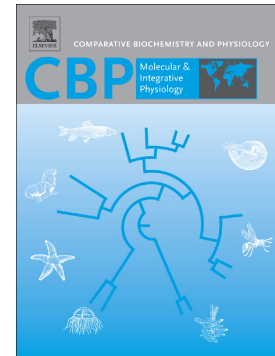


## Accepted Manuscript

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PII: S1095-6433(19)30279-X

DOI: <https://doi.org/10.1016/j.cbpa.2019.06.023>

Reference: CBA 10523

To appear in: *Comparative Biochemistry and Physiology, Part A*

Received date: 30 March 2019

Revised date: 23 May 2019

Accepted date: 26 June 2019

Please cite this article as: L.M. Zapata-Restrepo, C. Hauton, I.D. Williams, et al., 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*, <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 is an important influence on the 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<sub>2</sub>)- 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<sub>2</sub> 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*.

**Key words:** estradiol, gametogenesis, sex ratio, temperature, testosterone, *Ostrea edulis*

## 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 tonnes per year in 1950 to approximately 1,992 tonnes in 2016 (FAO, 2019). In response, the Oslo-Paris Convention included *O. edulis* as a Threatened Species (OSPAR (Agreement 2008-6)), 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 sexual types 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 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 cassostreid 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., 2007; 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., 2007; 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, 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., 2007; 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.

## Materials and methods

### 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. Oyster 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 1L/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).

### 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](http://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 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. Animals were fed *ad libitum* daily with 40000 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  $-20^{\circ}\text{C}$  until further analysis.

### Biological indices

Measurements of height (H), length (L), width (W); all to 0.01mm) for every animal were taken using a digital caliper. Shell cavity volume (Vol.), Fresh tissue weight (FW) and total weight (W, to the nearest 0.1g) 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:  $[(\text{total fresh tissue weight} / \text{total weight}) * 100]$  (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).

### 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 24h. 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- $\mu\text{m}$  using a rotary microtome (Leitz Wetzlar, model 1212), stained with hematoxylin/eosin (Cellpath Ltd) (Howard et al., 2004; Kim et al., 2006). Because maturation

is not an 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 with three different sections separated by 500  $\mu\text{m}$  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 female* (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).

### **Steroid Hormone Homologue Analysis**

Extraction and analysis of homologues of the sex hormones  $\text{E}_2$  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 (0.1g) from each animal was homogenized in  $\text{H}_2\text{O}$  (1:5 w:w) and sonicated twice for 30s. A volume of 400  $\mu\text{l}$  of 25mM HCl was added to 500  $\mu\text{l}$  homogenate and allowed to stand for 15 min at 40°C. Then, 1.25ml 0.07 M  $\text{Na}_2\text{HPO}_4$  (pH 7.4) was added before organic extraction. Homogenates were extracted twice with 14ml 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- $\mu\text{l}$  enzyme immuno-assay buffer.  $\text{E}_2$  and T concentrations were determined by competitive ELISA kits according to the manufacturer's instructions.  $\text{E}_2$  and T standards were prepared and determinations carried out in duplicate. Standard curves were carried out with  $\text{E}_2$  between 6.6 and 4,000 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  $\text{E}_2$  and T were 7.8-18.8 and 2.8-14.2, respectively. Mean intra-assay CVs for standards and samples were  $\leq 9.6\%$  for  $\text{E}_2$  and  $\leq 8.27\%$  for T. Mean inter-assay CVs were  $\leq 5.6\%$  and  $\leq 6.3\%$  for  $\text{E}_2$  and T, respectively.

### **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, the Windows 24.0 SPSS statistical pack 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  $\alpha=0.05$ .

## Results

### Temperature treatments

There was minimal variation in the actual temperature about the nominal set point for each treatment with mean values of  $10.13\pm0.70$ ,  $14.94\pm0.57$  and  $18.20\pm1.08$  recorded for the treatments 10, 14 and  $18^{\circ}\text{C}$ , 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 less than 5% in every treatment. In the third month, mortality was 5%, 7% and 10% for oysters kept at 10, 14 and  $18^{\circ}\text{C}$ , respectively, but in the fourth month mortality increased to 7%, 10% and 21%, respectively.

### 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, 1B, 1C, 1D, 1E and 1F). Condition index (CI) showed a significant effect at  $14^{\circ}\text{C}$  and  $18^{\circ}\text{C}$  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).

### 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).

### **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<sub>2</sub> and T standards, respectively (data not shown).

Gonad E<sub>2</sub> 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, 4D and 4F). After these months the E<sub>2</sub> concentration decreased until the end of the experiment. Significant differences in E<sub>2</sub> 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, 4D and 4F). 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 4E). 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<sub>2</sub> concentrations and weight ( $r_s = -0.198$ ;  $p = 0.023$ ) 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 identified.

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<sub>2</sub> and T concentrations during the temperature treatments. However, most of the female gonads presented T concentrations as high as males and E<sub>2</sub> 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<sub>2</sub> 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<sub>2</sub> 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<sub>2</sub>

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.

## Discussion

### 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.

### **Effect of temperature treatments on sex ratio**

Factors controlling the sex ratios 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 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 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 (Sastry, 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 (Lango-Reynoso et al., 1999; Chávez-Villalba et al., 2011). 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.

### **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<sub>2</sub> 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<sub>2</sub> levels found in the testis much higher than in the ovaries of *Mytilus edulis trossulus* were reported (Zabrzńska 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, 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., 2007; 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-17 $\beta$  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 of 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.

## 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.

### Conflict of interest

The authors declare no conflict of interest.

### Acknowledgements.

The authors thank the aquarium technician Robbie Robinson for his technical assistance for the maintenance of oysters in the National Oceanography Centre Southampton. LZP was supported by The University of Southampton Faculty of Engineering and the Environment Graduate School and a Colombian Government Scholarship (Colciencias).

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**Figure 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 ( $p < 0.05$ ) (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.

**Figure 2.** Proportion of *Ostrea edulis* 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) 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) and indeterminate (I).

**Figure 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) .

**Figure 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.

**Figure 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.

**Figure 6.** Hormone concentration 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). White bars: estradiol. Black bars: testosterone.

Graphical abstract



**Highlights**

- Rising sea temperatures may be contributing to skewed sex ratios in *Ostrea edulis* populations in European waters
- Temperature is an important factor determining the rate of gametogenesis in *O. edulis*
- Temperature influences sex ratio in *O. edulis*
- Gonadal development and maturation in *O. edulis* could be independent of steroid hormones
- Sex steroids may not be actively involved as endogenous modulators in sex determination in this species.

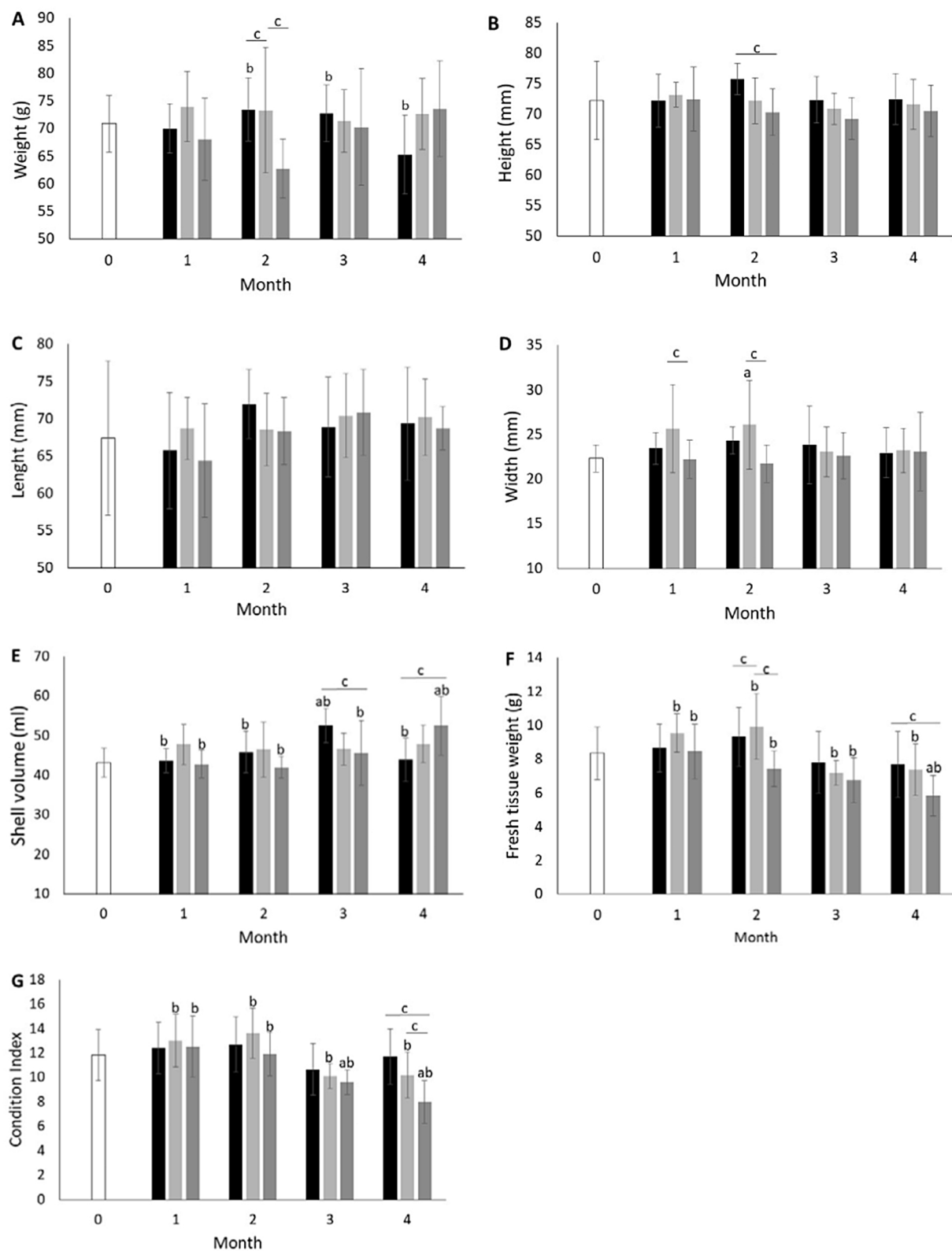


Figure 1

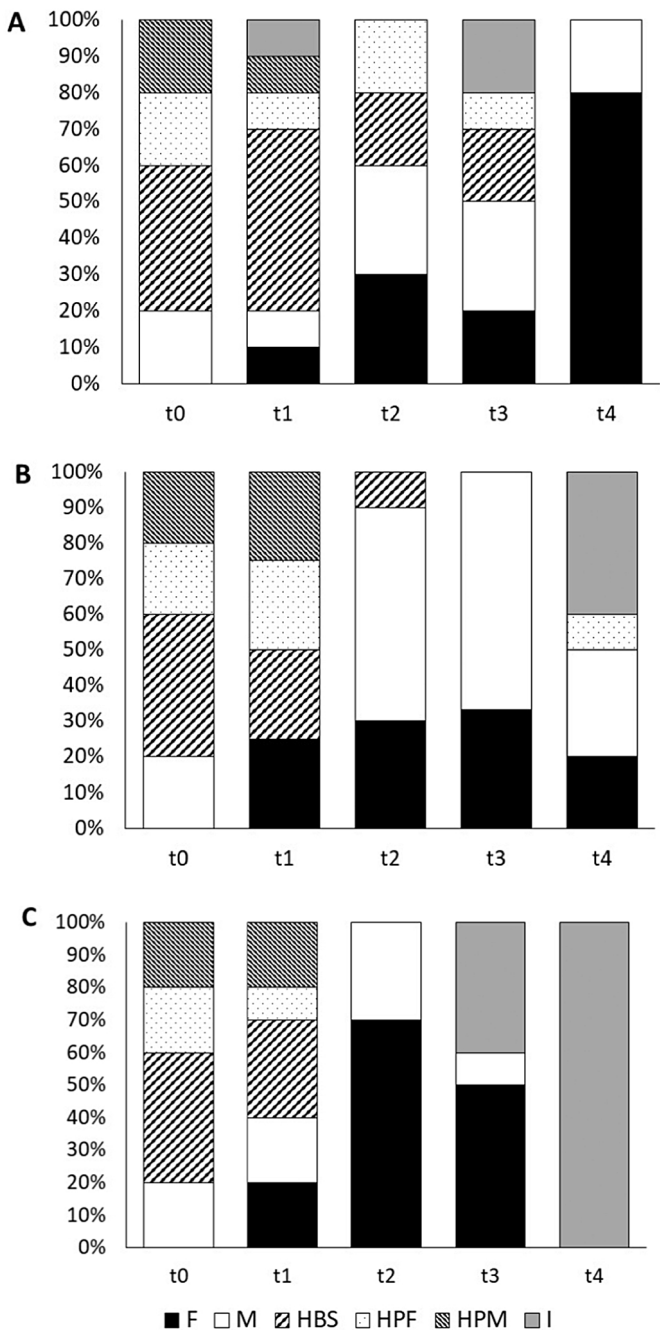
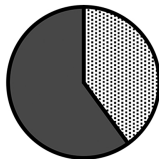


Figure 2

$t_0$

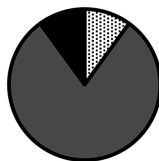
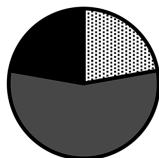
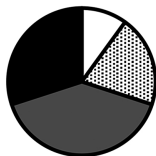


10°C

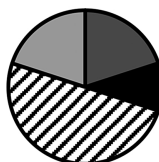
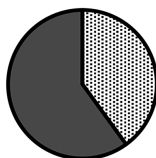
14°C

18°C

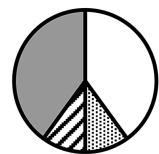
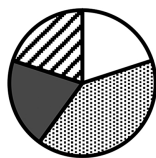
$t_1$



$t_2$



$t_3$



$t_4$

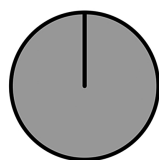
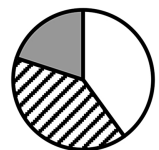
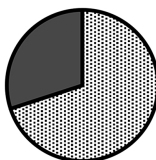


Figure 3

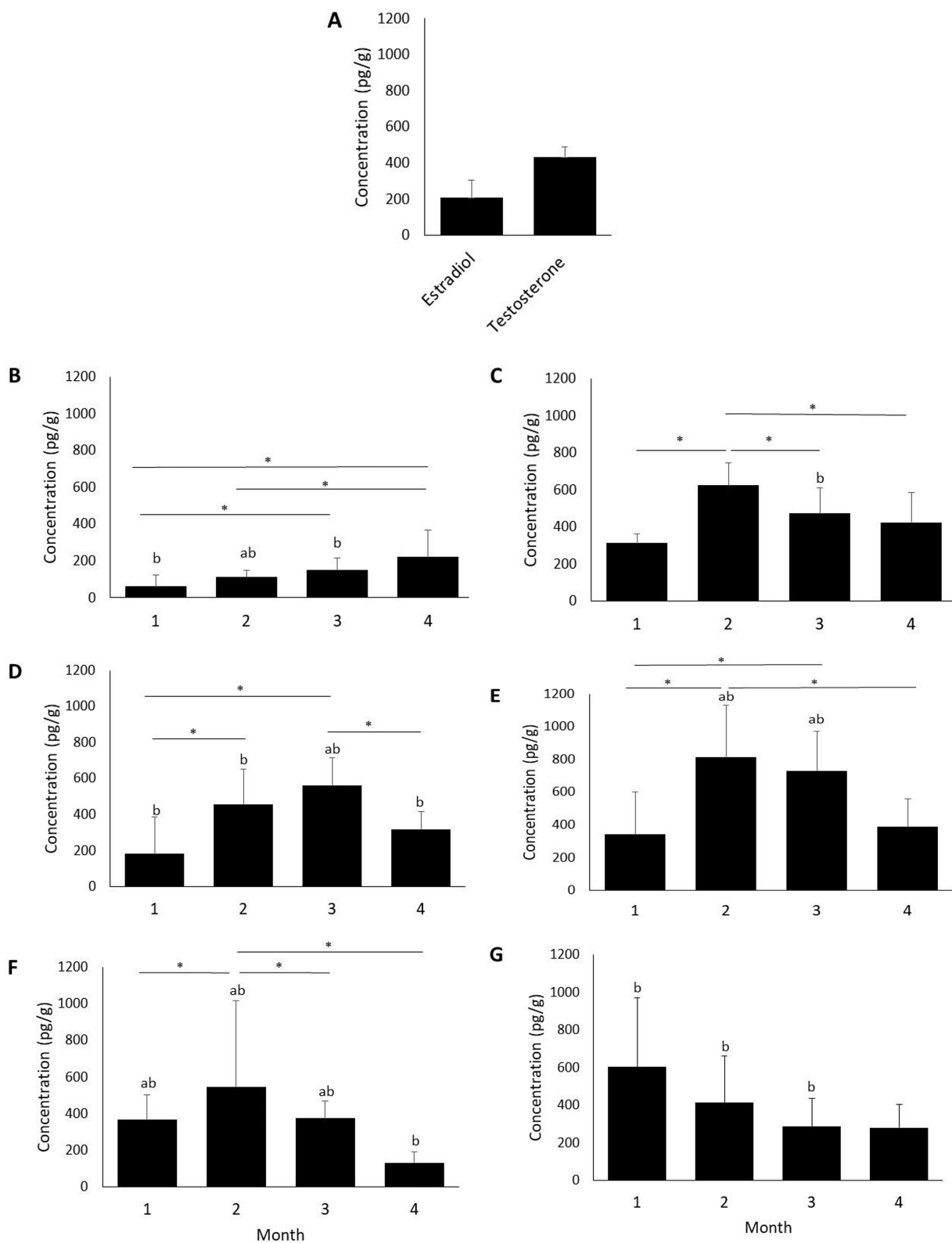


Figure 4

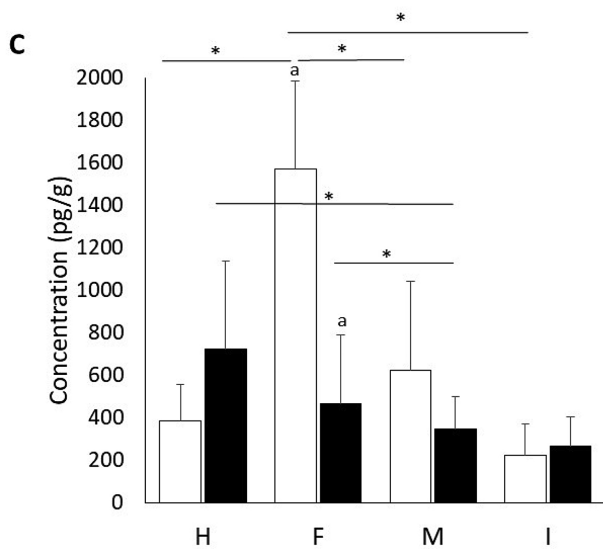
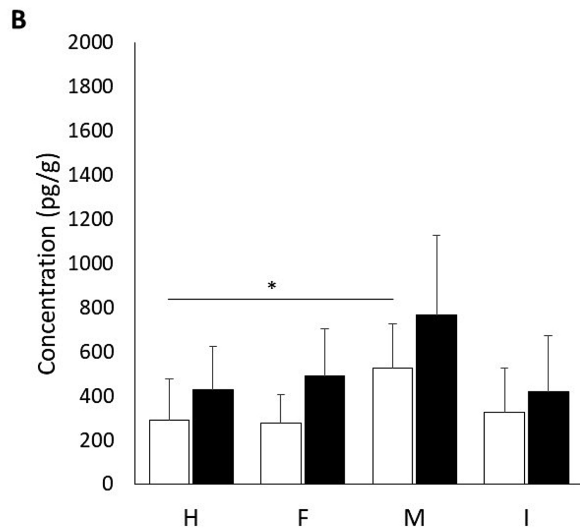
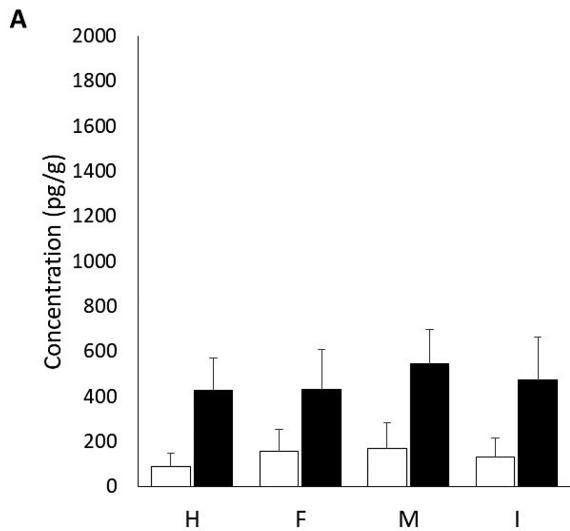


Figure 5

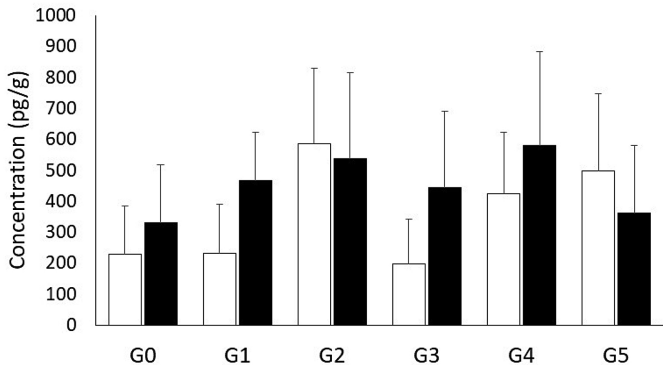


Figure 6