 monooxygenase enzymes (CYP) has been evaluated and it has been accepted that these molecules are present and have a role in metabolism of sex steroids in several invertebrate species (Le Curieux-Belfond *et al.*, 2001; Janer and Porte,

2007). The formation of androgen metabolites after exposure to testosterone and estradiol, and the activity of 5α-reductase has been reported in mussels and clams (Morcillo, Ronis and Porte, 1998; Janer *et al.*, 2005; Fernandes *et al.*, 2010). The injection of estradiol-17h in the adductor muscle of *Magallanas gigas* caused an in vivo transformation into estrone in the whole oyster and in its digestive gland confirming the activity of 17h-hydroxysteroid-dehydrogenase (Le Curieux-Belfond *et al.*, 2005). In the same manner the P-450 aromatase activity has been shown through the conversion of androstenedione to E_2 in the ovary and testis in the Japanese scallop, *Patinopecten yessoensis* (Osada, Tawarayama and Mori, 2004) and the aromatization of androgens into estrogens after exposure to exogenous estradiol with direct effects on this enzyme activity in a concentration-dependent manner (Janer, Lavado, *et al.*, 2004).

Evidence from radioactive tracer studies on molluscs (reviewed by Scott, 2012) shows that they have a very limited ability to carry out three important transformations in the steroid biosynthetic pathway mediated by P450 enzymes in vertebrates: cholesterol→pregnenolone; P→17-hydroxyprogesterone; and T→E_2. According to Scott (2012, 2018) P-450 aromatase homologue enzyme has been found in invertebrates but has sufficient differences in structure to suggest that it might not be as active as the vertebrate enzyme. So an apparent contradiction between genomic data and reports about the presence and activity of some enzymes can be found in the literature. This is the case for some of the enzymes involved in steroid synthesis pathways, e.g CYP450, whose activity has been shown in molluscs but the gene has not been reported (Scott, 2018). However several molluscan studies claim to have shown the transformation between intermediates in the sex steroid metabolic pathway (Janer, Lavado, *et al.*, 2004; Osada, Tawarayama and Mori, 2004).

It is relevant to mention that the enzymes involved in the steroidogenic pathway are inducible and have mixed functions (Peters and Livingstone, 2007). One of the most well-known functions of these enzymes is the detoxification role involving biotransformation and bioactivation of a wide range of xenobiotics (Peters and Livingstone, 2007). So the presence of enzymes involved in vertebrates-related sex steroid pathway reported by some authors as a decisive evidence of the synthesis of hormones in molluscs (Janer, Lavado, *et al.*, 2004; Osada, Tawarayama and Mori, 2004) requires a carefully interpretation due to a possible role in detoxifications process rather than a role in their metabolism.

A number of publications have provided evidence that steroids bind to proteins (sex steroid receptors or receptor-like proteins, membrane receptors and enzymes) in aquatic invertebrates (Thornton, Need and Crews, 2003; Janer and Porte, 2007; Cubero-Leon *et al.*, 2010). Steroid receptors (SRs) were originally thought to be a vertebrate-specific gene family but this changed when genes with clear sequence homology to SRs were discovered in molluscs (Thornton, Need

and Crews, 2003; Eick and Thornton, 2011). However, it has been discussed that the presence of a sex steroid receptor-like protein in invertebrates with properties (gene sequences, immuno-reactivity, ligand binding properties) comparable to sex steroid receptors in vertebrates does not necessarily indicate that it has comparable functions as it has in vertebrates (Köhler *et al.*, 2007; Scott, 2018).

Models of metabolic evolution provide explanations for the origin of multiple very ancient pathways related to the universal metabolism (Markov *et al.*, 2017). It seems that more derived metabolic pathways have evolved in animals due to multiple recruitments of enzymes from pre-existing pathways (Caetano-Anollés *et al.*, 2009). Genetic and phylogenetic analyses has suggested that vertebrate steroid receptors arose from a common ancestral gene in deuterostomes. The reconstruction of an ancient hormone/receptors has indicated that the side-chain cleavage is common to most vertebrate steroids, whereas aromatization was co-opted for estrogen synthesis from a more ancient pathway (Markov *et al.*, 2009, 2017). Markov *et al*. (2009, 2017) synthesized *de novo* a molecule comprising a cholesterol side chain and an aromatized A-ring which was able to bind to an ancestral steroid receptor *in vitro*. The results obtained by Markov *et al*. (2009, 2017) suggested coexistence of progestagens and similar ancestral molecules in basal vertebrates before the emergence of modern estrogens. The analysis of steroidogenic enzymes supports the hypothesis that nuclear receptors (NR) in vertebrates and arthropods evolved independently and the steroidogenic cytochrome P450 enzymes was not a hormone receptor but more likely a sensor to detoxify xenobiotics (Markov *et al.*, 2009). So many invertebrates could have SRs resembling that of estradiol but not specific just for this compound, and that aromatase could recognize steroid-like compounds as part of a detoxification response rather than as part of a reproductive pathway.

Androgen and estrogen metabolism in invertebrates is a controversial topic due to the lack of evidence on the synthesis and enzymatic pathways needed to metabolize sex steroid hormones (Scott, 2012, 2013). However the present study did not find all the intermediate molecules expected according to the vertebrate-sex steroid pathway from cholesterol. The absence of a clear pathway involving the enzymes responsible of hormones metabolism suggests this mechanism is not present in *O. edulis* and other molecular pathways are involved in reproduction in this species.

4.4.5 Sex steroids as aquatic environmental pollutants

Approximately 83,000 kg/yr estrogens enter into the aquatic environment from discharges by livestock in the United States and European Union, and 30,700 Kg/yr of natural and synthetic estrogens are discharged from birth control pill practices around the world (Adeel *et al.*, 2017). Studies have found that steroidal estrogens concentration in UK rivers plays a major role in sexual

disruption in wild fish populations (Jobling *et al.*, 2006). In the UK it has been suggested that direct excretion of steroid hormones by animals into water courses, or discharges from farmyard drains, are important sources of water contamination (Johnson, Williams and Matthiessen, 2006). In spite of the worldwide distribution of estrogens detected in rivers and surface water sites and the adverse effects of these exposures on plants and animals reported (Adeel *et al.*, 2017), these compounds are not included in the list of priorities for marine environmental standards. The situation is even worse for androgens and no proposal about maximum concentrations allowed or predicted effect concentrations are available for these compounds.

4.5 Conclusions

In this study the hormone concentrations in gonadal tissue of *O. edulis* significantly increased after exposure to exogenous E_2 and T in water, indicating that *O. edulis* can take and accumulate these hormones from the environment. Despite the lack of a clear effect of masculinization or feminization caused by testosterone and estradiol, these results suggest that environmental exposure to hormones, and potentially any mimicking compounds, might create endocrine misbalance affecting gametogenesis, sex determination and normal physiology of this species.

In this study, a decrease in energy reserves was found after two months of exposure to sex steroids suggesting a shift in glycogenolysis and the synthesis of proteins and lipids. These processes, alongside the breakdown of glucose, are the major metabolic processes occurring in the gonad during sexual maturation. So a lower content of energy reserves, a reduction in molecules such as glucose, energy carrier, and TCA cycle, suggest a reduction in the metabolic activity which slowed the gonadal maturation in this species.

The metabolism of sex steroids in invertebrates is a controversial topic due to the lack of evidence on the synthesis and enzymatic pathways needed to metabolize sex steroid hormones. Although when the present study identified the presence of some of the intermediate molecules predicted based on the vertebrate-sex steroid pathway from cholesterol, it is not possible to speculate a similar role in reproduction than in vertebrates. More research is needed in order to characterize these molecules and verify if the enzymes responsible for its synthesis and metabolism are present in invertebrates before making more conclusions about the reproductive effect of these compounds. Metabolite profiling has an important role to play in this. However there is still a long way from being able to routinely infer the underlying biochemistry from metabolic.

This chapter has shown an effect on biological, physiological and reproductive parameters of *O. edulis* exposed to exogenous estradiol and testosterone for 10 weeks. This suggests that

environmental exposure to sex steroids, and possible to steroids-mimicking chemicals, could be responsible for the declining populations in the Solent. Environmental pollution has been reported in this area, mainly by tributyltin widely used as a biocide in antifouling paint applied to the hulls of vessels. Therefore, the next chapter will be focused on the effect of this pollutant, a well-known EDC, on biological and reproductive parameters of *O. edulis* under laboratory conditions.

Chapter 5 TBT as a model endocrine disruptor and its effects on reproductive parameters in *Ostrea edulis*

5.1 Introduction

The presence of pollutants in the aquatic environment has led to an increasing number of endocrine disruption studies concerning both vertebrates and invertebrates and involving physiological processes controlled by steroid hormones (Davidson and Levine, 1972; Oehlmann and Schulte-Oehlmann, 2003). Although our knowledge of molluscan reproductive control is still limited, this phylum has provided evidence of endocrine disruption with the pseudo-hermaphroditism or imposex phenomena (Lafont and Mathieu, 2007). The limited understanding of normal endocrine processes in invertebrates makes an assessment of chemical endocrine disruption in the field extremely difficult (DeFur, 1999) and more studies need to be undertaken to clarify if environmental pollutants could affect biological and reproductive parameters in *Ostrea edulis*.

The most well-known organotin, tributyltin (TBT), and its derivates have shown adverse effects on mollusc reproduction in polluted areas and under laboratory conditions (Matthiesen & Gibbs, 1998 and literature cited therein). Organotin compounds, including TBTCl, have been extensively employed in a variety of industrial products including antifouling paints for boats to prevent the growth of organisms such as barnacles on the hulls of ships (Gadd, 2000; Pynaert and Speleers, 2000; Matthiessen, 2013). It has been also used in wood preservatives, biocides and plastic stabilizers (Gadd, 2000). Mono- and di-organotins also have been used as stabilizers in the manufacture of polyolefin plastics (polyvinyl chloride) and as catalysts in production of polyurethane (foam) and other polymers (Allsop and West, 2004; Hansen, 2014).

TBT is now a widespread global contaminant that persists in some UK estuaries and harbours. The introduction of legislation to reduce TBT inputs from vessels and the establishment of restrictions for using TBT as an anti-fouling paint occurred in the late 1980s (Fig 5.1), and successive bans were proposed since then. However, several years after the establishment of these restrictions, considerable concentrations of this pollutant were found in sediments in different locations with boating activities, ports and harbours (Dowson, Bubb and Lester, 1993). Studies carried out during the decade after the 1987 TBT restrictions showed a reduction in concentrations of this pollutant in water and bivalves (Waite *et al.*, 1991). However, sediments did not show a clear trend in the reduction of TBT and toxic effects were found on meiobenthic communities exposed to from different locations around the UK showing the ability to persist in the environment for several years (Waite *et al.*, 1991; Austen and McEvoy, 1997).

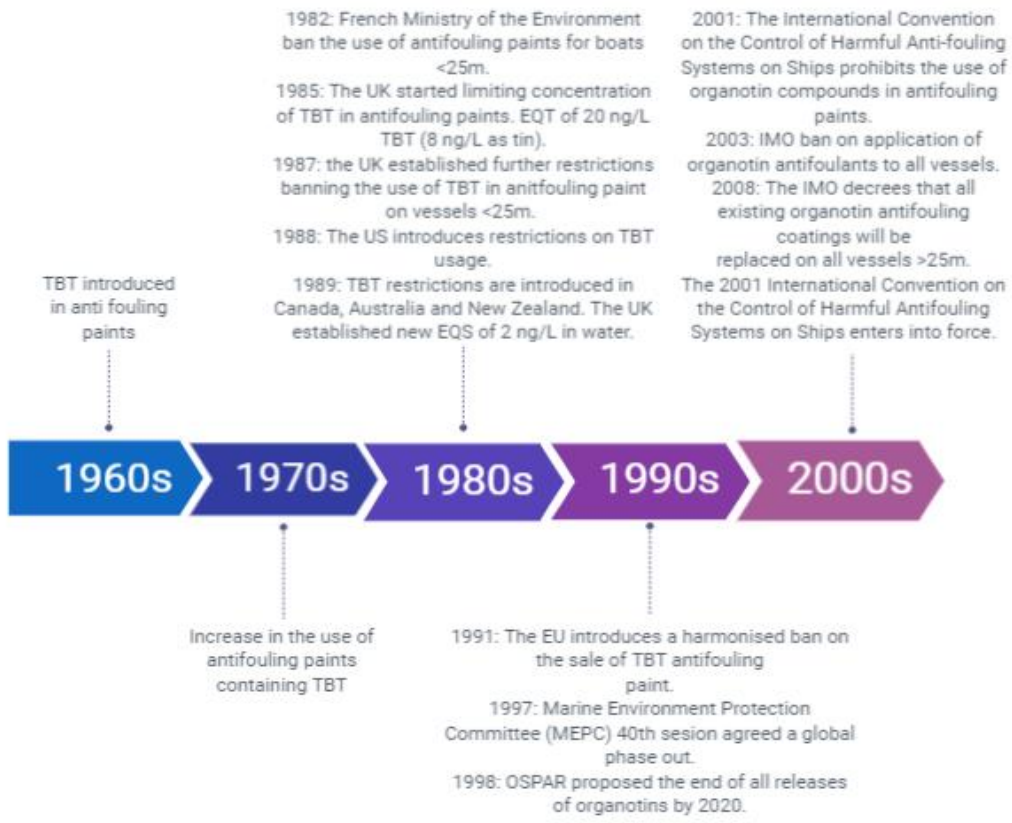

Figure 5.1 Timeline showing the key dates during the growth and banning of TBT Organotin (Alzieu, 2000; Cleary, 1991; MEPC, 2006; Santillo *et al*., 2001). Image created with Biorender (https://biorender.com/).

In the aquatic environment, TBT is quickly removed from the water column and adheres to sediment particles due to its high specific gravity (*i.e.* 1.2) and its capacity to bind to suspended particulate organic and inorganic materials (Gadd, 2000). The half-life of TBT in the water column is approximately 6–19 days but in marine sediments ranges from 0.91 to 5.2 years as its degradation rate is slower within sediments than in the water column (Seligman, Valkirs and Lee, 1986; Clark, Sterritt and Lester, 1988; Dowson, Bubb and Lester, 1996; Ayanda *et al.*, 2012). Laboratory-scale simulation experiments with TBT-contaminated sediment re-suspended in harbour water showed that environmental conditions such as pH, salinity and temperature have an influence in TBT-release (Pynaert & Speleers, 2004). Thus TBT could still represent a risk due to remobilization in the water phase when contaminated sediment particles are re-suspended by dredging or other activities.

TBT is the most toxic compound known to aquatic ecosystems (Gadd, 2000). This property is related to its high lipid solubility that provides easy cell penetration and association with intracellular sites (Gadd, 2000). TBT is transformed into less toxic compounds (DBT and MBT) by abiotic mechanisms

(such as chemical cleavage, thermal cleavage and UV irradiation) or biotransformation by bacteria, algae and fungi (Clark, Sterritt and Lester, 1988; Dowson, Bubb and Lester, 1996; Ayanda *et al.*, 2012). The presence and accumulation of TBT and its metabolites have been observed in mussels and oysters (Hsia and Liu, 2003; Meng *et al.*, 2005; Yang, Zhou and Jiang, 2006). Biological effects caused by TBT included abnormal shell development in *Magallanas gigas* and *Ostrea edulis* populations inhabiting polluted areas (Dyrynda, 1992; Axiak *et al.*, 1995). *O. edulis* has been shown to be sensitive to concentrations of TBT as low as 10 ng/L in seawater, exhibiting significant digestive cell atrophy correlated with autophagic mechanisms, catabolic metabolism, reduced bioenergetic balances and reduced somatic growth (Axiak *et al.*, 1995). Other changes in reproduction, physiology and mortality of some species, mainly gastropods, have been reported (Blaber, 1970; Gibbs and Bryan, 1986; Héral, Alzieu and Deslous-Paoli, 1989; Gibbs, 1993; Huet, Paulet and Le Pennec, 1996; Matthiessen and Gibbs, 1998; Morgan, Murphy and Lyons, 1998; Giusti *et al.*, 2013).

Of all the reproductive disruption effects caused by pollutants, particularly TBT and its metabolites, the most well-known is imposex. This refers to a virilisation phenomenon or a pseudohermaphroditic condition characterized by the development of sexual organs of the opposite sex in addition to the individual's own and the superimposition of non-functional male sexual characteristics (penis, vas deferens and/or seminiferous tubules) in female individuals, causing infertility, and in severe cases even leading to the death of the individual (Smith, 1971; Gibbs and Bryan, 1986; Gibbs *et al.*, 1987). It has been demonstrated that the intensity of female virilisation caused by TBT can be concentration-dependent but ultimately can lead to sterility (Matthiessen and Gibbs, 1998) with direct consequences on the reproductive capability of the population, limiting the numbers of juveniles and their recruitment to the adult population. Gastropod species that have low fecundity, limited mobility, and hatchlings that are not widely dispersed, suffer from reduction or elimination of populations at locations severely affected by this pollutant (Ketata *et al.*, 2007). While the ecological and population impacts of these compounds are well studied, the mechanism(s) through which they induce and promote the development of a penis-like structure and a vas deferens in female snails are still being deciphered.

The first reports identified gastropods with penis-bearing females developed in response to exposure to TBT in concentration as low as < 1 ng/L (Blaber, 1970; Smith, 1971; Gibbs, 1993; Huet, Paulet and Le Pennec, 1996; Ketata *et al.*, 2007). And after these findings most of the reviews and studies about the endocrine disruptor effect of TBT have been confined to gastropods (Bryan *et al.*, 1987; Gibbs, Pascoe and Burt, 1988; Spooner *et al.*, 1991; Oehlmann *et al.*, 1996; Matthiessen and Gibbs, 1998; Morgan, Murphy and Lyons, 1998; Oberdörster, Rittschof and McClellan-Green, 1998; Terlizzi, Geraci and Gibbs, 1999; Oberdörster and McClellan-Green, 2002; Gooding *et al.*, 2003;

Horiguchi, 2006). Evidence about effects on oysters is scarce and the underlying mechanism(s) remains unclear. The only laboratory-based study that has investigated the effect of TBT on reproduction in *O. edulis* showed that exposure to 240 ng/L of TBT for 74 days at 20-21°C reduced the proportion of a population that developed as females in this species via the inhibition or delay in the normal switch from male to female expected during gametogenesis (Thain, 1986). Other evidence showed that *O. edulis* and *M. gigas* inhabiting TBT-polluted areas showed a reduction in larval production with only 5 to 6% of individuals producing larvae, compared with 10 to 20% in uncontaminated areas (Thain and Waldock, 1986; Roberts *et al.*, 1987; Matthiessen and Gibbs, 1998). Despite this, there is not much information about the effect of TBT on *O. edulis* reproduction, or the mechanism through which it may have affected the normal switch from male to female reported by Thain (1986). An inhibition in the mechanism involved in this switch affecting the balance between males and females and creating a bias toward males at a population level might be expected in *O. edulis*. However, other effects such as toxicity, a delay in growth or a reduction in the ability to reach maturity (Thain, 1986; Ruiz, Bryan and Gibbs, 1995; Austen and McEvoy, 1997; Zhang *et al.*, 2013; Li, Li and Shi, 2015a).

It has been proposed that one of the mechanisms responsible for the reported androgenization/feminization phenomena caused by TBT could be related to the esterification of free hormones affecting levels of active steroids within tissues (Janer, Lavado, *et al.*, 2004). In fact, some contaminants can alter the levels of free and esterified steroids and esterification of testosterone has been considered as a possible site of action for the endocrine disrupter TBT (Spooner *et al.*, 1991; Gooding and LeBlanc, 2001; World Health Organization, 2013). Females of the gastropod *Marisa cornuarietis* exposed to TBT (10 ng/L) for 100 days showed a significant decrease in esterified steroids with a greater decrease in esterified testosterone than that of esterified estradiol (Janer *et al.*, 2006). Janer *et al*. (2006) reported a decrease in esterified steroids specific for TBT-exposed females as no change in esterified steroids was observed in TBT-exposed males. Individuals of *M. cornuarietis* showed the development of imposex and a reduction of fecundity, however the relationship with the observed decrease in esterified steroids or the mechanism by which TBT decreases the esterification of these hormones were not clear (Janer *et al.*, 2006).

There have been reports of the ability of TBT to interact with different steroid metabolic pathways- aromatase, and 5α-reductase- proposed as a mechanism to affect steroid pathways (Ronis and Mason, 1996; Morcillo, Ronis and Porte, 1998; Doering *et al.*, 2002; Oberdörster and McClellan-Green, 2002; World Health Organization, 2013). Although the mechanism is still unclear, the evidence shows that the TBT effect on invertebrates could be related to an effect on steroid pathways affecting esterification or conjugation processes.

Pollutants such as TBT are considered environmental stressors that can alter physiological and biochemical parameters including morphological indices, antioxidant responses and energy metabolic parameters (Roberts and Oris, 2004; Li *et al.*, 2013). It has been shown that TBT increases the vulnerability of fish and gastropods to oxidative stress, inducing not only endocrine disruption but also neurotoxicity and developmental toxicity (Wang *et al.*, 2006; Li, Li and Shi, 2015a, 2016; Primost *et al.*, 2017). Oxidative stress is the imbalance of oxidants and antioxidants in favour of the oxidants, potentially leading to cell damage (Davies, 1995; Lushchak, 2011). Biomarkers related to this imbalance are useful in terms of evaluating potential cytotoxic effects by oxidative damage and might be a mechanism of toxicity for aquatic organisms living in polluted areas (Lushchak, 2011).

An analysis of the current situation regarding reproductive parameters of the *O. edulis* population in the Solent has shown a significant reduction in the number of brooding female-phase oysters (Eagling, 2012), low values of fecundity of brooding oysters and skewed sex ratio towards male-phase oyster of 3:1 and 6:1 (Eagling, 2012; Kampahusen, 2012). Effects on the reproductive pathways and performances in hermaphroditic species are more difficult to understand and its changes are more subtle. This is probably why the effects of organotins in these animals have not been extensively investigated. It is important to define the main factors affecting the declining population in the Solent and if pollutants such as TBT, a well-known endocrine disruptor, could have an effect on physiological, biochemical and reproductive attributes of *O. edulis*.

Southampton Water is an area hosting commercial and naval ships, in addition to thousands of yachts and other small craft. Since 1986, prior to TBT restrictions, water samples, sediments and *Scrobicularia plana* showed evidence of pollution by this compound (Langston, Burt and Mingjiang, 1987). Females of *Nucella lapillus* inhabiting the English Channel also showed evidence of imposex by that time (Gibbs and Bryan, 1986; Bryan *et al.*, 1987). TBT concentrations higher than 100 ng/L (as Sn) were present in each of the Southampton Estuaries prior to restrictions in 1987 (Langston *et al.*, 2015). After the 1986 TBT restrictions, a slow decrease in concentrations of these pollutants was found in water, achieving in 1995 the proposed EQS of 2 ng/L (0.8 ng/L as Sn), initially projected for 1995 in the Hamble (Langston *et al.*, 2015). By 2009, TBT concentrations in clams from these waters were found indicating a persistent contribution to sub-lethal effects in species inhabiting this area (Langston *et al.*, 2015). The slow recovery from TBT pollution in Southampton water could reflect some release from sediments to the water column (supplemented by dredging), residual TBT on small boats, and continuing influence of large vessels in Southampton Water. By 2009 mean TBT seawater concentrations (ng/L as Sn) were reported between 4.5 and 10.4 in the Hamble Estuary, whilst mean sediment TBT concentrations (µg/g as Sn) were 0.02 in the same area (Langston *et al.*, 2015). TBT concentrations have been reported as high as 100 ng/L in the English Channel (Alzieu, 2000). The tin and organotin compounds can also be found in under-lying paint

layers on boats built before the prohibition of organotin compounds in antifouling paints and it has also reported that leaching of the old existing TBT layer paint from leisure boats occurs around the Baltic Sea (Eklund and Watermann, 2018). Furthermore, the illegal application remains unknown (Santillo, Johnston and Langston, 2001) and TBT concentrations could be even higher in countries that are not part of the IMO which have no restrictions. For instance, TBT pollution remains a serious environmental issue in the South American coastal areas (Castro, Perina and Fillmann, 2012; Batista, Castro and Fillmann, 2016) where only a few countries are signatories of the IMO antifouling systems convention.

Consequently, the aims of this chapter were to determine the effect of the exposure to TBT on survival, growth, and reproductive parameters of *O. edulis* after exposure to TBT under laboratory conditions. Understanding the alterations resulting from TBT exposure can help to clarify if pollution by this organotin could be one of the causes for the declining populations of *O. edulis* around the UK.

5.2 Methods

5.2.1 Biological Material

Ostrea edulis > 2y old (5-7 cm at their maximum diameter) were obtained from Galway Bay in Ireland in January 2018 and were transferred to the NOCS where they were acclimated for four weeks (see Section 4.2.1).

5.2.2 Feeding of oysters

During this period animals were fed *ad libitum* daily with 40,000 cells/ml of a mixed algae diet (40% *Tetraselmis suecica*, 40% *Pavlova lutheri* and 20% *Phaeodactylum tricornutum*) (see procedure in Section 2.2.2).

5.2.3 TBT treatments

After the acclimation process and before starting the exposure experiments, 6 oysters were taken as a control and these data are referred to as initial time (t0). One-hundred and twenty oysters were divided randomly among four treatment tanks: 20 ng/L Tributyiltin chloride (TBTCl), 200 ng/L TBTCl, 2000 ng/L TBTCl and a negative control. The lowest and medium concentrations are close to environmentally relevant values and have been reported in Southampton water samples (Langston *et al.*, 2015). The highest concentration tested in this study exceeded environmental levels but it has been shown that the concentration of TBT in oysters can be much higher than in environment

(for instance, tissue levels of oysters chronically exposed to 0.1 µg/L in water averaged about 7500 µg/L (Anderson, Unger and Burreson, 1996).

All the treatments were kept at 10°C because in the previous experiment two months of exposure at that temperature resulted in a similar proportion of females and males (Chapter 2). Also at that temperature gonadal development was slower so any response in the current experiment was a direct effect of the treatments rather than a maturation process resulting from incubation temperature. Water temperatures were controlled throughout the experiments using a free-standing chiller unit (TECO, model TR60). The salinity, pH, temperature, conductivity and dissolved oxygen were measured in every aquarium at least twice per week.

TBTCl (Sigma–Aldrich; Stenheim, Germany) dissolved in 100% ethanol, which was then dried under a nitrogen stream and diluted 1:100 with 1-µm filtered sterilized seawater to give a 0.5mg/mL stock solution. Processes such as adsorption of TBT into the wall of the glass containers and/or microbial degradation can occur so the water was renewed and spiked with TBTCl every 48h (renewal volume 80% of total aquarium water).

After 9 weeks of exposure, the individuals (n=13 per treatment) were sacrificed. The oysters were opened and dissected in saltwater in order to reduce stress and damage to tissues. Then samples were fixed in Bouin's solution for histological analysis or kept at -20°C for biochemical analysis.

5.2.4 Biological indices

Measurements of H, L, Wi, SVol, FW and W were taken following the same criteria referred to in Chapter 2 (see Section 2.2.4).

5.2.5 Histological analysis

For each oyster, gametogenic stages and sex were determined by histological examination of a 5-mm thick section of the visceral mass fixed in Bouin's solution (Howard *et al.*, 2004; Kim, Ashton-Alcox and Powell, 2006) (Sigma-Aldrich™, Dorset, UK) for 24h (see Section 2.2.5). Sex was recorded as indeterminate (I), female solely (F), male solely (M), hermaphrodite with both sexes equally represented (HBS), hermaphrodite predominantly male (HPM) and hermaphrodite predominantly male (HPF) according to (da Silva, Fuentes and Villalba, 2009). The gametogenic stage of the gonad was identified as inactive (G0), early gametogenesis (G1), advanced gametogenesis (G2), ripe gonad (G3), partially spawned gonad (G4) and reabsorbing gonad (G5) adopted by da Silva, Fuentes and Villalba (2009).

5.2.6 Energy reserves

Energy reserves were quantified in the gonad of each oyster following the same procedure explained in Section 3.2.5.

5.2.7 Steroid Hormone Homologue Analysis

Extraction and analysis of homologues of the sex hormones E_2 and T concentrations were quantified in the gonads of each oyster using enzyme-linked immunosorbent assay (ELISA) kits (Cayman Chemical Co.; Ann Arbor, MI, USA) as described by Gauthier-Clerc, Pellerin and Amiard (2006) (for more detail refer to Section 2.2.6). Mean intra-assay CVs for standards and samples were ≤ 8.6% for E_2 and ≤ 7.1% for T. Mean inter-assay CVs were ≤ 11.2% and ≤ 8.74% for E_2 and T, respectively.

5.2.8 Metabolomic profile of TBT-exposed *Ostrea edulis*

To characterize the metabolic changes that occurred in *O. edulis* during the treatments, gonadal samples (0.1 g) were immediately placed into liquid nitrogen and stored at -80°C until further analysis. The metabolomic profiles of all the animals per treatment was carried out by the McCullagh Metabolomics Laboratory for untargeted metabolomics, Department of Chemistry at the University of Oxford. See Section 4.2.8 for the description of the method.

5.2.9 Statistical analysis

The normality of data and homogeneity of variances were evaluated using the Shapiro Wilk and the Levene's tests, respectively. The assumptions of parametric tests were not met, so non-parametric tests were applied. The Kruskal-Wallis H-test was used to determine differences in mortality, biometric parameters (W, H, Wi, SVol, FW, CI), biochemical variables (lipids, carbohydrates, proteins, sex steroid hormones concentrations) and gonadal development. When non-parametric Kruskal and Wallis test was significant, differences were then evaluated using a non-parametric the Mann and Whitney test. Spearman's correlation was used to test the relationship between biochemical variables (lipids, carbohydrates, proteins, sex steroid hormones, Vtg-like proteins). Chi-square statistics were used to test sex ratios against a 1:1 ratio. Metabolomics results between the treatments and the control were analysed using univariate statistical analysis determining fold-change and t-tests between experimental groups for compound features and combined in volcano plots (FDR-adjusted p-values should be reported). PCA and PLS-DA were also used to analyse the patterns in metabolomic profiles among treatments (For more details of metabolomics analysis and data manipulation refer to section 4.2.9) Statistical significance was assigned at $p \leq 0.05$.

5.3 Results

5.3.1 *Ostrea edulis* mortality and growth

The mortality of oysters at the end of the trial was significantly ($p<0.05$) affected in all treatments compared to the control. At the end of the trial the highest mortality (34.3%) was observed for the highest concentration of TBT, followed by 26.9% at 200 ng/L and 21.8% at 20 ng/L (Fig 5.2). The lowest and medium TBT concentrations presented a similar increasing trend in mortality, but the highest concentration presented significant higher mortality ($p<0.05$) compared with the other treatments since the beginning of the exposure.

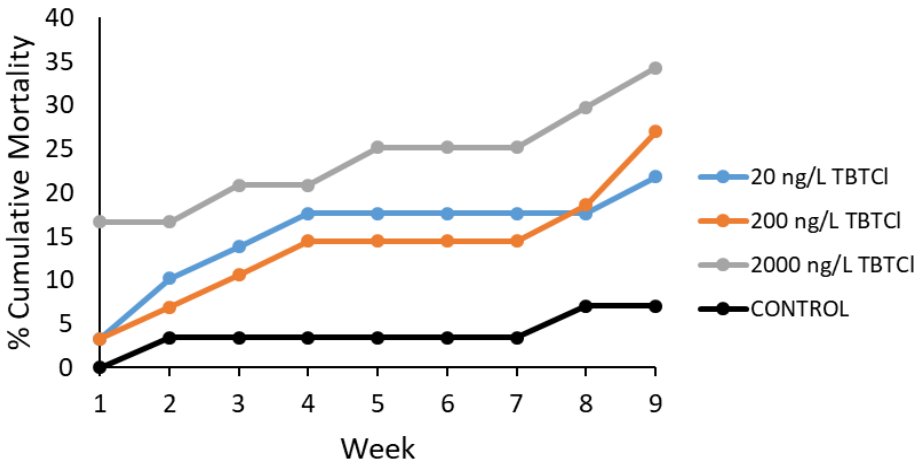

Figure 5.2 Cumulative mortality of *Ostrea edulis* treated with 20 ng/L (n=30), 200 ng/L (n=30), 2000 ng/L of TBTCl (n=30), and a negative control (n=30) during 9 weeks.

Table 5.1 *Ostrea edulis* biometric parameters (mean±SD) for total Weight (W), Height (H), length (L), width (Wi), Shell volume (SVol), flesh weight (FW) and Condition Index (CI) at the beginning of the experiment (t0), under different treatments (20 ng/L, 200 ng/L and 2000 ng/L of TBTCl), and a negative control after 9 weeks.

Treatment	n	W (g)	H (mm)	L (mm)	Wi (mm)	SVol (ml)	FW (g)	CI
t0	6	71.66±10.99	77.20±3.00	72.02±7.05	19.33±4.22	48.37±9.71	8.29±2.03	11.49±1.83
20 ng/L	13	63.30±7.05	70.56±10.43	71.22±4.92	20.96±3.04	45.46±6.12	6.83±0.80	10.86±1.27
200 ng/L	13	63.26±11.23	75.01±3.32	70.66±4.49	21.94±2.43	38.51±6.03	6.64±1.11	10.81±2.76
2000 ng/L	13	68.44±9.75	71.35±4.17	71.87±3.89	19.42±2.45	38.97±8.07	6.93±1.72	10.05±1.44
Control	13	65.51±10.09	73.51±5.46	71.44±3.93	20.00±2.50	35.91±6.63	7.41±1.18	11.40±1.75

No significant differences in any of the biometric parameters between the experimental and control groups were found (Table 5.1). TBT treatments did not show any detectable effects on the overall growth of the oysters or the soft body parts after nine weeks of exposure.

5.3.2 Effect of TBT treatments on gonadal development in *Ostrea edulis*

There was no significant difference in the gonad developmental between control oysters and those exposed to TBT (Mann Whitney U test, $p > 0.05$) (Fig 5.3). At the beginning of the experiment, 66.67% of the oysters were classified in gonadal stage G1 and 33.33% in G2. At the end of the exposure the control group 15.38% of the oysters were classified in stage G0, 61.54% in stage G1, 15.38% in stage G2 and 7.69% in stage G3. No oysters were found in stages G4 or G5 for the control group.

In the present study, exposure to TBTCl caused an increase in the percentage of animals classified as inactive with 33.33%, 30.77% and 69.23% for the lowest, medium and highest concentration, respectively. Overall the analysis of gonadal development showed an increase in early stages (G0, G1 and G2) for all the treatments compared to the beginning of the trial. At 200 ng/L of TBTCl an increase in oysters classified in G0 (33.33%) was found. The percentage of oysters in G1 (58.33%) at this treatment was similar compared to the t0 (66.67%) and the control group (61.54%).

Compared with the lowest concentration, oysters treated with 200 ng/L of TBTCl showed a similar percentage of oysters classified in stage G0 with fewer oysters at G1 (35.71%). At the medium concentration oysters classified in stage G2 (7.14%), G3 (14.29%) and G4 (14.29%) were found.

With the 2000 ng/L of TBTCl, a considerable increase in oysters classified in stage G0 (61.53%) was found compared to the other two treatments and the control group. At this treatment no oysters classified as G1 were found. Compared to the other treatments at this concentration a higher percentage of oysters classified in stage G2 (21.43%) was observed. At this treatment, 7.14% of oysters were classified in G3 and G4.

5.3.3 Effect of TBT treatments on sex ratio in *Ostrea edulis*

Exposure to TBTCl caused significant changes ($\chi^2 = 18.27$, df = 1, $p = 0.006$) in the proportion of sex categories in the experimental groups (Fig 5.4). The percentage of females decreased in all treatments with 33.33% at 20 ng/L, 23.08% at 200 ng/L and 23.08% at 2000 ng/L compared to 80% at t0 and 53.85% at the control group. Exposure of *O. edulis* to 20 and 200 ng/L of TBTCl increased the number of males over 9 weeks of exposure with 25% and 38.46% of the individuals classified as males, respectively. Interestingly at 2000 ng/L of TBTCl no males were found.

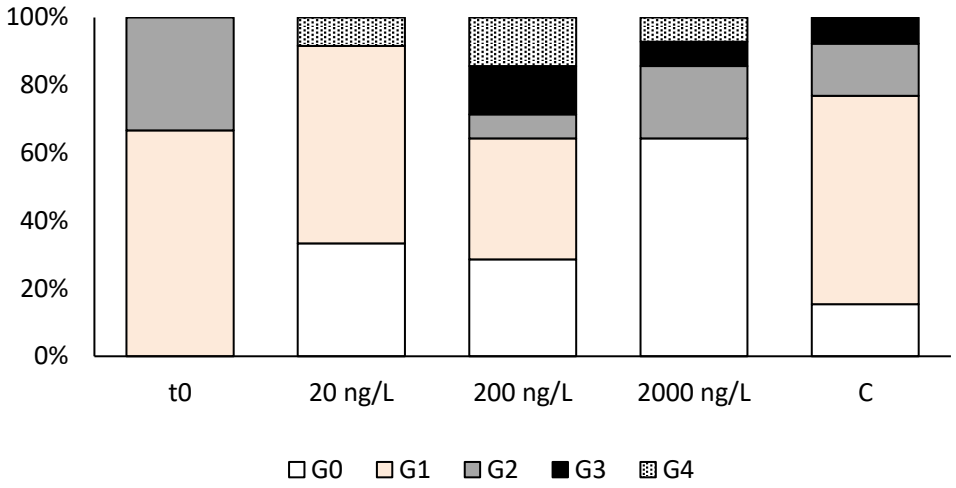

Figure 5.3 Distribution of *Ostrea edulis* at different stages of gonad development at the beginning of the experiment (t0; n=6), exposed to three different concentrations of TBTCl: 20 ng/L (n=13), 200 ng/L (n=13) and 2000 ng/L (n=13), and a negative control (n=13) kept under laboratory conditions for 9 weeks. According to (da Silva, Fuentes and Villalba, 2009) developmental stage was classified by the gametogenic stage of the gonad as inactive (G0), early gametogenesis (G1), advanced gametogenesis (G2), ripe gonad (G3), partially spawned gonad (G4) and reabsorbing gonad (G5).

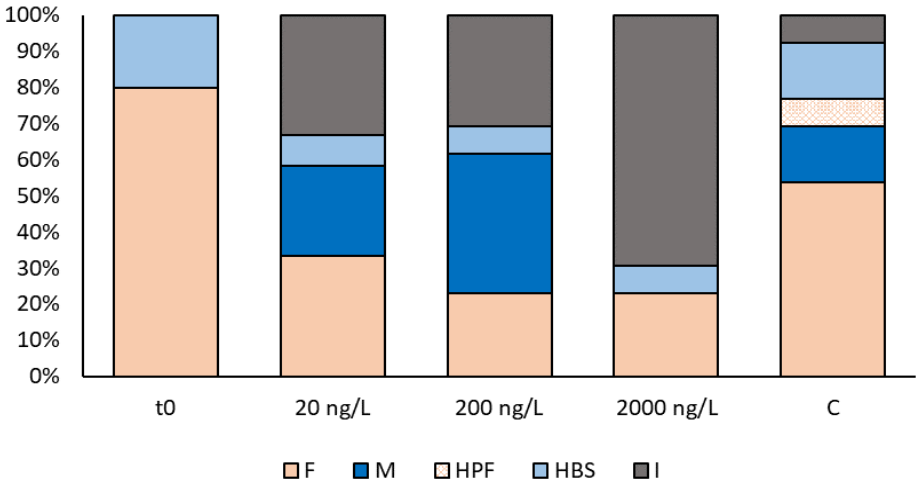

Figure 5.4 Proportion of sex categories in *Ostrea edulis* at the beginning of the experiment (t0; n=6) exposed to three different concentrations of TBTCl: 20 ng/L (n=13), 200 ng/L (n=13) and 2000 ng/L (n=13), and a negative control (n=13) kept under laboratory conditions for 9 weeks. Specimens were identified by histological examination as females (F), males (M), hermaphrodite with both sexes equally represented (HBS), hermaphrodite predominantly female (HPF) an indeterminate (I).

Hermaphrodites (HBS) were observed from the beginning of the exposure. A similar percentage was found at t0 (20%) and in the control group (15.38%) at nine weeks, but this value was reduced in all TBT exposure treatments (between 7-8%). Just one oyster was classified as hermaphrodite predominantly female (HPF) during the trial for the control group. No oysters classified as hermaphrodite predominantly male (HPF) were found in this study. Sex ratios (male:female) were not significantly shifted from the expected proportion of sexes (i.e. 1:1).

5.3.4 Effect of TBT treatments on sex steroids in *Ostrea edulis* gonadal tissue

E_2 and T concentrations analysed by ELISA increased at all treatments showing significant differences during the trial (Kruskal-Wallis test value = 41.673, $p<0.01$ for E_2, $p=0.01$ for T, N = 63) (Fig. 5.5). Oysters treated with 20 ng/L and 200 ng/L of TBTCl showed a significant decrease in E_2 (Mann Whitney U test, $p < 0.05$) compared to the control group. No significant differences in testosterone levels with respect to the control group were found.

The concentration of both hormones was very similar between treatments and was statistically significantly different between the lowest and the highest treatments. Although a trend was observed, the large variability detected among samples reduced the differences between treatments. No significant correlation between both hormone concentrations was found.

In this study, an increase in the concentration of free sex steroid homologues compared to the control was observed. However, the trend was different for both hormones. While the levels of free estradiol decreased in TBT-treated oysters, the concentration of free testosterone was similar between treatments.

It was evident that male and female phase oysters had similar hormone concentrations in all the TBT treatments and no significant differences between sexes were found (Table 5.2) indicating that there is no gender-specific response induced by TBT in *O. edulis.* This analysis was not possible for all the treatments because at the beginning and at the end of the trial at the highest concentration no males were found.

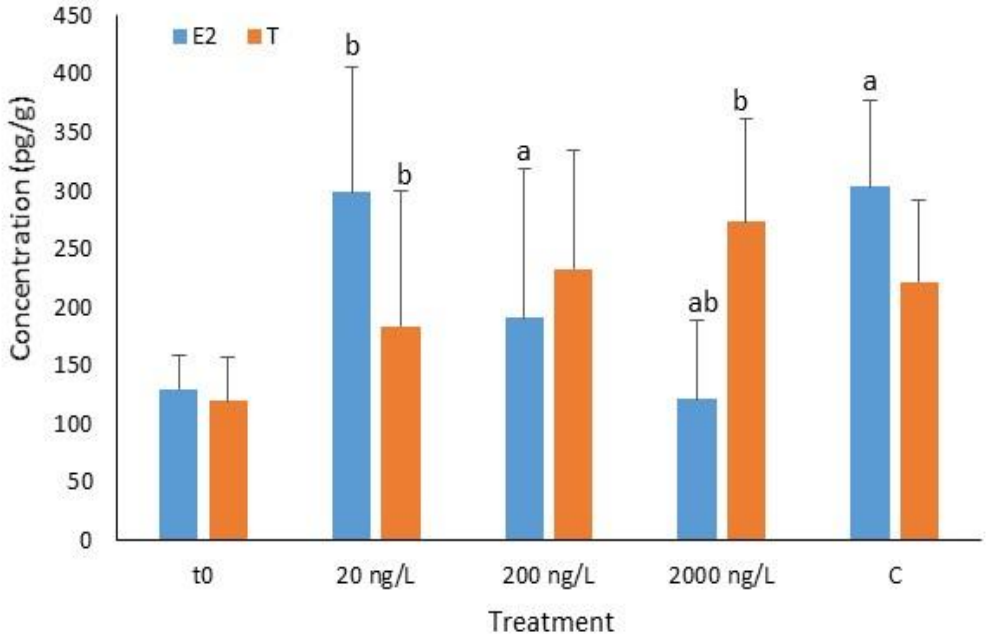

Figure 5.5 Hormone concentrations in *Ostrea edulis* at the beginning of the experiment (t0; n=6), exposed to three different concentrations of TBTCl: 20 ng/L (n=13), 200 ng/L (n=13) and 2000 ng/L (n=13), and a negative control (n=13) kept under laboratory conditions for 9 weeks. (a) Significant differences compared to the control group. (b) Significant differences between concentrations for the same hormone. Error bars denote standard deviation.

Table 5.2 Estradiol and testosterone concentrations (pg/g) for *Ostrea edulis* classified through histology as females (F) and males (M) at the beginning of the experiment (t0), exposed to three different concentrations of TBTCl: 20 ng/L (n=13), 200 ng/L (n=13) and 2000 ng/L (n=13), and a negative control (n=13) kept under laboratory conditions for 9 weeks. (*) significant differences for the same hormone at the same treatment.

Treatment	Estradiol (pg/g)		Testosterone (pg/g)	
	F	M	F	M
t0	135.89±28.95		123.19±43.18	
20 ng/L	497.39±180.00*	262.64±49.82*	179.42±27.47	118.06±42.49
200 ng/L	337.61±159.38	129.17±32.60	205.16±34.52	127.67±34.31
2000 ng/L	103.11±30.80		129.93±20.23	
Control	561.65±195.57*	188.70±116.85*	520.25±217.05*	150.14±0.96*

5.3.5 Change in the regulation of molecules related to vertebrate-sex steroid pathway on *Ostrea edulis* exposed to TBT

The metabolomic analyses of *O. edulis* exposed to different concentrations of TBTCl showed changes in ion profiles compared to the control group (Fig 5.6). The analysis of global biochemistry showed an overall change in the performance of oysters exposed to all treatments TBTCl compared to the control group suggesting that several pathways involved in survival and homeostasis could be affected by the exposure to TBT. The PLS-DA model was well-validated using a permutation test and presented a $Q^2 > 0.8$ in all cases. Combined, PCA components 1 and 2 explained 42.3% of the total variance among concentrations of TBTCl (Fig 5.6A). PLS-DA revealed separate clustering of all treatments, and components 1 and 2 explained 33.6% of the total variance among concentrations of TBTCl (Fig 5.6B).

There is a considerable amount of ions showing changes in oysters exposed to the different treatments. The general metabolomic profile shows an effect on biochemical pathways involved in diverse functions such as gluconeogenesis, energy balance, mitochondrial electron transport chain, etc. (see an example in Appendix G). From the total information obtained in this analysis, some of the main metabolites and the pathways affected were selected to understand the overall effect of exposure to TBTCl (see an example in Appendix H).

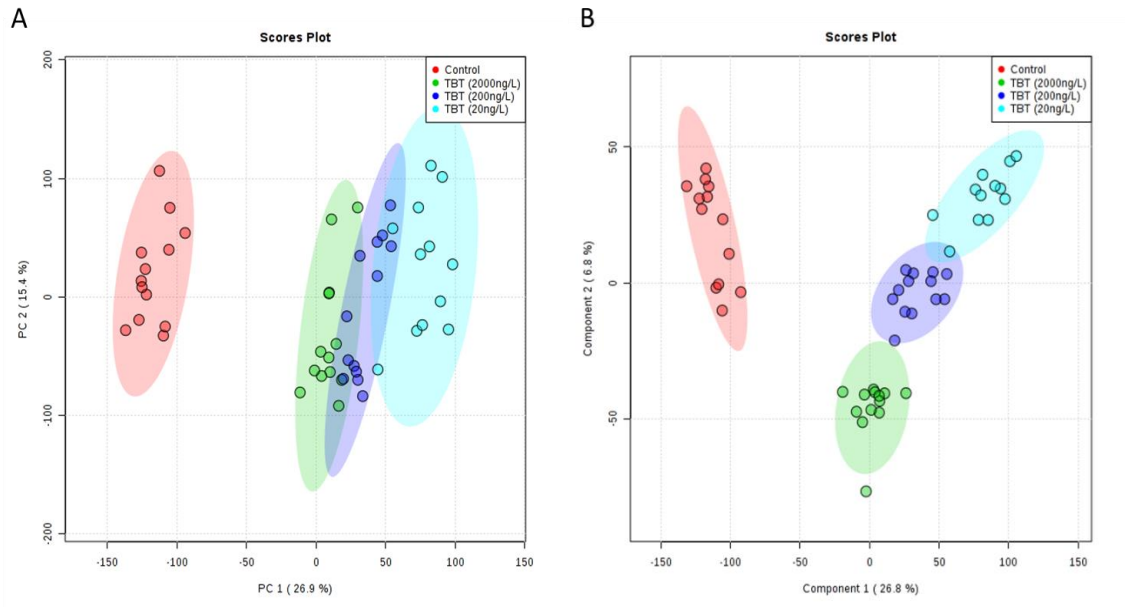

Figure 5.6 (A) Principal component analysis and (B) Partial least square discriminant analysis model comparing *Ostrea edulis* metabolomic profiles among TBTCl treatments: 20 ng/L (n=13), 200 ng/L (n=13) and 2000 ng/L (n=13), and a negative control (n=13) after 9 weeks of exposure.

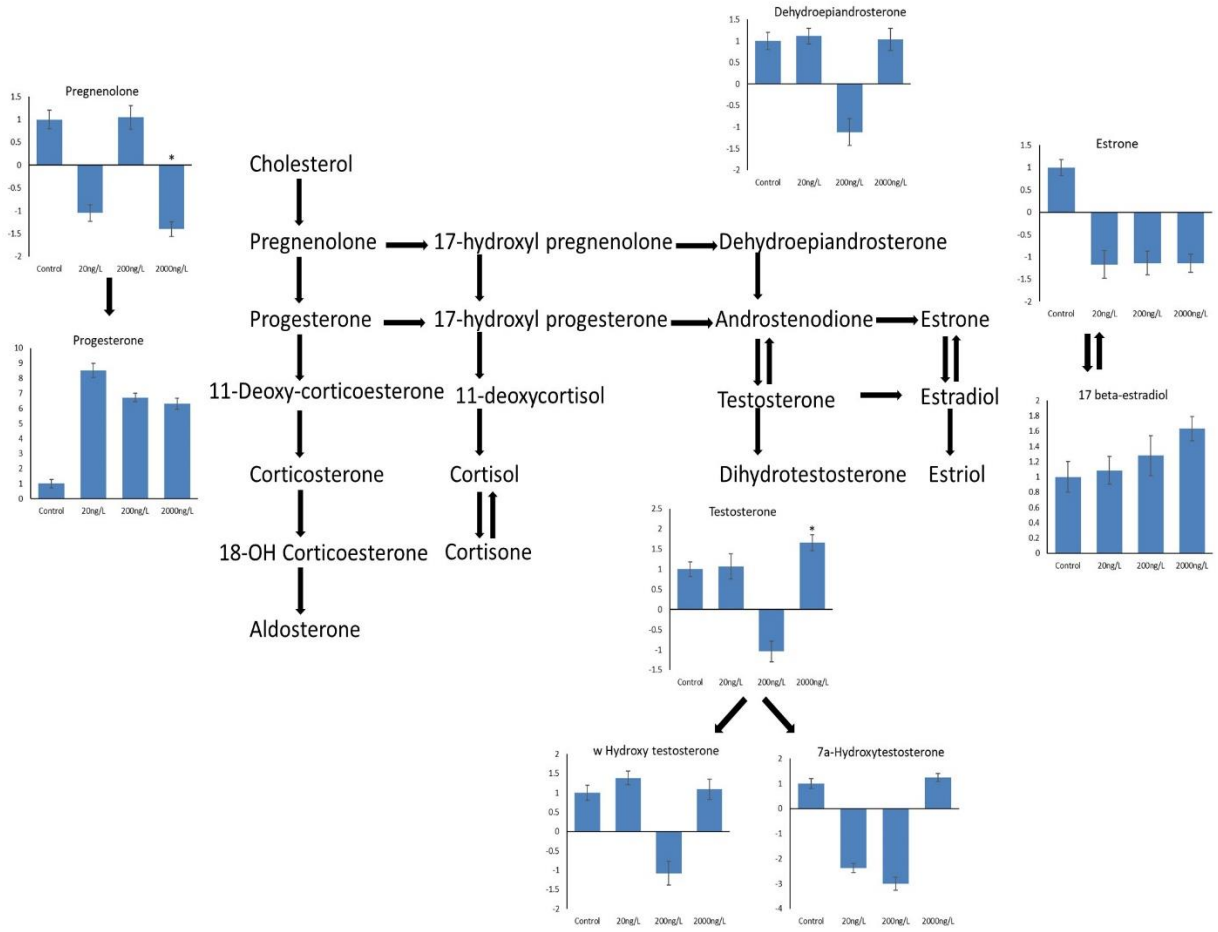

Figure 5.7 Presence and fold changes of some of the molecules expected according to the vertebrate-sex steroid pathway from cholesterol. *Ostrea edulis* exposed to three different concentrations of TBTCl: 20 ng/L (n=13), 200 ng/L (n=13) and 2000 ng/L (n=13), and a negative control (n=13) kept under laboratory conditions for 9 weeks. (*) Significant differences ($p<0.05$) respect to the control group.

Based upon the aims and objectives of this thesis, and the focussed pathway shown in Fig 1.5, Figure 5.7 describes fold changes on identified ions that could be considered as part of the vertebrate sex steroid pathway from a cholesterol starting substrate. There is no obvious trend in the up or down-regulation of these molecules in oysters treated with three different concentrations of TBTCl (Fig 57). The analysis of 17B-estradiol showed an increase in all the treatments compared to the control group (Fig 5.7). Testosterone was found to be increased up-regulated in oysters exposed at the lowest and highest concentration of TBT, but not for oysters exposed at 200 ng/L (Fig 5.7).

5.3.6 Changes in energy reserves and energy production in *Ostrea edulis* after exposure to TBT

The changes in the biochemical composition of gonad visceral mass of *O. edulis* throughout the experiment are shown in Fig. 5.8. Oysters treated for 9 weeks with the three concentrations of TBTCl showed a significant increase ($p<0.001$) in the total lipid content compared to the control group (40.58±11.27 %DW). The content of total fatty acids in TBT-treated oysters presented significant differences between treatments with the highest value reached at 200 ng/L (55.60±5.21 %DW), followed by treatments with 2000 ng/L (46.73±3.81 %DW) and 20 ng/L (30.31±0.89 %DW).

A slight decrease in carbohydrate content for all treatments compared to the t0 was observed (Fig. 5.8). However no significant differences were found compared to the control group (13.67±6.60 %DW). At the end of the trial, a decrease in carbohydrate content for the control group compared to t0 was observed.

The protein content of oysters treated with 2000 ng/LTBTCl for 9 weeks showed significant changes ($p<0.001$) with respect to the control group (34.74±7.49 %DW) (Fig. 5.8). Significant differences ($p<0.001$) in protein content between treatments were found. Oysters exposed to TBTCl showed an increase in protein content in a concentration-dependent manner with the highest value (54.63±16.40 %DW) observed at 2000 ng/L.

Analysis of metabolites showed that some of the main molecules affected by the treatments were those involved in the citric acid cycle pathway (Fig. 5.9). In summary, with all treatments a reduction of the TCA cycle intermediates such as citric acid, ketoglutaric acid, fumaric acid, malic acid and oxalacetic acid was observed suggesting a disturbance in TCA cycle metabolism. Furthermore, glucose - the preferred energy source for most body cells – showed a decrease in all the treatments compared to the control group. These changes could be related to the down regulation of the principal molecules for storing and transferring energy in cells (such as ATP and GTP) (Fig 5.10). Analysis of adenosine triphosphate (ATP) and guanosine triphosphate (GTP) and related metabolites showed a down-regulation in oysters treated with TBT compared with the control group (Fig 5.11). In accordance with the previous result showing that TCA cycle activity is less in oysters exposed to TBT a decrease in the ATP levels could be expected in these animals (Fig 5.10).

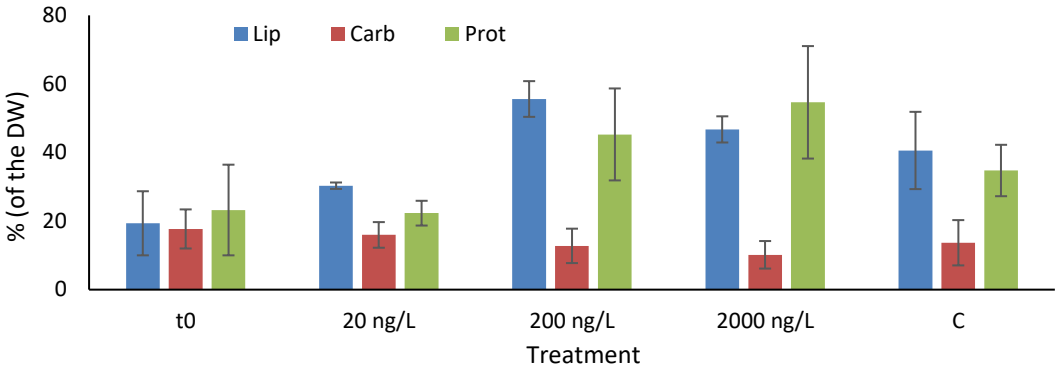

Figure 5.8 (A) Total lipids (% of dry weight, % DM), (B) total carbohydrates (% of dry weight, % DM) and (C) protein content (% of dry weight, % DM) of *Ostrea edulis* at the beginning of the experiment (t0), exposed to three different concentrations of TBTCl: 20 ng/L (n=13), 200 ng/L (n=13) and 2000 ng/L (n=13), and a negative control (n=13) kept under laboratory conditions for 9 weeks. Error bars denote standard deviation.

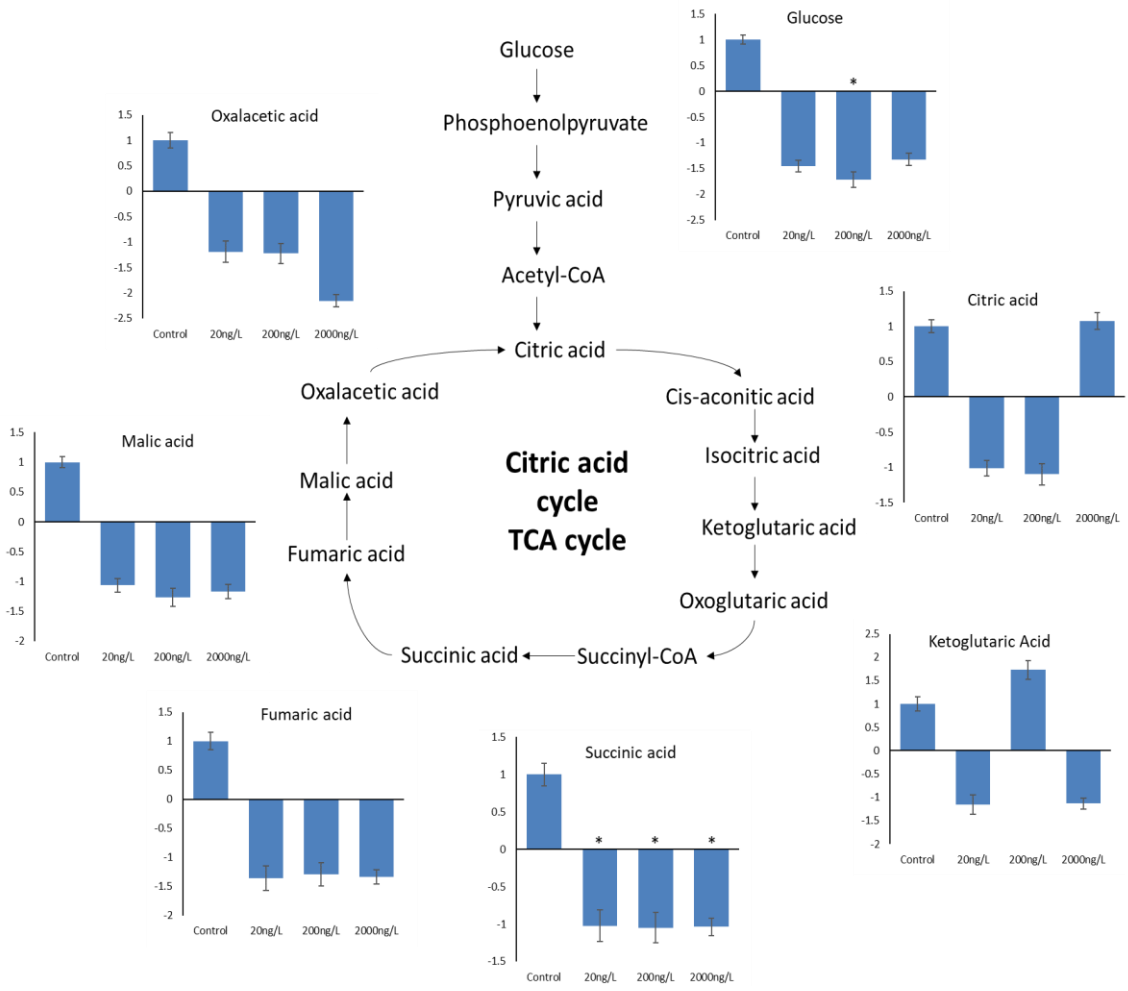

Figure 5.9 A summary of the biochemical pathway and fold changes in some of the molecules of TCA cycle metabolism in *Ostrea edulis* exposed to three different concentrations of TBTCl: 20 ng/L (n=13), 200 ng/L (n=13) and 2000 ng/L (n=13), and a negative control (n=13) kept under laboratory conditions for 9 weeks.

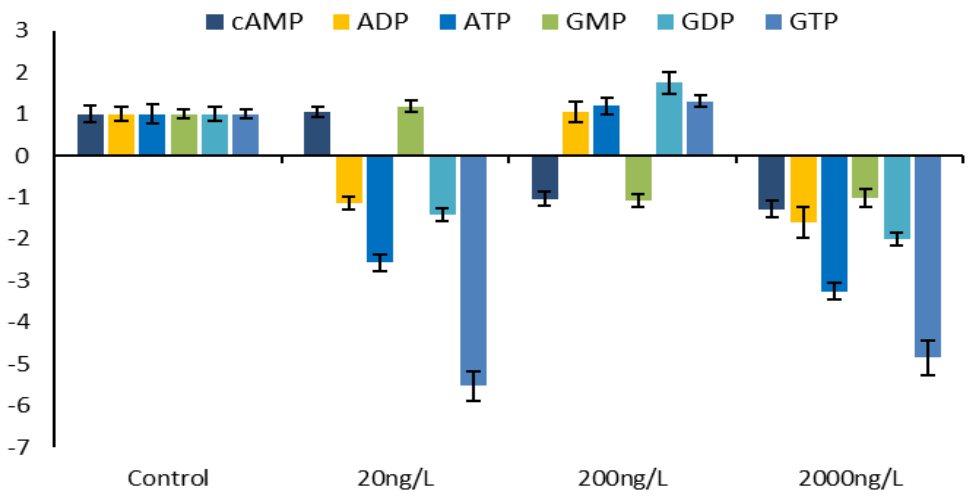

Figure 5.10 Fold changes for the energy molecules cAMP, ADP, ATP, GMP, GDP and GTP in *Ostrea edulis* exposed to three different concentrations of TBTCl: 20 ng/L (n=13), 200 ng/L (n=13) and 2000 ng/L (n=13), and a negative control (n=13) kept under laboratory conditions for 9 weeks.

5.3.7 Changes in oxidative stress and antioxidant defence molecules in *Ostrea edulis* exposed to TBT

8-Oxo-2'-deoxyguanosine-5'-Triphosphate (8-Oxo-dGTP), considered as a biomarker of oxidative stress and potential DNA damage, showed an increase compared to the control (Fig. 5.11A).

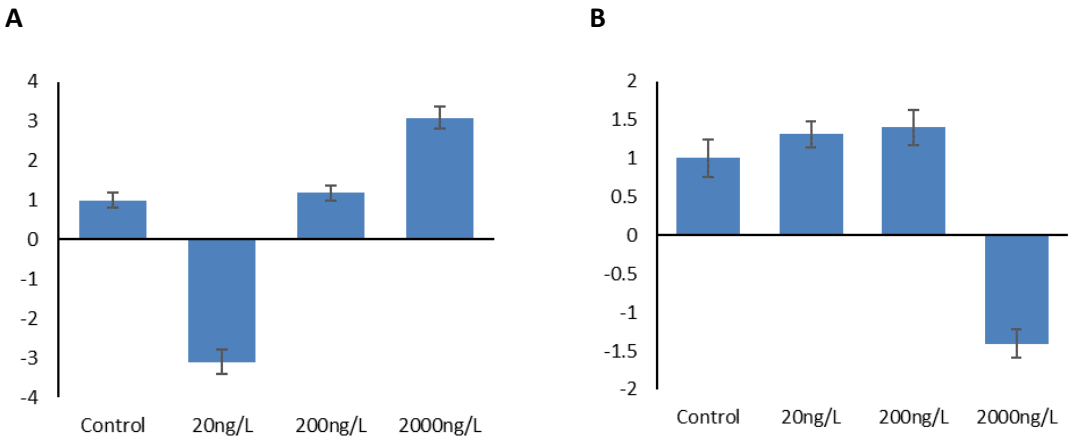

Figure 5.11 Fold changes of molecules involved in oxidative stress ((A)8-Oxo-deoxyguanosine) and antioxidant defense ((B)Glutathione) in *Ostrea edulis* exposed to three different concentrations of TBTCl: 20 ng/L (n=13), 200 ng/L (n=13) and 2000 ng/L (n=13), and a negative control (n=13) kept under laboratory conditions for 9 weeks.

Oysters treated with the lowest and medium concentrations of TBTCl showed an increase in the metabolite glutathione (Fig. 5.11B), which is part of the antioxidant system. In contrast, oysters treated with the highest of TBTCl showed a decrease in this metabolite at the end of the trial.

These results together suggest an increase in oxidative stress in *Ostrea edulis* exposed to different concentrations of TBTCl during 9 weeks.

5.4 Discussion

5.4.1 Effect of TBT treatments on survival and growth in *Ostrea edulis*

It has been reported that the half-life of TBT in the water column is approximately 6–19 days but the presence of TBT metabolites (DBT and MBT) can represent 35–56% of the butyltins detected in water (Seligman, Valkirs and Lee, 1986; Dowson, Bubb and Lester, 1996). Oysters take up butyltin compounds from the water column rapidly, quickly reach equilibrium, and then slowly depurate after an initial dose (Hall and Pinkney, 1985). Therefore, in this study the water containing the treatments was renewed and spiked with TBTCl every 48h to ensure a constant TBT concentration throughout the experiment.

It has been reported that TBT is chronically toxic for adult marine bivalves at concentrations as low as 0.2 ug/L (Héral, Alzieu and Deslous-Paoli, 1989). Thain and Waldock (1986) found that a concentration of 2.6 ug/L of TBT caused 50% mortality in *O. edulis* after 45 days exposure to TBT. In this study, *O. edulis* exposed to 20, 200 and 2000 ng/L for 9 weeks showed a significant increase in mortality compared to the control. This confirms the potential toxicity of this compound and its toxic effect on this species.

It has been reported that TBT can cause mortality in molluscs exposed to concentrations below 48-96h LC_{5O} through a mechanism of bioconcentration (Héral, Alzieu and Deslous-Paoli, 1989). Axiak *et al*. (1995) did not report mortality in *O. edulis* over the 96-h exposure period to TBT at 10 and 100 ng/l. In the same study it was reported that 20% of the animals died after 96-h exposure to 1000 ng/l of TBT (Axiak *et al.*, 1995). The high mortality observed in the present study could be a function of the duration of this study (nine weeks). This is supported by other studies carried out in oysters exposed chronically to TBTCl. For instance, adults oysters (*Crassotrea virginica*) exposed to TBT at 50, 100, 500, and 1000 ng/L for eight weeks did not show any effect on reproduction or gamete quality and the only effect observed was an increase in mortality at the highest exposure concentrations (Roberts *et al.*, 1987).

It has been shown that the growth of molluscs could be affected by exposure to TBT. Juveniles of *Scrobicularia plana* have shown a significant reduction in weight after exposure to this compound (Ruiz, Bryan and Gibbs, 1994). In this study a lack of effects on the overall growth of the oysters treated with TBTCl was observed. This could be related to the increase in stages G0 and G1 of sexual maturity for most of the animals treated with the three concentrations of TBT. The gonadal tissue is the main organ that varies throughout the reproductive cycle in bivalves (Filgueira *et al.*, 2013), so it could be expected that gonads that are not very developed (stages G0 and G1) can present a low change of weight which affects other biometric parameters.

5.4.2 Effect of TBT treatments on gonadal development and sex ratio in *Ostrea edulis*

Histological examination of the gonad showed normal development in the control animals according to what was expected at 10°C for two months at laboratory conditions (see Chapter 2). The appearance of males in the lowest and medium TBT-treated animals was expected in accordance with the reported TBT-related effects on masculinization observed in other molluscs (Bryan *et al.*, 1987; Spooner *et al.*, 1991; Oehlmann *et al.*, 1996; Matthiessen and Gibbs, 1998; Morgan, Murphy and Lyons, 1998; Oberdörster, Rittschof and McClellan-Green, 1998; Terlizzi, Geraci and Gibbs, 1999; Oberdörster and McClellan-Green, 2002; Horiguchi, 2006). This was also observed for *O. edulis* exposed to 0.24 ug/L of TBT where a predominance of males was found after 75 days of exposure (Thain, 1986).

In this study, no males were found at the highest TBT treatment and an increase of inactive individuals was observed. This was also observed by Thain (1986) in the same species exposed to a high concentration of TBT (2.6 ug/L) where little or no gonadal differentiation was observed after 75 days of exposure. Although the highest concentration used by Thain (1986) was higher (2.6 ug/L) than the highest concentration used in the present study (2 ug/L), it is clear that elevated levels of this compound cause an arrest in gonadal maturation in this species, which was reflected in the present study as an increase in oysters in stages G0 and G1. The increase in stages G0 and G1 was also observed for oysters treated with 20 and 200 ng/L of TBT in this study, although the proportion was lower.

Most of the available literature shows the masculinization effects of TBT measured in term of the imposex caused after exposure gastropods to TBT (Blaber, 1970; Smith, 1971; Bryan *et al.*, 1987; Spooner *et al.*, 1991; Gibbs, 1993; Oehlmann *et al.*, 1996; Huet, Paulet and Le Pennec, 1996; Matthiessen and Gibbs, 1998; Morgan, Murphy and Lyons, 1998; Oberdörster, Rittschof and McClellan-Green, 1998; Terlizzi, Geraci and Gibbs, 1999; Oberdörster and McClellan-Green, 2002; Horiguchi, 2006; Ketata *et al.*, 2007). However, due to the complexity and variety of

hermaphroditism strategies in bivalve molluscs (Pelseneer, 1894), changes are more subtle and a masculinization effect caused by organotin compounds is more difficult to understand. Other biological parameters need to be considered in hermaphroditic species to evaluate the effect of endocrine disruptors. For example, organotins have been shown to elicit abnormal ovarian spermatogenesis in species like the abalone *Haliotis gigantean* (Horiguchi *et al.*, 2002).

Other parameters that could be taken into consideration to evaluate the effect of these compounds in oysters are the larvae and embryonic development. Thain (1986) found that oysters exposed to 0.24 ug/L TBT went through retardation of sex change from male to female and no larvae were released from these oysters. Evidence of some deleterious effects of TBT on the embryonic development of *S. plana* has also been reported (Ruiz, Bryan and Gibbs, 1995).

5.4.3 Changes in sex steroids and their intermediate metabolites in tissue of *Ostrea edulis* after exposure to TBT

In this study, the concentration of free steroids in TBT-treated oysters increased compared to the beginning of the trial (Fig 5.5). An increase of free testosterone levels was recorded in the rockfish *Sebasticus marmoratus* after exposure to TBT (Zhang *et al.*, 2007, 2013). According to these authors, two mechanisms could be important in this increase:

1) TBT exposure inhibits the ovarian development, which relates to the increase of free testosterone:17β-estradiol ratio (Zhang *et al.*, 2007); and

2) the percentage of testosterone in an esterified form was significantly decreased in the ovaries by TBT exposure, which might be a mechanism by which free testosterone levels increased (Zhang *et al.*, 2013).

Some authors have suggested that the increase in testosterone levels in fish following TBT exposure may be caused by the inhibition of the aromatase enzyme which catalyzes estrogens from the conversion of aromatizable androgens (McAllister and Kime, 2003). In molluscs, it has been observed that TBT can alter testosterone esterification and elevate the free testosterone levels (Janer *et al.*, 2006).

Elevated free (unesterified) testosterone levels in snails have been associated with exposure to TBT, and direct exposure to testosterone has been shown to cause imposex (Gooding *et al.*, 2003). The same study found the production of testosterone-fatty acid esters decreased with increasing exposure concentration of TBT. Gooding *et al*. (2003) also found that decreased testosterone-fatty acid esterification among TBT-treated snails was not caused by direct inhibition of the acyl

coenzyme A testosterone acyltransferase (ATAT) enzyme responsible for testosterone esterification, nor by suppressed ATAT protein expression. According to the authors, these findings suggest that the observed effect for TBT in snails could be related with the testosterone fatty esterification process or a factor in the enhanced hydrolysis of the testosterone-fatty acid pool (Gooding *et al.*, 2003).

The evidence for the presence and activity of critical enzymes involved in the steroidogenic pathway such as 5α-reductase, 17β-HSD and Cytochrome P450 monooxygenase enzymes (CYP) has been evaluated and it has been accepted that these molecules are present and have a role in metabolism of sex steroids in several invertebrate species (Le Curieux-Belfond *et al.*, 2001; Janer and Porte, 2007). The formation of androgen metabolites after exposure to testosterone and estradiol, the activity of 5α-reductase and a steroid hydroxylase system similar to the one observed in vertebrate species have been reported in mussels and clams (Morcillo, Ronis and Porte, 1998; Janer *et al.*, 2005; Fernandes *et al.*, 2010). Some authors who have considered these enzymes as part of the steroidogenic reference pathway in molluscs, have reported the ability of TBT to interact with different molecules, such as aromatase and 5α-reductase, as the proposed mechanism to affect the steroid metabolic pathways (Ronis and Mason, 1996; Morcillo, Ronis and Porte, 1998; Doering *et al.*, 2002; Oberdörster and McClellan-Green, 2002; World Health Organization, 2013). In the same manner, cytochrome P450 aromatase (CYP19) is an essential steroidogenic enzyme responsible for the conversion of testosterone to estrogen and TBT has shown to inhibit its activity in fish (McAllister and Kime, 2003) and gastropods (Oberdörster and McClellan-Green, 2002).

Considering that conversion of testosterone to estradiol-17b is a key step in the steroid pathways in vertebrates, and assuming that invertebrates may present similar enzymes and pathways involved in the vertebrate sex steroids metabolism, some authors have studied and found evidence about the interaction of TBT with these molecules. For instance, Morcillo *et al*. (1998) found a significant increase in the formation of 6a-hydroxytestosterone and a dose-dependent decrease in the aromatization of testosterone to estrone and estradiol in TBT-exposed clams. These observations indicate a significant interaction of TBT with androgen metabolism in the clam *Ruditapes decussata* which may contribute to the masculinization of clam physiology (Morcillo, Ronis and Porte, 1998).

However, the androgen and estrogen metabolism in invertebrates is a controversial topic due to the lack of evidence on the synthesis and enzymatic pathways needed to metabolize sex steroid hormones (Scott, 2012, 2013, 2018). This is supported by other studies showing that homologues of both the estrogen receptor (ER) and the androgen receptor (AR) have not been found in invertebrates (Escriva, Delaunay and Laudet, 2000) and the composition of nuclear receptor family

members is very different between vertebrates and Invertebrates (Escriva *et al.*, 1997). So this evidence, alongside findings of other authors (e.g. Gooding *et al*. (2003)), indicates that testosterone fatty esterification is an effect of TBT exposure, suggesting that the observed changes in sex steroids caused by exposure to this organotin could be related to the ratio free/esterified steroid. Although esterified steroids do not bind steroid receptors, they are important because they could be considered as long-acting steroids, since they can be hydrolyzed by esterases (Hochberg *et al.*, 1991). The physiological role of esterified steroids can only be speculated upon. Recent studies showed that esterification might act as a homeostatic mechanism in molluscs to help them to maintain stable levels of free steroids (Gooding and LeBlanc, 2005; Janer *et al.*, 2005). The free steroids are able to diffuse throughout the organism and are available for a quicker excretion and metabolism, while esterified forms become immobilized in the fat for very long periods (with half-lives measured in weeks rather than days) (Scott, 2018). Although the mechanism is still unclear, this evidence could imply that the interference of TBT in the esterification or conjugation processes of free hormones can affect levels of active steroids within tissues, changing the balance between available free and esterified steroids, and may be one of the responsible mechanisms of the reported androgenization/feminization phenomena.

In the gastropod *Marisa cornuarietis,* exposure to TBT had a sex-related effect, with males presenting much higher concentration of esterified steroids than females, and females presenting lower levels than males by exposure to TBT (Janer *et al.*, 2006). In this study, no significant differences between sexes were found, indicating that no sex-specific response is induced by TBT in *O. edulis.* However, it was not possible to make the analysis for all TBT treatments due to the lack of males at some concentrations at the end of the trial.

Therefore, there are some doubts about the effect of organotins as inhibitors of enzymes that metabolize androgens in invertebrates. A mechanism of action of TBT or triphenyltin (TPT) on the development of imposex in gastropods was proposed, which was completely different from other mechanisms already proposed for imposex induction (Nishikawa *et al.*, 2004). Nishikawa *et al* (2004) showed that organotins were able to bind to human retinoic X receptor (hRXRs) with high affinity, and the injection of 9-*cis*-retinoic acid (9cisRA), the proposed natural ligand of the retinoid X receptor (RXR), was able to induce the development of imposex in the rock shells *Tritonia clavigera* (Nishikawa *et al.*, 2004). Castro *et al*. (2007) showed that 9cisRA was also able to induce imposex in females of *Nucella lapillus* to the same degree as tributyltin when administered at similar concentrations (1 µg/g body weight). Furthermore, the fact that male penises are also affected either by TBT or natural and synthetic RXR agonists, suggests that the normal process of accessory sex organ development in gastropods is retinoic dependent (Castro *et al.*, 2007). Retinoic acid (RA) has been implicated in the regulation of growth and patterning of many vertebrate structures

through the control of cell proliferation, differentiation and apoptosis (Filho *et al.*, 2010). Recent studies have shown that organotin compounds affect lipid homeostasis in vertebrates, probably through interaction with RXR receptors (Filho *et al.*, 2010; Delgado Filho *et al.*, 2011). Molluscs are sensitive species to the toxic effects of tributyltin (TBT), particularly to masculinization, and TBT has been recently shown to bind to molluscan RXR (Janer, Navarro and Porte, 2007). This suggests a different option and novel mechanism for organotin-induced toxic effects in invertebrates that require further exploration.

5.4.4 Effect of TBT treatments on energy reserves and energy production in *Ostrea edulis*

In the present study, there was an increase in lipid and carbohydrate content in TBT-treated oysters compared to the oysters at the beginning of the experiment (Fig 5.8). Abnormal lipid metabolism can induce negative effects on reproduction (Zhang *et al.*, 2013). TBT is recognized as a metabolic disruptor, and It has been reported that TBT exposure can influence the reproductive functions of fish through a lipotoxic mechanism (Zhang *et al.*, 2013). These authors showed that exposure of rockfish (*Sebastiscus marmoratus*) to TBT for 48 d induces a lipotoxicity response in the ovaries of this species, showing an increase of interstitial ectopic lipid accumulation and total lipids. An increase in lipid accumulation and altered fatty acid homeostasis were also observed in the digestive gland/gonad complex in the ramshorn snail (*M. cornuarietis*) exposed to 125 and 500 ng/L TBT (Janer, Navarro and Porte, 2007).

The implication of lipotoxicity varies according to the tissue analysed but the accumulation of excess lipids in nonadipose tissues leading to metabolic disorders has been reported (Weinberg, 2006). There is no evidence of an adverse effect of lipids accumulation in gonadal tissues of oysters but it is a key issue that requires further investigation. Studies in rodent models have shown that obesity could delay oocyte maturation, decrease *in vivo* fertilization rates and decrease developmental competence assessed as abnormal development to the blastocyst stage (Reviewed by Robker *et al*., 2011). Thus, an adverse effect could be expected in the accumulation of lipids in oyster tissues affecting development and reproductive attributes. In addition, the excessive accumulation of lipids in nonadipose tissues can exceed the cell's capacity to store or use them generating a lipotoxicity response, which is characterized by destruction of organelle membranes and the activation of stress pathways that can lead to apoptosis (Brookheart, Michel and Schaffer, 2009).

Energy reserves and lipid synthesis are processes required for gonadal maturation and animals use these reserves for to complete gametogenesis (Mori, 1969; Mori, Muramatsu and Nakamura, 1972;

Wang, 2000). Also during gametogenesis, glycogen stored in the gonad is broken down into glucose to produce ATP, NADPH, and NADH which are necessary for the synthesis of other organic compounds including fatty acids and nucleic acids (Wang, 2000). The analysis of molecules involved in the TCA cycle and the energy molecules (cAMP, ADP, ATP, GMP, GDP and GTP) showed a reduction of these ions in TBT-treated oysters (Fig 5.10 and 5.11). This could be related to the prevalence of early gonadal stages (G0 and G1) found in *O. edulis* (Fig 5.3), indicating an arrest in gonadal development due to a lack of energy available to go through this process. Taking together these results suggest that oyster exposed to TBT are not able to synthesize enough ATP to undergo gametogenesis successfully so hence the decrease in reaching maturity.

The perturbance in the TCA cycle observed in this study could be reflected in the high mortality presented by *O. edulis* exposed to TBT (Fig 5.9). The respiratory pathways of glycolysis, the tricarboxylic acid (TCA) cycle and the mitochondrial electron transport chain are essential for both energy provision in heterotrophic cells and a wide range of other physiological functions and survival mechanisms (Fernie, Carrari and Sweetlove, 2004). Enzymes and proteins that participate in these pathways are well known, but their regulation and control are much less well understood and just a few reports have shown the importance of those processes in invertebrates (Alp, Newsholme and Zammit, 1976).

The results of this study suggest that *O. edulis* exposed to 20, 200 and 2000 ng/L TBTCl go through a physiological imbalance affecting lipid homeostasis, while a reduction in energy molecules and a perturbance in TCA cycle occur in this species. This study suggests that exposure to TBT has a global effect involving lots of biological functions rather than just the steroidogenic pathways. Evidence suggests that these oysters could be struggling to survive and keep homeostasis rather than producing mature gonads, therefore “healthy” animals would not be able to produce gametes due to a lack of ATP and resources (from energy reserves) to invest in this process. According to these results a TBT mechanism of action in *O. edulis* is proposed in Figure 5.12.

5.4.5 Effect of TBT exposure on oxidative stress and antioxidant system in *Ostrea edulis*

In this study, the ATP decreased in a concentration-dependant manner in TBT-treated oysters compared with the control group (Fig 5.11) suggesting that animals exposed to this pollutant were going through an ATP imbalance. So it could be expected that organotins can trigger apoptosis via the mitochondrial pathway by blocking mitochondrial ATP synthesis and increasing the oxidative stress by promoting ROS production (Jaksic, 2012). It is known that organotin compounds can induce apoptosis (Aw *et al.*, 1990; Raffray and Cohen, 1991). Tributyltin chloride (TBTCl) and

triphenyltin chloride (TPhTCl) are able to trigger cytoskeletal modifications, apoptosis and disruption of mitochondrial function by cessation of ATP synthesis, leading to incomplete reduction of O_2 and subsequent production of reactive oxygen species (ROS) and the loss of mitochondrial membrane potential (ΔΨm) (Jaksic, 2012).

Blocking mitochondrial ATP synthesis is one of the vias by which organotins trigger apoptosis. These pollutants are able to bind to the ATP synthase complex and inhibit ATP synthesis (Jaksic, 2012). In this study, the ATP decreased in a concentration-dependant manner in TBT-treated oysters compared with the control group suggesting that animals exposed to this pollutant were going through an ATP imbalance (Fig 5.11 and 5.12). So it could be expected that organotins can trigger apoptosis via the mitochondrial pathway by blocking mitochondrial ATP synthesis and increasing the oxidative stress by promoting ROS production (Jaksic, 2012).

Another important process that contributes to increased oxidative stress caused by pollutants is the excess production an inadequate removal of reactive oxygen species (ROS) (Simon, Haj-Yehia and Levi-Schaffer, 2000; Elmore, 2007; Hongmei, 2012). ROS are mitochondria-derived molecules generated through the cellular oxidative metabolism and when in excess, they can target the mitochondrial membrane, increasing the mitochondrial membrane gating potential and promoting cytochrome c release as well as programmed cell death (Simon, Haj-Yehia and Levi-Schaffer, 2000).

Oxidative stress occurs when there is an imbalance between the production of free radicals and the cells ability to efficiently remove them (Simon, Haj-Yehia and Levi-Schaffer, 2000; Elmore, 2007; Hongmei, 2012). Glutathione (GSH) plays a key role in the detoxification of a large number of xenobiotics, and it has been reported alongside to other antioxidant enzymes in marine invertebrates as part of the antioxidant defences (Lee, 1988; Gamble *et al.*, 1995; Belcheva and Chelomin, 2011). Conjugation with GSH is one biotransformation process that generally results in less toxic products abut it could be affected by organotin compounds (Lee, 1996). It is an inducible system that is stimulated and increases following an oxidative stress event (Girard, Peynot and Lelièvre, 2018). TBT has shown an oxidative stress effect caused by inhibition of this enzyme in rat cell cultures (Ishihara *et al.*, 2012) and fish (Wang *et al.*, 2006; Li, Li and Shi, 2015b). However, some studies did not find any change in the activity of this enzyme after exposure to TBT compounds in *Daphnia magna* (Schmidt *et al.*, 2006) or the gastropod *Buccinanops globulosus* (Primost *et al.*, 2017). Further studies relating to the oxidative stress response in molluscs exposed to TBT because the information about its role in TBT-oxidative stress-related effects is still lacking. The antioxidant system can change in response to environmental stressors and seasonal changes as it has been shown in the bivalve *Aulacomya atra* (Di Salvatore *et al.*, 2013; Giarratano, Gil and Malanga, 2013)

so careful analysis is required to understand the changes in this system in response to a particular pollutant.

Several cell types and organotin compounds have been used in research on organotin-induced programmed cell death via oxidative stress (Jaksic, 2012). It has been reported that TBT can induce apoptosis via H_2O_2 generation in human cells (Gunasekar *et al.*, 2001; Tada-Oikawa *et al.*, 2008). This process involves a sequence of events such as loss of ΔΨm, mitochondrial permeability transition, membrane depolarization, cytochrome c release and caspase activation. The association between oxidative stress and the induction of apoptosis after exposure to TBT has been also shown in fish, for instance, in early-stage zebrafish *Danio rerio* retinal neuronal cells (Dong *et al.*, 2006) and *Sebasticus marmoratus* (Wang *et al.*, 2008; Zhang *et al.*, 2008). The effect of TBT in mitochondrial integrity was studied in *Mitylus edulis,* suggesting that exposure of this species to 0.5 ug/L of TBTCl for 60 days caused degeneration in the mitochondria and disappearance of the membrane (Huang and Wang, 1995). These results together suggest that the damage of mitochondria in molluscs could be one of the most important toxic mechanisms caused by TBT exposure.

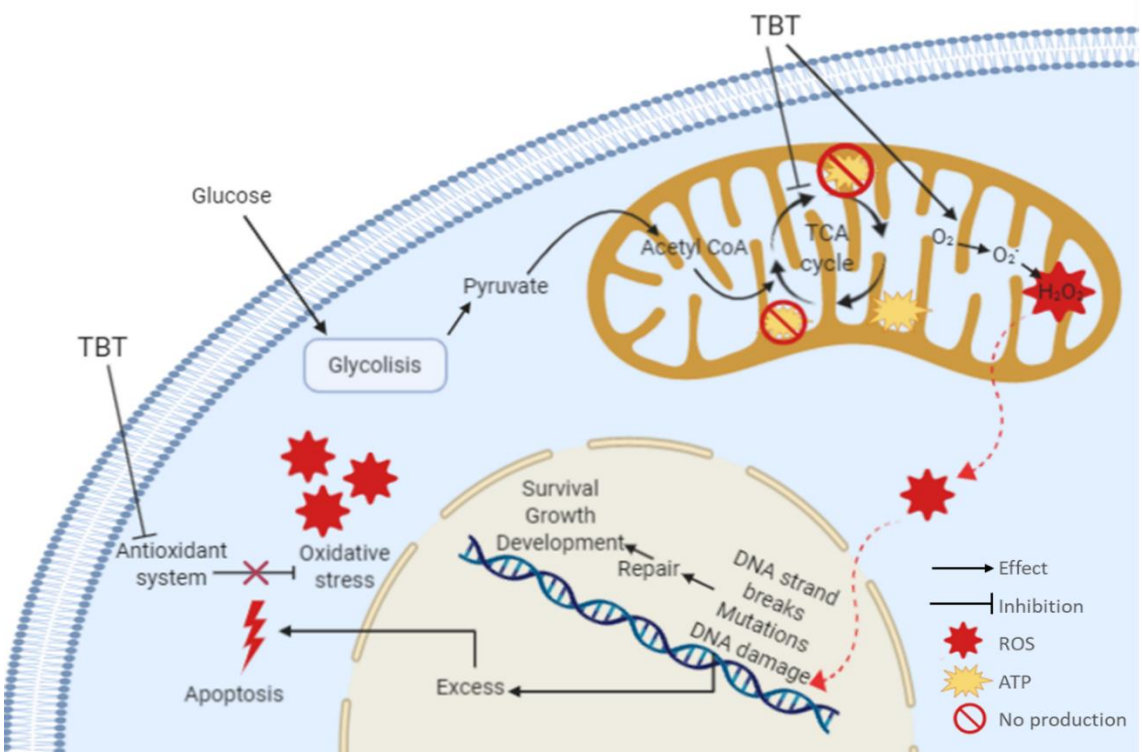

Figure 5.12 Schematic illustration with the proposed mechanism of action in *Ostrea edulis* exposed to different concentrations of TBT. Image created with Biorender (https://biorender.com/).

The analysis of the mutagenic and oxidative DNA damage product, 8-oxo-dGTP has been analysed in this study (Fig 5.11). 8-Oxo-2'-deoxyguanosine-5'-Triphosphate (8-Oxo-dGTP) is considered mutagenic dNTPs which can be incorporated into DNA. In brief, 8-Oxo-dGTP can be generated by endogenous oxidants arising in normal cell metabolism. Its mutagenicity results from mispairing properties of 8-oxoguanine (reviewed in (Grollman and Moriya, 1993; Sekiguchi, 1996)). If not decomposed, 8-oxo-dGTP can be incorporated into DNA opposite cytosine or adenine (Maki and Sekiguchi, 1992; Pavlov *et al.*, 1994; Kamath-Loeb *et al.*, 1997) generating an 8-oxo-G:A mispair. If this is not repaired, may result in AT→CG transversion in the genome (Cheng *et al.*, 1992). In this study the levels of mutagenic dNTP increased in oysters TBT-treated with the medium and highest concentrations compared to the oysters from the control group. This indicates that TBT could cause a mutagenic change in *O. edulis* chronically exposed.

Indeed, there is growing evidence that indicates that a range of pollutants can not only have endocrine disruptive effects interfering with the actions of endogenous hormones, but also possess mutagenic and carcinogenic activity (Choi, Yoo and Lee, 2004). It is well known that one of the adverse effects of ROS production is the DNA strand breaks (Simon, Haj-Yehia and Levi-Schaffer, 2000; Cadet and Wagner, 2013). Evidence suggests that TBT can cause genotoxic and clastogenic effects in molluscs. Genetic damage (single-strand DNA breaks, micronucleus (MN) formation) was induced in adult *M. edulis* exposed to TBT (Hagger, Depledge and Galloway, 2005). The fragmentation of DNA has been also induced by TBT in both human cells and gill cells of the mussel *Mytilus galloprovincialis* (Mičić *et al.*, 2002). Other authors have demonstrated that TBT produced mutagenic effects with an increase in DNA damage in the fish *Hoplias malabaricus* measured as single-strand DNA breaks, the induction of MN, and an increased incidence of chromosomal aberrations (Ferraro *et al.*, 2004). It was also shown that exposure of early life stages of the marine mollusc *Mytilus edulis* (Jha *et al.*, 2000) and the polychaete worm *Platynereis dumerilii* (Hagger *et al.*, 2002) to TBT caused DNA damage via the production of both chromosomal aberrations and sister chromatid exchanges (Jha *et al.*, 2000; Hagger *et al.*, 2002). The dog-whelk *Nucella lapillus* exposed to TBT showed evidence of DNA damage associated with the development of imposex (Hagger *et al.*, 2006). Interestingly Hagger *et al* (2006) found a very strong relationship between the degree of imposex and the extent of DNA damage (micronucleus formation) in hemocytes.

5.5 Conclusions

TBT has been widely recognized as an endocrine disruptor in gastropods. In this study, the lowest and medium concentration of TBTCl presented a masculinization effect evidenced by the increase of male oysters under these treatments at the end of the trial. However, oysters treated with 2000 ng/L TBTCl showed an increase in oysters classified as inactive and no males were found.

Signals of changes in the homeostasis of the oysters such as the down-regulation of molecules involved in the TCA cycle, increase in lipid and protein content, and a down-regulation in energy molecules were found for all the treatments. These last two processes are related to apoptotic pathways that could be related to the high mortality observed for TBT-treated oysters in the present study. In addition, an imbalance between oxidative stress and the antioxidant system supports the idea of toxicity related to the apoptotic process triggered by exposure to TBT toxicity in this species.

These effects could be related to a lack of change in overall growth and the increase in stages G0 and G1 of sexual maturity for oysters treated with TBTCl. This suggests that these animals were struggling to maintain homeostasis and survive rather than reaching sexual maturity.

The metabolism of sex steroids in invertebrates is a controversial topic due to the lack of evidence on the synthesis and enzymatic pathways needed to metabolize sex steroid hormones. In this study, a lack of effect between sex determination and the level of hormones was found. The changes in E_2 and T levels in TBT-treated oysters, along with evidence from other studies, suggest that TBT could interfere with the available free testosterone. The change in the balance between free and esterified steroids due to esterification or conjugation processes might be the responsible mechanism for the reported androgenization/feminization phenomena. To determine whether TBT interferes with the esterification of hormones, the total (free + esterified) and free steroid levels should be analysed in *O. edulis*.

Chapter 6 General discussion

6.1 Summary of thesis findings

Chapter 1 of the thesis critically reviewed and evaluated the academic literature and this highlighted the lack of recent information about the effects of environmental factors on reproduction in *O. edulis*. Further, there is contradictory evidence regarding the steroidogenesis pathway regarding the effect of vertebrate-related sex steroids and pollutants on reproduction in molluscs. The literature review brought this information together in order to establish the gaps in key biological and reproductive parameters in this species. The subsequent chapters in this thesis aimed to evaluate each of these aspects in *O. edulis* including environmental factors such as temperature (Chapters 2 and 3), vertebrate-related sex steroids (Chapter 4), and the most well-known EDC, TBT (Chapter 5), as some of the main aspects affecting reproduction in this species. The main reasons causing *Ostrea edulis* declining populations around the world was proposed in chapter 1 (Fig 1.1). This thesis has provided relevant information answering some of the gaps about biological and reproductive parameters in this species that could be involved in that decline (Fig 6.1). The contribution of this work in defining reproductive processes of *O. edulis* and some of the factors affecting them are relevant since the growing interest in the status of this species and the restoration programmes proposed and under development in some places across Europe.

A brief summary of the key findings presented in this thesis is provided below:

Chapter 2: Effect of temperature and endogenous steroid hormones on gametogenesis and sex ratio in *Ostrea edulis*

- There is a direct effect of temperature on the gonadal development of *O. edulis*, and the increase of temperature accelerates the gametogenesis process.
- The sex ratio is influenced by temperature.
- The lack of direct relationship between hormone concentrations in tissue with the gametogenesis and sex determination suggests that these processes are independent of sex steroids and other pathways could be involved.
- The Vtg-like protein assay did not show a relationship with sex determination.
- Other parameters - such as food availability - could play an important role in terms of energy allocation for sexual maturation.

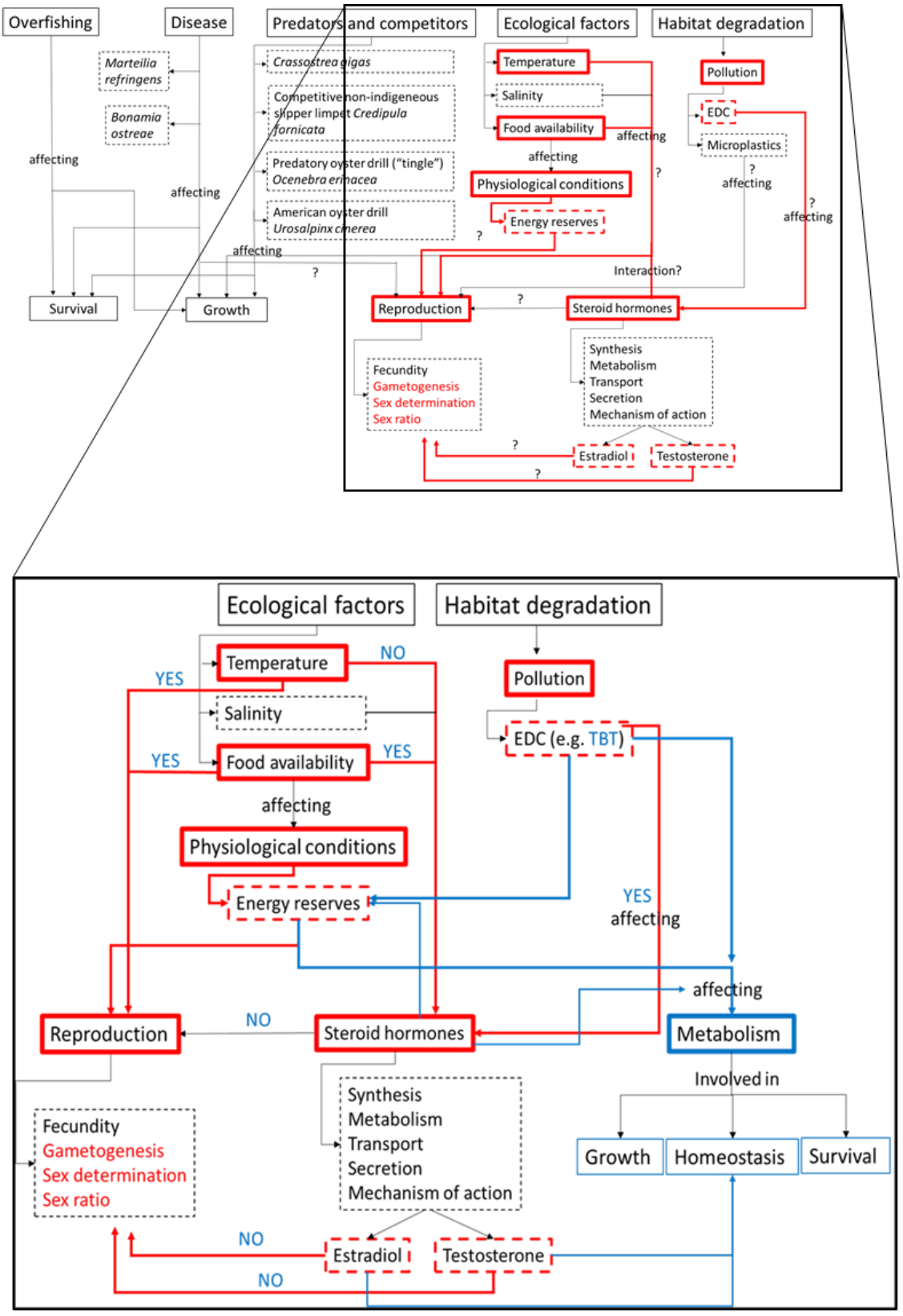

Figure 6.1 A summary showing biological and reproductive parameters affected by environmental factors. Red–marked sections show aspects considered in chapter 1 (Fig 1.1) as the main reasons and effects causing *Ostrea edulis* declining populations around the world. Blue–marked sections show the outcomes of this thesis in terms of interactions between factors and biological effects and new responses observed in this species.

Chapter 3: Seasonal changes in biochemical composition, gametogenesis, sex determination and endogenous steroids hormones in *Ostrea edulis*

- Temperature has an important effect on gametogenesis. A clear spawning period was observed during the months with the highest temperatures. On the other hand, gonad development decreased between August and November, when the temperature started to decrease.
- A significant effect of temperature and Chl α on gonadal maturation was identified.
- Lower temperatures were related to the increase of female individuals, whilst more males were found with an increase in the water temperature.
- Hermaphrodites were variable throughout the year.
- Energy reserves change throughout the year. Carbohydrates content was correlated with levels of Chl α, but lipids and proteins did not show any correlation with environmental variables.
- The relationship between Chl α and energy reserves in *O. edulis* suggests that the amount of energy used for gametogenesis activity in this species could depend more on food quality than on the nutrient storage.
- Lipids seem to be an important energy reserve used in sexual differentiation in females of *O. edulis*.
- Sex steroid hormones and Vtg-like proteins are not involved either in sex determination or gametogenesis in *O. edulis* during the annual cycle.

Chapter 4: Effect of exogenous steroids on survival, homoeostasis and reproduction of *Ostrea edulis:*

- *O. edulis* can uptake and accumulate estradiol and testosterone from the environment.
- Masculinization or feminization effects caused by testosterone and estradiol were not found confirming that sex determination could be regulated by other pathways independent of sex steroids.
- Glycogenolysis and the synthesis of proteins and lipids were affected by exposure to estradiol and testosterone in *O. edulis.*
- *O. edulis* exposed to estradiol and testosterone presented a reduction in metabolism evidenced by a lower content of energy reserves, down-regulation of molecules involved in the TCA cycle and energy molecules.
- The presence of some intermediate molecules in the steroidogenesis pathway was detected but their role in reproduction in invertebrates needs confirmation.

Chapter 5: TBT as a model endocrine disruptor pollutant and its effects on reproductive parameters in *O. edulis*

- TBT-treated oysters showed signs of gonadal maturation arrest in stages G0 and G1.
- Low and environmentally relevant concentrations of TBT showed a masculinization effect in *O. edulis.*
- High concentrations of TBT caused an increase of inactive oysters.
- TBT seems to interfere with available free testosterone.
- The high mortality, changes in metabolic pathways and an imbalance between molecules involved in oxidative stress and the antioxidant system suggest a TBT mechanism of action triggering apoptosis.

6.2 Environmental factors affecting sex determination and gametogenesis in *Ostrea edulis*

The duration of gametogenesis in *O. edulis* varies between locations and is highly dependent on water temperature and on food availability (Ruiz *et al.*, 1992; Shpigel, Barber and Mann, 1992). Temperature is a key factor determining gametogenesis during maturation of oysters and the initiation and duration of spawning are closely linked to temperature and food availability (Martínez, Aguilera and Mettifogo, 2000; González-Araya *et al*., 2012; González-Araya, Quillien and Robert, 2013; Teaniniuraitemoana *et al*., 2016). It has been reported that an algal diet rich in carbohydrates produces the best gonadal development in this bivalve (González-Araya *et al*., 2012), and a mixed-algal species diets support generally better growth and competence than single-species diets in *O. edulis* (Helm *et al*., 2004), showing the importance of this factor in biological and reproductive parameters in this species. In Chapters 2 and 3 an accelerated gametogenesis was more evident at higher temperatures. In the same manner, mortality and gonadal maturation seemed to be the biological responses more affected by temperature and Chl *a* making these two parameters the most relevant in terms of gonadal development.

The analysis of *O. edulis* collected in the Solent in previous years showed healthy oysters in good condition presented male-biased sex ratios (Kamphausen, Jensen and Hawkins, 2011) but the causes of this bias were not fully understood. Temperature plays an important role in sex determination in this species. The results in this study showed that exposure to lower temperatures is implicated in the development of female oysters which reflects what occurred with coldest water temperatures (under 12 °C), corresponding to the beginning of the breeding season, in oysters kept under semi-enclosed conditions; whereas more male gonads appeared when temperatures were warmer (Loosanoff and Davis, 1952; Loosanoff, 1962; Joyce *et al.*, 2013).

The sex ratio biased towards females observed at 18°C (Chapter 2) is not in accordance with what was expected at high temperatures but the potential reasons are discussed in Chapter 2. It has been reported that *O. edulis* has the ability to become a functionally mature female following an exceptional summer period because it needs a large quantity of energy to produce ovaries (Dodd *et al.*, 1937). Sex reversion to a male phase could be used as a strategy used for protandric species for saving energy producing the low-cost male gonads and allocating the energy reserves into survival or growth, and later when the environmental conditions become more favourable, they would be able to change to female (Pérez *et al.*, 2013; Santerre *et al.*, 2013). This means that an environment with poorly fed oysters and high energy demand (e.g. high temperatures) will cause a delay in gametogenesis and an imbalance between sexes affecting long term population dynamics. Consequently, aquaculture management and restoration projects should be able to establish or follow up relevant environmental conditions, such as temperature and food availability, to ensure a proper population development in terms of gametogenesis and change/balance between sexes. In this study, the spawning stages were earlier than expected in 2016 and 2017 suggesting that maybe additional parameters like environmental pollutants (such as hormones, organotins, PHAs, PCBs) or the quality of food are playing an important role on this behaviour.

The Solent *O. edulis* population has suffered several collapses and re-establishments. The intensive supplementation by re-laying spat and oysters from other areas and the use of additional larval supply from managed oyster beds have been techniques used to try to keep and maintain the oyster fishery in this area (Key and Davidson, 1981). Projects aimed at incorporating and restoring *O. edulis* populations should be able to continue these practices and consider the dynamic of temperature and food availability in locations proposed for restoration actions, to ensure adequate gametogenesis, spawning times and sex determination.

There was not a clear trend on masculinization or feminization after exposure to testosterone and estradiol in *O. edulis*, but exposure to these hormones induced an arrest in gonad maturation. There is a lack of evidence to support the role of vertebrate-related sex steroids in bivalves and contradictory information in this matter has been obtained from numerous studies in bivalves (Scott, 2018). The lack of a well-defined role as sex-determining hormones was confirmed in this study with the increase in males at the end of the exposure to the highest concentrations of T and the lowest concentration of E_2. However, the exposure to sex steroids was able to switch the balance and more hermaphrodites of *O. edulis* were found under all treatments. This confirms that under environmental exposure to estradiol, testosterone, and potentially other mimicking compounds, uptake of these pollutants might take place creating an endocrine misbalance affecting the normal development and function of organisms but not necessarily in the conventional pathways established for sex determination in vertebrates.

TBT is a pollutant with masculinising endocrine disruptor effects in other molluscs (Thain, 1986; Bryan *et al.*, 1987; Spooner *et al.*, 1991; Oehlmann *et al.*, 1996; Matthiessen and Gibbs, 1998; Morgan, Murphy and Lyons, 1998; Oberdörster, Rittschof and McClellan-Green, 1998; Terlizzi, Geraci and Gibbs, 1999; Oberdörster and McClellan-Green, 2002; Horiguchi, 2006). In this study the appearance of more males in the lowest and medium TBT-treated oysters was found, confirming that some pollutants can alter the balance between sexes in natural populations; however the mechanism is still not clear (see section 6.5 for more discussion about the potential mechanism of TBT in *O. edulis*). Gametogenesis was also affected by exposure to TBT causing an arrest on gonadal maturation in this species reflected as an increase in oysters in stages G0 and G1.

6.3 Energy reserves and metabolism in *Ostrea edulis*

The preparation and storage of energy reserves priori a reproductive period is crucial, so when food is abundant, nutrients are stored in various tissues in the form of glycogen, lipid and protein (Wang, 2000). Environmental factors affect the biochemical and physiological components involved in the maturation process of oysters. Gonadal development in *O. edulis*, as is typical in other bivalves, took place using energy reserves stored prior to the initiation of gametogenesis. This process is dependent on temperature and food availability. In addition, stored nutrient reserves are an important source of energy for development (Shpigel, Barber and Mann, 1992). In this study, *O. edulis* kept at 18°C showed rapid gametogenesis but the lowest values for most of the biometric measurements. It has been shown that high temperatures accelerate gametogenesis and gonadal development in bivalves, but also increase metabolism (Shpigel, Barber and Mann, 1992). Considering the trade-off between growth and reproduction (Pérez *et al.*, 2013) it is relevant to mention that some studies, including this study, have shown that the production of gametes results in a reduction in CI due to the demand for energy reserves obtained from carbohydrate, lipids and protein stored in tissues (Shpigel, Barber and Mann, 1992). The production of female gametes is believed to be more energetically costly than the production of male gametes so poorly fed oysters do not gain enough energy from the diet and reserves to initiate gametogenesis and produce eggs (Santerre *et al.*, 2013). The concentration of Chl α obtained in this study was according to the expected seasonal variations values in this area (Shi, 2000), but poor environmental quality could be related to the male-biased sex ratio found in previous years in the Solent.

Lipid, carbohydrates and proteins play an important role as energy reserves in reproduction. Thus it could be expected their variation during seasonal changes whilst oysters go through the maturation process. For oysters kept under semi-enclosed natural conditions, the lipids and carbohydrates presented a variable behaviour throughout the study period whereas protein

content was more constant. Carbohydrate content correlated with levels of Chl α during the year, but lipids and proteins were not affected by environmental variables. In the same manner, the carbohydrate content increases when lipid content decreased throughout late autumn and winter which could be attributed to glycogen conversion to lipids at the onset of gametogenesis (Mouneyrac *et al.*, 2008). This support the hypothesis that energy reserves are an important resource and can be used by *O. edulis* for growth and reproduction.

It seems that the mobilization of energy from storage tissues to gonad is a long-term process, probably under the regulation of steroids, especially estrogens in female bivalves (Wang, 2000). It has been shown that sex steroids could regulate the main metabolic pathways of the glycogen, proteins and lipids (Matute and Kalkhoff, 1971; Wang and Croll, 2004; Durou and Mouneyrac, 2007). This study showed a decrease lipid and protein content after two months of exposure to steroid hormones. This result, alongside the slow maturation process observed in these sex steroids-treated animals, suggests that after a long exposure with sex steroids the dynamic process including both anabolic and catabolic metabolism and the changes in energy reserves in these organisms are affected probably through changes in glycogenolysis and the synthesis *de novo* of proteins and lipids. Most of the studies reporting the health status of the Solent *O. edulis* populations consider growth parameters, gonad indices, larval development, presence/absence of bonamia, immunological parameters, or gonadal development (Kamphausen, Jensen and Hawkins, 2011; Eagling, 2012; Eagling *et al.*, 2018), but they have not determined the energy reserves and its changes throughout an annual cycle or after disturbance episodes by pollutants. According to the relationship between Chl α and energy reserves in *O. edulis*, this study suggests that the amount of energy used for gametogenesis activity in this species could depend more on available food than on the nutrient storage. Thus food availability and quality play an important role in the reproduction of *O. edulis,* but in case of scarce or poor quality food in the environment energy reserves could be decisive in reproduction and need to be taking in consideration to describe the condition and health of oyster populations.

The direct effects of steroids on glycolysis, the tricarboxylic acid (TCA) cycle and the mitochondrial electron transport have not been studied in molluscs and this thesis is the first report describing changes in metabolites involved in these processes in *O. edulis* (Fig 4.7 and 4.8). This study found that these processes were affected by sex steroids producing a down-regulation in important metabolites and molecules such as glucose, ATP and NADH. As a final outcome, the exposure to sex steroids affects not only reproduction but also other biological processes in the organisms exposed compromising important pathways related to the production of ATP in the cells and survival of exposed oysters. As a consequence it could be expected that any environmental contamination by

these hormones could cause an imbalance in energy reserves and its metabolism, affecting eventually the health of the population.

This study is the first study reporting the analysis of the global biochemistry using a metabolomic profile effect to understand the effect of exposure to exogenous sex steroids on biochemical pathways involved in diverse functions such as gluconeogenesis, energy balance, mitochondrial electron transport chain, etc (Fig 4.5, Appendix G). Metabolite profiling has an important role to play in understating general implications and the underlying biochemistry in response to exposure to environmental stressors.

The balance between the energy reserves is so important that some pollutants can act not only as toxic or endocrine disruptor agents but also as a metabolic disruptor. This is the case for TBT that showed a lipotoxic effect in TBT-treated oysters. An adverse effect could be expected following this accumulation of lipids in oyster tissues leading to apoptosis and eventually affecting development and reproductive attributes.

6.4 Presence and role of sex steroids in *Ostrea edulis* and implications for its populations inhabiting in the Solent

The existing evidence about the endogenous origin, metabolism, physiological/reproductive role and regulation of neuropeptides and peptide hormones in the Mollusca is still contradictory (LaFont, 2000; Janer and Porte, 2007; Ketata *et al.*, 2007; Scott, 2012, 2013). As was established in the literature review, E_2 and T has been proposed as regulators of gametogenesis and sex determination in bivalves, with the former related to feminizing effects and the later to masculinizing effects (Mori, Muramatsu and Nakamura, 1972; Le Curieux-Belfond *et al*., 2001; Gauthier-Clerc, Pellerin and Amiard, 2006; Wang and Croll, 2006; Ketata *et al*., 2007). However, the lack of a direct relation between gametogenesis and sex determination (from histology) and hormone concentrations for the oysters kept under laboratory conditions supports the idea of other biochemical pathways involved in the gonadal development and sex determination in bivalves (Scott, 2018). This was corroborated by the lack of any relationship between the annual variations in steroid content and reproductive cycle stages for oysters kept under semi-enclosed conditions in the Solent.

Oysters kept under semi-enclosed natural conditions showed a relationship between lipid content and estradiol and testosterone concentrations supporting the statement proposed by other authors about an increase in hormone concentrations related with the increase in energy reserves (fatty acid, lipids and protein content) in bivalves (Scott, 2012, 2013). Steroids are always present

in the animal's food in the environment as a product of physiological process in other animals or anthropogenic activities (see below) (Lafont and Mathieu, 2007) and this could be an external source for these hormones.

As already mentioned in Section 6.1, exposure to vertebrate-related sex steroids did not show evidence of masculinization or feminization effects in *O. edulis.* However, exposure to sex steroids was able to switch the balance and more hermaphrodites of *O. edulis* were found under all treatments. This study also reports a significant increase in hormone concentrations in gonadal tissue of *O. edulis* in all the sex steroid treatments, indicating that *O. edulis* can uptake and accumulate these hormones from the environment. Combined with this uptake, an increase in mortality, change in energy reserves and metabolism were found after exposure to these hormones. This confirms that under environmental exposure to estradiol, testosterone, and potentially other mimicking compounds, an uptake of these pollutants might happen to create an endocrine misbalance involved in physiological function, homeostatic processes and the normal development of organisms rather than in reproduction. In fact, the effects observed in shifts on gametogenesis and sex determination could be more related to a strategy to maintain homeostasis investing more energy in trying to survive than in reproductive parameters (Shpigel, Barber and Mann, 1992; Pérez *et al.*, 2013). Sex steroids taken up from the environment can be stored in the form of fatty acid esters for days or even months in the tissues of bivalves exposed (Scott, 2012) so there is a risk of accumulation of these hormones in tissues of oysters inhabiting areas with environmental pollution by sex steroids.

The present study identified some of the intermediate molecules expected according to the vertebrate-sex steroid pathway from cholesterol (Fig 6.2). This could indicate the uptake of these molecules from food or water (Scott, 2012), or the metabolism of some of these intermediates. However, more research is needed in order to confirm the presence of these molecules and the enzymes responsible for their metabolism.

Although androgen and estrogen metabolism in invertebrates is a controversial topic and homologues of both steroid receptors have not been found in invertebrates (Escriva, Delaunay and Laudet, 2000; Scott, 2012, 2013, 2018), exposure to some pollutants can affect reproduction via sex steroids. Evidence suggests that TBT can affect the ratio of free/esterified steroids (Gooding *et al*., 2003). Although esterified steroids do not bind steroid receptors, they are important because they could be considered as long-acting steroids, since they can be hydrolyzed by esterases (Hochberg *et al.*, 1991; Gooding and LeBlanc, 2005; Janer *et al.*, 2005), in counterpart to non-esterified (free) steroids that are able to diffuse throughout the organism for a short and quick effect (Scott, 2018).

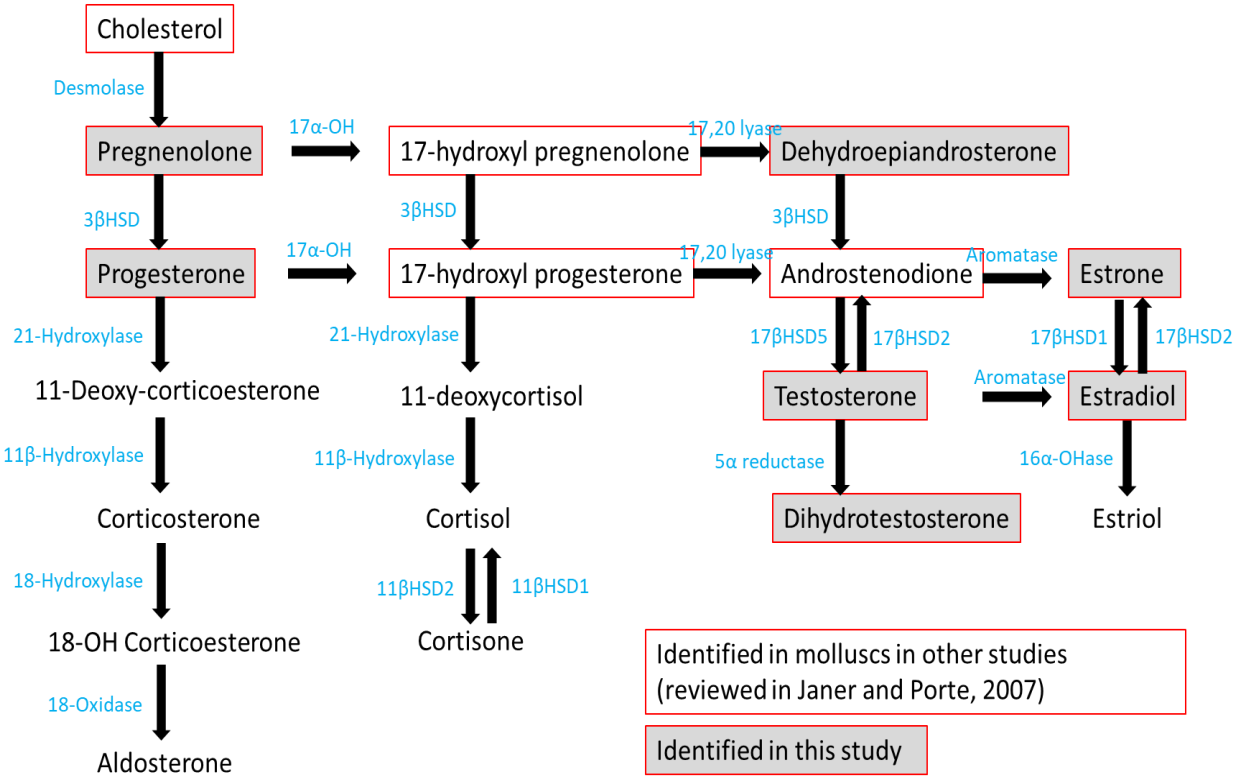

Figure 6.2 Steroidogenic and metabolic pathways described in vertebrates (adapted from Cole *et al.*, 2019; Medvei, 1982; Norris and Carr, 2013). Key enzymes (in blue) involved in steroid hormone synthesis reported in vertebrates. Metabolites that have been reported in molluscs (in red squares) according to Janer and Porte (2007). Metabolites identified in this study (grey). HSD=hydroxysteroid dehydrogenase.

The limited available information about the presence and concentration of sex steroids in aquatic environments in the UK comes mainly from environmental agencies and a few studies reporting pollution by these compounds. The Environment Agency (EA) in the UK started including estradiol as part in the water quality monitoring since 2012. The reports include values between 0.3 and 4 ng/L for 17β-oestradiol in some places where the monitoring programms are established (mainly around London and other main cities like Birmingham). Tarrant *et al.*, (2005) reported concentrations of E_2 from 15 to 260 ng/L in influents and from 1.0 to 76 ng/L in effluents in the UK. The case is worse for testosterone which is not even included in the environmental surveys. However environmental values for sex steroids are not monitored in the Solent.

6.5 Implications of TBT-related effects for *Ostrea edulis* populations inhabiting in the Solent

TBT was used as a paint antifouling agent on leisure and commercial craft until it was banned in 1987. Toxic substances of concern to the UK and European regulatory authorities established various lists of chemicals identified for priority action for their control, including TBT (Cole *et al.*, 1999). These measures reduced the routes of entry into the marine environment reducing environmental concentrations and allowing some wild populations to recover in places where pollution by TBT depleted natural populations in the UK many years ago (Bray *et al.*, 2012; Langston *et al.*, 2015). Nevertheless, TBT is strongly adsorbed to organic matter and sediments retain concentrations of TBT for long periods and it can be released to the water column when sediment is disturbed, for example by dredging which happens regularly on a very large scale in the Solent (Dowson, Bubb and Lester, 1996; Gadd, 2000; Pynaert and Speleers, 2000).

By 1987, TBT concentrations in waters and sediments of Poole Harbour were reported between 2-646 ng/L and 0.02-0.52 ug/g, respectively (Langston, Burt and Mingjiang, 1987). More than ten years after it was banned remaining concentrations of TBT were found in UK surface waters including the Crouch Estuary, Essex, Sutton Harbour, Plymouth and Southampton Water (Thomas *et al.*, 2001). Various samples from Southampton Water, especially around the dock area, showed higher concentrations than the UK environmental quality standard (EQS) of 2 ng/L (Thomas *et al.*, 2001). Since then the release of TBT into the marine environment from antifouling has long been of concern in the Solent. The Solent region is a centre for international shipping and maritime recreation activities and values of TBT above the Threshold levels are currently found in Southampton Water (e.g. in the Hamble Estuary) (Langston *et al.*, 2015; SEMS, 2019). With on-going dredging work in the docks potentially disturbing the TBT stored in the sediment, understanding the impacts of TBT on *O. edulis* should be a priority in this area where restoration programmes are currently in development. This is the case for the BLUE Marine Foundation's Solent Oyster Restoration Project aiming to return 10 million native oysters to the Solent by 2020 (Harding, Nelson and Glover, 2016)

Under the EQS for priority substances the proposed annual average for TBT is 0.0002 ug/L with a maximum allowable concentration of 0.0015 ug/L and 0.002 ug/L in the UK (Ports, 2011). However, the limit detection for the laboratory analysis of this contaminant makes difficult to assess compliance against the proposed standards. In the particular case of TBT, the UK is by far the least conservative in comparison with other European countries in terms of the action lists and methods used to detect this pollutant in the environment (MMO, 2015).

In the present study, the exposure of *O. edulis* to 20 ng/L and 200 ng/L of T increased the number of males over two months of exposure. This is in accordance to other studies showing the masculinization effect of exposure to testosterone (Wang and Croll, 2004; Fernandes *et al.*, 2010; Ruiz-Velásquez *et al.*, 2018). No male individuals were found and an increase of inactive animals was observed at the highest TBT treatment. Even when TBT showed an effect on sex steroids concentrations is not possible to demonstrate its role as an endocrine disruptor compound in *O. edulis.* There is not enough information about the endocrinology of this species and the implication of sex steroids and pollutants interfering with this pathway. This study suggests that *O. edulis* exposed to high levels of TBT go through a physiological imbalance affecting lipid homeostasis, while a reduction in energy molecules and a perturbance in TCA cycle occur in this species. It has been proposed that TBT can also act as a genotoxic, clastogenic and mutagenic agent via the imbalance between oxidative stress and the antioxidant system (Jha *et al.*, 2000; Simon, Haj-Yehia and Levi-Schaffer, 2000; Mičić *et al.*, 2002; Choi, Yoo and Lee, 2004; Hagger, Depledge and Galloway, 2005; Cadet and Wagner, 2013). The results of this study confirm these properties as well as the potential toxicity of this compound and its toxic effect on this species.

Therefore a toxic effect could be affecting the health of *O. edulis* populations inhabiting in the Solent. Some hypotheses have pointed to habitat degradation and pollution as additional factors involved in population decline (OSPAR, 2008a; Kamphausen, Jensen and Hawkins, 2011; Harding, Nelson and Glover, 2016; Chatterjee, 2017). The presence of TBT in the Solent adds additional environmental stress to this species influencing not only sex ratios towards males but also growth, development and survival. The decline in these populations in the Solent reported in previous studies could be explained, or at least partly explained, for the presence of remaining TBT in water and sediments in this area (Thomas *et al.*, 2001; Langston *et al.*, 2015; SEMS, 2019). Restorations efforts with numerous reintroduction schemes for *O. edulis* are in progress around the UK, but these results suggest that it is necessary to consider pollution by hormones and organotin compounds in water and sediments, changes in temperature patterns and food quality as important parameters to be considered in potential areas to relocate *O. edulis* populations. In addition the Solent has a history of exploitations, overfishing, predators pressure and diseases (Orton, 1927c; Laing, Walker and Areal, 2005; OSPAR, 2008a; Gravestock, James and Goulden, 2014; Harding, Nelson and Glover, 2016) which increase the selection pressure acting on this species and affecting population dynamics in this area.

In addition, organotins are also used more generally as fungicides in preservatives for wood, textiles, paper, leather, plastics and packaging and in food additives. Some TBTs are added to PVC plastics and other materials because of their stabilising and anti-corrosive properties (Dobson, Howe and Floyd, 2006). Remaining concentrations of TBT are still found in sediments around the

UK in concentrations between 3-103 µg/Kg (Environmental Agency, 2019) turning sediments in a continuous source of pollution by this compound.

This study has demonstrated the importance of a detailed investigation into the reproductive processes and the effect of environmental factors, including pollutants, of a population when considering its restoration. The skewed sex ratio reported in previous years in the Solent and the skewed sex ratio biased-males found after exposure to TBT does not make reproduction impossible, it does reduce the chances to survive in this areas and could be behind the recurrent recruitment failures described in other studies (Kamphausen, Jensen and Hawkins, 2011; Eagling, 2012).

6.6 Challenges and limitations

Many studies have reported the natural status of *O. edulis* in different locations around the world. These studies have confirmed the threatened status of this species with collapsing populations around Europe. Although this species has ecological and economic relevance, basic information about its biology and reproductive parameters is still scarce, contradictory and most of the available literature was published between the 1950's and 1990's. This made it difficult to compare the results obtained in this study with the available literature on the same species.

Pollution by estrogens, androgens and TBT have shown adverse effects on organisms. Disposal of waste water containing these pollutants are dangerous and well regulated and just a few companies offer the option to collect and treat the wastewater making this process slow and expensive. The work was limited by resources so the water containing the pollutants produced after treatments was collected in tanks outside the hatchery at NOCS. Then this water was treated with activated carbon and went through by-pass interceptors on-site which covers the quayside. These are emptied annually and collected for more treatment. This was a long and complicated process that limited the amount of water generated after treatments and therefore the number of replicates by treatment in this study.

An apparent contradiction between genomic data and reports about the presence and activity of some enzymes such as 5α-reductase, 17β-HSD and Cytochrome P450 monooxygenase enzymes (CYP) has been shown in bivalves (Scott, 2012, 2018). The endocrinology and molecular information in *O. edulis* is still scarce making very hard to generate final conclusions about the steroidogenic pathway and any potential effect of environmental factors in this species.

6.7 Recommendations for future study

The literature (Chapter 1) seemed to support the presence and role of vertebrate-related sex steroids in invertebrates, including bivalves. However, while investigating the reproductive processes and the role of hormones in *O. edulis*, more researches documented the lack of evidence supporting this role. This PhD project has laid the foundations for future studies by identifying some effects caused by the exposure to hormones, and also by confirming the absence of a sex-determining role for sex steroids in bivalves.

Oysters exposed chronically to sex steroids and TBT presented a change in the global metabolomics profile showing effects not only in molecules involved in reproduction but also in homeostasis and survival. This was also related to changes in energy reserves in these exposed animals. If the oysters eat less as a result of the exposure to pollutants this will create an imbalance in energy consumption vs. energy spend in changing/modifying gametes and sex. This could be evaluated under laboratory conditions measuring ^{13}C-labelled amino acids or lipids to understand the distribution of resources and the use of energy reserves under environmental stress caused by pollutants.

Estradiol and testosterone did not present any relationship with sex determination or gametogenesis. However, one of the biggest outcomes of this project is the presence of some metabolites involved in the steroidogenic pathway. This means that *O. edulis* could be using these compounds in biological processes directed to maintaining homoeostasis. Further confirmation and identification of these compounds are necessary in order to understand the molecular pathways where these compounds are involved in this species, or if these compounds are just taken up from the environment and stored in tissues for other processes. The ratio free/esterified steroids is also an important criterion to understand the effect of exposure to hormones, or compounds mimicking hormones, so it is necessary to include the analysis of esterified steroids in *O. edulis* as a mechanism to maintain endogenous levels of free steroids. The physiological role of steroid esters in molluscs and the mechanisms by which steroid esterification is regulated remain uncertain and further research could help to fill the gaps in this information providing better knowledge about the role of sex steroids in this species.

Most research on steroid concentrations in invertebrates, including this study, has used immunoassays as the detection system, relying on a cross-reactivity of these assay antibodies with other steroids (Porte *et al.*, 2006; Lafont and Mathieu, 2007). According to the manufacturer (Cayman Chemical Co.; Ann Arbor, MI, USA) these kits have 100% specificity but results obtained with the immunoassay methods should be interpreted with caution. Compared with these methods, mass spectrometry analysis, such as the liquid chromatography coupled with tandem

mass spectrometry (LC-MS/MS) or ultra-performance LC-MS/MS (UPLC-MS/MS), have higher sensitivity and greater specificity (Xu *et al.*, 2007; Gust *et al.*, 2010). GC/MS techniques are more expensive are require more sample preparation than immunoassay methods, but they are the standard method for identifying and quantifying sex steroids in complex environmental samples (Gust *et al.*, 2010).

To assess susceptibility by pollutants still requires research since every invertebrate taxon possibly has developed its own (and possibly sometimes unique) signalling system deriving from modifications of the first ancestral steroid receptor. The cloning of full-length sequences is another method that could be used in order to prove the presence of some of these compounds. Cloning of these genes will also provide us more information for the examination of the presence, origin and evolution of steroid receptors in invertebrates. Bringing together some of the 'omics' (genomics, proteomics, metabolomics) in the study of *O. edulis* reproduction will help to improve the better understanding of the basic biology of this species and the mechanisms involved in reproduction allowing the application of suitable strategies for aquaculture management and restorations programmes.

This project has also highlighted that we are still lacking a clear understanding of some aspects of fundamental oyster reproduction. If sex steroids are not involved in sex determination and gametogenesis, which molecular pathways are involved in these processes? Other pathways, such as the RA and RXR involved in imposex in gastropods, should be evaluated to understand the natural reproductive process occurring in *O. edulis*. That should be the first step before making more conclusions about the effect of pollutants acting as ED in this and other bivalve species.

Larval quality, settlement success and embryonic development are additional parameters where further research would greatly benefit the knowledge about how this species responds to changes in environmental factors, including pollution, and the best practises that should be considered for the management of oyster populations.

Chapter 7 Conclusions

Temperature is a key factor affecting sex determination and determining gametogenesis in *Ostrea edulis.* Under laboratory conditions lower temperatures were implicated in the development of female oysters whereas more male gonads appeared when temperatures were warmer. A similar response was observed in oysters kept under semi-enclosed natural conditions, showing the prevalence of females under coldest water temperatures corresponding to the beginning of the breeding season and then slowly changing the sex ratio to more males in summer when higher temperatures were reached. Food availability also shows a direct relationship with gonadal maturation, suggesting that this factor could play an important role in terms of energy allocation for sexual maturation.

The lack of a direct relationship between hormone concentrations (in tissue or exogenous exposure) with gametogenesis and sex determination suggests that these processes are independent of sex steroids and other pathways could be involved. However, exposure to exogenous sex steroids modified the concentration of estradiol and testosterone in gonadal tissue. This suggests that under environmental exposure to these compounds, and potentially other steroids-mimicking compounds, the uptake of these pollutants might take place creating an endocrine misbalance affecting the normal development and function of organisms but not necessarily in the conventional pathways established for sex determination in vertebrates. Combined with this uptake, an increase in mortality, and changes in energy reserves and metabolism were found after exposure to these compounds, suggesting a potential effect on the health of natural populations through a different mechanism affecting survival and physiological parameters rather than reproduction.

The results in Chapter 3 support the hypothesis that energy reserves are an important resource and can be used by *O. edulis* for growth and reproduction. Actually, lipids seem to be an important energy reserve used in sexual differentiation in females of *O. edulis*. This study showed significant changes in energy reserves content after two months of exposure to steroid hormones and TBT suggesting that exposure to pollutants could cause an imbalance in energy reserves and its metabolism, potentially affecting the health of natural populations in polluted environments.

The presence of some intermediate metabolites involved in the steroidogenesis pathway was detected but their role in reproduction in invertebrates needs confirmation. Although there is not enough information about the endocrinology of *O. edulis*, the implication of exposure to pollutants affecting specific molecular pathways is difficult. TBT-treated oysters showed a physiological imbalance affecting lipid homeostasis, while a reduction in energy molecules and a perturbance in the TCA cycle occurred. Evidence of oxidative stress and alterations in the antioxidant system were

also found in these oysters. Taking these results together, a TBT mechanism of action triggering apoptosis has been proposed in this study. Interestingly, treatments with TBT caused an increase of inactive oysters and signs of gonadal maturation arrest in stages G0 and G1, suggesting that probably these animals stopped the investment in gonadal maturation while they were struggling to maintain the homeostasis and physiological functions after two months of exposure to this compound.

It could be expected that the decline of *O. edulis* populations in the Solent, an area with remaining TBT concentrations reported in water and sediments, is related to the toxicity observed in TBT-treated oysters. In addition, "normal healthy" oysters in natural populations could be investing more energy in basic functions and survival rather than in reproduction, leading to the reduction in numbers of these animals in the area in a long term.

Restoration efforts with numerous reintroduction schemes for *O. edulis* are in progress around the UK and Europe, but this thesis suggests that it is necessary to consider the dynamic of temperature and food availability in locations proposed for restoration actions, to ensure the adequate gametogenesis, spawning times and sex determination. Also environmental pollution by hormones, organotin compounds and other EDCs in water and sediments needs to be considered in potential areas for restoration of *O. edulis* populations, to avoid a new collapse of the population due to failures in survival, homeostasis and reproduction.

Appendix A

Published paper Zapata-Restrepo, L.M, Hauton, C., Williams, I.D., Jensen, A.C., Hudson, M.D. (2019). 'Effects of the interaction between temperature and steroid hormones on gametogenesis and sex ratio in the European flat oyster (*Ostrea edulis*)'. Comparative Biochemistry and Physiology, Part A 236:110523. DOI: https://doi.org/10.1016/j.cbpa.2019.06.023

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Effects of the interaction between temperature and steroid hormones on gametogenesis and sex ratio in the European flat oyster (*Ostrea edulis*)

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ABSTRACT

Throughout Europe, populations of *Ostrea edulis* have been in decline since the 1970s. Temperature has an important influence on physiological, biochemical and reproductive attributes of oysters. It is also the most easily modulated environmental factor in hatcheries, so it is useful to understand the implications of temperature variation in driving gametogenesis and sex development in a protandrous sequential hermaphrodites such as *O. edulis*. To understand the effect of temperature on gametogenesis and sex ratio, as well as the potential mechanism of sex determination through the role of steroid hormone homologues, oysters were exposed to three temperatures (10, 14, and 18 °C) for four months. Gametogenic stage and sex ratio were assessed histologically for each treatment. In parallel, concentrations of estradiol (E_2)- and testosterone (T)- were determined in developing gonads. Our data show that by some biometric parameters, gametogenesis and sex ratio were significantly influenced by temperature during the experiment. There was a weak but significant correlation between E_2 and T concentration during the treatments. However, and importantly, a direct relation between gonadal maturation, sex determination and hormones concentration was not found. These results suggest that gametogenesis and sex determination are predominantly affected by temperature in this species, and that steroids may not be actively involved as endogenous modulators in sex determination. Rising sea water temperatures and warmer condition through the year could cause an accelerated gametogenesis and skewed sex ratios in natural populations of *O. edulis*.

1. Introduction

The European flat oyster (*Ostrea edulis*), which is naturally distributed around Northern Europe, has been extensively harvested for human consumption since the 1800s and it is still a commercially important marine resource (Kamphausen et al., 2011; Smith et al., 2006; Southern IFCA, 2015). According to the Food and Agriculture Organisation of the United Nations (FAO), *O. edulis* production has suffered a reduction in Europe from 13,580 t per year in 1950 to approximately 1992 t in 2016 (FAO, 2019). In response, the Oslo-Paris Convention included *O. edulis* as a Threatened Species (OSPAR, 2006), and since this time there has been growing interest across Europe in the status of this species and it is now the focus of several separate restoration programmes.

O. edulis is a protandrous alternating hermaphrodite (Cole, 1942a; Mann, 1979; Orton, 1927a, 1927b; Sparck, 1925). Each oyster can complete one male and one female phase each year under favourable conditions releasing sperm and eggs at different times in each reproductive season (Coe, 1943; Korringa, 1957). The lack of synchronicity in wild populations results in a mixed sex population and makes it possible to find individuals of both sexes during the spawning season (Coe, 1943; Loosanoff, 1962).

Sex ratio is a fundamental indicator for population reproductive success but the nature of oyster reproduction generates natural populations displaying sex ratios different to the proportion 1:1 (Fisher, 1930). Although sex parity in protandric species inhabiting the waters of Britain and Ireland was reported the last century (Cole, 1941; Mann, 1979; Orton, 1927b), early field studies reported a male-skewed ratio for *O. edulis* in natural populations (Cole, 1942b; Millar, 1964). More recent investigations demonstrated a significant reduction in the number of brooding female-phase oysters and sex ratios biased towards male-phase in members of the family Ostreidae, including the *Ostrea*

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edulis population in the Solent, with ratios as high as 7:1 (Acarli et al., 2015; da Silva et al., 2009; Eagling, 2012; Hassan et al., 2018; Kamphausen et al., 2011). A cyclically skewed sex ratio, especially a male-biased sex ratio, could decrease the effective breeding population size (Baeza et al., 2010) making the populations susceptible to other external factors that precipitate population declines. It is thus important to identify and understand the factors that may trigger or affect sex changes in this species.

Changes in some reproductive parameters such as gametogenesis and sex ratio in response to seasonal variations in the family Ostreidae have been studied (Acarli et al., 2015; Eagling et al., 2018; Hassan et al., 2018). Abiotic factors, such as temperature, may be important in terms of influence on physiological, biochemical and reproductive attributes of oysters (Mann, 1979; Newell et al., 1977; Newell and Branch, 1980). Early evidence suggested that temperature plays an important role in *O. edulis* by keeping this species in a resting stage during winter temperatures with higher temperatures in the summer being associated with adult spawning (Korringa, 1952; Loosanoff, 1962; Loosanoff and Davis, 1952; Mann, 1979).

Changing ocean temperatures and the rise in sea temperatures in temperate latitudes, including seasonal differences in warming trends, could have impacts on natural populations influencing the behaviour, growth, reproduction and survival of many marine species (Marine Climate Change Impacts Partnership, 2015). Moreover, in the case of commercially exploited bivalves, including ostreid and crassostreid oysters, temperature is the most readily modulated environmental factor in hatcheries. A more refined understanding of the direct influence of temperature on gametogenesis and sex determination remains essential for the prediction of the fate of *O. edulis* in a changing marine climate (Joyce et al., 2013).

Additional evidence has suggested that temperature combined with hormonal control could be involved in sexual maturation and sex determination in other species of bivalve (Mori et al., 1972; Teaniniuraitemoana et al., 2016). However, the exact mechanism by which temperature triggers these processes and the role of steroid hormones in gametogenesis, sex change and sex ratio in molluscs is not completely understood (Ketata et al., 2008; Morishita et al., 2010). Evidence of the presence of steroid hormones (e.g., oestrogens, testosterone) or other molecules involved in reproduction, development and maturation, especially in early life stages (i.e., gametes, larvae, and juveniles) in bivalves is scarce and inconclusive. Existing evidence supports a possible presence and role of neuropeptides and peptide hormones with physiological functions in some taxa (Ketata et al., 2008; Lafont and Mathieu, 2007). However, for some authors, the supporting evidence remains equivocal and they suggest that more rigorous studies need to be conducted in order to resolve current uncertainties (Fernandes et al., 2011; Scott, 2013, Scott, 2012). There is not enough information about the role of sex steroids in *O. edulis* and the exact controls for alternating sex change in this species have not yet been resolved satisfactorily (Ketata et al., 2008; Morishita et al., 2010). At this time, additional experimental studies are required to understand the potential function and mode of action of estradiol and other hormones on sex ratio and gametogenesis.

Although populations have declined in recent years, *O. edulis* remains economically important in the areas where wild or cultivated stocks are present. In this study, this species was used to establish the role of temperature in gametogenesis and sex determination in a controlled laboratory experiment and to elucidate if endogenous steroids hormones homologues are involved in these processes in this species.

2. Materials and methods

2.1. Oysters

Oysters (*Ostrea edulis*) were provided by the Loch Ryan Oyster Company, a Centre for Environment, Fisheries and Aquaculture Science (Cefas) certified *Bonamia* sp. free location. Oysters were > 2y old, and were 5–7 cm at their maximum diameter. Four-hundred and fifty oysters were transferred to the aquarium of the National Oceanography Centre Southampton at the beginning of March 2016. They were placed in seawater tanks (about 1 L/oyster) with continuous aeration at the same temperature (8 °C) and salinity (33.1) as at the hatchery site. After four weeks, oysters were divided randomly among three treatment tanks with 49 oysters per treatment tank and three replicates per treatment. Oysters were kept according to the ethics guidelines and under the approval of the Ethics and Research Governance Online system (ERGO) run by the University of Southampton (Project ID: 20658).

2.2. Temperature treatments

Three temperatures (10, 14 and 18 °C) were used in the experimental design from April 2016 to August 2016. Temperature was raised at a rate of 1 °C per day until the target temperature was reached for all the treatments. The temperatures were chosen so as to remain within the range of temperatures recently reported for the Solent during a year (www.seatemperature.org) whilst accounting for the observation that the maximum scope for growth, optimal filtration and reproduction in *Ostrea* has been reported at 20 °C (Newell et al., 1977). The aquaria were kept under static conditions with aerated filtered seawater being circulated through particulate filters and protein skimmers and with an 80% water change twice per week. Dead animals were counted and removed. During the experimental period, mean salinity was 34.5, pH range was 7.8–8.0 and dissolved oxygen was always > 98%. Water temperatures were controlled throughout the experiments using either a free standing chiller unit (TECO, model TR60) or using a constant temperature room. Animals were fed ad libitum daily with 40,000 cells/mL of a mixed algae diet (40% *Tetraselmis suecica*, 40% *Pavlova lutheri* and 20% *Phaedactylum tricornutum*). These algal species typically show adequate characteristics as food for several bivalve species and complementary profiles in essential fatty acids (Pernet et al., 2003).

At the beginning of the experiment (t_0) and at each sampling point for four months (t_1-t_4), 10 oysters were dissected from each temperature treatment into discrete tissues. Oysters were opened and dissected in salt water to reduce stress and damage of tissues. Samples of the visceral mass were immediately fixed in Bouin's solution for histological examination and the other tissues were kept in the freezer at −20 °C until further analysis.

2.3. Biological indices

Measurements of height (H), length (L), width (Wi; all to 0.01 mm) for every animal were taken using a digital caliper. Shell cavity volume (Vol.), Fresh tissue weight (FW) and total weight (W, to the nearest 0.1 g) were measured using a Denver instrument SI-603 balance with a precision of 1 mg. Maximum antero-posterior length were taken as length, maximum length in the dorso-ventral axis from umbo as depth (height) and maximum thickness of the oyster when both valves were closed as width (Gaspar et al., 2002). After removal of the shells, the Condition Index (CI) was calculated for each bivalve: ([total fresh tissue weight/total weight] * 100) (Lawrence and Scott, 1982; Walne, 1976). This index is a standard method widely used as a health and fitness state used to evaluate the condition of oysters (Crosby and Gale, 1990).

2.4. Histological analysis

Sections of the visceral mass were sampled for histological examination following a standard protocol (Howard et al., 2004; Kim et al., 2006). After dissection, 5-mm thick sections were cut along the sagittal plane containing gill, gonad, digestive gland, and mantle lobes and were fixed in Bouin's solution (Sigma-Aldrich™, Dorset, UK) for 24 h. The samples were dehydrated through an ethanol series (70%,

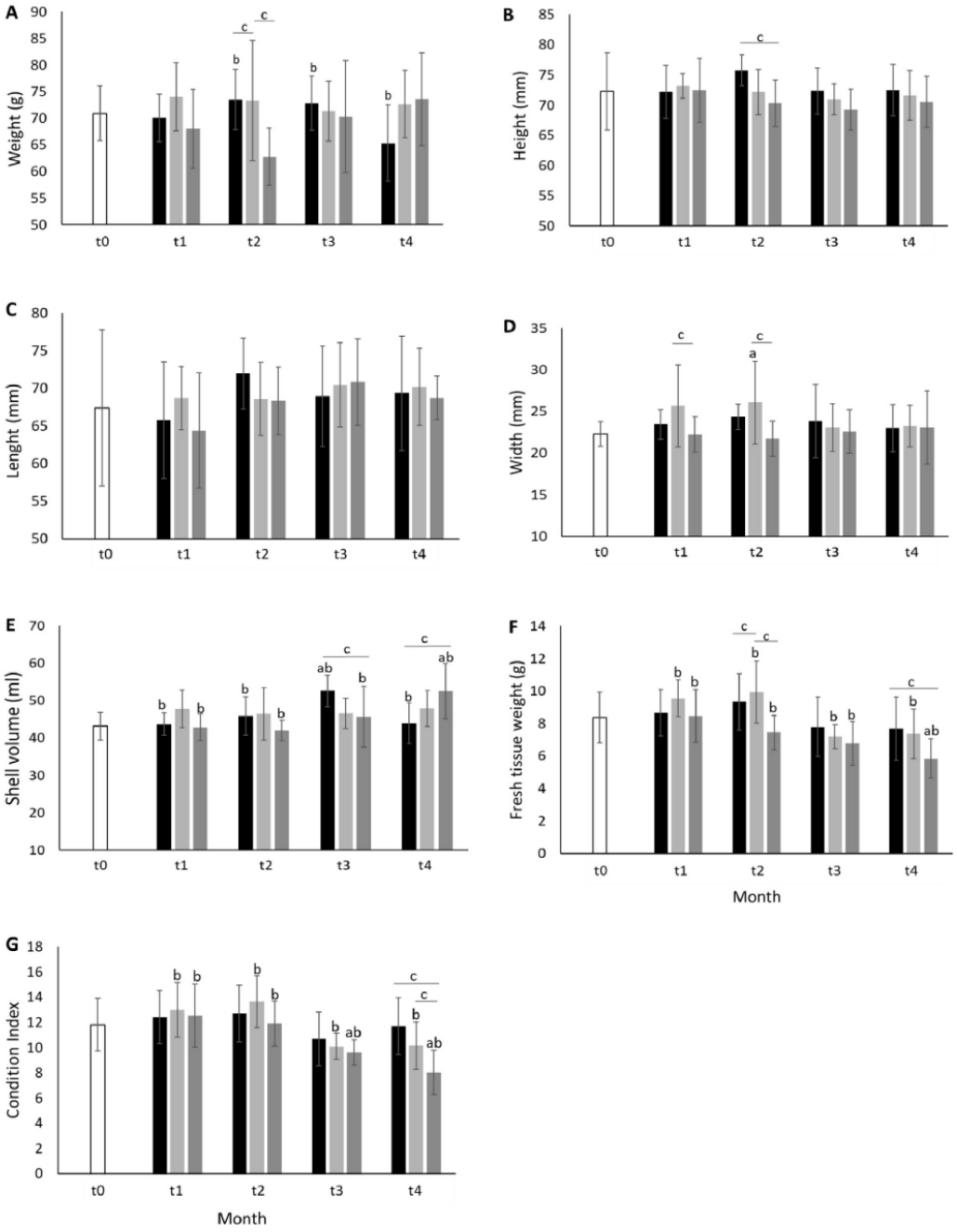

Fig. 1. Biometric parameters including total weight (A), height (B), length (C), width (D), shell volume (E), flesh weight (F) and condition index (G) measured during temperature treatments under laboratory conditions. Different letters bars are significantly different ($p < .05$) (a) Significant differences compared to the beginning of the experiment (t0), (b) Significant differences between time of treatment at the same temperature, and (c) Significant differences between temperatures at the same time. White bar: beginning of the experiment (t0), black bar: 10 °C, light grey: 14 °C, and dark grey: 18 °C.

80%, 90% and dehydrated ethanol) overnight for each concentration. The samples were embedded in paraffin, and the wax blocks were sectioned at 6-μm using a rotary microtome (*Leitz Wetzler, model 1212*), stained with hematoxylin/eosin (Cellpath Ltd) (Howard et al., 2004; Kim et al., 2006). Because maturation is not a homogenous process and female and male gametes can be present in different follicles at different maturation stages at the same time (Coe, 1932; Korringa, 1952; Loosanoff, 1962; Sparck, 1925), three slides per animal were prepared

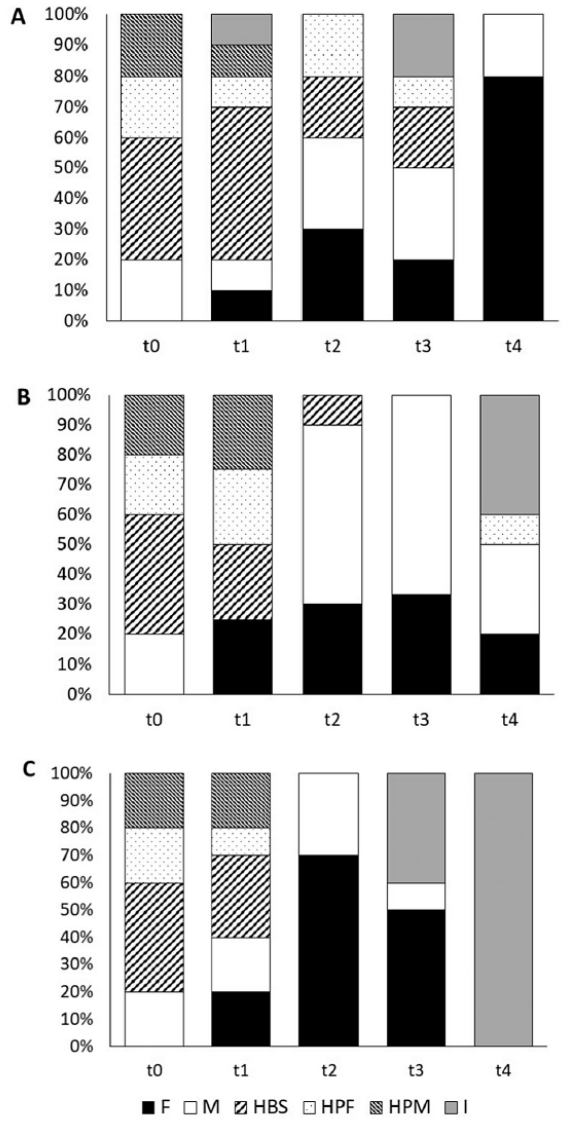

Fig. 2. Proportion of sex categories in *Ostrea edulis* exposed to (A) 10 °C, (B) 14 °C and (C) 18 °C during four months. Specimens samples (n = 10 per treatment) were identified by histological examination as females (F), males (M), hermaphrodite with both sexes equally represented (HBS), hermaphrodite predominantly male (HPM), hermaphrodite predominantly male (HPF) an indeterminate (I).

with three different sections separated by 500 µm to determine sex and developmental stage of the gonad. All microscope analysis was carried out using an Olympus BH-2-RFCA microscope fitted with a Nikon Coolpix E4500 microscope camera. Sex was recorded as *indeterminate* (I), *female solely* (F), *male solely* (M), *hermaphrodite with both sexes equally represented* (HBS), *hermaphrodite predominantly male* (HPM) and *hermaphrodite predominantly male* (HPF) according to (da Silva et al., 2009). The developmental stage was classified by the gametogenic stage of the gonad as *inactive* (G0), *early gametogenesis* (G1), *advanced gametogenesis* (G2), *ripe gonad* (G3), *partially spawned gonad* (G4) and *reabsorbing gonad* (G5) adopted by da Silva et al. (2009).

2.5. Steroid hormone homologue analysis

Extraction and analysis of homologues of the sex hormones E_2 and T concentrations were quantified in the gonads of each oyster using enzyme-linked immunosorbent assay (ELISA) kits (Cayman Chemical Co.; Ann Arbor, MI, USA) as described by Gauthier-Clerc et al. (2006). Gonad tissue (0.1 g) from each animal was homogenized in H_2O (1:5 w:w) and sonicated twice for 30s. A volume of 400 µL of 25 mM HCl was added to 500 µL homogenate and allowed to stand for 15 min at 40 °C. Then, 1.25 mL 0.07 M Na_2HPO_4 (pH 7.4) was added before organic extraction. Homogenates were extracted twice with 14 mL dichloromethane and organic extracts were evaporated to dryness under a nitrogen stream at room temperature (20–25 °C). The resulting pellet was dissolved in 250-µL enzyme immuno-assay buffer. E_2 and T concentrations were determined by competitive ELISA kits according to the manufacturer's instructions. E_2 and T standards were prepared and determinations carried out in duplicate. Standard curves were carried out with E_2 between 6.6 and 4000 pg/mL and T between 3.9 and 500 pg/mL. *A priori* criteria for intra-assay CVs reported by the manufacturer using a reference standard curve for E_2 and T were 7.8–18.8 and 2.8–14.2, respectively. Mean intra-assay CVs for standards and samples were ≤ 9.6% for E_2 and ≤ 8.27% for T. Mean inter-assay CVs were ≤ 5.6% and ≤ 6.3% for E_2 and T, respectively.

2.6. Statistical analysis

The normality of the data and the homogeneity of variances were evaluated using the Shapiro Wilk and Levene's tests, respectively. The assumptions of parametric tests were not met, so non-parametric tests were applied. Differences between biometric parameters (W, H, Wi, Vol, FW), CI and each steroid hormone concentration between different treatments were tested using the non-parametric Kruskal-Wallis H test. Spearman correlation was performed to evaluate the correlation between biometric parameters and hormone concentrations. For the statistical analysis, Windows 24.0 SPSS was used. The Kruskal-Wallis H-test was also used for the comparison between male, female and hermaphrodite hormonal concentration. In the same manner comparison for hormonal concentration between stages of gonadal development (G0-G5) was undertaken. Chi-square statistics were used to test sex ratios against a 1:1 ratio. Statistical significance was assigned at $\alpha = 0.05$.

3. Results

3.1. Temperature treatments

There was minimal variation in the actual temperature about the nominal set point for each treatment with mean values of 10.13 ± 0.70, 14.94 ± 0.57 and 18.20 ± 1.08 recorded for the treatments 10, 14 and 18 °C, respectively. The mortality during the acclimation process was 2% but was variable during the treatments (Appendix A). During the first two months of the experiment mortality was < 5% in every treatment. In the third month, mortality was 5%, 7% and 10% for oysters kept at 10, 14 and 18 °C, respectively, but in the fourth month mortality increased to 7%, 10% and 21%, respectively.

3.2. Biometric measurements after temperature treatment

Overall, the oysters at each sampling point showed homogeneity in the biometric parameters analysed indicating no significant or considerable effects of temperature in animal condition and growth during the treatments (Fig. 1A, B, C, D, E and F). Condition index (CI) showed a significant effect at 14 °C and 18 °C during the experiment with values significantly lower for oysters kept at the highest temperature at months three and four compared to the beginning of the experiment (Fig. 1G).

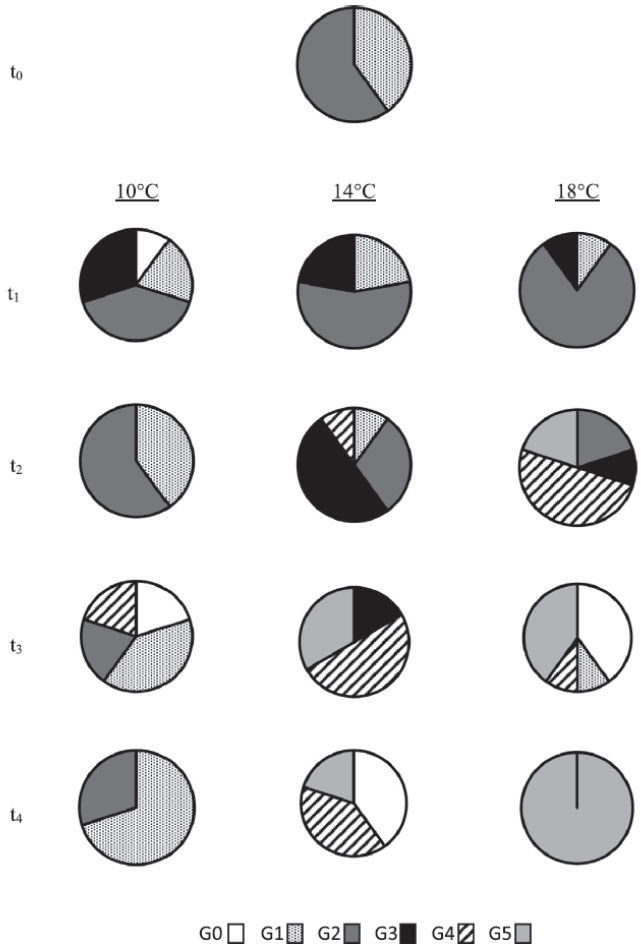

Fig. 3. Proportion of *Ostrea edulis* at different stages of gonad development (n = 10 per treatment) under different temperature treatments (10, 14 and 18 °C) during four months. According to da Silva et al. (2009) developmental stage was classified by the gametogenic stage of the gonad as inactive (G0), early gametogenesis (G1), advanced gametogenesis (G2), ripe gonad (G3), partially spawned gonad (G4) and reabsorbing gonad (G5).

3.3. Effect of temperature treatments on gonadal development and sex ratio

According to histological examination of gonadal tissue (Appendix B) sex ratio (males:females) changed throughout the experiment suggesting that it was significantly influenced by temperature and time during the treatments. At the beginning of the experiment 90% of oysters were hermaphrodites and 10% males (Fig. 2). Thereafter different sex proportions (Fig. 2) and different stages of gonad development (Fig. 3) were identified depending on treatment temperature.

At 10 °C the ratio of females increased by the end of the experiment. The proportion of males and females was not significantly different from 1:1 during the first two months, but it was different by the third and fourth months with sex ratios of 1.5:1 and 0.25:1, respectively (Fig. 3). At the end of the treatment at 10 °C, 80% of oysters had developed as females and just 20% as males. The percentage of hermaphrodites decreased over time with HPF occurring in similar proportion during the exposure and HPM only found at the initial time and first month.

At 14 °C the percentage of females and males increased during the first two months at the same time that the percentage of hermaphrodites decreased. Throughout the exposure there was a smaller proportion of females compared with males. Incubation at 14 °C had the greatest effect on stimulating male gonad development, with sex ratios (M:F) significantly different from 1:1 at the second (2:1), third (2:1) and fourth months (1.5:1) (Fig. 2). At the end of the fourth month at 14 °C, just 10% of oysters were identified as females, 30% as males, 10% as HPF and 50% were in an inactive or undifferentiated state of gonadal development (Fig. 2 and Fig. 3).

At 18 °C the proportion of females increased until the end of the second month and then decreased at the end of the third month. The proportion of males was similar during the treatment (Fig. 2). The sex ratios (M:F) at this temperature showed significant differences from 1:1 at the second (0.43:1) and third (0.2:1) months. Hermaphrodites decreased at the end of first month and they disappeared at the second and third months of treatment (Fig. 2). The first undifferentiated animals were observed again at the third month showing an inactive state of gonadal development. At the end of the treatment all the oysters were spent.

Gametogenic changes showed an effect of temperature on the gonadal maturation in *O. edulis* (Fig. 3). The asynchrony among individuals was evident showing a high variability among the individual oysters in all the treatments. In spite of this variability, it was possible to follow the gonadal maturation process at each temperature. At the beginning of the experiment, 40% of the oysters analysed were classified in gonadal stage G1 and 60% in stage G2. At 10 °C, a slow progress in gonadal maturation was observed during the first month, presenting

mainly stages determined as G1 and G2 and around 30% of the animals were classified as G3. Then the gonad follicles were filled mainly by a few oogonia and spermatogonia, and the follicles gradually became larger with more developed cells. By the third month of treatment at 10 °C, the percentage of animals with gonads in G2 decreased and 20% of the animals were classified as G4. The proportion of oysters in G1 and G2 increased by the fourth month at this temperature.

Accelerated gametogenesis was more evident at 14 °C. The percent classified as G1, G2 and G3 were similar compared to 10 °C during the first month (Fig. 3). By the second month the percent classified as G3 increased to 50% and G4 classified oysters were observed for the first time. The third month was characterized by animals in the later maturation stages (G3, G4 and G5) showing well developed gonads. Oysters with gonads in G0 were found at the end of the treatment at 14 °C reflecting the preparation of the oysters at this temperature to start a new cycle again.

At 18 °C gametogenesis was faster than at 14 °C and 10 °C. At the second month of treatment 20%, 10%, 50% and 20% of the oysters were in the stages G2, G3, G4 and G5, respectively (Fig. 3). Subsequently empty gonad follicles showed complete absorption of residual gametes, with 40% of the oysters staged as G0 by the end of the third month. By the fourth month, 100% of the animals were staged at G0 indicating they were ready to start the gonadal maturation process again (Fig. 3).

3.4. Effect of temperature treatments on endogenous hormones

The standardization of the protocol for hormonal analysis ELISA kits produced linear standard curves of R value of 0.999 and 0.988 for E_2 and T standards, respectively (data not shown).

Gonad E_2 concentrations increased at all temperatures showing significant differences compared to the beginning of the experiment (Fig. 4A). The maximum peak concentration for this hormone was the fourth, third and second month at 10, 14 and 18 °C, respectively (Fig. 4B, D and F). After these months the E_2 concentration decreased until the end of the experiment. Significant differences in E_2 were found between all the temperatures analysed for the same month except at the fourth month when significant differences were found only between 14 and 18 °C (Fig. 4B, D and F). The pattern observed for T concentration was different, with an increase at the beginning of the experiment until the second month when a maximum peak was reached for 10 and 14 °C. Then the T concentration decreased for the third and fourth month of treatments (Fig. 4C and E). On the contrary the maximum peak for T concentration at 18 °C was detected at the end of the first month of treatment and it decreased until the end of the experiment (Fig. 4G).

Some biometric parameters were correlated with hormone concentrations. A significant weak negative relation between E_2 concentrations and weight ($r_s = -0.198$; $p = .023$) was found. Significant weak positive relations between T concentration and width ($r_s = 0.179$; $p < .04$), fresh tissue weight ($r_s = 0.174$; $p = .046$) and CI ($r_s = 0.204$; $p = .019$) were identified.

Hormone homologue concentrations in gonadal tissue of *O. edulis* showed a weak but highly significant correlation ($r_s = 0.311$; $p = .0003$) between E_2 and T concentrations during the temperature treatments. However, most of the female gonads presented T concentrations as high as males and E_2 concentrations lower than T levels in most of the samples (data not shown). A direct relation between sex determination results through histology and hormone concentrations for all the temperatures was not found. However, a tendency was observed with oysters kept at 14 °C showing the highest values for T concentrations and more individuals classified as males at this temperature. In the same way, at 18 °C more individuals were classified as females and these individuals exhibited the highest values for E_2 concentrations.

The presence of both hormones at 10 °C was very similar, without any significant difference in concentration recorded between sexes (Fig. 5A). At 14 °C the concentration of testosterone homologues was always higher than estradiol for all the sex categories with the highest values presented by individuals classified through histology as males (Fig. 5B). However no significant differences were found for T concentration between sexes at this temperature. There was a significant difference for E_2 concentration for males compared with hermaphrodites, but not between males and females oysters kept at 14 °C.

Estradiol concentrations at 18 °C were significantly higher than testosterone for females (Fig. 5C). Although males showed the same tendency no significant difference was found. Animals classified through histology as females at this temperature exhibited the highest values for E_2 between all the treatments (Fig. 5C). A significant difference for both hormones between males, females and hermaphrodites was found at 18 °C.

No significant differences between hormone concentrations were found between hermaphrodites (HBS, HPM and HPF) at any temperature so they were treated in the same group (as hermaphrodites) for comparison with the other sex categories.

The comparison of hormone concentrations between stages of gonadal maturation showed an increase from G0 to G2, then showed a slight decrease for both hormones at G3 and finally a second increase was observed at G4 and G5 (Fig. 6). However, no significant differences in hormone concentrations were found between stages.

4. Discussion

4.1. Effect of temperature treatments on gametogenesis

Temperature has been shown to be a key factor during the maturation of oysters; affecting biochemical and physiological components involved in the maturation process (Korringa, 1957; Mann, 1979; Newell et al., 1977; Newell and Branch, 1980). At colder temperatures *O. edulis* remains in a resting stage, with no gonadal development reported at temperatures below 7 °C, but formation of eggs or sperm is triggered in the spring when temperatures start to increase (Korringa, 1952; Loosanoff and Davis, 1952; Mann, 1979). A positive effect of temperature on the gonadal development of *O. edulis* has been shown in this study. Accelerated gametogenesis was more evident at the highest temperature compared with the other treatments and by the fourth month of treatment all the oysters were in an inactive state of gonadal development. It is known that elevated temperatures increase metabolism, accelerate rates of oxygen consumption and ammonia excretion but also accelerate gametogenesis and gonadal development in bivalves (Chávez-Villalba et al., 2003; Pérez et al., 2013; Santerre et al., 2013; Shpigel et al., 1992; Teaniniuraitemoana et al., 2016). Indeed, others have previously reported that the gonadal volume occupied by germinal cells almost doubled for oysters conditioned under a gradient of temperature between 14 and 18 °C compared to those oysters kept at 15 °C, indicating that change in temperature could trigger and accelerate this process (Maneiro et al., 2017).

Gonadal development in bivalves normally takes place using reserves of carbohydrate and lipids stored prior to the initiation of gametogenesis (Shpigel et al., 1992). This makes the process of gametogenesis dependent on temperature and the availability of stored nutrient reserves. A reduction in CI related to the production of gametes due to the demand for energy reserves stored in tissues has been observed (Shpigel et al., 1992). Accordingly, oysters kept at 18 °C showed the lowest values for most of the biometric measurements. Hereby the relation between temperature and the energy allocation to initiate gametogenesis in bivalves could explain the rapid maturation and the accelerated gametogenesis process observed for oysters kept at 18 °C in this study.

4.2. Effect of temperature treatments on sex ratio

Factors controlling the sex ratios in natural populations of oysters

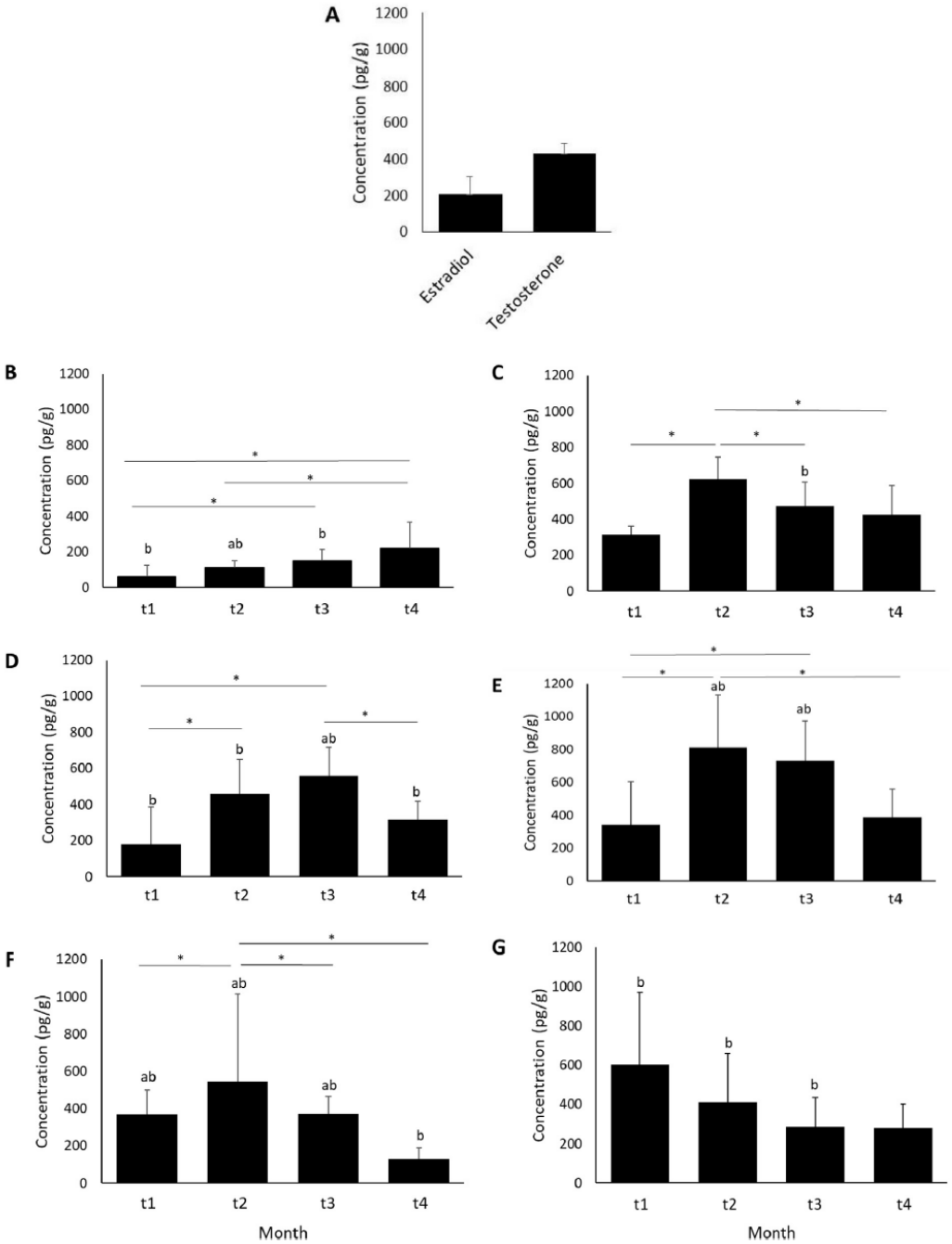

Fig. 4. Hormones concentrations per treatment (A) at the beginning of the experiment (t0) and during four months of treatment at 10 °C (B, C), 14 °C (D, E) and 18 °C (F, G). Estradiol concentration (B, D, F) and Testosterone concentration (C, E, G). (a) significant differences compared to the beginning of the experiment (t0), (b) significant differences between temperatures at the same time, and (*) significant differences between times at the same temperature.

remain unclear. Some field studies on oysters in the family Ostreidae have reported the effects of environmental factors, such as temperature, salinity and food availability, among others, on sex ratio (Acarli et al., 2015; Eagling et al., 2018; Hassan et al., 2018). In natural populations biased sex ratios are expected and frequently observed for strict sequential hermaphrodites (sex-changing species) and in species where sex is determined by environmental conditions experienced during pre-adult development (Charnov and Bull, 1989). Modelling has

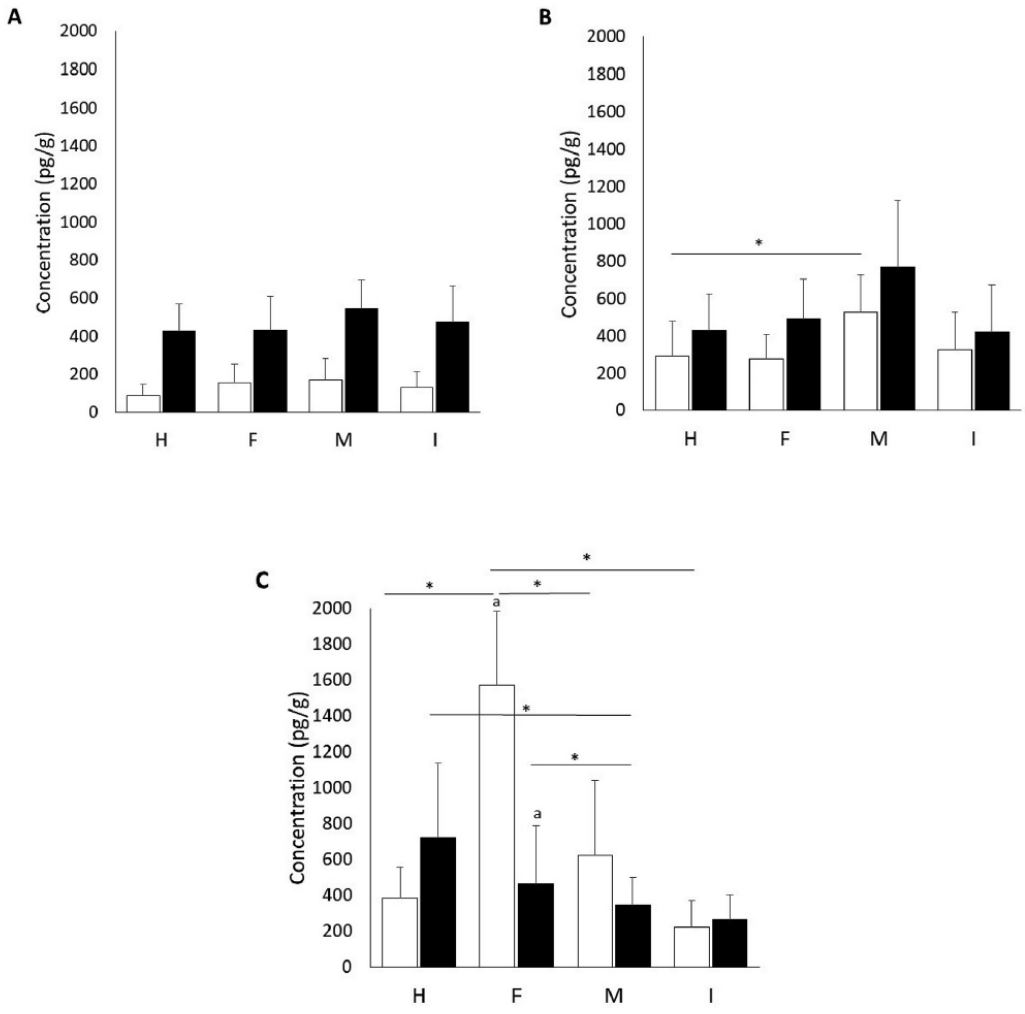

Fig. 5. Hormones concentrations for animals classified through histology as hermaphrodites (H), females (F), males (M) or in an inactive stage (I) under a treatment of (A) 10 °C, (B) 14 °C and (C) 18 °C. White bars: estradiol. Black bars: testosterone. (*) significant differences for the same hormone between sex categories at the same temperature. (a) significant differences between hormones for the same sex category.

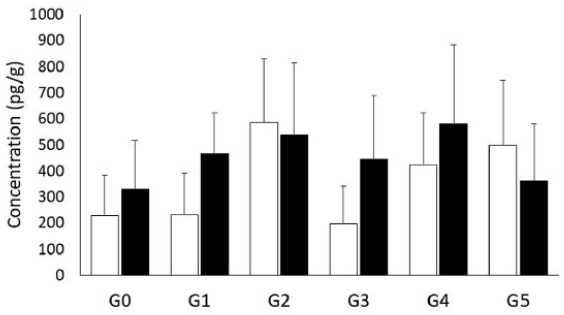

Fig. 6. Hormone concentration according to the stage of gondal maturation in *Ostrea edulis* classified as inactive (G0), early gametogenesis (G1), advanced gametogenesis (G2), ripe gonad (G3), partially spawned gonad (G4) and re-absorbing gonad (G5) according to da Silva et al. (2009). White bars: estradiol. Black bars: testosterone.

demonstrated a trend to present a skewed sex ratio towards the first sex in some sequential hermaphrodite species (Baeza et al., 2010; Charnov and Bull, 1989); however, the sex ratio and other sex allocation parameters differ between species. For instance, not all the protandric species featured a male-skewed sex ratio in the adult life showing a large range in sex ratio variation (Allsop and West, 2004; Collin, 2006).

O. edulis usually first undergo gametogenesis as a male and, when older, the oyster can alternate between female and male functions (Cole, 1942b; Loosanoff, 1962; Loosanoff and Davis, 1952; Orton, 1927a, 1927b) but the influence of temperature on this process and the determination of sex ratios in natural populations remains unclear in this species. Few studies have experimentally evaluated the effect of temperature in *O. edulis*, and the results have indicated that lower temperatures are implicated in the development of female germinal cell lines causing a female-bias at the beginning of the breeding season with coldest water temperatures, whereas male gonads appeared when temperatures were warmer (Joyce et al., 2013; Loosanoff, 1962; Loosanoff and Davis, 1952).

These earlier reports are supported by our study, which demonstrated a higher proportion of females found at the lowest temperature (10 °C) and a higher proportion of males at 14 °C. It has been suggested that there is an energetic cost related to sex inversion and the production of female gametes could be more energetically costly than the production of male gametes (Pérez et al., 2013; Wright, 1988). In environments with a high energy demand the oysters could not gain enough energy from the diet and reserves to initiate gametogenesis (Santerre et al., 2013). In such a case, a protandric species will save energy by producing the low cost male gonads and allocating the energy reserves into survival or growth, and later when the environmental conditions become more favourable, they would be able to change to female (Pérez et al., 2013; Santerre et al., 2013). For instance, it has been shown that *Aulacomya atra* and *Scrobicularia plana* males and females reach a similar energy content of the mantle-gonad (ECMG), but they use this energy in a different way: males have gonads of larger size but with lower energy per unit of mass than females (Mouneyrac et al., 2008; Pérez et al., 2013).

In this study, the sex ratio was biased towards females at 18 °C. This behaviour contradicts the expected response that at high temperatures protandric species will save energy through the production of the low cost male gonads (Pérez et al., 2013; Wright, 1988). However, it has been shown that food availability can affect reproduction, with high concentrations of microalgae promoting gonadal cell proliferation and gamete maturation in *P. margaritifera* (Teaniniuraitemoana et al., 2016). In that study, the animals kept at high temperature (28 °C) showed females transitioning into males after exposure to low food availability and females presenting male and female gametes together under a high food treatment. The same behaviour has been shown in other bivalves. In a similar manner, *Aequipecten irradians concentricus* showed that the oogonia differentiation started when a minimum temperature in warmer waters was reached but the fecundity and gonadal size were determined mainly by food availability (Sastryz, 1965).

The oysters used in this study were fed ad libitum to cover the energy demands needed to go through gametogenesis. This could suggest that under favourable conditions and in an environment with enough food to supply a high energy demand *O. edulis* could be expected to go through a faster gametogenesis which favours female gonadal production. It has been reported that under exceptionally favourable conditions, *O. edulis* have the potential to reach maturity and spawn several times during the same season because even just a few hours after releasing eggs or sperm the gonads can begin to change into the opposite gender (Korringa, 1957). Furthermore, sex ratios in oysters could favour females when food availability is high (Chávez-Villalba et al., 2003). It has been reported that *O. edulis* only has the ability to become a functionally mature female following an exceptional summer period because it needs a large quantity of energy to produce ovaries (Dodd et al., 1937). This evidence supports the female-biased sex ratio observed in this study at the highest temperature but further studies with larger sample sizes would help in understanding natural sex ratios in this species.

Sea-surface temperatures in the north east Atlantic and UK coastal waters have been rising since the 1980s by around 0.2–0.9 °C per decade with the most rapid rises occurring in the southern North Sea and the English Channel (Holliday et al., 2008), with ongoing rises predicted (Marine Climate Change Impacts Partnership, 2015). The year 2006 was the second-warmest year in UK coastal waters since records began in 1870 and seven of the 10 warmest years have occurred in the last decade. This could be implicated with the skewed sex ratio towards male-phase oyster of 3:1 and 6:1 found by Eagling (2012) and Kampahusen (2012) in oyster populations in the Solent.

The reason why different species show a different effect in gametogenesis and gender determination in response to changes in environmental conditions is not clear and more studies are needed. Some species have a fixed size at sex change and others have plastic responses (Benvenuto et al., 2017; Hamilton et al., 2007). Fisheries exploiting hermaphroditic species may affect sex ratios by skewing these towards the sex that matures first, producing a population with smaller and younger individuals (Hamilton et al., 2007). Thus, finding *O. edulis* populations in the Solent with a considerable skew towards males (Eagling, 2012; Kamphausen et al., 2011) raises the question of whether fishery practice is responsible for the removal of larger individuals leaving only smaller individuals with the first sex (males), and/or if the changes in local environmental temperatures are modifying the reproductive behaviour of this species.

4.3. Concentration of endogenous hormones levels under different temperature treatments

Most research on steroid concentrations in invertebrates, including this study, has used immunoassays as the detection system, with the possibility of having cross-reactivity of these assays with other steroids (Janer and Porte, 2007; Lafont and Mathieu, 2007). According to the manufacturer (Cayman Chemical Co.; Ann Arbor, MI, USA) these kits have 100% specificity and the percentage of detection for hormone homologues is low. Thus, it is strongly recommended to include the detection and characterization of homologues as an important step in this type of study.

It has been reported that environmental factors, especially temperature, combining with hormonal control are involved in the gender determination of adult pearl oysters (*Pinctata margaritifera*) (Teaniniuraitemoana et al., 2016). However, the lack of a direct relation between sex determination results (from histology) and endogenous hormone concentrations for the oysters at the end of the current study indicates that other biochemical pathways are involved in the gonadal development and that maturation in *O. edulis* are independent of steroid hormones.

The lower E_2 concentrations compared with T levels found in most of the samples of this study (data not shown) has been also reported in other species. Female gonads presenting a higher level of T than testis and E_2 levels found in the testis much higher than in the ovaries of *Mytilus edulis trossulus* were reported (Zabrzańska et al., 2015). This result, along with the similar hormone concentrations found for female and male gonads in most of the treatments and sex categories, indicate a lack of a direct relation between the different stages of gonadal maturation. Sex determination results through histology and hormone concentrations could indicate that these steroids may not be actively involved as endogenous modulators in gonadal maturation and sex determination in this species. A lack of differences in testosterone and estradiol content has been shown in other bivalve species such as *Mya arenaria* (Gauthier-Clerc et al., 2006), *Mytilus edulis* (Reis-Henriques et al., 1990), and *Patinopecten yessoensis* (Osada et al., 2004).

Several reviews have reported evidence about the presence, metabolism and enzymatic pathways of sex steroids, e.g., testosterone, androstenedione, and estradiol occurring in several invertebrate species (Fernandes et al., 2011; Janer and Porte, 2007; Lafont and Mathieu, 2007; Le Curieux-Belfond et al., 2001). Fluctuations in levels of sex steroids have been found to be correlated with the sexual maturation cycle in a number of bivalves, thus suggesting that sex steroids may play important stimulatory roles in their reproductive regulation (Gauthier-Clerc et al., 2006; Ketata et al., 2008; Le Curieux-Belfond et al., 2001). In this context, some studies have concluded the central role of estrogens in the natural gametogenic cycle in oysters, scallops, and clams (Gauthier-Clerc et al., 2006; Mori et al., 1972). In the soft clam *Mya arenaria*, Gauthier-Clerc et al. (2006) suggest that estradiol-17β and testosterone act as endogenous regulators of gametogenesis, and other studies suggest similar, though species-specific, roles in oyster *Magallana gigas* (formerly *Crassostrea gigas*) (Mori, 1969; Mori et al., 1972) and scallops *Placopecten magellanicus* (Wang and Croll, 2006).

However, others have questioned the role and endogenous origin of vertebrate-type steroids, their regulation and synthesis in molluscs (Lafont and Mathieu, 2007; Fernandes et al., 2011; Scott, 2013, 2012).

Scott (2012) argued that the seasonal changes in hormone concentrations reported in some studies could be more related to an increase in fatty acids, lipids and proteins during reproductive maturation, with hormones taken up from the environment or through a dietary source and stored in the form of fatty acid esters for days or even months. In fact, various steroids are always present in the animal's food and in the environment as a product of physiological process in other animals or anthropogenic activities (Lafont and Mathieu, 2007) and this could be an external source for these hormones identified in the current study. It may well be that the presence of E2 and T in the gonad tissues of these oysters was a function of accumulation of these steroid homologues from the phytoplankton food source. The potential for the exogenous origin of steroid homologues and the presence of steroid pathways in oysters represents the focus of on-going study by this research team.

5. Conclusions

A positive effect of temperature on the gonadal development of *O. edulis* was found. At the highest temperature treatment, the oysters went through a faster gametogenesis process and all of them were in an inactive state of gonadal development at the end of the treatment. Furthermore, in this study, the sex ratios (males:females) changed throughout the experiment suggesting that the ratio was significantly influenced by temperature and time during the treatments. The lowest and highest temperatures analysed in this study caused a female-biased sex ratio in adults, but at 14 °C a higher proportion of males than females was found. The results from this study also suggest that sex determination could be affected by other parameters such as food availability, indicating a complex relation in terms of energy allocation for sexual maturation. It could therefore be expected that a rise in sea temperatures and warmer conditions in European waters through the year, potentially combined with differences in phytoplankton food supply (species assemblage and concentration), could influence the processes of gametogenesis, sex determination and sex ratios, affecting the long term health of populations. Although it has been reported that environmental factors, especially temperature, combined with hormonal control are involved in the gender determination and the sexual maturation cycle of other bivalves, this study demonstrated the lack of a direct strong relation between sex determination results through histology and endogenous hormone concentrations at three different temperatures. These results together could indicate that other biochemical pathways are involved in the gonadal development and maturation in *O. edulis* independent of steroid hormones.

Declaration of Competing Interests

The authors declare no conflict of interest.

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Appendices. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cbpa.2019.06.023.

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Appendix B

STANDARD OPERATING PROCEDURE OCEAN AND EARTH SCIENCE RESEARCH AQUARIUM	
SOP: Bulk Algal Culture	ORIGINATOR NAME AND DATE: Nicola Pratt 10/07/2014
VERSION NUMBER: 3	REVIEWED BY, AND DATE: Nicola Pratt 08/07/2016
ASSOCIATED DOCUMENTS: Bulk Algal Culture Risk Assessment, Bulk Algal Culture COSHH Forms	
PPE REQUIRED: Laboratory Coat, Gloves, Safety Glasses	
Location: 491/02, 454/07 and 456/01	

Protocol for Preparing Reagents:

a) Algal Nutrient Medium Stock Solution (from Helm *et al*., 1979).

This protocol is carried out in 454/07 or 456/01. A laboratory coat, safety glasses and nitrile gloves are required.

The following chemicals are weighed out (following handling guidelines in Bulk Algal Culture COSHH) and are added to approximately 4.5 litres of de-ionised water in a 5 litre conical flask:

Chemical	**Weight (g)**
Iron (III) chloride 6-hydrate	6.5
Manganese (II) chloride 4-hydrate	1.8
Boric acid	168.0
Ethylenediaminetetraacetic acid (disodium salt) (EDTA)	225.0
Sodium dihydrogen orthophosphate 2-hydrate	100.0
Sodium nitrate	500.0
Trace metal solution (see below)	5.0 (ml)

The conical flask is placed on a magnetic stirrer unit and a magnetic stir bar is added. The flask is then covered with Parafilm and the solution is left to stir until all the chemicals have dissolved (approximately 8 hours or overnight). Once all the chemicals have dissolved, the solution is made up

to 5 L with de-ionised water and the magnetic stir bar is retrieved. The resulting solution is decanted in 1 L aliquots into 5 x 1 L Duran bottles. The lids are placed on loosely and the bottles are sterilised in the autoclave according to the instructions provided on the unit. Once the autoclave has finished its sterilising cycle and has cooled down, the bottles are removed, lids tightened and stored in the yellow chemicals cabinet in room 491/02.

b) Trace Metal Solution.

This protocol is carried out in 454/07 or 456/01. A laboratory coat, safety glasses and nitrile gloves are required.

The following chemicals are weighed out (following handling guidelines in Bulk Algal Culture COSHH) and are added to approximately 80 ml of deionised water in a 200 ml Duran bottle. The bottle is placed on a magnetic stirrer unit and a magnetic stir bar is added. The lid is placed on loosely and the solution is left to stir until all the chemicals have dissolved (a few minutes).

Chemical	**Weight (g)**
Zinc chloride	2.1
Cobalt (II) chloride 6-hydrate	2.0
Ammonium molybdate 4-hydrate	0.9
Copper sulphate 5-hydrate	2.0

This reagent needs to be acidified to produce a clear solution. Small aliquots of dilute (0.1M) hydrochloric acid are added using a Pasteur pipette whilst on the stir plate until the solution becomes clear. Once the solution clears, the solution is made up to 100 ml with de-ionised water and the magnetic stir bar is retrieved. The lid is placed on loosely and the bottle is sterilised in the autoclave according to the instructions provided on the unit. Once the autoclave has finished its sterilising cycle and has cooled down, the bottle is removed, lid tightened and stored in the yellow chemicals cabinet in room 491/02.

c) Silicate Solution (required for the diatoms).

This protocol is carried out in 454/07 or 456/01. A laboratory coat, safety glasses and nitrile gloves are required.

40 g of sodium metasilicate 5-hydrate are weighed out (following handling guidelines in Bulk Algal Culture COSHH) and are added to approximately 800 ml of deionised water in a 1 L Duran bottle. The bottle is placed on a magnetic stirrer unit and a magnetic stir bar is added. The lid is placed on loosely

and the solution is left to stir until all the sodium metasilicate has dissolved (a few minutes). Once all the sodium metasilicate has dissolved, the solution is made up to 1 L with de-ionised water and the magnetic stir bar is retrieved. The lid is placed on loosely and the bottle is sterilised in the autoclave according to the instructions provided on the unit. Once the autoclave has finished its sterilising cycle and has cooled down, the bottle is removed, lid tightened and stored in the yellow chemicals cabinet in room 491/02.

d) Thiosulphate stock solution.

This protocol is carried out in 454/07 or 456/01. A lab coat is required.

1 kg of sodium thiosulphate 5-hydrate is weighed out and added to approximately 4.5 litres of de-ionised water in a 5 litre conical flask. The conical flask is placed on a magnetic stirrer unit and a magnetic stir bar is added. The flask is then covered with Parafilm and the solution is left to stir until all the sodium thiosulphate has dissolved (a few minutes). Once all the sodium thiosulphate 5-hydrate has dissolved, the solution is made up to 5 L with de-ionised water and the magnetic stir bar is retrieved. The resulting solution is decanted in 1 L aliquots into 5 x 1 L Duran bottles. The lids are placed on loosely and the bottles are sterilised in the autoclave according to the instructions provided on the unit. Once the autoclave has finished its sterilising cycle and has cooled down, the bottles are removed, lids tightened and stored in the yellow chemicals cabinet in room 491/02.

2. Protocol for Sub-culturing Bulk Algal Culture:

This protocol is carried out in 194/02, following handling guidelines in Bulk Algal Culture COSHH when using sodium hypochlorite and ethanol. A laboratory coat, safety glasses and nitrile gloves are required when handling these chemicals.

The algae are sub-cultured by saving 5 L of a stationary phase culture and using it to start 2 new culture flasks.

A 5 L conical flask is sterilised by carefully rinsing the inside with 100% ethanol. The ethanol is poured off into a bottle and used repeatedly. 5 L of a stationary phase culture are carefully poured into the conical flask from a 20 L Nalgene bottle - the air supply to which has been turned off. The conical flask is then stoppered with a rubber bung. The remaining algae in the Nalgene flasks is then transferred to the aquarium for any required feeding (follow manual handling guidelines when carrying Nalgene flasks as outlined in Bulk Algal Culture RA). Once empty, the flasks are rinsed with hot water and scrubbed using the bottle brushes provided. The flasks are refilled with 20 L of aquarium seawater via the seawater tap located next to the sink in the culture room.

In the culture room 25 ml of the algal nutrient stock solution and 50 ml of sodium hypochlorite solution are added carefully to each flask. For the diatom species 10 ml of sodium metasilicate solution are also added. All pipettes should be sterilised with ethanol before use to avoid contamination of stock solutions. The bungs and air lines are cleaned in the sink using hot tap water and are then placed back in the Nalgene flasks. The flasks are then gently swirled to ensure that the bleach rinses the whole of the interior. The flasks are then transferred back to the shelving and are allowed to stand for at least five hours. This gives time for the bleach to sterilise the water. After five hours, a 25 ml pipette is sterilised with 100% ethanol and is used to add 25 ml of the sodium thiosulphate solution to each of the Nalgene flasks. The bottles are carefully given a swirl to ensure that all of the bleach is neutralised. The cultures are then restarted by carefully pouring 2.5 L of the reserved algae from the conical flask into both of the Nalgene flasks. The flasks are then placed back on their shelves, airlines re-attached and air supplies re-started.

Note:

When removing bungs from the culture vessels to take algae for experiments, ensure that the work surface is thoroughly decontaminated with 70% ethanol, before placing the bung on the work surface. This is to minimise accidental contamination of the culture when the bung is placed back on the vessel.

A B C

20L bottles with single algae culture used in mixed diet for feeding *O. edulis*. (A) *Tetraselmis suecica*, (B) *Pavlova lutheri* and (C) *Phaedactylum tricornutum*.

Appendix C

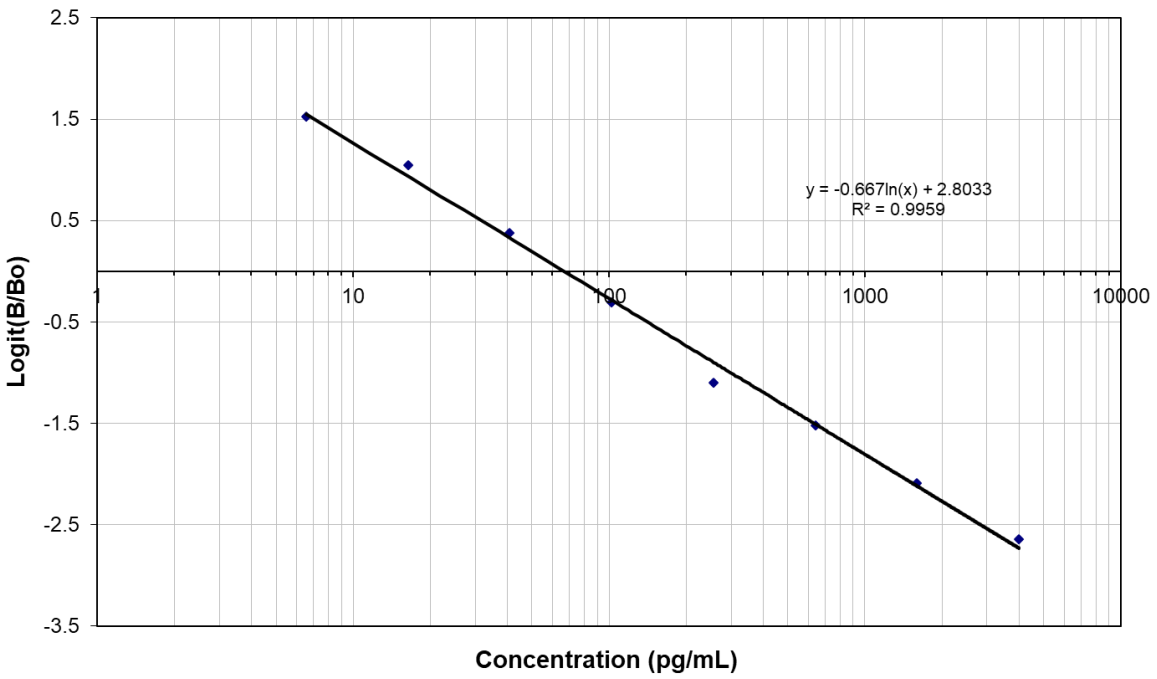
Oysters Total Weight (W), Height (H), length (L), width (Wi), Shell volume (Vol), flesh weight (FW) and Condition Index (CI) at different temperature treatments during four months. Values are expressed as means ± standard deviation

Time (month)	Temp. (C)	W (g)	H (mm)	L (mm)	Wi (mm)	Vol (ml)	FW (g)	CI
0		70.88±5.14	72.26±6.44	67.38±10.37	22.32±1.49	43.15±3.69	8.35±1.54	11.80±2.08
1	10°C	70.03±4.49	72.20±4.38	65.73±7.75	23.45±1.79	43.67±3.00	8.66±1.43	12.40±2.11
	14°C	73.987±6.41	73.18±2.02	68.70±4.17	25.675±4.92	47.79±5.03	9.54±1.12	12.99±2.17
	18°C	68.05±7.44	72.48±5.27	64.38±7.64	22.225±2.13	42.81±3.56	8.46±1.61	12.52±2.50
2	10°C	73.48±5.69	75.75±2.55	71.93±4.68	24.34±1.51	45.83±5.20	9.32±1.74	12.70±2.25
	14°C	73.34±11.33	72.18±3.73	68.52±4.84	26.09±4.94	46.41±6.98	9.93±1.91	13.62±2.06
	18°C	62.78±5.38	70.33±3.83	68.33±4.50	21.74±2.09	41.99±2.68	7.44±1.06	11.89±1.78
3	10°C	72.79±5.12	72.34±3.83	68.93±6.69	23.84±4.38	52.58±4.29	7.79±1.82	10.67±2.11
	14°C	71.36±5.66	70.93±2.56	70.41±5.63	23.06±2.83	46.57±4.02	7.18±0.72	10.09±1.03
	18°C	70.28±10.54	69.25±3.43	70.81±5.77	22.62±2.61	45.68±8.16	6.76±1.34	9.60±1.01
4	10°C	65.32±7.13	72.47±4.21	69.33±7.59	22.97±2.78	43.89±5.45	7.69±1.94	11.69±2.25
	14°C	72.65±6.38	71.61±4.11	70.18±5.13	23.22±2.48	47.86±4.75	7.36±1.52	10.16±1.86
	18°C	73.58±8.66	70.51±4.22	68.71±2.91	23.05±4.39	52.54±7.41	5.84±1.21	8.00±1.75

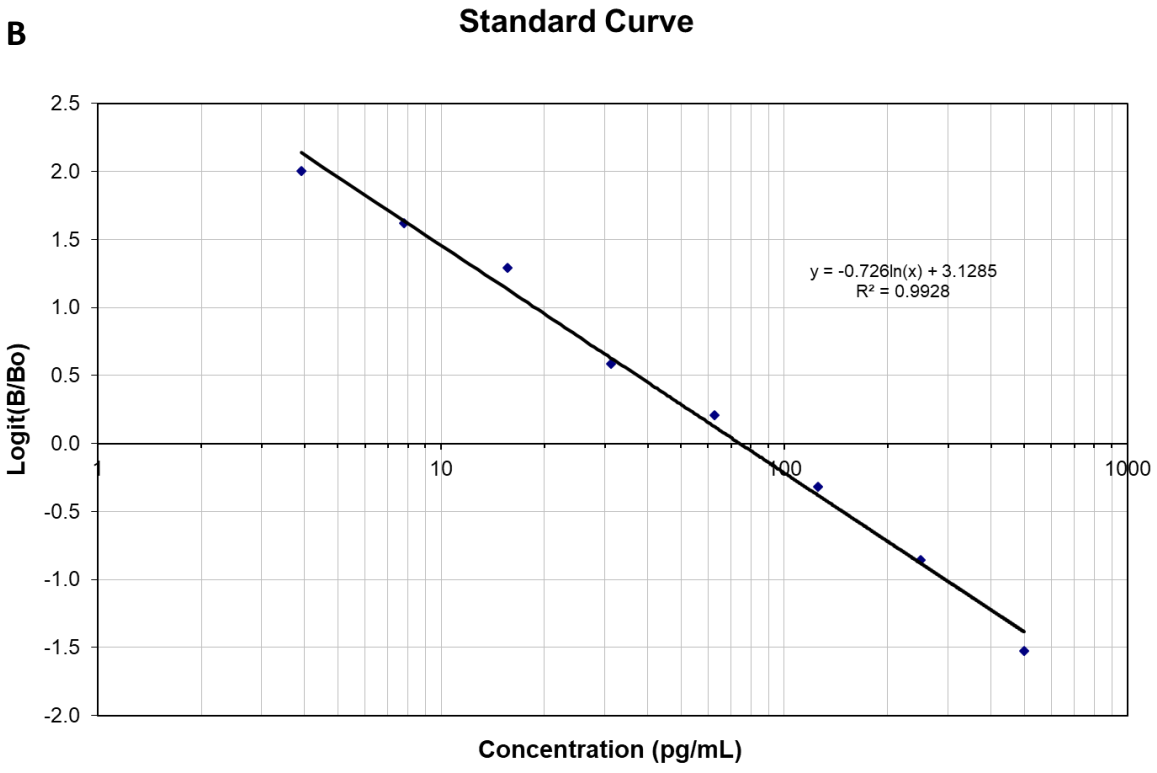
Appendix D

Examples of standardization curves for ELISA assays for (A) E_2 and (B) T analysis.

A



B



Appendix E

Oysters Total Weight (W), Height (H), New growth (NG), length (L), width (Wi), Shell volume (Vol), flesh weight (FW) and Condition Index (CI) of *O. edulis* kept in the dock side tank at NOCS from May 2016 to June 2017. Values are expressed as means ± standard deviation.

Month	W (g)	H (mm)	NG (mm)	L (mm)	Wi (mm)	Vol (ml)	FW (g)	CI
May 2016	69.18±4.15	72.14±1.30	0	64.08±4.56	24.58±44.2	44.2±2.45	8.96±1.40	13.00±2.19
Jun 2016	82.45±10.80	78.6±4.70	1.4±1.98	72.16±6.52	24.74±3.91	53.78±7.34	8.27±1.29	10.21±2.47
Jul 2016	78.91±9.82	82.10±6.94	9.94±5.38	77.93±6.66	25.26±4.56	49.66±5.82	8.82±0.96	11.22±0.85
Aug 2016	77.32±10.35	81.80±2.92	7.13±5.82	75.61±5.08	22.73±4.26	61.90±7.71	8.82±0.55	11.50±0.91
Sep 2016	82.92±10.70	84.95±4.22	10.50±2.90	79.63±10.84	22.75±1.89	53.55±7.55	10.40±1.36	12.56±0.64
Oct 2016	74.69±5.54	80.21±5.82	3.90±2.75	72.63±4.91	24.32±3.19	48.27±2.80	11.28±1.34	15.11±1.45
Nov 2016	85.77±10.38	83.60±4.00	1.90±2.29	78.44±8.21	21.90±2.87	54.35±6.48	12.06±1.28	14.10±0.90
Dec 2016	81.68±12.61	81.31±6.00	3.30±3.81	76.78±9.77	25.56±1.81	61.71±11.75	11.24±0.91	13.91±1.28
Jan 2017	76.47±8.00	76.72±5.90	1.27±1.97	70.38±7.66	22.73±1.40	56.42±7.60	10.55±1.48	13.86±2.03
Feb 2017	82.97±10.87	80.32±7.94	0.60±1.46	74.42±8.24	22.89±1.84	58.92±11.28	10.24±1.19	12.41±1.10
Mar 2017	79.34±9.81	78.26±5.07	0.52±1.27	73.88±5.27	22.76±1.18	50.72±5.72	8.64±2.02	10.80±1.60
Apr 2017	79.99±6.75	79.61±7.24	0.00±0.00	77.13±8.72	21.76±1.96	50.59±4.60	9.76±0.71	12.23±0.81
May 2017	81.64±8.92	79.26±4.56	1.09±1.71	78.41±7.45	22.12±1.22	51.94±6.18	8.93±2.34	10.81±1.82
Jun 2017	83.66±5.03	80.32±7.94	0.60±1.46	74.42±8.24	22.89±1.84	58.92±11.28	10.24±1.19	12.41±1.10

Appendix F

RDA analysis for environmental factors and biological variables in *O. edulis*

Scores of biological responses on the two axis of the RDA analysis

```
WCanoImp produced data file
 RDA    Canonical axes:        4      Covariables:     0      Scaling:        2
Cent./stand.   by samples:     0      0      by species:     1      0
No transformation
Spec: Species scores (adjusted for species variance)
```

N	NAME	AX1	AX2	AX3	AX4	WEIGHT	1
	EIG	0.1022	0.0644	0.0174	0.0153		
1	Mortalit	0.7515	-0.3713	-0.0537	0.3373	1.00	1.00
2	Weight	-0.2317	-0.2133	0.1861	-0.0081	1.00	1.00
3	Height	-0.1365	-0.3888	-0.0822	0.1333	1.00	1.00
4	new grow	0.1986	-0.6075	-0.2680	-0.0631	1.00	1.00
5	Length	-0.1234	-0.3418	0.0608	0.0437	1.00	1.00
6	Width	0.1795	-0.0620	-0.0094	-0.0610	1.00	1.00
7	Shell vol	-0.2990	-0.1257	-0.1376	-0.2039	1.00	1.00
8	Fresh tissue	-0.2933	0.0026	-0.2852	0.3639	1.00	1.00
9	CI	-0.1356	0.1617	-0.4577	0.3840	1.00	1.00
10	Sex	0.0326	-0.0898	-0.1491	0.2778	1.00	1.00
11	Gonad stage	0.3379	-0.1453	0.3023	-0.3198	1.00	1.00
12	Estradiol	-0.4322	-0.4450	-0.1106	0.4191	1.00	1.00
13	Testosterone	-0.3790	0.1267	0.1959	0.1214	1.00	1.00
14	Vtg-like prot	-0.4389	0.1052	-0.0305	0.0699	1.00	1.00
15	Lipids	-0.0603	0.0021	-0.1127	0.0589	1.00	1.00
16	Carbohydrates	0.4167	-0.2350	-0.1487	0.0497	1.00	1.00
17	Proteins	-0.0130	0.1410	0.3155	0.0223	1.00	1.00

Regression/canonical coeficients for environmental variables

```
WCanoImp produced data file
 RDA    Canonical axes:        4      Covariables:     0      Scaling:        2
Cent./stand.   by samples:     0      0      by species:     1      0
No transformation
Regr: Regression/canonical coefficients for standardized variables
```

N	NAME	AX1	AX2	AX3	AX4
	EIG	0.1022	0.0644	0.0174	0.0153
1	Temperat	-1.6806	0.0175	3.4513	4.3278
2	Conducti	2.1859	0.6069	-3.8890	-3.1046
3	Salinity	-0.0486	0.2406	0.2475	1.4889
4	LDO	-0.0571	1.3363	-0.3385	2.6593
5	O2	0.3618	0.0803	0.8537	-1.2990
6	Ch a	0.4792	-0.3766	0.1030	0.1389

Correlations of environmental variables with axes

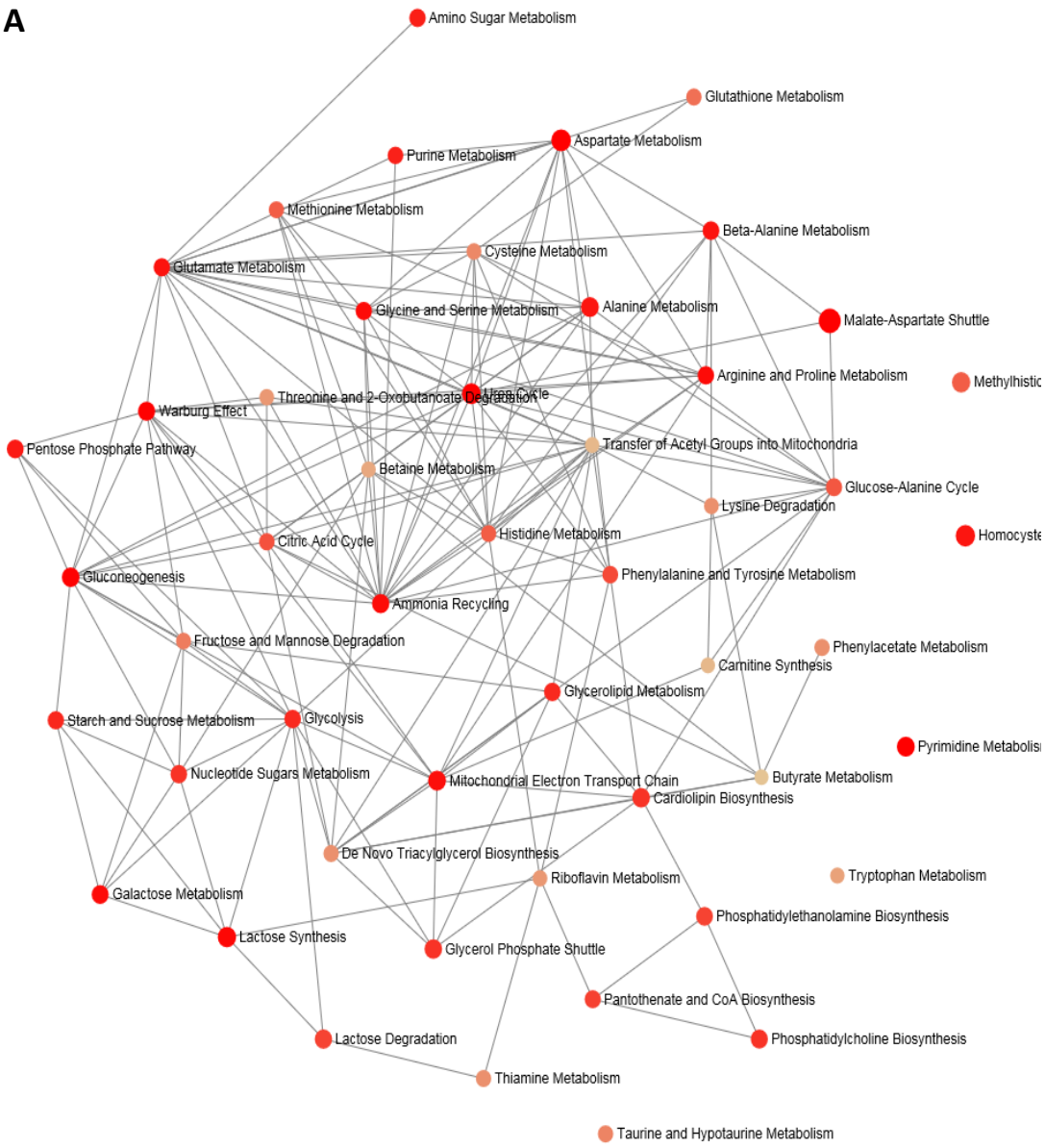
```
WCanoImp produced data file
 RDA    Canonical axes:        4       Covariables:     0      Scaling:        2
Cent./stand.   by samples:      0       0      by species:      1       0
No transformation
CorE: Inter set correlations of environmental variables with axes
```

N	NAME	AX1	AX2	AX3	AX4
	FR EXTRACTED	0.0911	0.0726	0.0266	0.0131
1	Temperat	0.3015	-0.2824	0.0259	0.0167
2	Conducti	0.3665	-0.2184	-0.0712	0.0174
3	Salinity	0.3416	-0.0257	-0.1437	0.2571
4	LDO	-0.1496	0.3932	0.0921	-0.0314
5	O2	0.2508	0.3347	0.3002	-0.0828
6	Ch a	0.3456	-0.2028	0.1855	0.0640

Appendix G

Metabolite enrichment analysis for oysters exposed to (A) testosterone and (B) estradiol

A

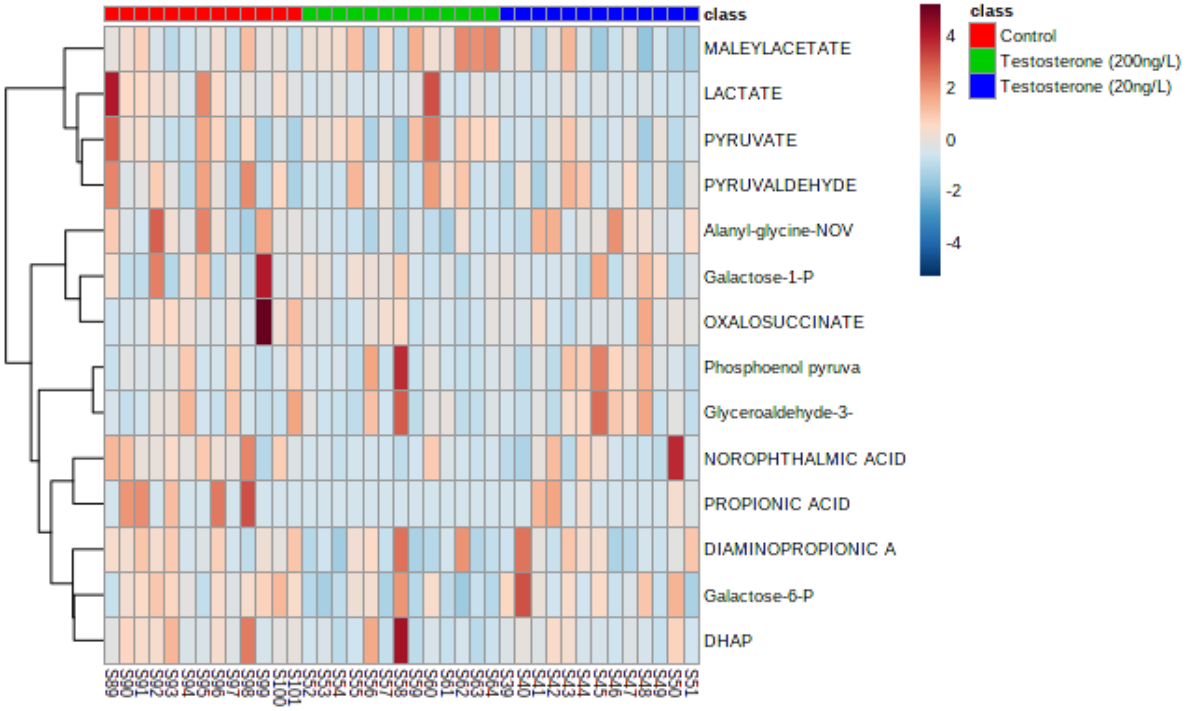


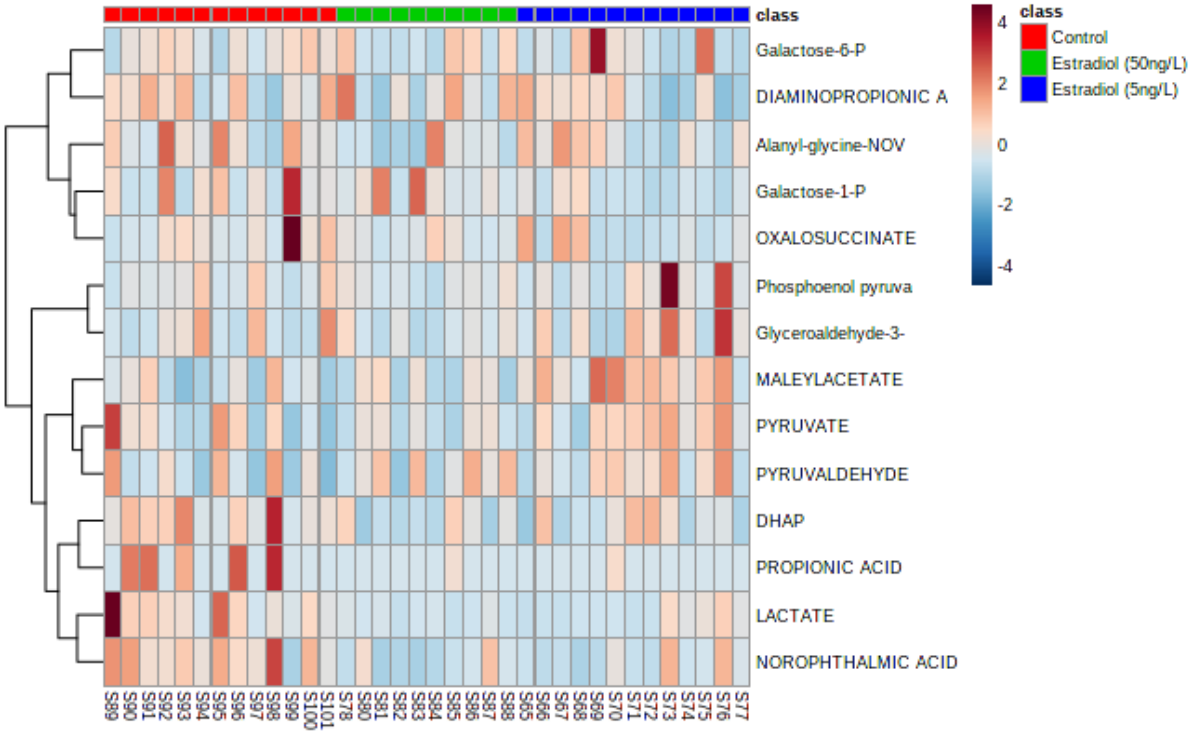
B

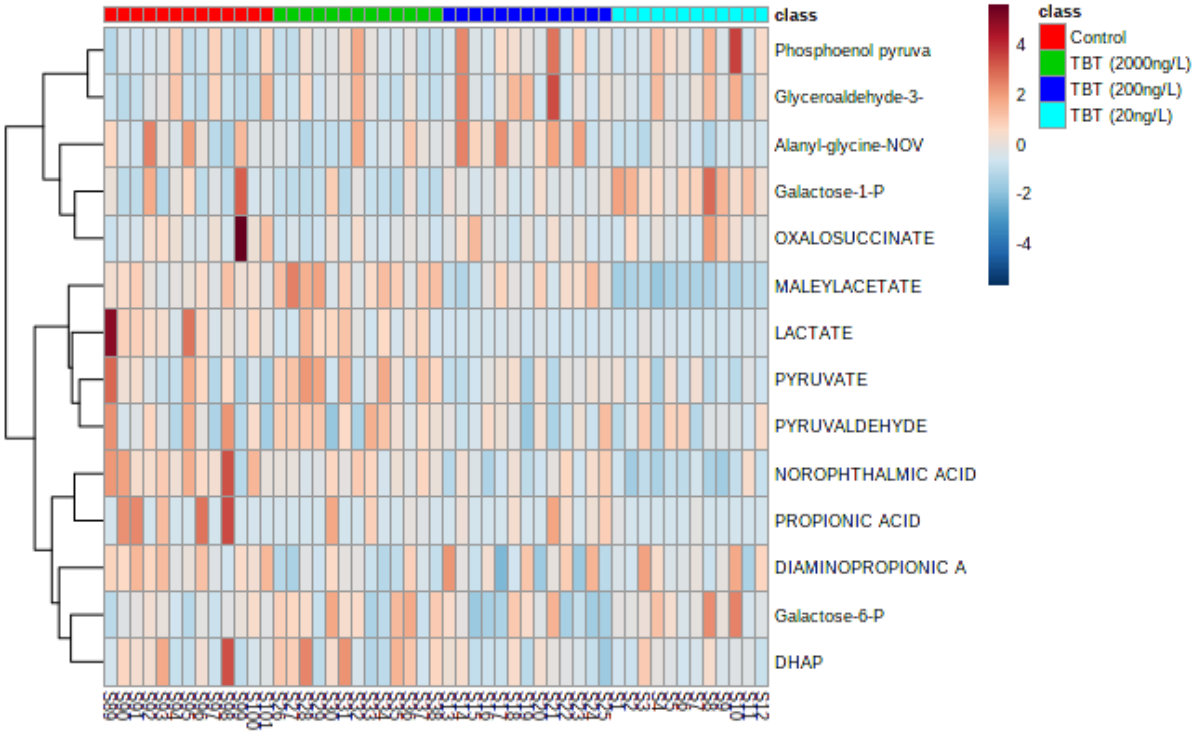

Malate-Aspartate Shuttle
Glutathione Metabolism
Amino Sugar Metabolism
Glucose-Alanine Cycle
Beta-Alanine Metabolism
Alanine Metabolism
Glutamate Metabolism
Taurine ar
Aspartate Metabolism
Lysine Degradation
Urea Cycle
Carnitine Synthesis
Cysteine Metabolism
Purine Metabolism
Citric Acid Cycle
Threonine and 2-Oxobutanoate Degradation
Glycine and Serine Metabolism
Tryptop
Ammonia Recycling
Transfer of Acetyl Groups into Mitochondria
Phenylalanine and Tyrosine
Gluconeogenesis
Arginine and Proline Metabolism
Mitochondrial Electron Transport Chain
Glycerol Phosphate Shuttle
Betaine Metabolism
Methionine Metabolism
Methylhistidin
De Novo Triacylglycerol Biosynthesis
Histidine Metabolism
Warburg Effect
Cardiolipin Biosynthesis
Nucleotide Sugars Metabolism
Riboflavin Metabolism
Glycerolipid Metabolism
Fructose and Mannose Degradation
Pyruvaldehyde De
Lactose Degradation
Glycolysis
Pentose Phosphate Pathway
Pyrimidine Metabolism
Galactose Metabolism
Lactose Synthesis
Thiamine Metabolism
Starch and Sucrose Metabolism
Phosphatidylethanolamine Biosynthesis
Phenylacetate Metabolism
Homocysteine Degradation
Phosphatidylcholine Biosynthesis
Pantothenate and CoA Biosynthesis

Appendix H

Sample Heatmap - Hierarchical analysis with additional identified ion features for testosterone, estradiol and TBT treatments






class
Phosphoenol pyruva
Glyceroaldehyde-3-
Alanyl-glycine-NOV
Galactose-1-P
OXALOSUCCINATE
MALEYLACETATE
LACTATE
PYRUVATE
PYRUVALDEHYDE
NOROPHTHALMIC ACID
PROPIONIC ACID
DIAMINOPROPIONIC A
Galactose-6-P
DHAP
class
Control
TBT (2000ng/L)
TBT (200ng/L)
TBT (20ng/L)
4
2
0
-2
-4

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