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Fish and sex steroids – Do juvenile fish have a distinct sex-specific steroid hormone profile? Observational study using the brown trout (*Salmo trutta fario*) as a model organism

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“Happiness can be found, even in the darkest of times, if one only remembers to turn on the light”

- **Albus Dumbledore**

***in* Harry Potter and the Prisoner of Azkaban *by* J.K.Rowling**

Abstract

Salmonids are a well-known fish group, with special relevance ecologically, economically, culturally and scientifically as they are used as a model organism in distinct research areas. In the last decades, it has been noticed that salmonids, and other animal species, have started to be profoundly affected by several impacts of anthropological origin and, in particular, by pollutants present in the water systems such as endocrine-disrupting compounds (EDCs). These EDCs constitute a large variety of contaminants, which are involved in the disruption of the endocrine system of animals, by altering their normal hormonal balance and causing problems in their health, development and overall well-being. Humans can also be affected by these compounds, by several direct or more indirect routes, namely through fish consumption.

The early life stages such as embryonic stages, larvae and also juveniles are considered more sensitive to the impacts of these compounds and it has been shown that even a short exposure to such pollutants at these stages can lead to consequences in the fish adult form and 2nd generations. Therefore, juvenile fish are one of the common fish stages to be used for the study of the effects of these compounds. However, not much is currently known about the baseline hormonal levels of these animals, and of how they differ from adult fish, and between genders and different maturation levels.

The objective of this work was precisely to provide data concerning the plasma levels of several steroid hormones in both male and female juvenile brown trout (*Salmo trutta fario*) of 1.5 years of age. The investigated hormones (Pregnenolone - Pn, Progesterone - P, Androstenedione - A, 11-keto-Testosterone - 11-kT, Testosterone - T, Estrone - E_1, 17β-Estradiol - E_2, 17α-Hydroxypregnenolone - 17-OHPn, 17α-Hydroxyprogesterone - 17-OHP and 17α,20β-Dihydroxy-4-pregnen-3-one - 17,20β-P) are linked with the gonadal/sexual development of animals and few data exist on their levels in brown trout juveniles. Furthermore, the other purpose of this study was to establish a possible relation between plasma hormone levels and gonadal maturation status (through histological evaluation).

For the measurement of the plasma steroid levels, the method selected was based on a solid-phase extraction and gas chromatography-mass spectrometry technique that had been previously successfully applied in adult rainbow trout (*Oncorhynchus mykiss*). The method was optimized for juvenile brown trout and, despite the low steroid levels of these young fish (for which the overall mean ranged between 0.17 and 0.52 ng/mL), all ten steroids were successfully measured in both male and female fish plasma. For most of the hormones, the detection rate was between 50% and 100%, with only the steroids E_1 and 17-OHP being below 50%. The steroid levels measured were within the same ranges of

those that were possible to find in literature for other salmonid species and other fish species.

No differences were found between sexes on the plasma steroid levels, showing that both genders at such a young age still present a similar steroid profile. Steroids such as E_2 and 11-kT presented the highest total mean levels (0.42 and 0.52 ng/mL, respectably) which were expectable since E_2 is associated with growth in developing fish and 11-kT is the main active androgen, having an essential role in male maturation, and being thought to have a role in female maturation potentially. T presented the lowest concentration values (total mean value was 0.17 ng/mL) since at this stage it is not yet largely produced in males as they are not mature, and it is also present in females since it is a relevant intermediate steroid. The total mean values of concentration of steroids such as E_1 (0.29 ng/mL), Pn (0.29 ng/mL), P (0.19 ng/mL), A (0.31 ng/mL), 17-OHPn (0.34 ng/mL) and 17-OHP (0.25 ng/mL) were similar and consistent, since the majority of these hormones are at the base of the steroid metabolism pathway. 17,20β-OHP also had a total mean concentration value similar to these hormones (0.25 ng/mL) and a consistent detection rate of 100%, despite being a hormone typically associated with later maturation phases. The quantification of 17,20β-OHP at those levels could be an indicator of the start of a more advanced maturation state for some individuals, as confirmed by the histological analyses of the gonads.

Additionally, no correlation was found between both the body and liver weight of the sampled animals and their plasma steroid levels. Furthermore, both the GSI (gonadosomatic index) and the gonad weight presented high variance within each gender group, with GSI having a coefficient of variation (CV) of 141.87 % in males and 30.85% in females and the gonad weight (GW) having a CV of 139.95% in males and 36.36% in females. The histological findings confirmed the existence of specific maturation differences between individuals of the same sex, but, despite this, no correlation was found between steroid levels and either the gonad weight or the GSI values.

Future studies should focus on a more in-depth gonad histological evaluation of the juvenile fish analysed here and of other (older and younger) age groups, in order to correctly associate the maturation status of the fish with their plasma steroid levels at non-mature ages. Additional studies should also focus on later stages of development to obtain data on the levels of a larger variety of steroids in fish closer to adulthood and compare those levels to those here obtained. It would also be important to determine at what point of development significant differences in steroid levels can be observed between genders. With robust base information, it would be then crucial to evaluate how these steroid profiles could be affected by pollutants such as EDCs.

Resumo

De entre os diferentes grupos de peixes, os salmonideos constituem um dos grupos mais conhecidos e com maior relevância tanto a nível ecológico como a nível económico cultural e científico. Este grupo é inclusivamente muito utilizado como organismo modelo para várias áreas de investigação científica. Nos últimos anos, o grupo dos Salmonídeos, e outras espécies de animais, têm vindo a ser bastante afetados por impactos de origem antropológica, e, em especial, pela presença de poluentes na água tais como os compostos desrugalodores endócrinos (CDEs), que têm origem em atividades humanas. Estes CDEs constituem um grupo de contaminantes variados, que são capazes de causar a disrupção do sistema endócrino do animal ao alterarem o seu balanço hormonal regular, levando a vários problemas de saúde, desenvolvimento e mal-estar para o organismo. O próprio ser humano pode ser afetado por estes compostos com os quais está exposto por várias vias, e das principais é precisamente pelo consumo de peixe.

As fases da vida mais jovens, como as fases embrinárias, larvar e juvenil são mais sensíveis aos impactos destes compostos e sabe-se que mesmo uma exposição curta a estes poluentes nestas fases pode causar consequências mais tarde na fase adulta e nas gerações seguintes. Por isso, os peixes juvenis são os mais utilizados para o estudo dos efeitos destes compostos. No entanto, pouca informação existe acerca dos níveis hormonais base destes animais e de como estes níveis diferem do peixe adulto, entre gêneros e entre níveis diferentes de maturação.

Logo, o objetivo deste trabalho foi precisamente tentar obter esta informação em relação aos esteroides em circulação de peixes do sexo feminino e masculino de truta fario (*Salmo trutta fario*) juvenil com 1,5 anos de idade. Este estudo avaliou os níveis plasmáticos de dez diferentes esteroides ligados ao desenvolvimento sexual e da gónada: Pregnenolona – Pn, Progesterona – P, Androstenediona – A, 11-keto-Testosterona – 11-kT, Testosterona – T, Estrona – E_1, 17β-Estradiol – E_2, 17α-Hydroxypregnenolona – 17-OHPn, 17α-Hydroxyprogesterona – 17-OHP e 17α,20β-Diidroxi-4-pregnen-3-ona – 17,20β-P e estabelecer uma possível relação entre o estado de maturação do animal (avaliado histologicamente) e os seus níveis hormonais.

O método usado para a medição dos níveis plasmáticos dos esteroides foi baseado numa técnica de extração de fase sólida e de cromatografia gasosa – espectrometria de massa que já tinha sido previamente utilizada com sucesso em truta arco-íris (*Oncorhynchus mykiss*) adulta. O método foi otimizado para a truta fario juvenil e, apesar de estes animais terem níveis baixos de hormonas (a média comum varia entre os 0,17 e os 0,52 ng/mL), foi possível medir com sucesso os níveis de todas as hormonas tanto em

machos como em fêmeas. Para a maioria das hormonas, a taxa de deteção estava entre 50% e 100%, com apenas os esteróides E_1 e 17-OHP a surgir abaixo de 50%. Os níveis de esteróides medidos foram semelhantes aos que foram possíveis de encontrar na literatura para outras espécies de salmonídeos e outras espécies de peixes.

Não foram encontradas diferenças entre os sexos nos níveis de esteróides no plasma, mostrando que os peixes jovens apresentam um perfil semelhante de esteroides independentemente do gênero. Os esteróides como a E_2 e a 11-kT apresentaram os níveis médios gerais mais altos no plasma (0,42 e 0,52 ng/mL, respetivamente) o que era expectável visto que a E_2 está normalmente associada ao processo de crescimento em peixes em desenvolvimento e a 11-kT é o androgénio principal em peixes, tendo um papel importante na maturação dos machos e possivelmente também na maturação das fêmeas. A T foi a hormona com o nível mais baixo (nível médio total foi de 0,17 ng/mL), uma vez que nesta fase jovem do peixe é pouco produzida nos machos e a sua presença nas fêmeas é devida a este esteroide ser um importante intermediário no metabolismo dos esteroides. As hormonas como a E_1 (0,29 ng/mL), Pn (0,29 ng/mL), P (0,19 ng/mL), A (0,31 ng/mL), 17-OHPn (0,34 ng/mL) e 17-OHP (0,25 ng/mL) apresentaram valores médios totais semelhantes uma vez que se encontram na sua maioria na base da cadeia metabólica dos esteroides. A 17,20β-OHP (0,25 ng/mL) apresenta também valores médios totais semelhantes a estas hormonas anteriores assim como uma deteção de 100% apesar de ser um esteroide normalmente associado a fases mais tardias de maturação. A quantificação de 17,20β-OHP poderá ser indicadora de que alguns indivíduos poderão estar em fases mais avançadas de maturação, o que também foi observado na análise histológica das gónadas.

Adicionalmente, não foi encontrada qualquer correlação entre o tamanho corporal e peso do fígado dos animais e os seus níveis de esteroides. Além disso, o índice gonadossomático (GSI), assim como o peso da gónada apresentaram bastante variabilidade dentro do grupo dos machos e dentro do grupo das fêmeas, com o GSI a ter um coeficiente de variação de 141,87% no sexo masculino e 30,85% no sexo feminino e peso da gônada a ter uma variação de 139,95% no sexo masculino e 36,36% no sexo feminino. Esta variação foi confirmada pela observação histológica, que revelou existirem ligeiras diferenças na maturidade de diferentes indivíduos do mesmo sexo, mas mesmo assim não foi encontrada uma correlação entre os níveis de esteroides e os valores de peso da gónada.

Sugere-se que estudos futuros se devam debruçar nos detalhes histológicos destes peixes juvenis, e de outros, mais novos e com maior idade para se tentar compreender se

de facto o nível de maturação do peixe pode ter uma associação com os seus níveis de hormonas no sangue. Adicionalmente, seria também importante que os estudos acerca dos níveis de esteroides no plasma fossem realizados em fases mais tardias do desenvolvimento, já mais perto da idade adulta, de forma a verificar que diferenças existem em relação aos níveis dos vários esteroides aqui medidos dos peixes mais jovens e de forma a determinar em que nível de maturação é que se começam a observar diferenças nos níveis de esteroides entre machos e fêmeas. Com essa grande base de informação acerca dos níveis de esteroides será crucial posteriormente avançar com estudos que foquem como estes podem ser impactados por poluentes tais como os CDEs.

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List of abbreviations

A – 4-Androstenedione

ATZ – Atrazine

BPA – Bisphenol A

BPS – Bisphenol S

CHD – Coronary heart disease

Cyp19a – Aromatase

17,20β-OHP - 17α,20β-Dihydroxy-4-pregnen-3-one

E_1 – Estrone

E_2 - 17β-estradiol

EDC – Endocrine-disrupting compound

EE_2 – Ethynylestradiol

ELISA – Enzyme-linked immunosorbent assay

GC – Gas chromatography

GSI – Gonadosomatic index

GTH-I – Gonadotropin type I

17-OHP – 17α-Hydroxyprogesterone

17-OHPn - 17α-Hydroxypregnenolone

17α-OH – 17α-Hydroxylase

17β-HSD - 17β-Hydroxysteroid dehydrogenase

3β-HSD - 3β-Hydroxysteroid dehydrogenase

HSI – Hepatosomatic index

K – Fulton's condition factor

11-kT – 11- Ketotestosterone

LC – Liquid chromatography

LOD – Limit of detection

LOQ – Limit of quantification

MS – Mass spectrometry

MSTFA – N-Methyl-N (trimethylsilyl) trifluoroacetamide

NP – Nonylphenol

NPE – Nonylphenol ethoxylate

P – Progesterone

P450scc – Cytochrome P450 side-chain cleavage

PAH – Polycyclic aromatic hydrocarbons

PCB – Polychlorinated biphenyls

PFOA – Perfluorooctonic acid

PFOS – Perfluorooctone sulfonate

Pn – Pregnenolone

RIA – Radioimmunoassay

RT – Retention time

SPE – Solid-phase extraction

T – Testosterone

TBOEP – Tris (2-butoxyethyl) phosphate

TBT – Tributyltin

TCEP – Tris (2-chloroethyl) phosphate

Chapter 1. Introduction

1. The Salmonidae role in ecology, economy and culture

1.1. Ecological relevance

The Salmonidae family is one of the most well-known fish groups in the world, containing around 77 different species (Moyle and Cech 2004, Pennel and Prouzet 2009). It can be divided into three groups (usually considered the sub-families) which are the Salmoninae (includes salmon and trout species), the Coregoninae (includes the whitefishes) and the Thymallinae (includes the graylings) (Moyle and Cech 2004). Within these groups, the Salmoninae have a particular relevance either economically, culturally and even scientifically, with some of the most well-studied fish belonging to the *Oncorhynchus* and *Salmo* genus (Guiguen 2018). The Salmoninae group includes a large variety of species and some of the most well-known can be found represented in Table 1.

Salmonids are originally from the northern regions of the globe (Moyle and Cech 2004), but throughout the years several species have spread from their original homelands, having now a vast distribution, from North and South America to Europe and Asia (Table 1). With high adaptability, the fish within this group can occupy a large variety of both freshwater habitats (such as rivers and lakes) and also saltwater habitats (Moyle and Cech 2004).

Appearance-wise they are usually characterised by having a streamlined body with a forked tail and an adipose fin (Moyle and Cech 2004). Despite these main characteristics, they can vary in certain body aspects such as size, and life traits such as in their frequency of reproduction and their method of parental care (Willson 1997). One important common characteristic of all salmonids is the fact that they all spawn in freshwater habitats, reproducing via external fertilisation, with juveniles then either remaining in these freshwater systems or migrating to the sea (Buschmann and Muñoz 2016, Guiguen 2018).

Table 1. Different important species of salmonids and their respective original birthplace location and current distribution.

Species	Original location	Current distribution	Reference
Atlantic Salmon (*Salmo salar*) 	North Atlantic	Europe, North America	(Hendry and Cragg-Hine 2003, IUCN 1996), Image by (Knepp 2018)
Brook Trout (*Salvelinus fontinalis*) 	North America	Africa, Asia, Europe, Oceania, South America, Sub Antarctica	(FAO 1995), Image by (Reece 2019)
Brown Trout (*Salmo trutta*) 	Europe, Russia, Africa	Europe, Africa, Asia	(Behnke 2007, MacCrimmon and Marshall 1968), Image by (Raver 2017)
Chinook Salmon (*Oncorhynchus tshawytscha*) 	North America, Asia	Oceania, North America, South America	(Behnke *et al.* 2010), Image by (NOAAFisheries 2019a)
Pink Salmon (*Oncorhynchus gorbusha*) 	North America	Europe, North America, South America	(Crawford and Muir 2008), Image by (NOAAFisheries 2019b)
Rainbow Trout (*Oncorhynchus mykiss*) 	North America	Africa, Asia, Europe, North America, Oceania, South America	(Cowx 2005), Image by (Reece 2019)

Being so widely spread, these fish species have entirely adapted to their environment, becoming part of the ecosystem and having an ecological relevance, both from their effects as invasive species (Kitano 2004) and from their role in the ecologic balance of the habitat (Holmlund and Hammer 1999). In western North America for example, the Pacific trout

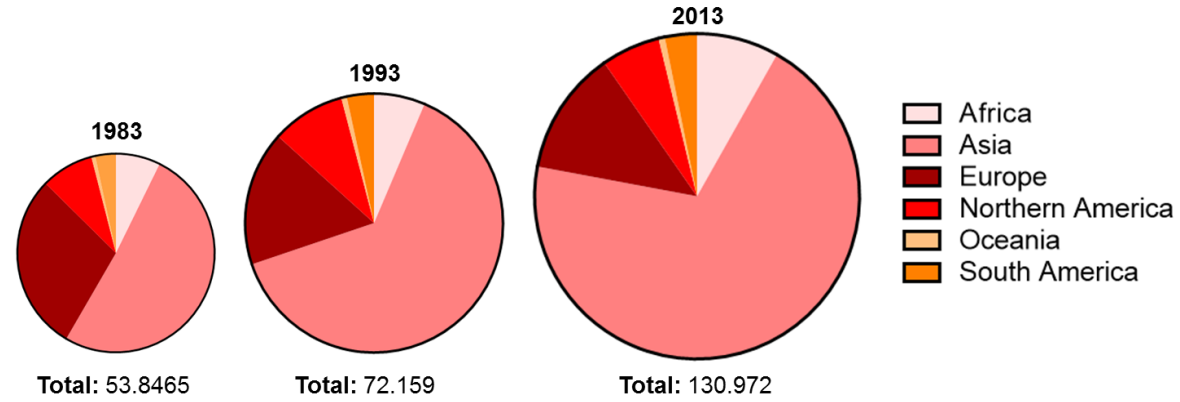
(*Oncorhynchus* spp.) is vital in rivers and lakes as a predator, contributing for the maintenance of the food webs, and is also a significant nutrient transporter from the sea into freshwater habitats (Quinn 2011). The presence of carcasses of salmonids in ecosystems of rivers and lakes is known to comprise a good source of nutrients from the sea such as nitrates and phosphates (Levings 2016). Another example is some species of salmon (*Oncorhynchus* spp.) that have been known to have a significant role on the diet of various terrestrial wild animals and even having an influence on the riparian vegetation that surrounds streams (Hilderbrand *et al.* 2004). Species such as the rainbow trout (*Oncorhynchus mykiss*), the brown trout (*Salmo trutta fario*) and the brook trout (*Salvelinus fontinalis*) for example, have shown adverse effects on the number of native species of fish in Japanese inland waters, resultant from predation, resource competition and interspecific hybridization (Kitano 2004).

Both the negative and positive effects that these animals can cause on many freshwater habitats show their ecological importance and how crucial it is to conserve these species in their natural ecosystems. *H*owever, the majority of fish from these species are currently suffering from a variety of impacts, including habitat degradation and effects coming from pollutants in the water (Levings 2016), such as the endocrine disruptors. To help the preservation and well-being of these species, and maintain their ecological benefits, it becomes crucial to understand the physiology of these animals during their life cycle, so that it is easier to perceive how they can be affected by the impacts mentioned above.

1.2. Economic and cultural importance

Humans have long consumed aquatic products such as fish and crustaceans and so, through the years, both fishing techniques, as well as production techniques of these products, have increased and been improved (Pillay and Kutty 2005) in order to respond to the high levels of their consumption in the world (Figure 1.A). In fact, for some areas of the world, such as Asia (particularly countries such as China and India, Figure 1.A), the contribution of aquatic products as a food supply appears to be of particular relevance when compared to other types of food products (FAO 2018a)

With these high consumption values of fish and other seafood, the economic sector of fisheries and aquaculture has been not just evolving, but also taking the top seat together with other food production industries in the world, making nowadays many countries rely significantly on the economic outcome of the production of aquatic products, which can provide a great source of income and employment for various countries (FAO 2018a). Among these seafood products, salmonids have through the years reached a high economic significance that has spread through the world, as various species are produced and consumed in a large variety of different countries (Figure 1.B).



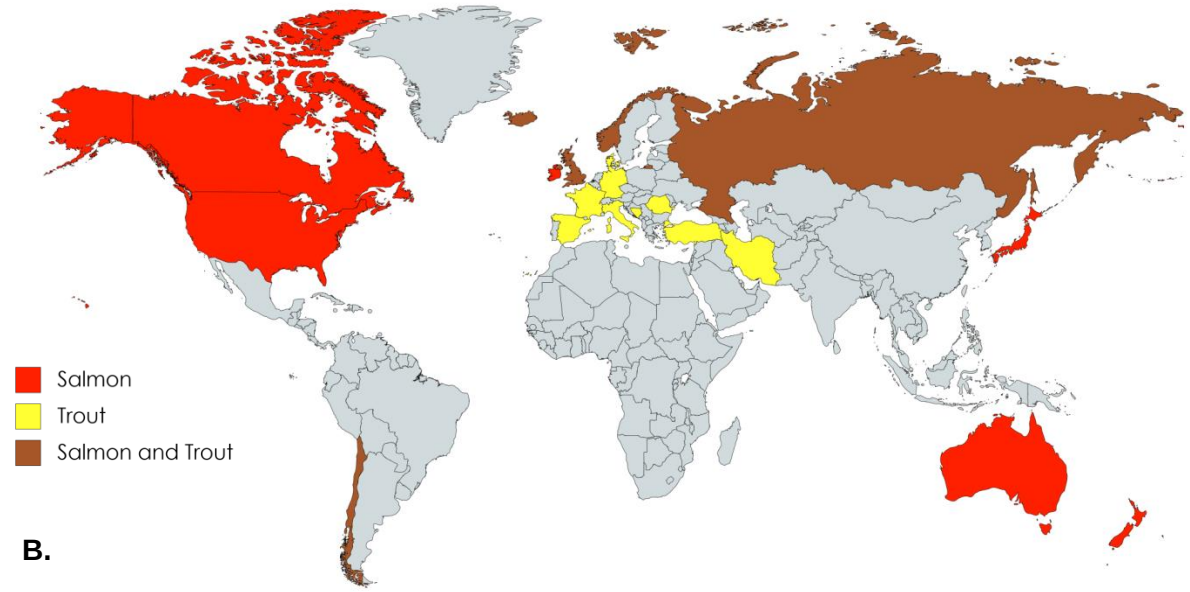


Figure 1. A. Seafood product food supply quantity (in million tonnes) of the different main regions of the globe in the years of 1983, 1993 and 2013. Data from (FAO 2018a). **B**. World map with the main countries of production of salmon (red), trout (yellow) and both salmon and trout (brown). Data from (European Commission 2012, Sabatini 2016, Vandeputte and Labbé 2012). Created with mapchart.net.

Salmonids have not only become a worldwide produced product but through the years their husbandry and exportation have increased sharply (Figure 2.A and 2.B). Species such as the Atlantic salmon (*Salmo salar*) and the rainbow trout are amongst the 20 most-produced species in aquaculture in the world, accounting for 4% and 2%, respectively, of total world aquaculture production (FAO 2018b). Besides their economic value as a product for consumption, in various countries, salmonids have also reached an excellent relevance for those who enjoy the sport of recreational fishing.

The farming and fishing of salmonid species has, therefore, become a large global industry, with high demand, particularly in the United States, Europe and Japan (Buschmann and Muñoz 2016). So, with these fish species having such a significant economic and cultural role in many countries, it is crucial to guarantee their health and overall well-being, assuring by this way the quality of the fish product, as well as, its abundance.

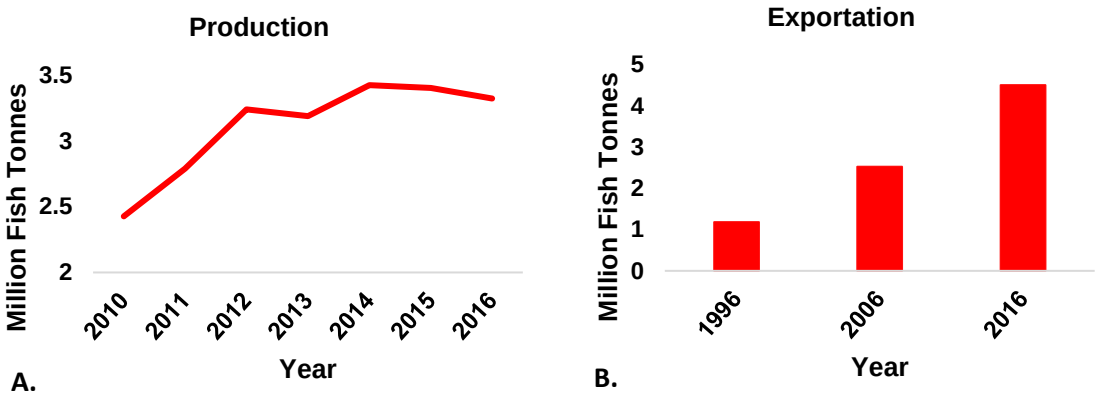


Figure 2. A. World aquaculture production of salmon, trout and smelt in million tonnes of fish between the years of 2010 and 2016. **B.** International exportation of salmon, trout and smelt in million tonnes of fish in the years of 1996, 2006 and 2016. Data from (FAO 2018b).

1.3. The consumption of salmonids in the human diet

For many years the use of aquatic food products both from fishing and aquaculture production has had a central role in the human diet (Tacon and Metian 2013). These aquatic food products, such as fish and seafood, are known to be highly nutritious, containing proper levels of protein, lipids and other vital micronutrients (Tilami and Sampels 2018). In fact, one of the most essential and abundant nutrients provided by aquatic food is the n-3 long-chain polyunsaturated fatty acids (n-3 LC-PUFA) but other critical nutritional components can also be found, such as taurine, choline, D_3 and B_{12} vitamins, calcium, phosphorus, iodine and selenium (Szlinder-Richert *et al.* 2011, Tilami and Sampels 2018). Several farmed salmonid species have relevant amounts of these various nutrients, having inclusively some of these nutrients, such as the omega-3 fatty acids, in higher values than other farmed fish species (Figure 3).

All these nutrients are highly relevant to help maintain a healthy and functional human organism (Szlinder-Richert *et al*. 2011). In fact, over the years several studies show that the intake of fish and seafood is related with the decrease of several cardiovascular-related complications, such as coronary heart disease (CHD), high blood pressure and strokes (Lund 2013). It has also been shown to help with other health problems such as rheumatoid arthritis and inflammatory diseases (Lund 2013). Specifically associated with salmonid consumption, it has been shown that the consumption of Atlantic salmon led to a decreased risk for cardiovascular disease due to healthy levels of fatty acids n-3 and n-6 (Raatz *et al*. 2013).

In the last 10 years, several studies have also shown benefits of consumption of salmon during pregnancy due to the increase of plasmatic essential amino acids (AA), which produce a positive effect on maternal health (Rossary *et al*. 2014) and have anti-inflammatory properties (Van den Elsen *et al.* 2011). Besides, the intake of salmon during gestation also improves the quality of breast milk during early lactation, by increasing its n-3 LC-PUFA concentration (Urwin *et al.* 2012).

Therefore, the consumption of fish from salmonid species is not only quite elevated in many parts of the world, but it also brings a large number of health benefits for humans. However, the escalating presence of EDCs in these organisms can potentially compromise any beneficial effect in their consumption and turn, what is a known healthy food choice, into a dangerous and forbidden one as it will be shown further on.

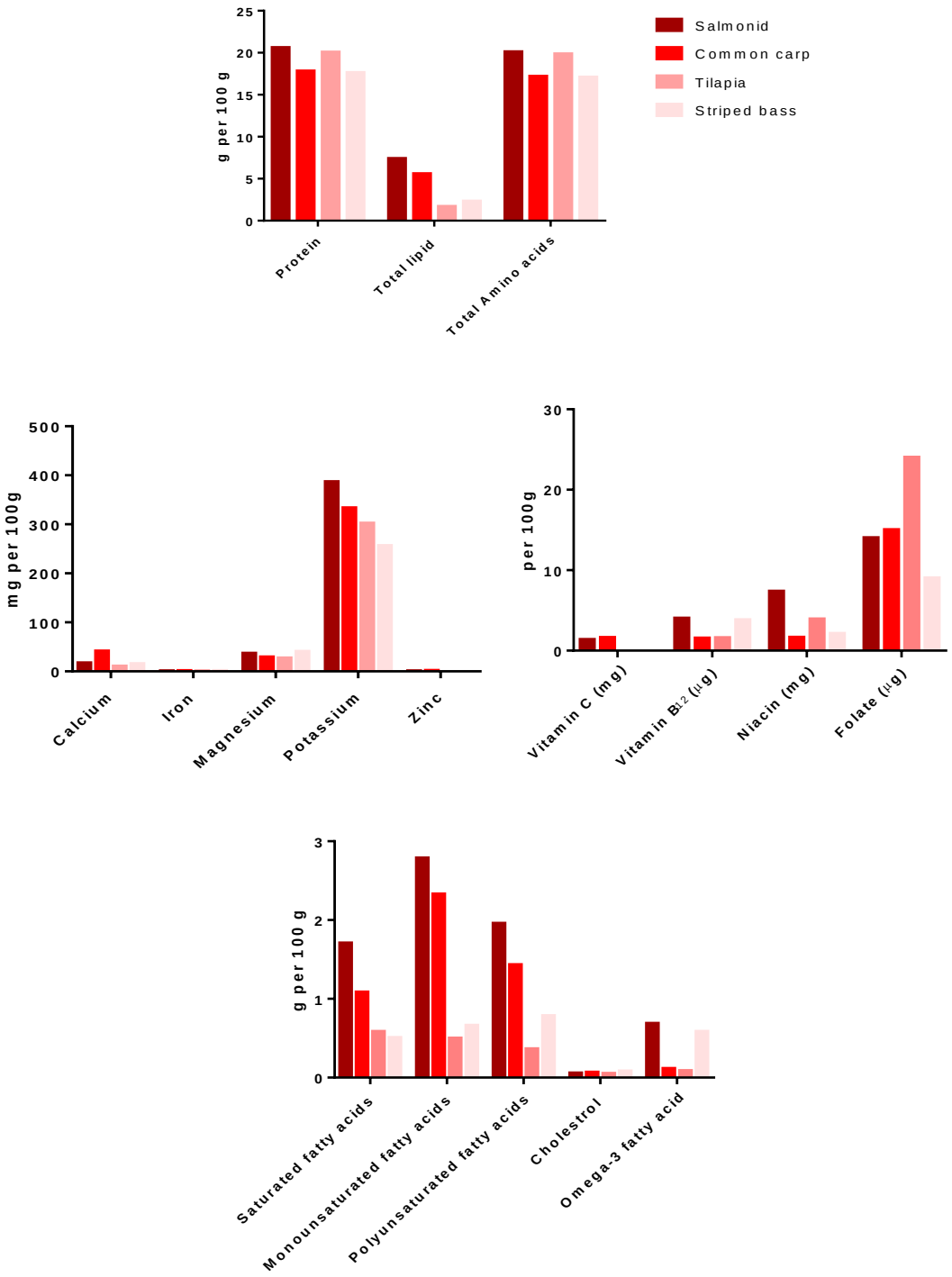


Figure 3. Different nutrient composition in 100 g of the edible portion of salmonids (mean of different salmonid species) and 3 other fish species, the common carp (*Cyprinus carpio*), the tilapia (*Oreochromis* spp) and the striped bass (*Morone saxatilis*). Data from (Tacon and Metian 2013).

2. Steroid metabolism in freshwater fish – Main aspects

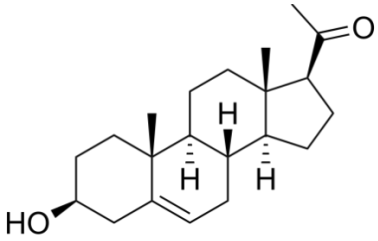
Steroids have an essential role in the regulation of several physiological functions in fish (James 2011). They are central for the proper occurrence of puberty, and the processes of oogenesis in females and spermatogenesis in males during their adulthood (Piferrer 2011). Furthermore, they are also vital for the stimulation of reproductive behaviour and are involved in the development of secondary sexual characteristics (Moyle and Cech 2004).

Generally, in fish, three types of steroids can be found (Figure 4): the androgens, the estrogens and the progestogens (Piferrer 2011). These steroids are synthesized from cholesterol (Figure 5) and although they are mainly produced in the gonads of the fish, other organs, such as the liver and the interrenal glands, are also capable of their syntheses (Piferrer 2011).

Although both females and males have all types of steroids, androgens usually determine masculine sexual characteristics, while estrogens are commonly associated with those of females (Piferrer 2011). For this reason, the balance of these steroids in both genders and their accurate regulation is highly relevant due to their involvement in sexual development and reproductive behaviour.

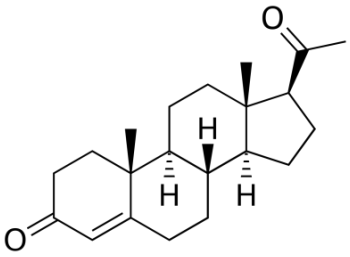
Pregnenolone

(Pn)



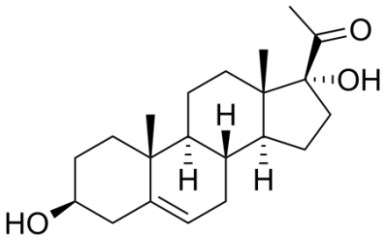
Progesterone

(P)



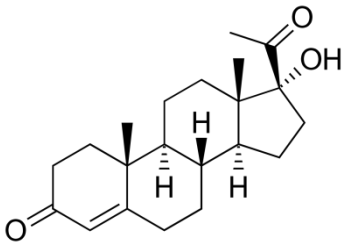
17α-Hydroxypregnenolone

(17-OHPn)



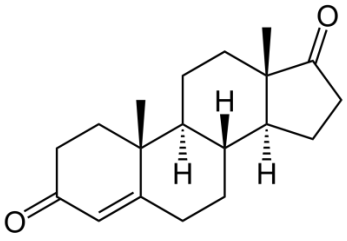
17α-Hydroxyprogesterone

(17-OHP)



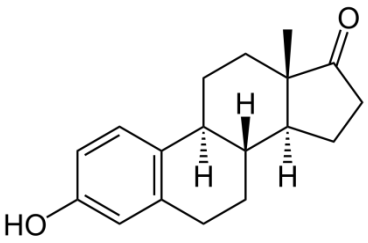
4-Androstenedione

(A)



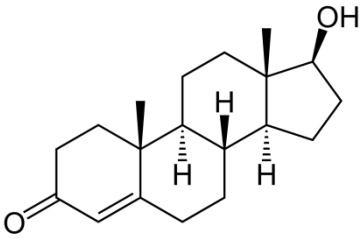
Estrone

(E_1)



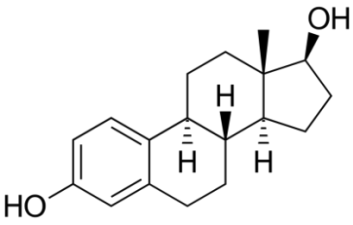
Testosterone

(T)



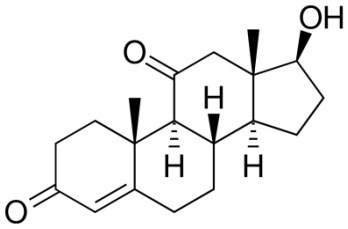
17β-Estradiol

(E_2)



11-Ketotestosterone

(11-kT)



17α,20β-Dihydroxy-4-pregnen-3-one

(17,20β-OHP)

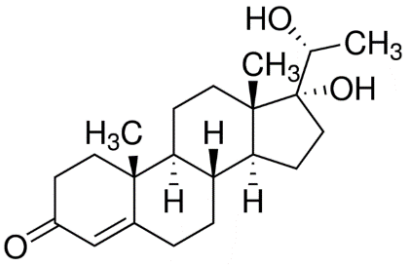


Figure 4. Chemical structures of the main steroids present in freshwater fish.

2.1. Endocrine regulation of steroid hormones in juvenile salmonid

In the life stage of young fish, during which the gonads become mature, the organism produces the gonadal steroid hormones, usually as a result of the stimulation by gonadotropin hormones (produced in the pituitary gland)(Moyle and Cech 2004). In salmonids, this gonadotropin is typically called the type I gonadotropin (GTH-I), and will act upon the steroid producing sites (Nagahama *et al.* 1995). However, contrary to other kinds of hormones, the sex steroids once synthesised cannot be stored (Piferrer 2011). Therefore, they are always present in the circulation and can be found in varying concentrations depending on their production levels and weight of the animal (Table 2). So, the regulation of the concentration of these steroids relies on the activity of the steroidogenic enzymes (Figure 5) involved in the steroid production (Piferrer 2011). Their action in developmental stages, such as the juvenile stage, can be a critical factor in the determination of the sex of the organism. It is stressed that enzymes such as the cytochrome P450 aromatase convert androgens into estrogens defining the equilibrium between the levels of these two groups of steroid hormones (Piferrer 2011, Rehman *et al*. 2017).

In females, estrogens such as 17β-estradiol are involved in the hepatic synthesis and release of vitellogenin (Nagahama *et al*. 1995), which is an essential protein for oocyte maturation during the development of the female fish (Arukwe and Goksoyr 2003). This hormone is commonly produced by the ovarian follicles, usually in response to the rise of GTH-I in blood. When this occurs, there is the synthesis (in the thecal cell layer) of androgens that are further converted into 17β-estradiol in the granulosa cell layer (Nagahama et al. 1995). During this process, the cytochrome P450 aromatase, as well as 17β-hydroxysteroid dehydrogenase (17β-HSD) and others (Figure 5) are the key factors that convert steroids and so determine the amount of each of these hormones in the organism (Nagahama *et al.* 1995, Piferrer 2011).

In males, Leydig cells are the structures containing higher amounts of enzymes involved in the synthesis of steroid hormones (Nagahama 1994). These cells present in male testicles also respond to the stimulus of gonadotropins and produce both 17β-estradiol and 11-kT (Piferrer 2011). Steroids such as these have an essential role in this sex, particularly on the proper functioning of the different processes within the spermatogenesis (Maugars 2007). For example, the divisions of spermatogonia are regulated by 17β-estradiol, whilst the production in male Sertoli cells of important mediators of the spermatogenesis, such as activin B, is controlled by 11-kT (Piferrer 2011).

Table 2. Plasma concentration levels of various steroid hormones of juvenile and adult salmonid fish in the rainbow trout and Atlantic salmon species, with respective weight.

Hormone	Species	Weight (g)	Concentration (ng/mL)	Reference
Pn	Rainbow Trout (*Oncorhynchus mykiss)*	200 - 300	Approx. 2.8 * (Male) Approx. 1.7 * (Female)	(Ellis *et al.* 2004)
P	Rainbow Trout (*Oncorhynchus mykiss)*	150	Approx. 0.5 * (Female)	(Atteke *et al.* 2003)
T	Rainbow Trout (*Oncorhynchus mykiss)*	200 - 300	Approx. 0.24 * (Male) Approx. 0.09 * (Female)	(Ellis *et al.* 2004)
		145 ± 2.5	Approx. 7.5 * (Male) Approx. 0.35 * (Female)	(Naderi *et al.* 2015)
		10.6 ± 3.4	Approx. 0.0023 *	(Riar *et al*. 2013)
		22 - 56	1.2 ± 0.3	(Hou *et al*. 1999)
	Atlantic salmon (*Salmo salar*)	70 ± 20	0.0335 ± 0.0026	(Mortensen *et al.* 2011)
11-kT	Rainbow Trout (*Oncorhynchus mykiss)*	10.6 ± 3.4	Approx. 0.0033 *	(Riar *et al.* 2013)
E_1	Atlantic salmon (*Salmo salar*)	70 ± 20	0.2255 ± 0.0428	(Mortensen *et al*. 2011)
	Rainbow Trout (*Oncorhynchus mykiss)*	150	Approx. 0.03 * (female)	(Atteke *et al.* 2003)
E_2	Rainbow trout (*Oncorhynchus mykiss*)	50 ± 6	Approx. 0.7 *	(Tintos *et al.* 2008)
		60 ± 5	Approx. 0.52 * (Female)	(Tintos *et al*. 2007)
		145 ± 2.5	Approx. 0.12 * (Male) Approx. 0.96 * (Female)	(Naderi *et a*l. 2015)
		10.6 ± 3.4	Approx. 0.0035 *	(Riar *et al*. 2013)
		22 - 56	1.4 ± 0.1	(Hou *et al.* 1999)
		150	Approx. 0.5 * (female)	(Atteke *et al.* 2003)
17-OHP	Not found			
17-OHPn	Not found			
A	Not found			

* Approximate mean value from graphic /image

With all this, it becomes clear that both female and male fish rely strongly on the steady presence of sex steroids for their proper development and later for their reproduction. The fact that these fish have well organized physiological systems for the synthesis of these hormones shows how crucial the regulation of their balance is, and how easily this balance can be disturbed by external factors, such as EDCs.

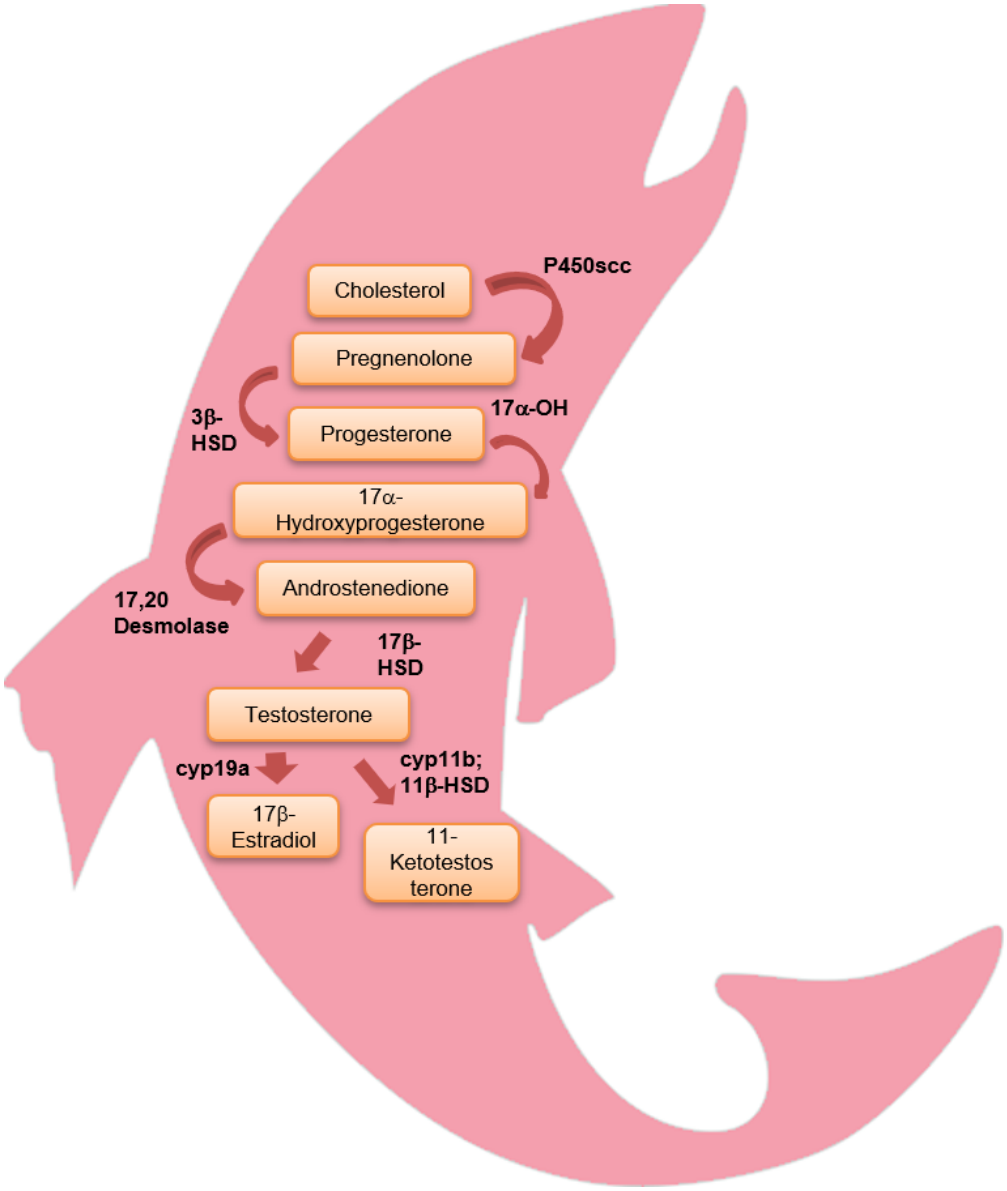


Figure 5. Pathway of the metabolism of the main steroids in fish, including the main steroidogenic enzymes involved: P450scc (cytochrome P450 side-chain cleavage); 3β-HSD (3β-hydroxysteroid-dehydrogenase); 17α-OH (17α-hydroxylase); 17β-HSD (17β-hydroxysteroid-dehydrogenase); cyp11b (11β-hydroxylase); 11β-HSD (11β-hydroxysteroid-dehydrogenase); cyp19a (aromatase). Data from (Budzinski *et al.* 2006, Nagahama *et al.* 1995, Piferrer 2011).

3. EDCs impacts on juvenile salmonid gonadal development

Salmonids, like many other fish groups, are increasingly being affected by several factors, mainly of anthropological origin, that compromise the fish survival and well-being, and consequently affect their ecological and economic value for humans (Mills and Chichester 2005).

One of these factors is, for example, the destruction and alteration of habitats, which is one of the main threats for salmon and trout and that has been at the base of several extinct populations (Moyle and Cech 2004). These habitat problems occur since many aquatic habitats are a target for human interference, both by land-use (Cunjak 1996) and placement of anthropological structures, which happens for example with the canalisation of streams (Millidine *et al*. 2012). This interference eventually results in an alteration of the original characteristics of the habitat. These altered features, such as a variation of the stream substrate (Cunjak 1996, Millidine *et al*. 2012) or alteration of the streamflow (Nislow and Armstrong 2012, Warren *et al.* 2015) can end up having an impact in the different life stages of salmonids. Other human activities, such as overfishing and the introduction of exotic species can also lead to a decline in some salmonid populations (Marschall and Crowder 1996). Another concerning factor in recent years has also been global warming, which has been causing a negative effect on aquatic organisms such as salmonids mainly by changing the stream temperatures and affecting the population's distribution, abundance and persistence (Isaak *et al*. 2010).

Although all these factors contribute to disturbing salmonid populations, probably one of the major concerns nowadays for these fish species is that they have been increasingly affected by concerning levels of pollutants in the water which are commonly originated from different industries and wastewaters, and agriculture (Rehman *et al.* 2017). Some of these widely used chemicals when present in the environment pose a risk both for wild and human lives (Kraak *et al.* 2001). For several years, studies have been made on these contaminants and on how they can affect juvenile salmonids, focusing on various effects from impacts on development and reproduction, to impacts on immunity and behaviour. One study shows, for example, that certain pollutants such as PAHs (polycyclic aromatic hydrocarbons) and PCBs (polychlorinated biphenyls), can affect the immunity of the juvenile chinook salmon (*Oncorhynchus tshawytscha*), acting as immunosuppressors and increasing their disease susceptibility (Arkoosh *et al*. 1998). Other studies made in the juvenile rainbow trout, show that wastewater effluents with metals and pharmaceutical drugs can affect their tissue metabolic capacities, their plasma cortisol levels and consequently affect their response to an acute stressor (Best *et al*. 2014, Ings *et al.* 2011, Ings *et al*. 2012).

One of the most impactful contaminants, however, are usually referred to as endocrine-disrupting compounds (EDCs), which consist of compounds that can affect the organism by interfering with its hormonal balance (Rehman *et al.* 2017). This can end up causing problems not only on the health of the organism but also on its offspring (Damstra *et al*. 2002). Because of the presence of several receptors for EDCs in most animals, these chemicals can disturb various organs, leading not only to reproductive and development issues but also to other health problems, such as tumours and nervous damage (Rehman *et al*. 2017).

There is a large variety of different contaminants that act as EDCs (such as pesticides, xenoestrogens, and others) (Rehman *et al*. 2017) and such compounds are typically highly present in aquatic habitats (Mills and Chichester 2005). They can come from many different sources which can be either synthetic sources or natural sources. Synthetic sources include compounds such as pesticides, plastics, detergents and contraceptive drugs that usually originate by my man input from sewages and industrial releases (Goksoyr 2006). Natural sources include compounds such as human and animal hormones, which can also be easily found coming from sewages, as well as agricultural runoff (Goksoyr 2006). Although these chemicals can affect various types of environments and many different animals, they seem to be of particular concern for fish, since once the pollutants are present in the water the animals are permanently exposed to them (Jobling and Tyler 2003). The main routes of exposition include the gills, skin and through their diet when they feed on other contaminated organisms (Rehman *et al.* 2017).

Additionally, fish can also be vulnerable to EDCs from the accumulation of these compounds in the lipid reserves of their egg due to maternal transfer of these compounds during the ovary development (Jobling and Tyler 2003). The early life stages, such as embryonic stages, larvae and juveniles are usually considered more sensitive to the impacts of these chemicals and it has been shown that even a short exposure to such pollutants at these life stages might have consequences later on the adult form and second generations (Kime 1998). In addition to being crucial stages of fish development, these early life phases are also less capable of metabolising and eliminating these toxic chemicals (Jobling and Tyler 2003).

The EDCs can affect the endocrine system of fish species in several ways. These include for example mimicking the body's original hormones (acting either as agonists or antagonists); affecting the levels of natural hormones by affecting either their production, secretion, transport or metabolism; and finally interfering with the function or production of the hormone receptors (Goksoyr 2006). It is important to note as well that different compounds can have different mechanisms to affect the organism, but in some cases, one single

compound can be capable of acting by using not only one but several or all these mentioned mechanisms (Goksoyr 2006).

During steroid metabolism, several of these chemicals have been found to interfere with many of the essential enzymes (Thibaut and Porte 2004) as the ones mentioned previously. As an example, studies have shown that certain fungicides in rainbow trout and certain polycyclic aromatic hydrocarbons (PAHs) in flounder (*Platichthys flesus*) can inhibit steroidogenic enzymes such as P450 aromatase and 17β-HSD (Monod *et al.* 1993, Rocha Monteiro *et al*. 2000). Another study showed that various estrogenic and androgenic EDCs can have either an inhibitory or an activation effect on different necessary enzymes in carp (*Cyprinus carpio*) steroidogenesis (Thibaut and Porte 2004). It is also known that generally the processes of sex differentiation and smoltification in salmonids are particularly susceptible to the action of EDCs (Jobling and Tyler 2003). Within the next sub-sections, an overview will be given regarding the negative effects of juvenile salmonid fish caused by the main types of EDCs.

3.1. Estrogenic EDCs

Estrogenic EDCs such as bisphenol A (BPA) and nonylphenol (NP) are thought to imitate the action of the endogenous estrogen, 17β-estradiol, and thus start cellular processes that are estrogen-dependent (Arukwe *et al*. 2001, Yoon *et al*. 2014).

In the case of the nonylphenol, this EDC is a product of the microbial breakdown of nonylphenol ethoxylate (NPE), which is a compound usually used in the production of cleaning agents, pesticides, plastics and paints (Vincent and Sneddon 2009). Because of its extensive use, NPE is frequently present in wastewaters from industries and urban areas and therefore has a consistent presence in the aquatic environment (Mao *et al.* 2012). In juvenile salmonids, studies have shown that this chemical induces several physiological responses. For example, a survey with juvenile Caspian brown trout (*Salmo trutta caspius*), indicates that NP induces unusual synthesis of vitellogenin, as well as, possible hyperplasia and hypertrophy of the hepatocytes (Shirdel and Kalbassi 2016). In this study, it was also observed that NP caused histopathological lesions in both gill and intestine tissues (Shirdel and Kalbassi 2016). Another study, using juvenile rainbow trout, showed that injection of NP leads to several warning signs, including the increase in plasma levels of 17β-estradiol (in females), T (in males), cortisol, and vitellogenin (Naderi *et al.* 2015). Also, a decrease in triiodothyronine and thyroxine plasma levels were noted (Naderi *et al*. 2015).

Similarly to NP, BPA is also widely used for the production of plastics, flame retardants, powder paints, adhesives, amongst many other industrial products (Staples *et al*. 1998). Recently, bisphenol S (BPS) replaced BPA (Ribera 2015). Both compounds commonly reach the aquatic environment through wastewaters, and/or by accidental release during their processing, handling and transportation (Staples *et al.* 1998). Presently there are not many studies concerning the effect of BPA and BPS on juvenile salmonids. However, these EDCs induce in juvenile brown trout an increase in plasma concentrations of vitellogenin (Ribera 2015).

An additional well-known estrogenic EDC is ethynylestradiol (EE_2). EE_2 is the main component of synthetic contraceptive pills (Solé *et al*. 2000), and thus is usually present in high quantities in effluents and consequently in the aquatic environment (Verslycke *et al*. 2002). Studies so far have only shown that when exposed to EE_2 juvenile salmonids, such as the brown trout (Bjerregaard *et al.* 2008, Korner *et al*. 2008) and the rainbow trout (Verslycke *et al.* 2002), have increased plasma levels and synthesis of vitellogenin.

As seen, the type of alterations that these estrogenic EDCs cause in the juvenile salmonids can be of concern, especially since they can lead mainly, but not only, to reproductive and growth issues in these fish (Naderi *et al.* 2015).

3.2. Androgens

Despite most studies focusing on the presence and effects of estrogenic EDCs in the aquatic environment, various other studies also show the presence and impact of androgenic compounds (Sumpter 2005). The androgenic steroids are known for stimulating and controlling the development of male characteristics (Streck 2009) and therefore, can lead to the masculinization of fish (Sumpter 2005). Both natural and synthetic androgens (some used in growth therapy of humans and animals) can enter the aquatic environment through wastewaters and paper mill effluents or via the microbial degradation of phytosterols (Streck 2009).

In juvenile salmonids, two indispensable androgens, dehydroepiandrosterone and A, have been shown to increase the plasma levels of vitellogenin in juvenile rainbow trout (Shilling and Williams 2000). In the same study, also dihydrostestosterone could decrease significantly the number of enzymes of the P450 family (Shilling and Williams 2000), which can be concerning for the metabolism of steroids. Another study, made with juvenile Atlantic salmon, showed that the exposure to T and adrenosterone (11-ketoandrostenedione) lead to an increase in the plasma levels of 11-kT and T that would stimulate the spermatogonial differentiation in testis tissue of immature salmon (Melo *et al*. 2015). Studies like these clearly

show the possible dangers for salmonid species that can arise from the presence of androgens in aquatic habitats.

3.3. Polycyclic aromatic hydrocarbons (PAHs)

Polycyclic aromatic hydrocarbons (PAHs) are well known environmental contaminants that can come from natural sources, such as volcanic eruptions and forest fires, or from human activities, including emissions from industries (for example of petroleum and aluminium), as well as domestic emissions from activities, such as smoking and wood burning, with one of the primary sources being vehicle engine combustion (Ramesh *et al*. 2012). These contaminants are already of profound concern due to their mutagenic and carcinogenic nature and are known to cause reproductive issues in several organisms, including fish (Nicolas 1999).

Despite this concern, not many studies focus on the effect of PAHs in juvenile salmonids. One of the few existent studies, which consisted on the injection of two PAHs, β-naphthoflavone and benzo(a)pyrene in juvenile Rainbow trout, revealed that these contaminants could cause an increase in the plasma levels of cortisol and alterations in the liver metabolism (Tintos *et al*. 2008).

3.4. Pesticides

In agriculture production, there is active use of pesticides as a way to prevent and reduce pests and diseases that might compromise the levels of production and the quality of the products (Damalas and Eleftherohorinos 2011). Despite nowadays their use being highly regulated to minimise possible impacts for the environment and human health, some of these pesticides still end up in the aquatic environment, mainly through agricultural runoffs (Damalas and Eleftherohorinos 2011). Inclusively, studies have shown that, even after the banning of certain pesticides, these compounds remain in the aquatic food webs, bioaccumulating in fish and still affecting their development and reproduction long after they have stopped being used (Donohoe and Curtis 1996).

In opposition to PAHs, there is an increase in information on the impacts of pesticides in juvenile salmonids, however most works focus on effects such as mortality (Riar *et al*. 2013), behaviour (Saunders 1969) and other physiological traits (Velíšek *et al*. 2007) and not as much on their particular effects on the development and reproduction. Still, some studies do show that there is a possible impact of certain pesticides in the reproductive processes of these

juvenile fish. One of these pesticides is, for example, the widely used herbicide atrazine (ATZ), which has been shown to induce in juvenile rainbow trout the enzyme aromatase and cause the increase of E2 and vitellogenin, and the impairment of hepatic metabolism (Salaberria *et al*. 2009). Also, DDT pesticides induce zonagenesis and inhibit the induction of vitellogenesis in juvenile Atlantic salmon (Celius and Walther 1998). Furthermore, estrogenic organochlorine insecticides and DDT (including DDT degradation products), induce vitellogenesis in the juvenile rainbow trout (Donohoe and Curtis 1996).

3.5. Metals

In several aquatic ecosystems, it is possible to find relatively high concentrations of metals, due mainly to their release by industrial sources (such as the plastic and paint industries), agriculture and mineral mining (Daudt 2005, Vetillard and Bailhache 2005). Some examples of these metals are cadmium, copper, lead, mercury, nickel and zinc, which can be found naturally in low concentrations in the water, however, due to human activity, these concentrations have been increasing dangerously (Hewitt and Servos 2001). Several studies have considered several prejudicial effects that metals produce on fish from freshwater ecosystems (Scott and Sloman 2004).

When it comes to juvenile salmonid fish in specific, cadmium is one of the metals that stands out for its effect in these juvenile's endocrine system. Studies have shown that this metal can be a crucial EDC by affecting various estradiol signalling pathways (Vetillard and Bailhache 2005), causing, for instance, the increase in the plasma levels of 11-kT (in males) and of E_2 (in females) (Daudt 2005).

Other metals cause impacts on juvenile salmonids; however, few works exist on how these affect specifically the juvenile's development and reproduction. Instead, most studies focus on other consequences following metal exposure, such as juvenile survival (De Schamphelaere and Janssen 2004) and effects on social behaviour (Sloman *et al.* 2003).

3.6. Other EDCs

The last sections show the main problematic pollutant groups affecting juvenile salmonids. The EDCs within the previous groups are the most frequently found to have an impact on juvenile salmonids and therefore, the most regularly studied. However, the diversity of chemicals that can cause issues on these juveniles is full, and several compounds exist outside of these groups that are still important to recognise.

An example of such compounds are industrial surfactants, such as perfluorooctonoic acid (PFOA) and perfluorooctone sulfonate (PFOS) (Mortensen *et al.* 2011). PFOA and PFOS have been shown to lead to significant decreases in the plasma levels of E1, testosterone and cortisol in juvenile Atlantic salmon (Mortensen *et al.* 2011). Another example are the organophosphates tris(2-butoxyethyl) phosphate (TBOEP) and tris(2-chloroethyl) phosphate (TCEP), that have been used as flame retardants and that have been shown to cause changes in juvenile Atlantic salmon specifically in the transcription of isoforms of key steroidogenic enzymes such as P450scc, cyp11β and cyp19 (Figure 4) (Arukwe *et al*. 2016). These changes can then lead to several problems in smoltification, sexual maturation and reproduction in these salmonids (Arukwe *et al*. 2016).

Finally, one last example is tributyltin (TBT), a biocide used in antifouling paints for ships. Although TBT is banned, due to its impactful toxicity (Mortensen and Arukwe 2007), this chemical has been shown to affect hormonal signalling pathways in juvenile Atlantic salmon (Mortensen and Arukwe 2007) as well as decrease its transcript levels of cyp11β (Kortner *et al*. 2010).

As seen, juvenile salmonid fish can be affected by a large variety of EDCs, and many of these toxic chemicals have a relatively frequent presence in the aquatic environment, primarily due to human activities. However, the problems that arise from these contaminants are not limited to the aquatic environment and the fish populations. As it will be shown in the next section, through fish, the prejudicial effects of these toxic compounds can reach the human being, making the consumption of fish a possible danger to human health.

4. From juvenile salmonid to human

4.1. Bioaccumulation of EDCs in salmonid fish

Aquatic organisms and in particular salmonid fish, are exposed to various types of pollutants that cause several prejudicial consequences that include development, reproduction and behaviour disruption. However, pollutants in aquatic systems and fish can end up having consequences not just for these organisms but also for those that consume them. The last observation happens mainly due to the bioaccumulation and biomagnification processes. The process of bioaccumulation is commonly known as the “uptake, storage and accumulation of organic and inorganic contaminants by organisms from their environment” (Streit 1998), whilst the process of biomagnification is usually known as “the tendency of pollutants to concentrate as they move from one trophic level to the next” (Blowes *et al.* 2003).

These processes lead the compounds not only to spread through food networks but also to appear in higher concentrations as they progress across such systems (Voutsas *et al*. 2002).

Depending on the contaminant and on the type of fish in question, bioaccumulation and biomaginification can occur differently. For example certain types of metals such as isotopes, tend to accumulate mostly in the bone structures of the fish whilst other metals such as mercury accumulate in the more soft tissue of the organism, meaning that it will pass along the food chain more quickly than the compounds accumulated at the bony structures (Streit 1998). Bioaccumulation and toxicity can also vary with the lipid content of the fish since lipophilic contaminants are usually found in higher levels in fish with more lipid content. Such content usually differs not only between species but also within species, as in the case of the salmonid fish, in which for example more lipid content can be found before spawning occurs and even within the organism itself, with some organs having more lipid content than others (Streit 1998). Because of these processes, the consumption of salmonid fish has become a possible way of entrance of compounds such as EDCs into the human body, and much like in the case of fish, these compounds may produce consequences on the health and well-being of humans.

4.2. Effects of EDCS on human health

The increase of pollution in both freshwater and marine habitats, and the tendency of fish to accumulate water pollutants (Skerfving 1995) turn their consumption, in some situations, in potential risk to human health. This last occurrence materialises when the quantity of toxic organic substances in fish exceeds those recommended for human consumption (Swain 1988). In this sense, several incidents of this were reported in European countries (Alvarez-Munoz *et al*. 2018).

In one case, for example, the consumption of sport fish from a contaminated lake resulted in the exposure of toxic compounds, such as PCBs, which showed consequences in the endocrine regulation of humans such as diminished menstrual cycles in females (Buck *et al.* 1997). Another case shows that elevated consumption of fatty fish (including salmonids) from specific regions such as the Baltic sea constitutes a risk of exposure to high levels of polychlorinated dibenzodioxins and dibenzofurans (Svensson *et al*. 1991). These compounds are known to have carcinogenic effects as well as reproductive and immunosuppression effects (Skerfving 1995). Additionally, it was also shown that breast-fed infants were at particular risk since such compounds if consumed by the mother can easily pass to the infant through breast milk (Svensson *et al.* 1991). One more compound that can also pass via human milk is DDT, which has also been a compound that was found in high levels in fatty fish and

consequently in a high blood level in humans which consumed elevated amounts of these fish (Skerfving 1995).

Although there are other ways in which humans can enter contact with EDCs, besides the consumption of fish, it becomes vital to understand of how these compounds enter and interact with aquatic organisms, since aquatic food products have such an essential role in the human diet.

5. Analytical methods for sex-steroids quantification in fish plasma samples: why gas chromatography-mass spectrometry

In order to measure sex steroids in plasma samples, several analytic methods have been used. Initially, the most commonly used techniques were immunoassays, which varied in type, from conventional radioimmunoassays (RIAs) to direct immunoassays and enzyme immunoassays such as ELISA (Enzyme-Linked Immunosorbent Assay) (Kidd *et al*. 2010, Stanczyk and Clarke 2010). These methods are highly reliable when adequately validated since they have high selectivity and specificity (Hoga *et al*. 2018). The advances undertaken in the last years for techniques such as ELISA have also allowed the development of commercial kits that are easier and quicker to use (Bonwick and Smith 2004).

However, despite their advantages, both RIAs and ELISA assays present some challenges in the measurement of sex-steroids. They can be, for example, in some cases, quite time-consuming and expensive due to the need of large sample volumes; are only able to measure one metabolite at a time; lack the sensitivity to measure with accuracy certain steroids; and one kit cannot be applied to all species, with few kits being available for fish species (Sink *et al*. 2008, Hoga *et al.* 2018, Hosseini *et al*. 2018). Nonetheless, both RIA and ELISA were, respectively, successfully used to measure sex steroid profiles of Coho salmon during early development (Feist *et al*. 1990) and plasma levels of steroids in various fish species (Barannikova *et al.* 2002, Metcalfe *et al.* 2018).

Recently, with the improvement in mass spectrometry (MS) techniques, many research laboratories have been switching their use to these analytical methods, which have facilitated for many the analysis of steroid hormones (Stanczyk and Clarke 2010). The most common chromatographic methods used for the study of steroids are liquid chromatography (LC) and gas chromatography (GC) coupled with MS (Stanczyk and Clarke 2010). These methods allow the analysis of several compounds at one time, needing a few amounts of sample and little sample preparation before analysis (Hoga *et al.* 2018). One of the significant differences between these two mass spectrometry methods is that GC is only applicable to volatile samples, while LC is capable of analysing non-volatile compounds (Shimada *et al.* 2001).

Because of this, all non-volatile compounds need to be subjected to a derivatisation step before GC analysis (Díaz-Cruz *et al*. 2003). Therefore, the GC-MS method is more time-consuming than LC-MS. However, GC-MS still has some advantages when compared to LC-MS, since it has a slightly higher sensitivity that allows reaching low limits of quantification and it has less difficulty separating sample analytes with similar structures (Hoga et al. 2018, Stanczyk and Clarke 2010). Due to this, although LC-MS has been successfully applied to measure sex steroids in fish (Han and Liu 2019), until now it seems to be more commonly used in humans and mammals (Koren *et al.* 2012, Yuan *et al*. 2019), whilst GC-MS seems to be more often applied in fish (Budzinski *et al.* 2006, Lu *et al.* 2013).

6. Main dissertation objectives

Considering that juveniles are extremely sensitive to the presence of EDCs, that are known to interfere with normal pathways of steroid hormones, knowing the normal sex steroid profiles in plasmas of juvenile fish, namely salmonids, becomes crucial. Thus, the main objectives of this study were to:

(1) Implement in our facilities a GC-MS method, previously developed for the evaluation of ten steroid hormones in rainbow trout (Budzinski *et al*. 2006). The steroids evaluated were: Pn, P, A, T, 11-kT, E_1, E_2, 17-OHP, 17-OHPn, and 17,20-OHP;

(2) Evaluate, for the first time, the concentration levels of the last referred steroids in juvenile (1.5 years) brown trout plasma, considering both males and females;

(3) Establish a possible relation between plasma hormones and gonadal maturation (histologic evaluation);

(4) Investigate if there are different hormonal profiles between genders at a juvenile stage.

Chapter 2. Materials and Methods

1. Chemicals and materials

Acetonitrile (C_2H_3N) (CAS 75-05-8; ≥99.9%GC) methanol (CH_4O) (CAS 67-56-1; puriss p.a. ≥99.8%, GC) and hexane (C_6H_{14}) (CAS: 110-54-3; absolute ≥99.0%, GC) were purchased from Sigma-Aldrich (Germany). Isooctane (2,2,4-Trimethylpentane) (CAS 540-84-1) was acquired from VWR Chemicals (EC). MSTFA (N-Methyl-N-(trimethylsilyl)trifluoroacetamide) (CAS 24589-78-4; 97% GC) was purchased from Fluka. Ammonium iodide (NH_4I) (CAS 12027-06-4; 99.0% granular) and 2-mercaptoethanol (C_2H_6OS for synthesis, 99%, GC), were bought from Alfa Aesar (Germany) and Merck-Schuchardt, respectively. Ultrapure water was purified through a Milli-Q system (Millipore, São Paulo, Brazil; conductivity = 0.054 µS/cm, at 25 ºC).

The hormones used in this study were obtained from Sigma Aldrich; Pn (CAS 145-13-1), P (CAS 57-83-0), A (CAS 63-05-8), T (CAS 58-22-0), 11-kT (CAS 564-35-2), E_2 (CAS 50-28-2), E1 (CAS 53-16-7),17-OHP (CAS 68-96-2), 17-OHPn (CAS 387-79-1) and 17,20-OHP (CAS 1662-06-2).

For the solid phase extraction (SPE) protocol, SPE cartridges (SPE C_{18}; 1000 mg/6 ml; pK/30) from Teknokroma (Spain) were attached to an extraction manifold (20 inserts) from Waters, Milford, Massachusetts USA, coupled to a vacuum pump (220/240 V, 50Hz) from Waters, USA.

For the GC-MS analysis, the vials (1.5 ml with wide opening of 6 mm; dimensions 1.6 x 32 mm; B70-pK100), were bought from Specanalitica (Portugal) and flat bottom glass inserts (0.2 ml; dimensions 31 x 6 mm; pK100) were from BGB Analytik Vertrieb GmbH (Germany). The GC-MS equipment was a Trace GC ultra-gas chromatograph, fitted with a TG-5MS GC column (30 m x 0.25 mm x 0.25 µm) coupled to a mass detector (ITQ 1100) from Thermo Scientific.

Animals were anaesthetized with ethylene glycol monophenyl ether (CAS 122-99-6) obtained from EMD Millipore (Germany). Sterile syringes (0.50 x 0.16 mm, 25 G) and Eppendorf tubes (2 ml) were coated with heparin (B. Braun). Bouin's solution (CAS 464-B-3001) was purchased from VWR Chemicals (Belgium). Other materials and chemicals used in the histological process are referred in Appendix 1.

2. Fish collection

Juvenile trout with approximately 1.5 years in age, were obtained from the aquaculture facilities of Torno (Marão mountains, Amarante, Portugal) (Fig.6. A) during early August. Fishes were maintained in several wide exterior tanks (Fig. 6. B), with water temperature ranging from 15 to 16 ºC. In total, 38 fish were randomly sampled, from which 22 were male and 16 were female.

Before necropsy, each fish was placed in a small container with approximately 5 L of water (Fig. 6. C) and euthanised with an overdose of 5 mL of ethylene glycol monophenyl ether. Then, fish were weighted (g) and measured, considering the total and standard lengths (cm). The total blood was sampled from the caudal vein with an insulin syringe and immediately transferred to a heparinised Eppendorf tube. Then, the blood was centrifuged at 1000 rotations during 8 min in a microcentrifuge (Micro Star 12, VWR, Denmark) and the plasma obtained was collected and stored in ice.

Trout necropsy was performed on a proper dissecting plate (Fig. 6.D). The gonads and the liver were extracted and weighted and then stored in flasks containing Bouin's solution during 24 h, being afterwards stored at 70% ethanol.

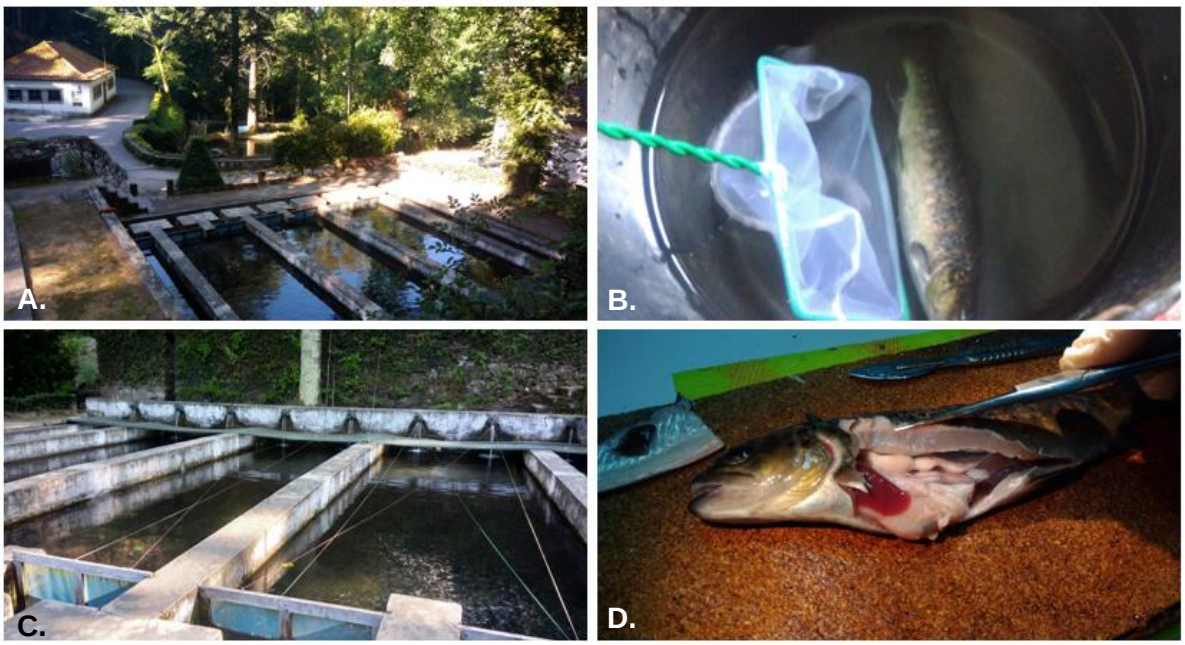


Fig. 6. Pictures of: **A**. Aquaculture Farm of Torno at Marão Mountains, Amarante, Portugal**. B.** Juvenile trout being collected after euthanasia. **C.** Fish tanks with juvenile trout. **D**. Fish necropsy and organ collection.

3. Histological analysis – Methacrylate procedures of the gonads

After fixation, the gonad samples were then processed for impregnation using Technovit® H7100 Embedding Kit (Kulzer 14653, Germany). The protocol is fully detailed in Appendix 1.

The methacrylate blocks were then sectioned at 4 µm, using a fully rotary microtome (Leica RH2255, Germany). Sections were collected and extended in a 25 ºC distilled water bath, and then carefully moved to glass microscope slides which were placed in a plaque at 45 ºC until dry. After this step, the slides were stained with haematoxylin and eosin (H&E) (Appendix 2) and mounted with mounting medium (Coverquick 2000, VWR International, France) and a glass coverslip (Medite Medzntechnik, Germany). For qualitative histological analyses, the slides were observed under a light microscope (Olympus BX50, Japan), and photographed with a digital camera (Olympus DP20, Japan).

4. Optimisation of the analytical procedures

4.1. Preparation of standard solutions

Individual standard solutions, containing each analysed hormone or internal standard (IS) were prepared in acetonitrile at 1000 µg/L (Table 3). Additionally, mixtures containing all hormones were also prepared in eight different concentrations ranging from 0.50 µg/L to 50 µg/L (Table 3).

Table 3. Standard solutions prepared in acetonitrile and stored at -20 ºC.

Individual hormones and IS standards at 1000 µg/L	Pn
	P
	A
	T
	11-kT
	E_1
	E_2
	17-OHPn
	17-OHP
	17,20β-OHP
	Estradiol-d_2 (E_2-d_2)
	Bisphenol A-d_{16} (BPA-d_{16})
Mix standard solutions	Mix C1 - 50.00 µg/L
	Mix C2 - 25.00 µg/L
	Mix C3 - 10.00 µg/L
	Mix C4 - 5.00 µg/L
	Mix C5 - 2.50 µg/L
	Mix C6 - 1.00 µg/L
	Mix C7 - 0.50 µg/L
	Mix C8 - 0.25 µg/L

4.2. SPE extraction

The method used for the SPE extraction was adapted from Budzinski *et al.*(2006). For Each SEPACK C_{18} column extracted 1 g of plasma. Firstly, each plasma sample was diluted in 2 mL of acetate buffer (0.1 M; pH 5). Secondly, the columns were placed in an extraction manifold (Fig.7 A), and with the help of a vacuum pump, they were conditioned, first with 3 mL of MeOH and then with 2 mL of ultrapure H_2O. Subsequently, each of the diluted plasma samples was loaded on to the C_{18} SPE column, and the column was then rinsed with 3 mL of ultra-pure water and left to dry under vacuum for 60 min. Finally, the steroids were extracted from the columns using 2 mL of MeOH and then were evaporated until dry with under nitrogen flow.

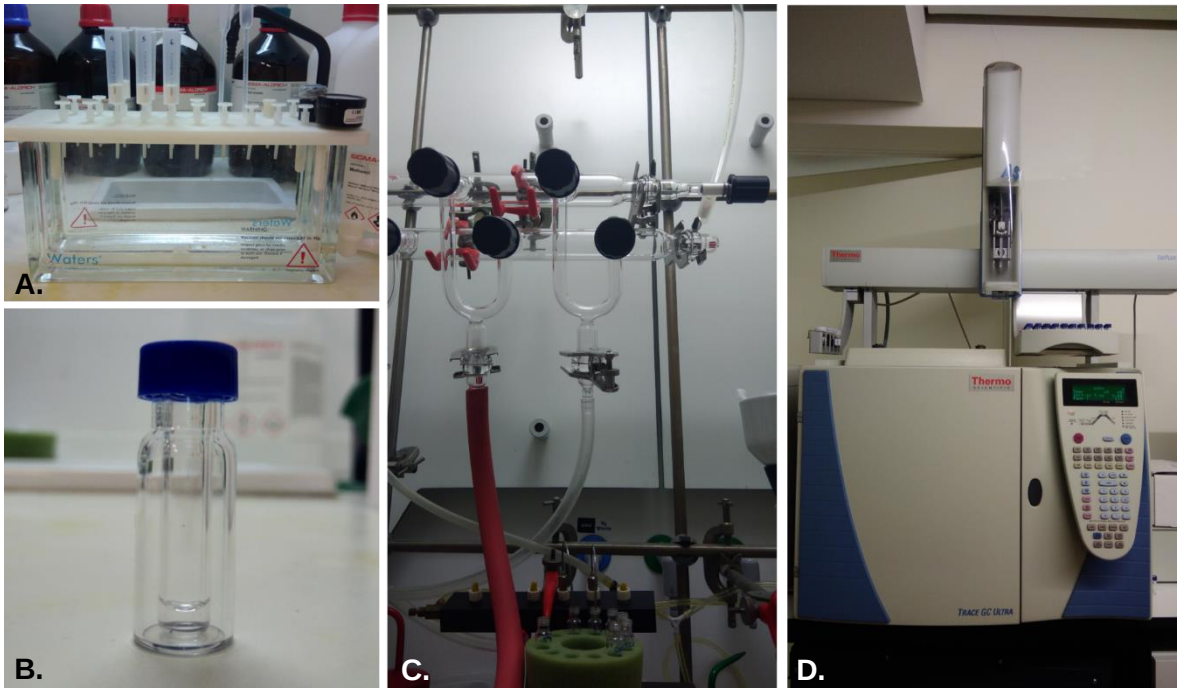


Figure 7. Photography of: **A.** C_{18} SPE columns attached to the extraction manifold for SPE extraction. **B.** GC vial with flat bottom glass insert. **C.** Samples were evaporated to dryness over nitrogen flow. **D.** Thermo Scientifc Trace GC Ultra.

4.3. Derivatisation and preparation of calibration curve samples for GC-MS

Before GC-MS analysis, the extracts were dried under N_2 flux and reconstituted in isooctane (100 µL per extract). Then, 25 µL of the extract was transferred to each of 8 different GC vials with flat bottom glass inserts (one vial per level of the calibration curve) (Fig. 7.B) and evaporated to dryness. Subsequently, to each vial, 25 µL of each Mix standard solution (Table 3) was added, and once again, each vial was evaporated to dryness (Fig. 7.C).

Derivatization was achieved using 7.5 µL of a mixture of MSTFA (250 µL) + β-mercaptoethanol (15 µL) + NH_4I (10 mg) (99.1%:0.5%:0.4) with pure MSTFA (1/9). Then, the vials were capped, stirred in the vortex for 1 min, and then kept at 60 ºC for 30 min. After cooling at room temperature, the samples/standards were evaporated to dryness over a N_2 flux (Fig. 7.C) and reconstituted with 25 µL of isooctane.

4.4. Plasma analysis in Gas chromatography/mass spectrometry (GC/MS)

Plasma retrieved from juvenile trout during samplings were grouped in pools of 2 animals, of the same gender and same weight, in order to guarantee the required amount of plasma for GC/MS analysis (both the SPE processing and derivatisation steps are described

above in sections 4.2 and 4.3). Individual juveniles from which it was possible to obtain 1 g of plasma were considered as a pool.

4.5. Gas chromatography/mass spectrometry (GC/MS) analysis

All analyses were carried out using a gas chromatograph (Trace GC Ultra, Thermo Finnigan Electron Corporation), coupled with an ion trap mass spectrometer (Thermo Scientific ITQ™ 1100 GC-MSn) and an autosampler (Thermo Scientific TriPlus™). The GC column was A Trace GOLD (TG-5MS, length - 30 m, ID - 0.25 mm, film thickness - 0.25 µm). The flux of the He carrier gas (99.9999 % purity) had a constant flow rate of 1.3 mL/min. The oven temperatures were as follows: from 90 ºC (initial equilibrium time, 1 min) to 290 ºC at 7.5 ºC/min and maintained at 290 ºC for 5 min (Budzinski *et al.* 2006).

A solvent delay time of 20 min was used to protect the ion multiplier of the MS instrument from saturation. Both MS transfer line and ion source were at 280 °C. Sample injection (2 µL) occurred in a 250 ºC injector programmed in splitless mode using an 80 mm injection needle. Quantitative analysis was performed in a selected ion monitoring mode (SIM) using external calibration. A summary of the analytic parameters of the GC-MS is in Table 4.

Table 4. Experimental retention times (RT) and selected ions (m/z) for the quantification of Pn, P, A, T, 11-kT, E1, E2, 17-OHPn, 17-OHP and 17,20-OHP.

Target steroid hormones	RT (min)	Precursor ion (m/z)	Qualifier ions		Segment time
			Q1 (m/z)	Q2 (m/z)	
E_1	25.52	414	399	342	24.00 – 26.15
A	26.50	430	432	415	26.15 – 26.80
E_2	26.60	285	416	129	
T	26.60	432	417	327	
11-kT	27.80	503	518	169	26.80 – 29.00
Pn	28.20	445	460	446	
P	29.60	458	443	157	29.00 – 32.70
OHPn	30.13	548	230	404	
17α,20β-P	31.71	431	548	-	
OHP	31.96	546	316	301	

4.6. Recovery protocol

In order to verify the recovery percentage of each steroid after the SPE method proposed by Budzinski *et al.* (2006), 0.5 g of adult trout plasma was fortified with 50 µL of the standard solution Mix C7 (Table 3), and then extracted (as described in section 2.3.), derivatized and analysed in the GC-MS equipment as referred above in sections 4.3 and 4.5, respectively.

5. Data analyses and Statistic

The gonadosomatic index (GSI), the hepatosomatic index (HSI) and Fulton's condition factor (K) (Ricker 1975) were calculated for each animal using the following mathematic quotients:

$$GSI = \frac{Total\ Gonad\ Weight\ (g)}{Total\ Body\ Weight\ (g)} \times 100$$

$$HSI = \frac{Total\ Liver\ Weight\ (g)}{Total\ Body\ Weight\ (g)} \times 100$$

$$K = \frac{Total\ Body\ Weight\ (g)}{Total\ Lenght^3\ (cm)} \times 100$$

Linearity was evaluated using three independent calibration curves, each with eight nominal calibration standard mixtures (ranging from 0.5 to 50 µg/L of each steroid hormone.

The limit of detection (LOD) and quantification (LOQ) were calculated with the same curves, using the following formulas: LOD = 3.3 α/S and LOQ = 10 α/S, where α is the standard deviation of the response and *S* is the average slope of the calibration curves.

For statistical analysis the software used was PAST 3.26 (Paleontological Statistics software package for education and data analysis). To compare biometry and plasma hormone levels between male and female fish t-tests were applied. Previously to using the t-tests, all data were tested for normality (using the Shapiro-Wilk test) and for homogeneity of variances (using the Welch F test for equal variances). For the data that did not pass the homogeneity of variances test (data for gonadal weight and P and E_1 plasma levels), the unequal variances t-test was applied instead of the t-test. When the data did not pass the normality tests with or without applying transformations (GSI data), a non-parametric test was applied (Mann-Whitney test).

Correlation between biometry data and plasma hormone levels was also tested using Pearson's correlation test after, once again, confirming normality and homogeneity of the data.

Chapter 3. Results

1. GC/MS method optimisation

The linearity obtained from the independent calibration curves was of 0.99. The values of LOD obtained for the steroid levels ranged between 0.05 and 0.21 ng/mL, and the values of LOQ ranged between 0.16 and 0.69 ng/mL (Table 5).

The recovery protocol performed with adult trout plasma and the standard solution, permitted the calculation of the recovery percentage for each steroid and all the recoveries ranged between 57.4 % and 110 % which confirmed the validation of the method for the analysis of the plasma steroid levels (Table 5)

As an example, figure 8 shows three typical chromatograms with different samples.

Table 5. Values of LOD (Limit of detection), LOQ (Limit of quantitation) and recovery for each hormone.

Hormone	LOD (ng/mL)	LOQ (ng/mL)	Recovery (%)
Pn	0.21	0.69	83.4
P	0.12	0.40	93.1
A	0.13	0.42	95.8
T	0.11	0.36	110.0
11-kT	0.17	0.58	94.5
E1	0.18	0.60	110.0
E2	0.16	0.55	97.2
17-OHPn	0.12	0.41	99.6
17-OHP	0.19	0.64	57.4
17,20β-OHP	0.05	0.16	105.0

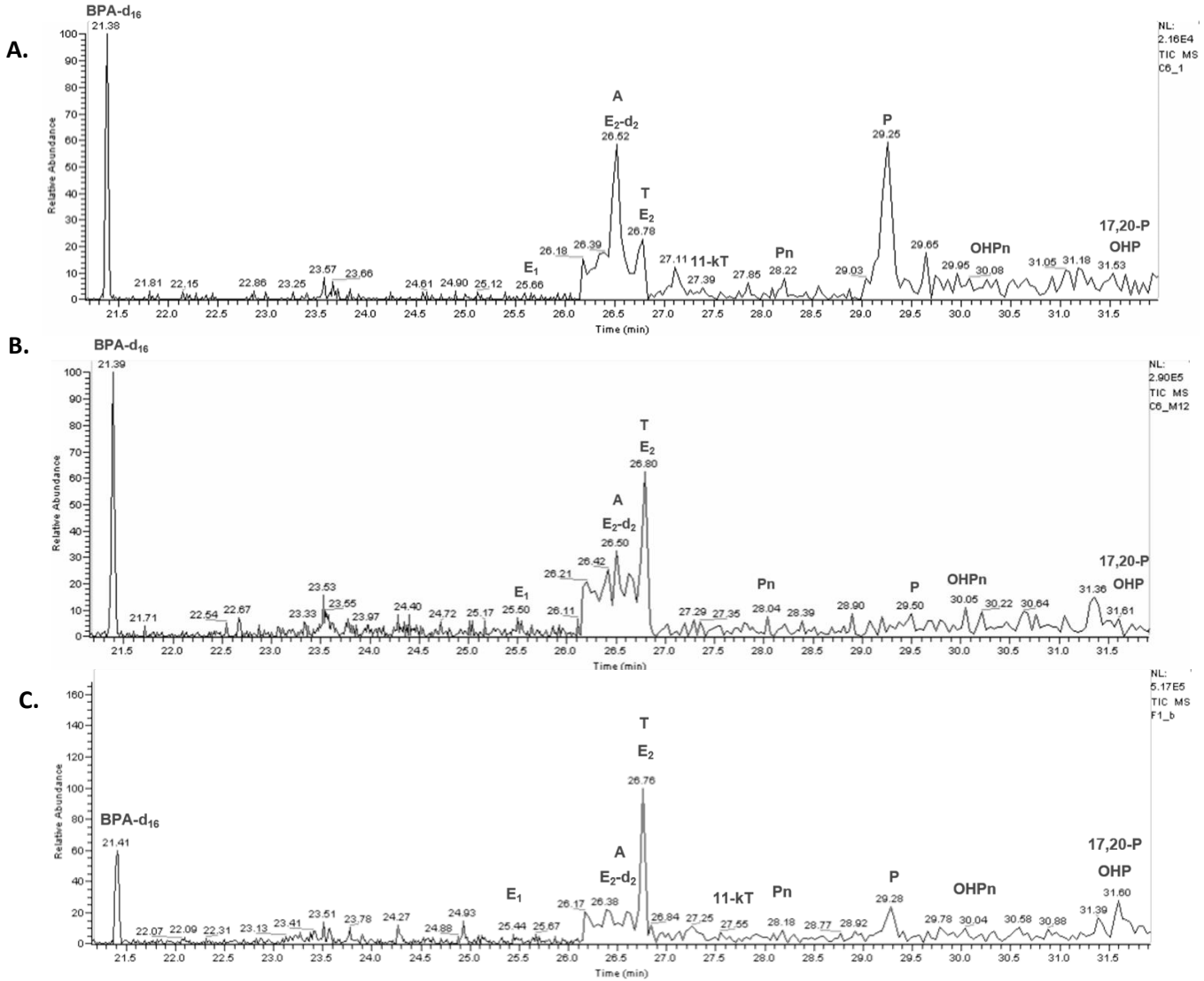


Figure 8. GC-MS chromatogram, in SIM mode, of (**A.**) the standard solution C6 (1 µg/L), (**B.**) a plasma sample of juvenile male trout fortified with the ten steroid hormones analysed in this study at a final concentration of 0.5 µg/L and (**C**.) a plasma sample of juvenile female trout, added with internal standards BPA-d_{16} and E2-d_2 (30 µg/L).

2. Animals biometry

The biometry data for both female and male juvenile brown trout is plotted in figures 9 and 10 and is presented in detail in Appendix 3. The female mean total length and body weight was of 19.94 cm and 86.7 g, respectably. In male fish, the total length mean was 19.83 cm and body weight mean 87.58 g. Mean gonad weight was 0.30 g in males and 0.18 g in females while the mean GSI was 0.34 in males and 0.21 in females. In males, the mean liver weight and HSI were 1.25 g and 1.44, respectably. In female fish, the mean liver weight was 1.17 g, and HSI was 1.35. Finally, K mean value was 1.09 in females and 1.12 in males.

Variability of the biometry data within each gender group was also calculated and reached elevated values in some parameters. For males, the coefficient of variation (CV) of the gonad weight was of 139.96 % and for GSI was of 141.81 %. In females, the CVs for the gonad weight, GSI and liver weight were 36.36 %, 30.85 % and 32.97 %, respectably. The remaining biometry parameters presented a variability lower than 30% in both genders.

The statistical analysis of the biometric data revealed that for juvenile brown trout, there are no significant differences between sexes concerning both weight (p=0.8373) and total length (p=0.7195). Similarly, gonadal weight (p=0.9373) and liver weight (p=0.4836) also showed no significant differences between genders.

Relatively to the GSI, the non-parametric test of Mann-Whitney showed no differences between females and males (p=0.7374). The t-test relative to the HSI (p=0.4822) and the one relative to K (p=0.3673) also showed no differences between genders.

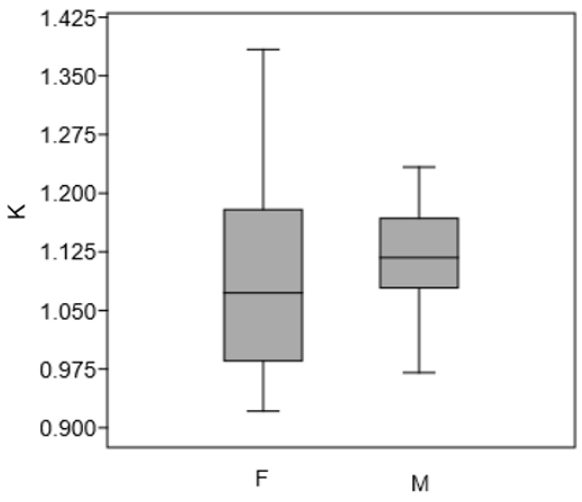


Figure 9. Fulton's condition factor (K) in female (F) and male (M) juvenile brown trout. Data is given as minimum, first-quartile,median, third-quartile and maximum values.

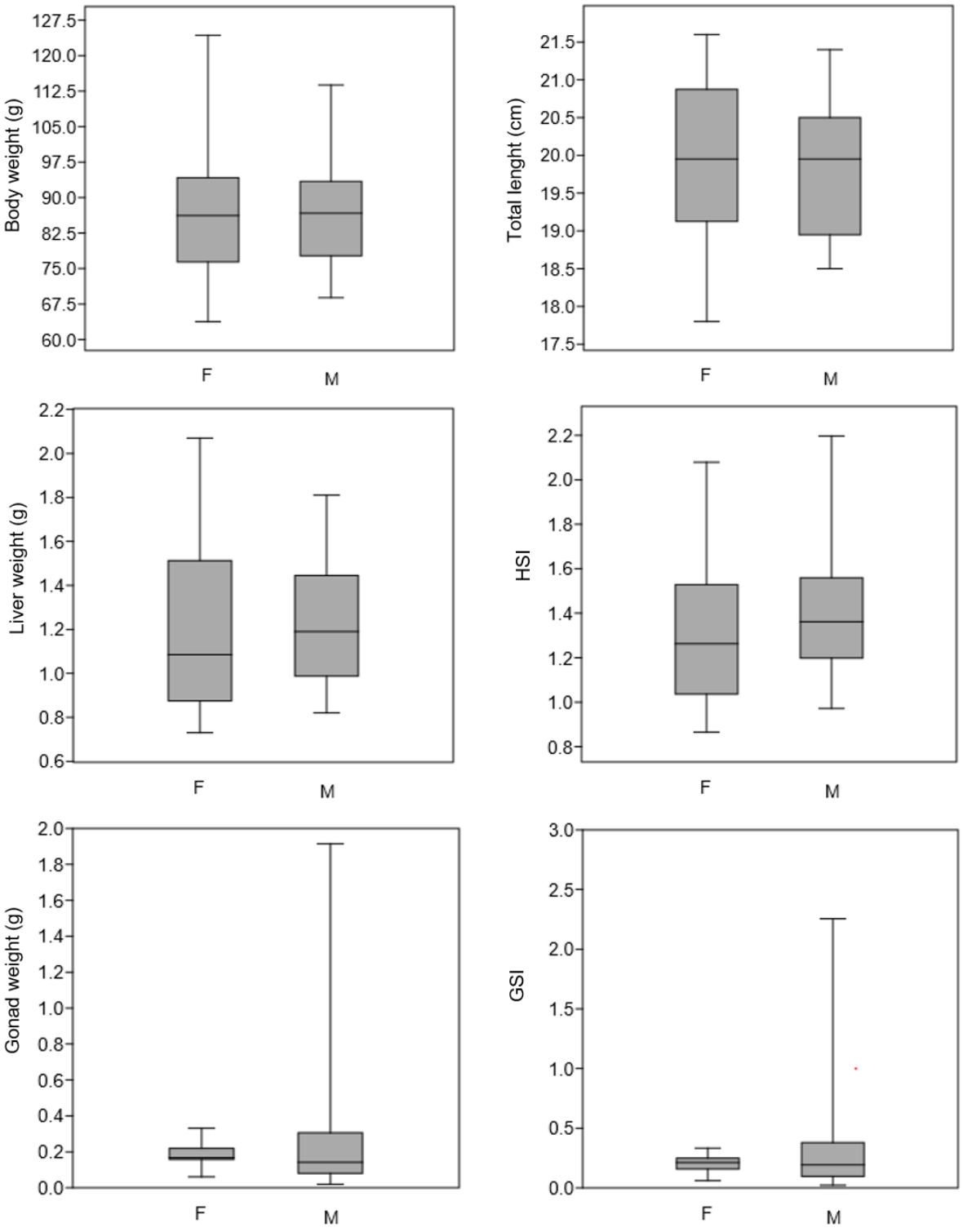


Figure 10. Biometry data of both female (F) and male (M) juvenile brown trout. GSI (Gonadosomatic index), HSI (Hepatosomatic index). Data is given as minimum, first-quartile,median, third-quartile and maximum values.for each parameter.

3. Plasma steroid levels

The plasma steroid levels are presented for both female and male juvenile brown trout in table 6 and 7, respectably. In both female and male juveniles, all steroid hormones measured presented values above the respective LOD (Table 5), with detection rates ranging from 50% to 100 % for most hormones. E_1 and 17-OHP presented values below 50% for both genders and P as well for males (Tables 6 and 7).

Plasma levels obtained varied between 0.12 and 0.75 ng/mL in females (Table 6) and 0.08 and 1.06 ng/mL in males (Table 7). The hormones with the highest mean values (above 0.40 ng/mL) were 11-kT and E_2 for both female (Table 6) and male (Table 7) juvenile brown trout while P and T showed the lowest mean concentration values (bellow 0.23 ng/mL) in both genders. For the remaining hormones, the values obtained remained between 0.23 and 0.40 ng/mL in males and females.

Statistical analysis was performed in order to compare the plasma steroid levels between males and females. The levels of Pn ($p=0.7705$), P ($p=0.3029$), A ($p=0.7491$), T ($p=0.9786$), 11-kT ($p=0.6503$), E_1 ($p=0.3609$), E_2 ($p=0.7186$), 17-OHPn ($p=0.1235$), 17-OHP ($p=0.2385$) and 17,20-OHP ($p=0.4627$) all showed no significant differences between juvenile genders.

Furthermore, correlations were tested between the concentrations of each hormone and the steroid levels, body, liver and gonad weight. After normality tests, the Pearson's correlation test demonstrated that there is no correlation between the assayed steroids levels and the mentioned biometric data.

Table 6. Range, Detection rate (DR), Mean (Standard error, SE), and Coefficient of variation (CV) of female juvenile trout steroid plasmatic concentration (ng/mL).

Steroid	Plasmatic concentrations (ng/mL) ♀ n= 9 pools and 16 animals				
	Min - Max	**DR (%)**	**Mean (SE)**	**Median**	**CV (%)**
Pn	0.207 – 0.388	55.6	0.280 (0.036)	0.267	28.7
P	0.121 – 0.222	55.6	0.159 (0.017)	0.144	24.5
A	0.147 – 0.722	88.9	0.310 (0.065)	0.267	59.7
T	0.118 – 0.209	66.7	0.167 (0.015)	0.165	21.6
11-kT	0.322 – 0.748	88.9	0.556 (0.065)	0.570	33.3
E1	0.240 – 0.264	33.3	0.256 (0.008)	0.262	5.4
E2	0.179 – 0.707	88.9	0.398 (0.073)	0.318	51.9
17-OHPn	0.144 – 0.522	100.0	0.286 (0.038)	0.254	39.7
17-OHP	0.209 – 0.246	44.4	0.228 (0.008)	0.228	7.0
17,20β-OHP	0.115 – 0.331	100.0	0.234 (0.024)	0.238	30.9

Table 7. Range, Detection rate (DR), Mean (Standard error, SE), Median and Coefficient of variation (CV) of male juvenile trout steroid plasmatic concentration (ng/mL).

Steroid	Plasmatic concentrations (ng/mL) ♂ n= 12 pools and 22 animals				
	Min - Max	**DR (%)**	**Mean (SE)**	**Median**	**CV (%)**
Pn	0.202 – 0.429	50.0	0.296 (0.036)	0.289	30.1
P	0.126 – 0.428	41.7	0.225 (0.055)	0.205	54.2
A	0.181 – 0.429	83.3	0.305 (0.030)	0.306	30.6
T	0.122 – 0.254	66.7	0.167 (0.017)	0.151	28.2
11-kT	0.169 – 1.058	100.0	0.501 (0.086)	0.374	59.1
E1	0.196 – 0.413	41.7	0.315 (0.055)	0.325	34.9
E2	0.207 – 0.671	83.3	0.431 (0.055)	0.375	51.9
17-OHPn	0.167 – 0.575	100.0	0.374 (0.038)	0.383	35.0
17-OHP	0.225 – 0.271	33.3	0.244 (0.010)	0.241	8.03
17,20β-OHP	0.078 – 0.441	100.0	0.265 (0.032)	0.272	41.5

Taking into consideration the lack of significant difference in plasma steroid levels between female and male juvenile trout, table 8 shows a summary of the values for all sampled fish, without distinction between genders.

Table 8. Average steroid plasma levels of all sampled juvenile trout, CV (Coefficient of variation).

Steroid hormones	Pn	P	A	T	11-kT	E_1	E_2	17-OHPn	17-OHP	17,20-OHP
Mean (ng/mL)	0.29	0.19	0.31	0.17	0.52	0.29	0.42	0.34	0.24	0.25
Median (ng/mL)	0.25	0.16	0.28	0.16	0.45	0.26	0.36	0.33	0.24	0.24
CV (%)	28.2	48.1	44.5	24.6	48.5	29.2	44.2	38.3	7.9	37.7

4. Histological analyses

The sex of the juveniles was classified *in situ* and then further confirmed by histology. The last procedure allowed sex confirmation and observation of the gonads for the detection of gametes with different maturation stages. Sex confirmation was possible in all individuals, as female and male gametes were easily identified. Although most individuals presented gonads with gametes in a less matured stage (75% of females and 68% of males), certain animals presented gonads with some gametes in a more advanced maturation stage. The differences between the gonads of these animals can be seen in Figures 11 to 14.

Figure 11 shows an example of females with gonads containing cells associated with primary growth (primary oocytes and early endogenous vitellogenesis) and Figure 12 demonstrates a specimen in a more advanced maturation stage (with endogenous and early exogenous vitellogenic oocytes).

As for the males, it can be seen in Figure 13 an example of a male individual which essentially presents spermatogonia and some (rare) spermatocytes. In Figure 14 it is possible to observe already an individual which contains spermatids and even spermatozoa.

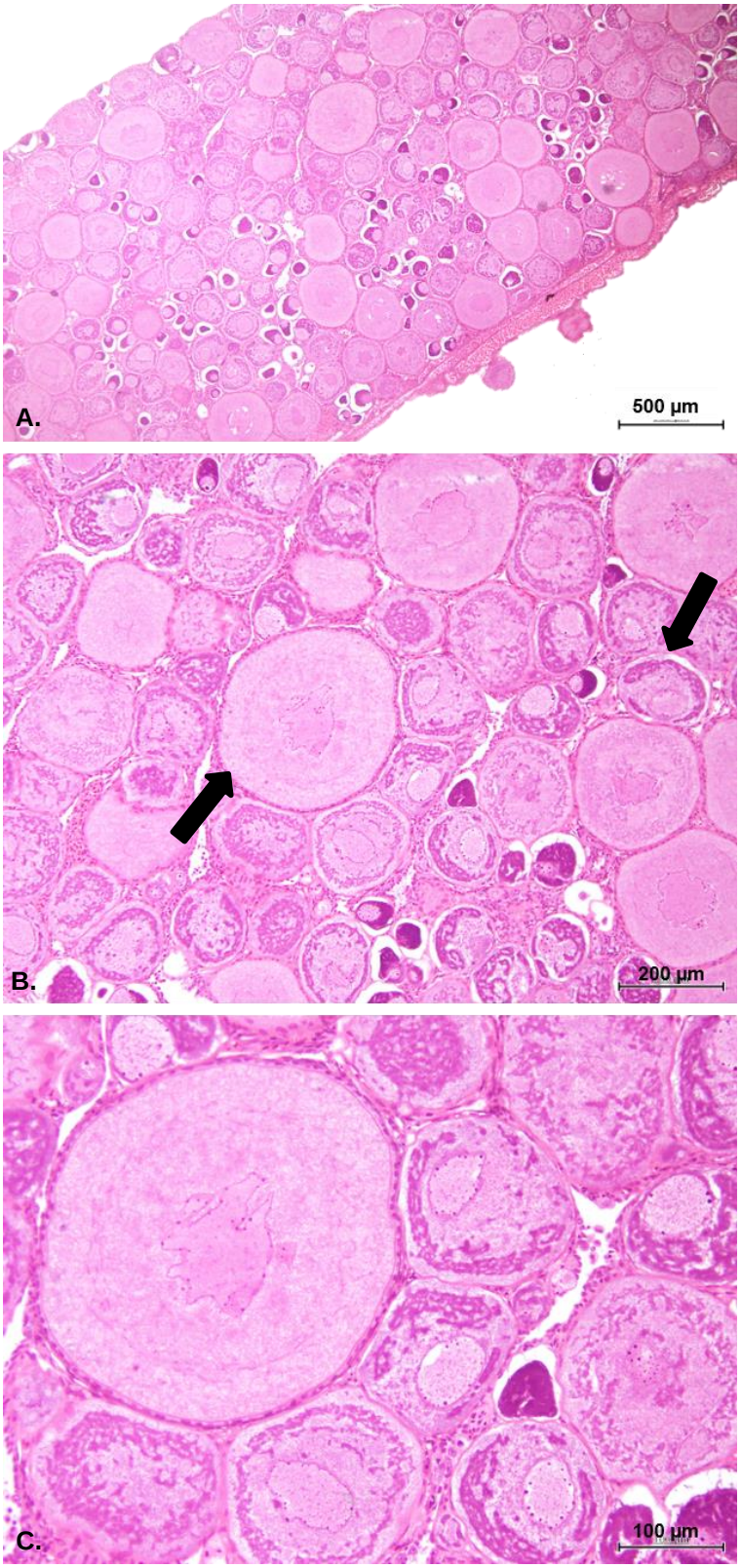


Figure 11. Histological images of juvenile female trout with less developed reproductive cells, mostly primary endogenous previtellogenic oocytes and some reaching very early vitellogenisis (Arrows). (H&E staining)

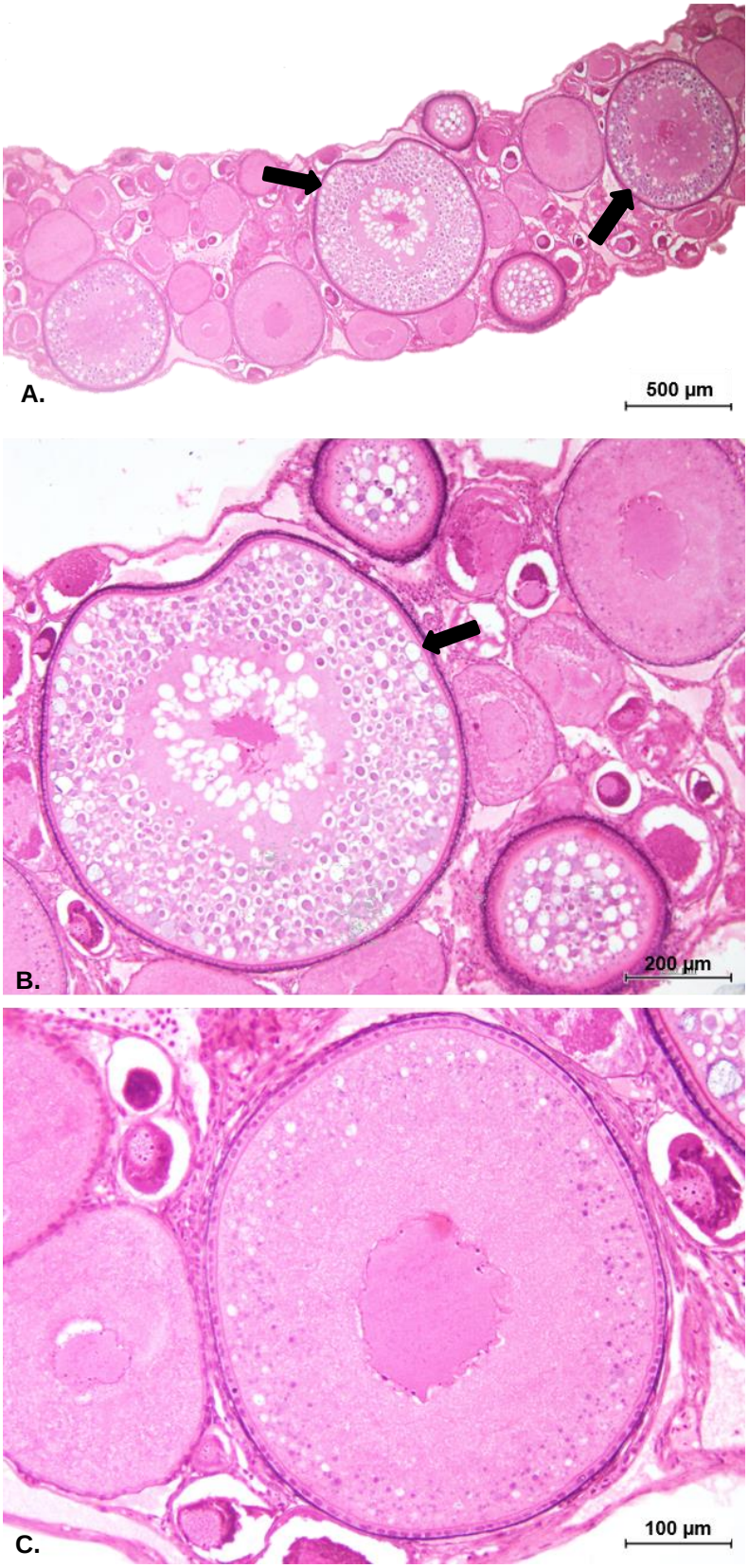


Figure 12. Histological images of juvenile female trout with more developed reproductive cells and endogenous (central in C) and early exogenous vitellogenic oocytes (Arrows). (H&E staining)

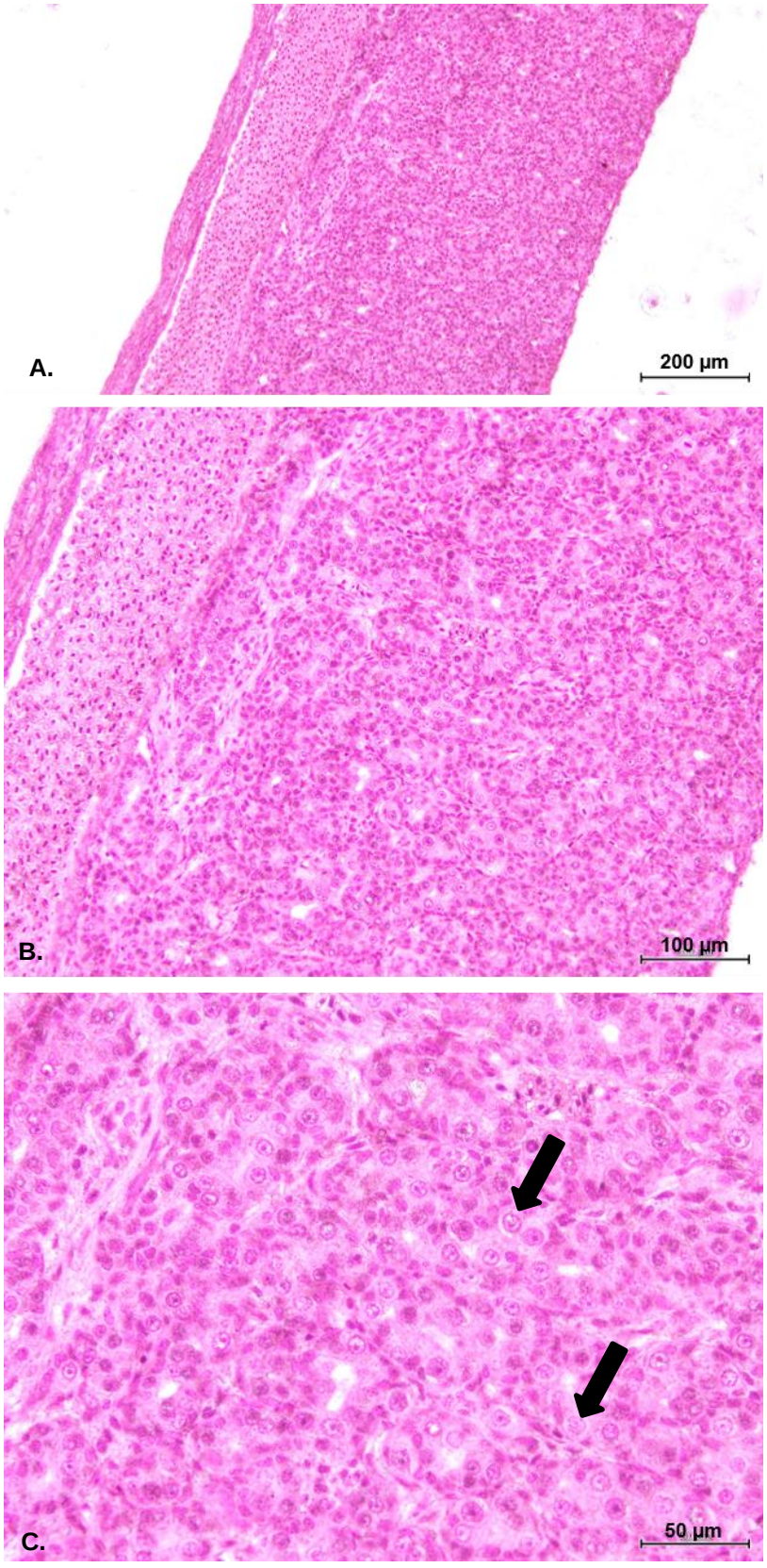


Figure 13. Histological images of juvenile trout male gonad with less developed stages of reproductive cells which mostly appear as spermatogonia (arrows). (H&E stainin)..

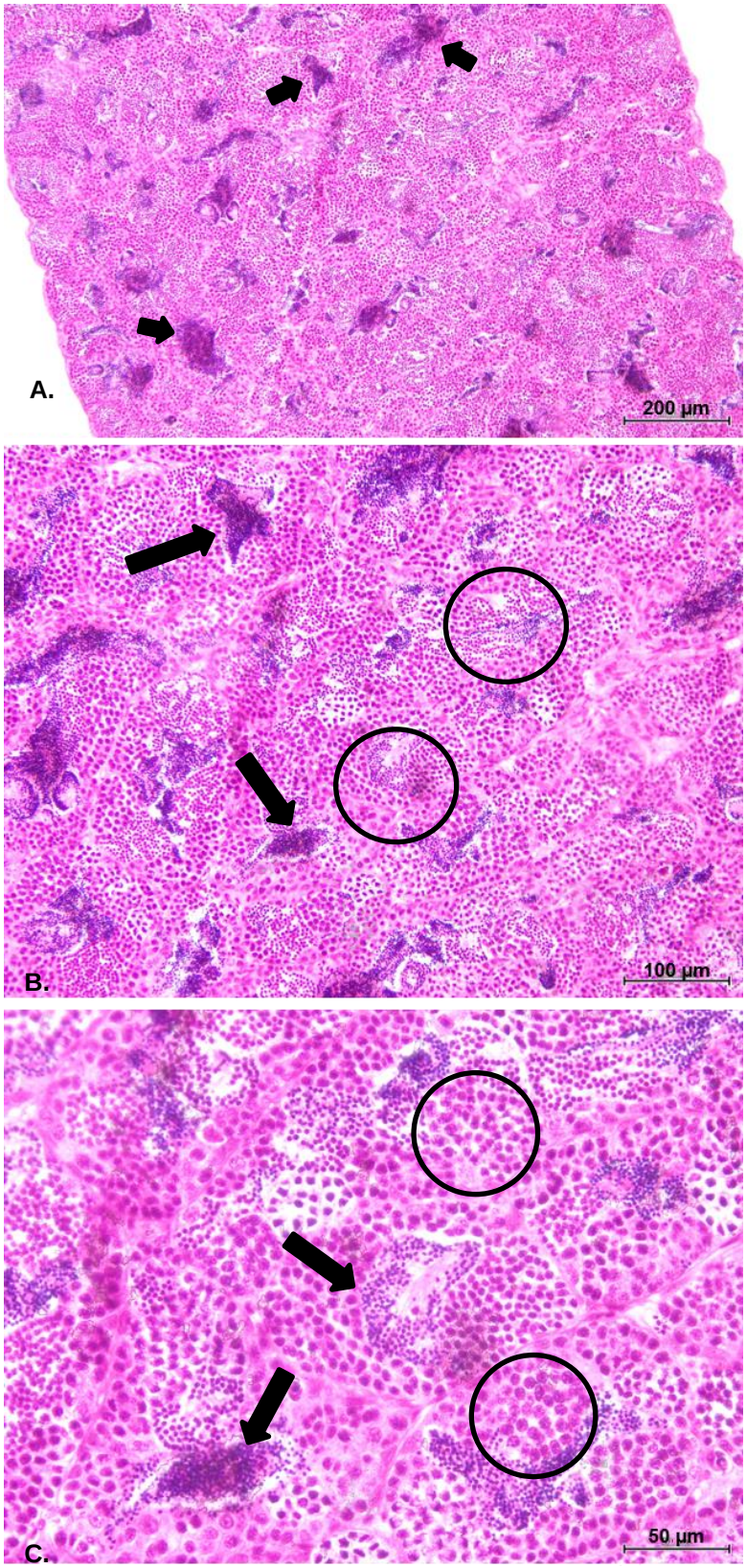


Figure 14. Histological images of juvenile trout male gonad with more developed reproductive cells such as spermatocyte (circles) and spermatozoa (arrows). (H&E staining)

Chapter 4. Discussion and Conclusions

Salmonids are one of the most studied groups of fish worldwide (Moyle and Cech 2004), with the juveniles being widely used in scientific studies (Bunde and House 1993, Korner *et al.* 2008, Naderi *et al*. 2015, Ribera 2015, Yadetie and Male 2002, Yu *et al*. 1979). However, the endocrine physiology of this stage is poorly documented, as it is stated in Table 2 of Chapter 1. Taking in mind this occurrence, this study aimed to provide unique data about the levels of ten steroid hormones in brown trout juveniles. For this purpose, a SPE-GC/MS methodology, previously validated for the measurement of steroids in fish plasma (Budzinski *et al.* 2006), was implemented for juvenile brown trout. This method was here successfully applied, since it allowed the evaluation of low concentrations of hormones in plasma (ng/mL) in a relatively fast and less expensive way compared to other techniques, such as RIAs or ELISAs that are inclusively species-specific (Bidwell *et al.* 1976). Using this strategy, it was possible to identify and quantify, simultaneously, ten different steroid hormones.

It was possible to obtain values above the LOD for all hormones, with some steroids presenting higher detection rates than others (Tables 6 and 7, chapter 3). Particularly E_1, showed a low detection rate in both male and female juveniles. One possible explanation for this comes from the fact that E_1 is known to be a weaker estrogen and so it mostly acts as a precursor of E_2 (Kuhl 2005). A similar case appears to happen with 17-OHP, that also presents lower DR, and once again is known to act as a precursor for other steroids (Figure 4, chapter 1). Apart from these two cases, most of the measured hormones seemed to have an overall DR between 50% and 100% (Tables 6 and 7, chapter 3).

The concentrations of all ten steroids showed no significant differences between female and male fish, indicating that, at this developing stage, juvenile brown trout present similar plasma steroid profiles. Of the steroids measured, the ones that had a higher concentration were E_2 and 11-kT, which had total mean values of 0.42 ng/mL and 0.52 ng/mL, respectably (Table 8, chapter 3). Juvenile fish are expected to present higher levels of E_2, as this steroid is known to stimulate growth hormones that promote the growth of the animal (Piferrer 2011). In the case of 11-kT, this is the most effective androgen in teleost fish (Piferrer 2011), which explains the values of 11-kT being higher than those of T. Although 11-kT is mainly linked with male differentiation, being produced in the Leydig cells and being responsible for the regulation of the processes of spermatogenesis and spermiogenesis (Piferrer 2011), it is present in the same levels in females too. The latter situation could occur because 11-kT is believed to be involved in female maturation, as it is suggested for example in the eels (*Anguilla* spp.), where this steroid appeared to have an important role in the

previtellogenic oocyte growth (Matsubara 2003). However, further research is needed to understand the role of 11-kT in female fish maturation.

The plasma levels of T were relatively low, reaching a mean value of 0.17 ng/mL in both male and female fish (Tables 6 and 7, chapter 3). Although females, as mentioned in previous chapters, produce mainly estrogen steroids, T is also produced, and it acts as a central intermediate steroid (Moyle and Cech 2004). While estrogens are more related to females and androgens with males, the similarity of concentration levels of steroids such as T and E_2 among genders could be related to the fact that at this young life stage, fish are not fully matured and differentiated, and therefore genders are not yet entirely different from each other at a hormonal level.

Hormones such as E_1, A, P, Pn, 17-OHP and 17-OHPn were all detected at similar levels (Table 8, chapter 3). These hormones are present at the basis of the pathway for steroid metabolism, and therefore their presence is expected at relatively similar and consistent levels. 17,20β-OHP also had a concentration value similar to these hormones (Table 8, chapter 3), as well as a consistent DR of 100% (Tables 6 and 7, chapter 3). This steroid is usually related to more mature animals, as it is typically a hormone present at later maturation stages, associated with follicular maturation and ovulation in females; and with the start of the meiotic division of spermatogonia, the control of spermatozoa maturation and the control of spermiation in males (Piferrer 2011). In this study, the quantification of 17,20β-OHP at these levels could be an indicator of the start of a more advanced maturation state for some individuals, as shown as well by the histological analyses of the gonads (Figures 12 and 14, chapter 3).

Very few studies focus on the plasma steroid levels of juvenile fish (Budzinski *et al*. 2006). In Table 2 of chapter 1, it was shown some of the few data found for several of the measured steroids. These data are within the same range of those obtained in this study for juvenile brown trout. For example, in previous studies, it was shown that individuals of 70 g of body weight presented E_1 levels of 0.23 ng/mL (Mortensen *et al.* 2011), and individuals of 60 g presented values of 0.52 ng/mL of E_2 (Tintos *et al.* 2007).

Beyond salmonids, also for other species, there is not much information concerning the plasma levels of steroid hormones. For instance, the grey mullet (*Mugil cephalus*), with approximate weight to the fish used in this work, show values of E_2, T and OHP similar to those reported for the brown trout (Chang *et al.* 1995). Juvenile grey mullet presented values between 0.3 and 0.5 ng/mL for E_2, between 0.2 and 0.3 ng/mL for T and between 0.2 and 0.4 for 17-OHP (Chang *et al*. 1995). Besides the similarities of these values, it is important to note as well that the mentioned study also reported no differences between the steroid values of

female and male fish. Another study, this time using sturgeons (*Acipenser baerii*), also showed similar values to those observed in this work for hormones such as E_2 (0.62 ng/mL) (Vizziano-Cantonnet 2018). The lack of studies in this subject makes it difficult to compare, for most hormones, the values of steroid levels obtained here, as most studies usually focus only a few steroids (such as E_2 and T) and are mainly interested in changes that occur along the reproductive cycle at adult phases (Sulistyo *et al.* 1998).

The absence of differences in the plasma steroid levels between genders could be highly relevant for studies of steroid levels which use groups of juvenile fish without gender distinction. Since males and females present similar levels and detection rates for all steroids, when studying for example factors that could influence the steroid levels of fish (such as EDCs), juvenile salmonids used in experiments can be all grouped without the need to take into consideration their gender. However, it is important to note that although it is shown here that, at a young age, trout fish seem to not vary in steroid levels between genders, it remains unknown if the steroid metabolism and regulation of each gender is affected differently by external factors. The last topic also deserves to be further studied as it would be valuable information for future research studies.

In agreement with the last observations, also the biometric data showed that there are no differences between both genders. However, it is significant to highlight that within each gender group, the biometric parameters, particularly those related to the gonads, have high variability (Figures 9 and 10, Chapter 3). The last observation shows that among fish of the same age, gender and similar size, the gonadal development is different as some juveniles seem to be maturing faster than others. The qualitative analysis confirms this hypothesis, being visible that some individuals show gametes in different maturing stages. In females, for example, some individuals had some oocytes in early endogenous vitellogenic stage (Figure 11, Chapter 3) while others showed already some in early exogenous vitellogenesis (Figure 12, Chapter 3). This fact is not enough however, to consider the ovary as in a fully late vitellogenic stage (Brown-Peterson *et al*. 2011, Johnson *et al*. 2009). Similarly, in the male group, it is observed that some animals were also riper than others. These individuals already presented spermatids and even spermatozoa (Figure 14, Chapter 3) showing their advance in maturation, while others presented only mostly spermatogonia (Figure 13, Chapter 3) and so appear to be more immature.

An important note to take here is that although it is possible to have a maturation grading of the gonads in these juvenile fish, it is rare for it to be applied in research studies as most consider the juvenile phase a completely homogeneous phase (Johnson *et al.* 2009). However, as it happens, these juvenile animals, not considered adults as they lack

reproductive ability, cannot be considered entirely immature, seen as it is easily possible to determine their sex through histological analysis. The previous observation led to the observation of a development stage between immature fish and adult fish which is poorly studied, and for which not many gonad grading systems exist. Therefore, it would be very plausible to perform a detailed histological characterisation of fish gonads in this age group.

Despite these histological differences and the high inter-animal variability of the GSI (CV = 30.85% in females and 141.87% in males) and gonad weight (CV = 36.36% in females and 139.95% in males), no linear correlation was found between each steroid level and the GSI or gonad weight values. These results show that, although some females have more maturely advanced oocytes and some males already present spermatozoa, the steroid levels do not seem to proportionally increase as it could be expected, considering the apparent more advanced maturity of the animal.

The outcome of this work brings important data for further research studies in the area. The concentration levels of steroids in juvenile trout could serve as crucial information for future studies, particularly for studies focusing on how such levels can be affected by pollutants such as EDCs. The lack of differences between male and female juvenile steroid profiles could also prove to help facilitate such studies that could use juvenile brown trout without gender distinction. Adding to the further studies already mentioned, it could also be of interest to evaluate the levels of more grown and mature fish, now starting to enter the adult phase, in order to verify what differences exist in the steroid levels compared to the ones found in this work, as well as determine at what point of development significant differences in steroid levels can be observed between genders.

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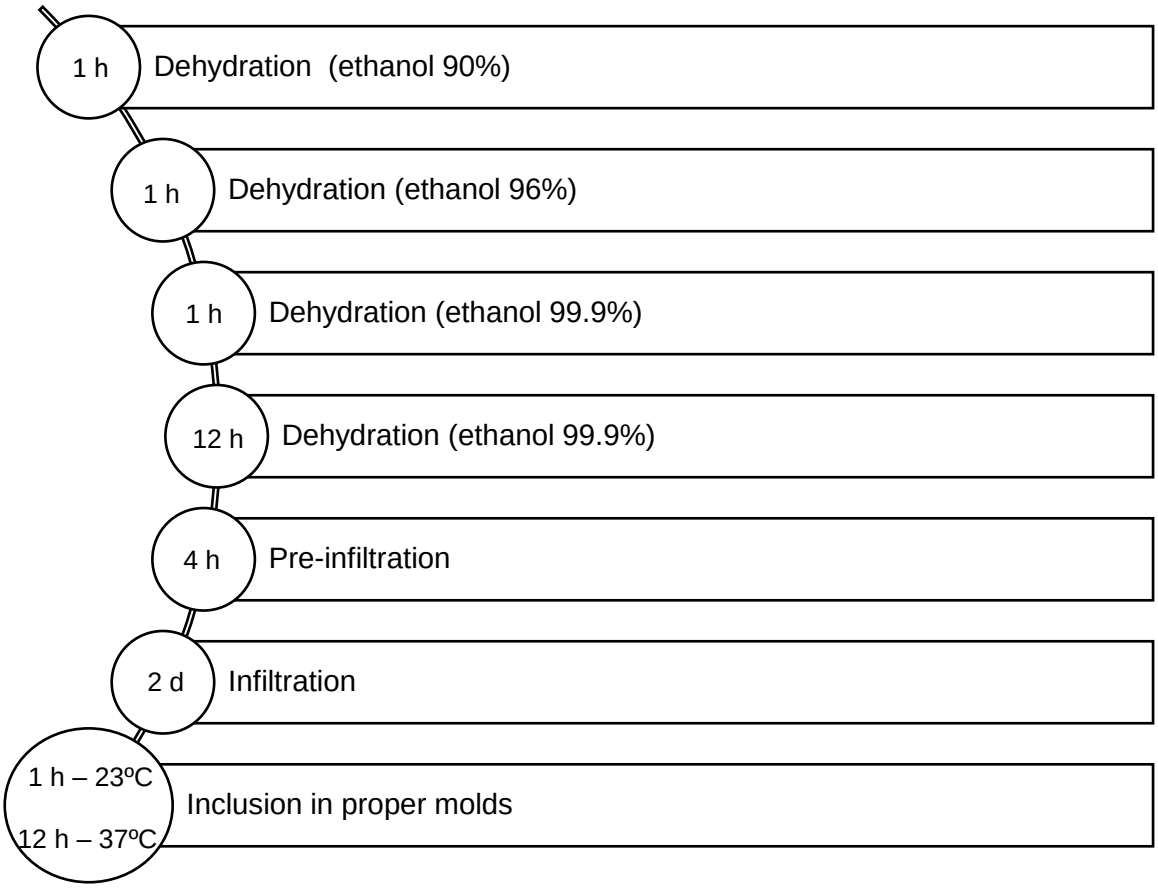
Appendix 1

Methacrylate Manual Processing Protocol



Solutions used:

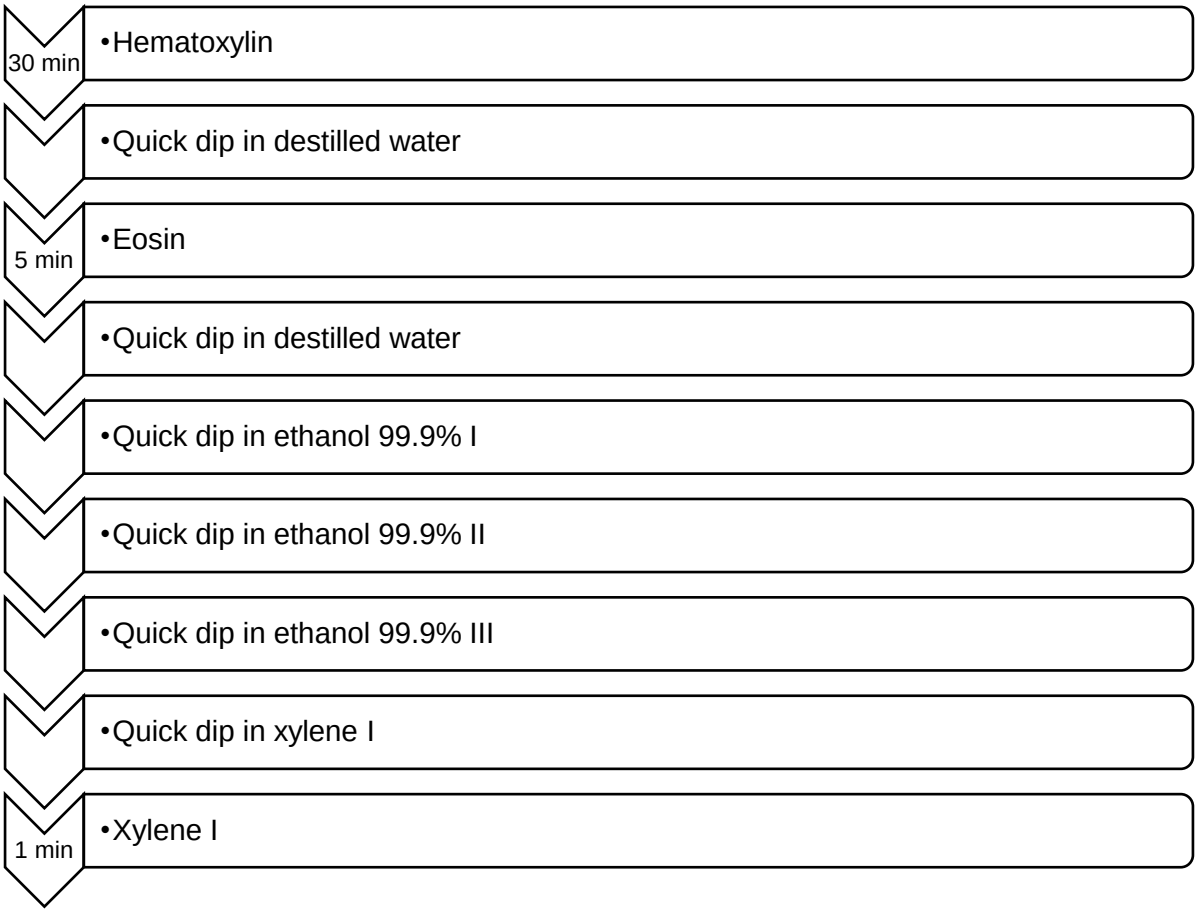
- **Infiltration solution:** 100 mL of Technovit + 1 g of hardener I (shake for 10 min)
- **Pre-infiltration solution:** mix 1:1 – infiltration solution: ethanol 99.9%
- **Inclusion solution:** mix 15:1 – 15 mL of infiltration solution: 1 mL of hardener II

Material used:

- **Technovit 7100 Kit:** Kulzer (Mitsui Chemicals Group) (Hanau, Germany)
- **Ethanol 99.9%:** (CAS: 64-17-5) Manuel Vieira & Cª (Irmão) Sucurs. LDA. (Portugal)
- **Ethanol 96%:** (CAS: 64-17-5) Manuel Vieira & Cª (Irmão) Sucurs. LDA. (Portugal)

Appendix 2

Hematoxylin and Eosin (H&E) Staining Protocol



Materials used:

- **Hematoxylin**: Merk Millipore (Darmstadt, Germany)
- **Eosin Y:** (CAS 17372-87-1), Merk Millipore (Darmstadt, Germany)
- **Ethanol 99.9%:** (CAS 64-17-5) Manuel Vieira & Cª (Irmão) Sucurs. LDA. (Portugal)
- **Xylene:** (CAS 1330-20-7) VWR International (Fontenay-sous-boise, France)

Appendix 3

Biometry data

Table 1. Biometry data of each sampled female juvenile trout. In the bottom, gender mean and %CV for each biometric parameter. GSI (Gonadosomatic index), HIS (Hepatosomatic index), K (Fulton's condition factor), CV (Coefficient of variation).

Animal ♀	Total length (cm)	Body weight (g)	Gonad weight (g)	Liver weight (g)	GSI	HSI	K
1	19.00	78.01	0.17	1.62	0.21	2.08	1.14
2	21.50	100.62	0.06	0.87	0.06	0.86	1.01
3	21.00	90.42	0.23	0.89	0.26	0.98	0.98
4	17.80	63.75	0.09	0.77	0.15	1.22	1.13
5	19.80	92.58	0.24	1.17	0.26	1.26	1.19
6	18.50	87.62	0.16	1.67	0.18	1.91	1.38
7	19.50	75.88	0.17	0.74	0.22	0.98	1.02
8	19.60	80.45	0.17	1.05	0.21	1.31	1.07
9	20.00	99.56	0.33	2.07	0.33	2.08	1.24
10	18.50	70.56	0.16	0.97	0.23	1.37	1.11
11	19.90	84.82	0.17	1.34	0.21	1.58	1.08
12	21.40	94.74	0.27	1.13	0.29	1.19	0.97
13	20.00	74.23	0.16	0.73	0.21	0.98	0.93
14	20.50	79.36	0.17	1.03	0.22	1.30	0.92
15	20.50	90.31	0.14	1.12	0.15	1.24	1.05
16	21.60	124.32	0.18	1.57	0.14	1.26	1.23
Mean	19.94	86.70	0.18	1.17	0.21	1.35	1.09
CV (%)	5.65	16.67	36.36	32.97	30.85	27.71	11.48

Table 2. Biometry data of each sampled male juvenile trout. In the bottom, gender mean and %CV for each biometric parameter. GSI (Gonadosomatic index), HSI (Hepatosomatic index), K (Fulton's condition factor), CV (Coefficient of variation).

Animal ♂	Total length (cm)	Body weight (g)	Gonad weight (g)	Liver weight (g)	GSI	HSI	K
1	18.50	78.08	0.14	1.21	0.18	1.55	1.23
2	20.00	92.64	0.23	0.90	0.24	0.97	1.16
3	20.00	81.00	0.08	1.09	0.10	1.35	1.01
4	21.00	113.80	0.27	1.38	0.24	1.21	1.23
5	20.50	95.76	0.36	1.15	0.38	1.20	1.11
6	21.00	102.36	0.06	1.43	0.05	1.40	1.11
7	21.00	105.42	0.12	1.43	0.11	1.36	1.14
8	18.80	80.03	0.06	1.27	0.07	1.59	1.2
9	19.00	75.40	0.29	1.03	0.38	1.37	1.1
10	18.80	74.66	0.19	0.92	0.26	1.23	1.12
11	19.70	91.55	0.19	1.01	0.21	1.10	1.2
12	21.40	105.85	0.80	1.49	0.75	1.41	1.08
13	19.00	82.38	0.40	1.81	0.49	2.20	1.2
14	19.90	76.48	0.13	0.84	0.18	1.10	0.97
15	19.80	88.52	0.73	1.80	0.83	2.03	1.14
16	20.20	90.24	0.07	0.90	0.08	1.00	1.09
17	19.50	84.91	1.92	1.74	2.26	2.05	1.15
18	20.50	92.63	0.13	1.39	0.14	1.50	1.08
19	20.00	82.13	0.06	1.72	0.08	2.09	1.03
20	18.80	74.96	0.13	1.05	0.17	1.40	1.13
21	20.10	89.14	0.02	1.17	0.02	1.31	1.1
22	18.70	68.86	0.14	0.82	0.20	1.19	1.05
Mean	19.83	87.58	0.30	1.25	0.34	1.44	1.12
CV (%)	4.33	13.43	139.95	25.45	141.87	24.42	6.15