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Cycle XXXIII

The reproductive biology of the swordfish *Xiphias gladius* and the Atlantic bluefin tuna *Thunnus thynnus* in the Mediterranean Sea: bases for a sustainable fishery management

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Abstract

Investigating the reproductive biology and the evolution contributes to define a conceptual model able to explain the processes that allowed any species to succeed. The emergence of innovative anatomical, developmental and physiological features which entail a selective advantage is indeed tightly linked to the success of the reproductive strategy because this favours the species to survive. Furthermore, nowadays, understanding the reproductive strategies is a key for a sustainable use of the biological resources and to respect the principle of intergenerational equity, which consists in the preservation of natural resources and the environment for the benefit of future generations.

The overall objectives of the present doctoral dissertation were 1) to investigate the reproductive biology of the swordfish *Xiphias gladius* and the Atlantic bluefin tuna *Thunnus thynnus* in the Mediterranean Sea in the context of the fishery management and 2) to shed light onto the evolution of some peculiar anatomical, behavioral and physiological features of the two species. The detailed objectives are provided for each chapter as follows

1) Maturity assignment based on histology-validated macroscopic criteria: tackling the stock decline of the Mediterranean swordfish (*Xiphias gladius*)
    - Aims: i) improving the knowledge of the swordfish reproduction in the central and western Mediterranean in terms of histological description of the ovarian stages, reproductive timing and size at first maturity (L_{50}) ii) highlight the importance of the gonadic index (GI) as useful macroscopic indicator through its calibration with the histological approach and the definition of a GI threshold to discriminate sexually immature from mature females
2) A *de novo* transcriptome assembly approach elucidates the dynamics of ovarian maturation in the swordfish (*Xiphias gladius*)
    - Aims: i) elucidating the molecular processes driving the ovarian maturation ii) identifying the main genes of the molecular pathway of steroidogenesis and draw an *ad-hoc* pathway for the vitellogenin synthesis and uptake integrated with gene expression results iii) characterizing a candidate vitellogenin receptor iv) making the results freely available through a dedicated online portal
3) Some like it hot: the heater tissue transcriptome of the swordfish *Xiphias gladius*

- Aims: i) providing a molecular toolbox to understand the evolution of the regional endothermy in the swordfish ii) expanding the species-specific findings towards the broader exploration of the evolution of endothermy in vertebrates iii) contributing to the knowledge on the heater tissue at a histological level

4) *De novo* transcriptome assembly, functional annotation and characterization of the Atlantic bluefin tuna (*Thunnus thynnus*) larval stage
    - Aims: i) providing the scientific community currently working on the larval rearing with a ready-to-use and annotated transcriptome representative of the larval stage ii) identifying the molecular pathways underlying the high larval growth rates through a comparative approach with other fish species iii) detecting the expanding protein families iv) exploring the biological functions associated with transcripts having higher codon usage bias
5) A comparison of reproductive potential in young and old females: a case study on the Atlantic bluefin tuna in the Mediterranean Sea
    - Aims: i) Elucidating the reproductive potential of old and young females in the Mediterranean Sea ii) characterizing the expression of mir-202 in young and old females and between reproductive and non-reproductive periods iii) identifying the mir-202 ovarian localization in vitellogenic ovaries

INTRODUCTION

Reproductive biology of fish

The Actinopterygian ray-finned fish, with 28,000 registered species so far (www.fishbase.org), can be considered one of the most successful group of animals on Earth. They diverged from the Sarcopterygian lobe-finned fish about 400 millions of years ago and remained linked to the aquatic habitat since then (Benton & Donoghue, 2006; Betancur-R et al. 2017). It is believed that one of the reasons of such an evolutionary success is represented by the additional round of genome duplication (3R), which allowed the evolution of new functions through novel duplicate genes which arose during this event (Meyer & Van de Peer, 2005). Yet, for any species to succeed, the reproductive strategy and its trade-off with energy availability have to be successful. Indeed, before puberty onset, individuals allocate most of its energy balance to grow and survive but, once puberty is reached, part of this energy fuels gamete production and reproductive processes, therefore originating a key balance between somatic growth and reproduction that will last for the rest of the lifetime of individual fish (Saborido-Rey, 2016) (Fig. 1). This balance depends on a variety of factors, some of them internally (e.g. physiology, genetics) and environmentally (e.g. temperature, feeding) driven. These and other processes shape the striking diversity of reproductive strategies observed in fish (Murua, 2014), in terms of:

- Gender system: although gonochorism (i.e. sexes are separated) is the most common situation in fish, hermaphroditism is also observed. Noteworthy, hermaphroditism is observed in some species of great commercial interest such as the gilthead sea bream (*Sparus aurata*) and groupers (*Epinephelus spp*.). Hermaphroditism can be either sequential, namely protandrous and protogynous, or simultaneous.
- Spawning dynamics: the most common situation is represented by iteroparity, that is multiple reproductive cycles during the individual's lifetime, although some iconic cases of semelparity, that is spawning following by death, are observed such as some salmonids and eels (*Anguilla spp.*).
- Fertilization modes: external fertilization is by far the most widespread strategy observed in fish, in which diploid gametes are released in the aquatic environment

where fertilization occurs. However, internal fertilization is observed in several species of fish as guppies (*Poecilia spp.*).

- Parental cares: they occur in many fish species and exhibit a high diversity of strategies, ranging from hiding to guarding eggs. They may include nest construction (e.g. *Labrus bergylta*), mouthbrooding (e.g. *Oreochromis spp.*) or carrying embryos in specialized body structures (e.g. seahorses). Often, parental care is unisexual and provided by the male.
- Buoyancy of the eggs: the osmolarity of the surrounding water deeply affects the content of the eggs that will be released. Indeed, most seawater pelagophil species spawn fatty floating eggs containing a relevant pool of free amino acids that helps to balance the osmolarity gradient. Among the representative examples of this strategy, there are pelagic species such as the bluefin tunas (genera: *Thunnus*) and the swordfish (*Xiphias gladius*).

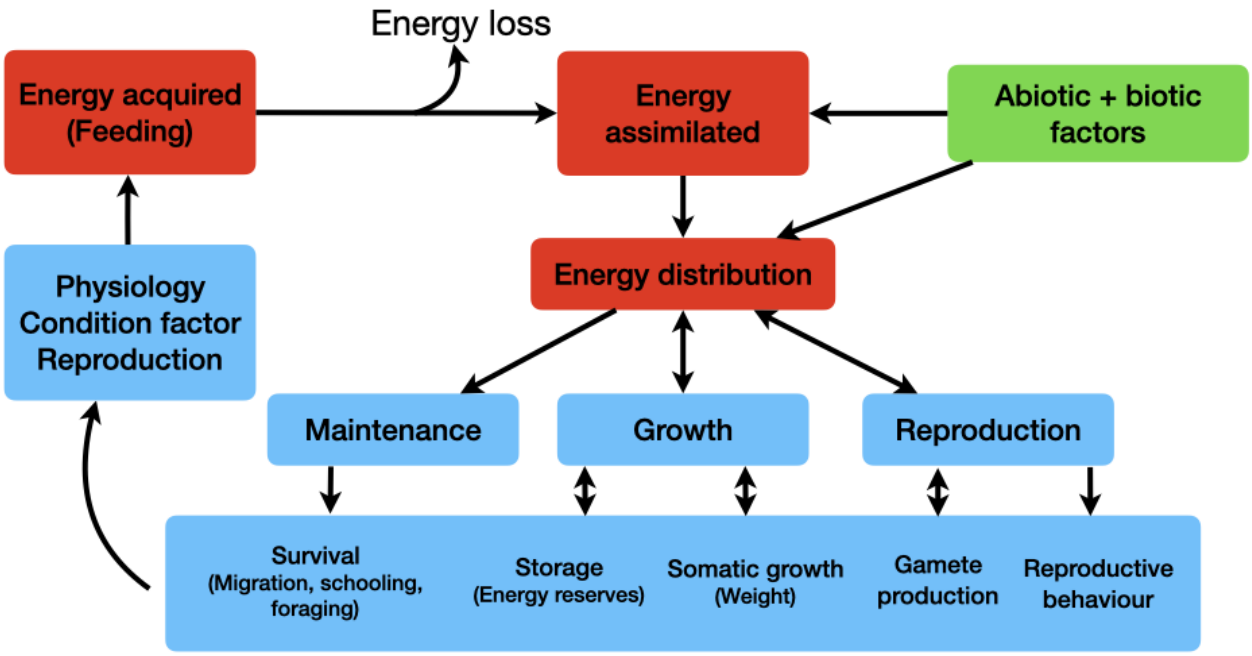

Figure 1. Schematic representation of the energetic flows in fish. Energy allocated for growth, maintenance and reproduction is partitioned between survival, energetic reserves and gametogenesis, which in turn affect how energy flows among compartments. External factors such as food availability and environmental conditions affect energy intake and allocation routes. The scheme is taken from Saborido-Rey (2016) with some minor modifications.

Despite such a diversity regarding the reproductive biology of fish, the underlying processes driving reproduction are well conserved not only among fish but also in vertebrates (Muñoz-Cueto et al., 2020; Whitlock et al., 2019). The regulation of complex processes like gametogenesis is achieved by endocrine and neuroendocrine systems (Kah & Dufour, 2011; Muñoz-Cueto et al., 2020; Nagahama, 1994). Generally, the neuroendocrine system refers to that series of biological processes which leads to the synthesis of neurohormones by the nervous system while the endocrine system refers to hormones produced by certain tissues which are not part of the nervous system (Kah & Dufour, 2011). The tremendous advantage of the neuroendocrine system is the ability to integrate external (e.g. environmental, social cues) and internal (e.g. metabolic) information to control over timing, intensity and success of reproduction, therefore plays a crucial role in the adaptive response of fish according to the experienced conditions (Dufour et al., 2020; Shahjahan et al., 2014). Hence, hormones are the chemical messenger transported through the bloodstream and their actions are mediated by relative receptors located on target tissues, where the density of available receptor determine the entity of downstream responses. After more than half-century of research on these topics, it is nowadays clearly established that the primary hormones driving gametogenesis in vertebrates are the gonadotropins Follicle Stimulating Hormone (Fsh) and the Luteinizing Hormone (Lh) produced in the pituitary gland, within the so-called Hypothalamus-Pituitary-Gonad (HPG) axis (Zohar et al., 2010).

Hypothalamus–Pituitary–Gonad axis

The pituitary gland, also known as the hypophysis, plays a central role in the regulation of a variety of physiological processes being at the interface of the nervous and endocrine systems (Fontaine et al., 2020). The pituitary gland can be divided into a glandular and a neurohemal parts. The glandular part is called the adenohypophysis and can be divided into the pars distalis, pars intermedia and pars tuberalis (Kah & Dufour, 2011). The neurohemal part is called the neurohypophysis, which in fish consists of axons of neurosecretory cells originating in the brain that enter the pituitary (Kah & Dufour, 2011). Indeed, a unique feature of the fish pituitary is the lack of the blood portal system typical of mammals which is instead replaced by a direct innervation of the pituitary with neuronal projections originating in the hypothalamus (Fontaine et al., 2020; Kah & Dufour, 2011; Peter et al., 1990; Zohar et al., 2010). The two regions directly communicate as a result of specific neurones that deliver neuroendocrine signals in the surroundings of relative target cells. Indeed, several discrete

populations of pituitary cells are regionalized and specialized to secrete a particular hormone such as the case of the lactotropes secreting prolactin, corticotropes secreting corticotropin, somatotropes secreting growth hormone and gonadotropes secreting gonadotropins (Kioussi et al., 1999). The secreted hormones are released in the bloodstream to reach target organs and trigger downstream physiological responses. In the case of the reproductive system, the central hormones released by the pituitary are the gonadotropins Fsh and Lh, with the gonads as the target organs. Following this scheme, the secretion of pituitary gonadotropins is mainly regulated, beyond any doubts, by the Gonadotropin-Releasing Hormone (GnRH) originating in the hypothalamus. Certainly, besides the GnRH, a plethora of factors are at play both at hypothalamic and pituitary level to activate and/or inhibit the production of Fsh and Lh such as the dopamine (Da), γ aminobutyric acid (Gaba), pituitary adenylate cyclase-activating peptide (Pacap), neuropeptide Y (Npy), and kisspeptin (Kiss) in addition to feedback loops from gonadal steroids and peptides (Akazome et al., 2010; Muñoz-Cueto et al., 2017; Yaron et al., 2003). For this reason, the release of gonadotropins can be considered multifactorial (Kah & Dufour, 2011). Altogether, this series of signals and feedbacks originating in the brain and ending at the gonadal level is known as Hypothalamus–Pituitary–Gonad axis.

The GnRH is a 10 amino acids long neuropeptide on which most of the research on vertebrate reproduction concentrated its efforts over the last decades. Indeed, its secreting GnRH neurones, which originate in the brain, can integrate sensory information from the internal and external environment as well as social clues to regulate the reproductive activity (Chen & Fernald, 2008). Malfunctioning of this GnRH system impairs gametogenesis and spawning (Mylonas & Zohar, 2000, 2009; Zohar & Mylonas, 2001), therefore, elucidating the underlying mechanisms of this system is tightly linked to the control of the reproduction of species of commercial interest in captivity and the flourishing of the aquaculture sector. Our understanding of the functional roles of the GnRH is further puzzled by the discoveries of multiple and distinct forms encoded by different genes, reflecting a complex and lineage-specific evolutionary history (Muñoz-Cueto et al., 2020). After a period of nomenclature based on the species in which the GnRH form was found, which generated confusion in the literature, today the classification is based on the sequence of the corresponding genes and their relative location in the genome (i.e. synteny), clarifying the naming of the GnRH forms (Fernald & White, 1999). Following pioneering discoveries of species-specific GnRH isoforms and advancements in molecular techniques, it is today well-recognized that fish possess from two (salmonids, cyprinids) to three (Perciformes, Pleuronectiformes and many

others) distinct forms depending on the evolutionary history of the lineage (Muñoz-Cueto et al., 2020). In most vertebrates, the true hypophysiotropic form, the one essential for puberty onset and controlling the release of gonadotropins from the pituitary, is the GnRH1 and its neurones are localized in the preoptic area (POA) of the hypothalamus with their axons entering directly the pituitary (Zohar et al., 2010). The other two GnRH systems are the GnRH2 and the fish-specific GnRH3 and axons from their secreting neuronal extrahypothalamic populations do not enter directly the pituitary, rather project widely throughout the brain (Kah et al., 2007; Lethimonier et al., 2004; Yamamoto et al., 1995). Accordingly, the GnRH2 is thought to be involved into the control of food intake and behaviours associated with reproduction (Desaulniers et al., 2017) while the GnRH3 has usually neuromodulatory functions, although it might modulate specific parts of the nervous system linked to social behaviours important for reproduction (Oka, 2009). In addition to a different anatomical organization of these neuronal GnRH populations, differences in terms of electrical activity highlight divergent functions. Indeed, in fish, the activity of GnRH1 neurones in the POA resemble those of mammals, with irregular and sporadic spontaneous electrical activity with circadian fluctuations in the firing frequency (Oka, 2009). This irregular pattern is in sharp contrast with that of GnRH2 and GnRH3 neurones which display regular pacemaker activity, further supporting the idea of neuromodulatory and/or indirect hypophysiotropic functions (Oka, 2010).

The gonadotropins Fsh and Lh are the primary hormones that directly control nearly all aspects of gonadal activities such as gonadal and germ cells development, gonadal steroid production, ovulation, spermiation and spawning (Biran & Levavi-Sivan, 2018; Levavi-Sivan et al., 2010; Zohar et al., 2010). Secondary hormones produced in the adenohypophysis which regulate other physiological systems that indirectly, but deeply, affect reproductive processes include the adrenocorticotropic hormone (Acth) and thyroid-stimulating hormone (Tsh) (Blanton & Specker, 2007; MacKenzie et al., 2009; Sousa et al., 2015). Structurally, fish gonadotropins are heterodimeric glycoproteins formed by two subunits, namely α and β, with the common α subunit non-covalently but strongly linked to a specific β subunit, which is responsible for the specific biological activity of the hormone (Pierce & Parson, 1981). In the gonads, they act at the level of the steroidogenic cells (i.e. the granulosa and theca cells in the ovary and the Leydig cells in the testis). Once stimulated by the gonadotropins, the steroidogenic cells synthesize in turns sexual hormones such as the 17β-estradiol and the 11-ketotestosterone, the two main fish female and male sex hormones, respectively (Nagahama, 1994). With the synthesis of steroid hormones at gonadal level

upon stimulation by gonadotropins, the HPG is fully activated and, from this point on, the gametogenesis proceeds.

Regulation of folliculogenesis

The folliculogenesis, the biological process leading to the production of viable eggs, is the result of a complex series of events mainly occurring at endocrine and, secondarily, at autocrine and paracrine level. In fish, during the oocyte growth, a wide array of changes in terms of biochemical composition, size, epigenetics, transcription, enzymatic activities, water content and osmotic pressure occurs (Cerdà et al., 2007, 2008; Lubzens et al., 2017). Among the main events behind these spectacular changes, there is the uptake of vitellogenin and lipids, which will form the future yolk in a process known as vitellogenesis, an aspect that will be covered in the next pages. Therefore, it is convenient to adopt a broad conceptual separation to separate the primary and the secondary growth phase, with the vitellogenesis as the key process diving the two and marking the entry into the secondary phase (Kagawa, 2013; Lubzens et al., 2010; Wallace & Selman, 1981).

Oogonial proliferation and primary growth

One of the hallmarks of the HPG activation is the synthesis of sex steroids upon gonadotropins stimulation. In turn, sex steroids indirectly and directly affect oocyte growth in a stage-dependent fashion (Lubzens et al., 2010; Nagahama, 1994; Nagahama & Yamashita, 2008; Rajakumar & Senthilkumaran, 2020). Despite the action of gonadotropins as the main effector to promote ovarian development (e.g. vitellogenesis, final maturation, ovulation) is well understood, our understanding of the regulation of early stages is still limited (Fig. 2).

In the Japanese huchen (*Hucho perryi*) and common carp (*Cyprinus carpio*), the early steps of oogenesis were influenced by both the 17β-estradiol and the Maturing Inducing Hormone (MIH) 17,20β-dihydroxy-4-pregnen-3-one (17,20βP), resulting in oogonial mitotic proliferation and entry into meiosis (Higashino et al., 2003; Miura et al., 2007). The authors observed that oogonial mitotic proliferation was triggered by both hormones but, on top of that, the MIH was also effective in inducing the first meiotic division (meiotic prophase I), leading to the entry into the so-called primary growth phase. However, one may argue that steroidogenesis is far from being fully activated at this stage, especially regarding the

synthesis of MIH and, in fact, the underlying processes driving the early synthesis of these sex hormones are largely unknown. Evidence of precocious steroidogenic capacity comes from the developing ovaries of medaka *Oryzias latipes*, in which transcripts of the key enzymes *hsd3b*, *star*, *cyp11a1* and *aromatase cyp19a1* were detected in follicular cells, although their spatial and temporal pattern of expression suggest a different regulation at transcription level (Nakamoto et al., 2012). Furthermore, high intraovarian levels of 17,20βP in the previtellogenic ovary of the gilthead sea bream *Sparus aurata* and the fact that oogonia express the progestin receptor, further strengthen the hypothesis that these early stages are driven by sex steroids under the control of gonadotropins, possibly Fsh (Zapater et al., 2012, 2013). On the other hand, the metabolic mediator Igf-I might be a good candidate to participate in the first steps of oogenesis through endocrine or autocrine/paracrine signalling (reviewed by Reinecke, 2010) and, in this scenario, it may represent either a mediator of steroid actions or integrate their effects under the control of the Growth Hormone (Gh). Indeed, Igf-I was able to stimulate steroidogenesis, oocyte meiosis and growth in multiple fish species (Berishvili et al., 2006; Kagawa et al., 2003; Lokman et al., 2003; Weber et al., 2007), highlighting important roles in reproduction other than metabolism.

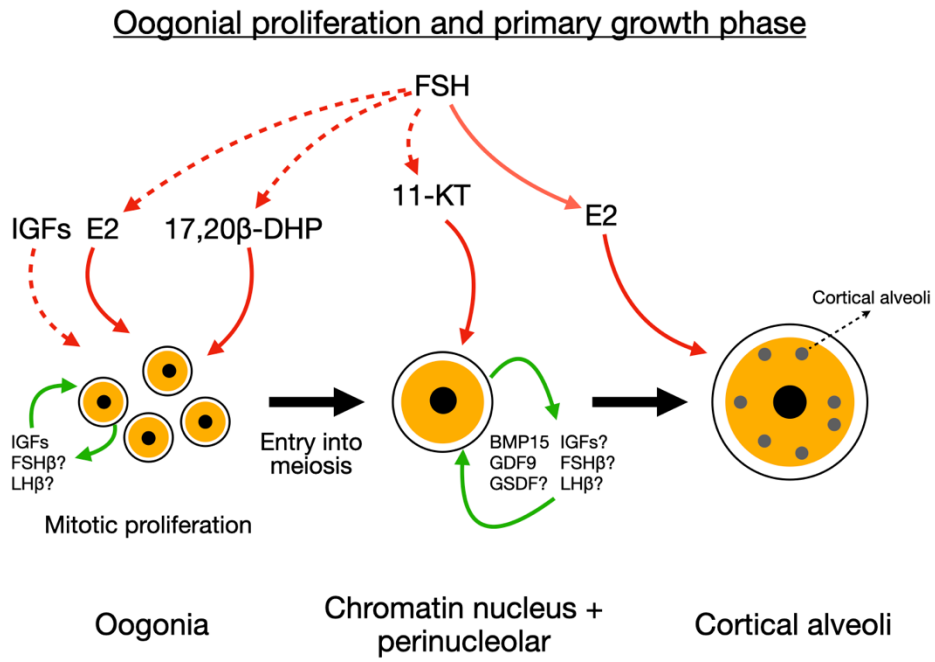

Figure 2. Schematic representation of the primary growth phase of oogenesis. Red arrows=systemic signals; green arrows=autocrine/paracrine signals; dotted red arrows= putative systemic signals from which an experimental validation is still lacking.

Following oogonial proliferation and entry into meiosis and subsequent arrest at prophase I, the oocyte begins the primary growth phase. The main events occurring at this phase are i) the formation of the granulosa and theca cells, which together form the follicle ii) an intense synthesis and storage of RNAs, histones, proteins and the formation of the Balbiani's vitelline body iii) the beginning of biochemical communication follicle-oocyte iv) the formation of the cortical alveoli, a process in between the primary and the secondary growth phase which precedes the vitellogenesis. Endocrine signals driving the primary growth are not fully understood and this phase is commonly defined as gonadotropin-independent as pioneering hypophysectomy studies evidenced a normal oocyte development until the early cortical alveoli stage (Khoo, 1979; Pickford & Atz, 1957). However, it would more correct to define this stage as pituitary-independent because both *fshβ* and *lhβ* transcripts and proteins were found in primary and secondary oocytes of the gilthead seabream *Sparus aurata* (Wong & Zohar, 2004), although their functional role in the ovary has not been elucidated yet. Furthermore, the nonaromatizable 11-KT elicited increases in perinucleolar oocytes size in the short-finned eel *Anguilla australis* (Lokman et al., 2007) and the coho salmon *Oncorhynchus kisutch* (Forsgren & Young, 2012).

It is commonly accepted that autocrine and/or paracrine mechanisms might play relevant roles to regulate primary oocyte growth. Accordingly, two members of the wide TGF-β family, namely the *growth and differentiation factor 9* (*gdf9*) and *bone morphogenetic factor 15* (*bmp15*), were found to control oocyte progression at this stage. In fact, the *bmp15* was found to be highly expressed at both germinal (i.e. oocyte) and somatic (i.e. follicle) level in previtellogenic oocytes with a subsequent decline during vitellogenesis in the European sea bass *Dicentrarchus labrax* (García-López et al., 2011; Halm et al., 2008), catfish *Clarias batrachus* (Yadav & Lal, 2019) and gibel carp *Carassius auratus gibelio* (Chen et al., 2012), with a more consistent expression within the oocyte if compared with follicle cells. Conversely, mRNA levels were similar between previtellogenic and vitellogenic oocytes in zebrafish *Danio rerio* (Clelland et al., 2006). Mechanisms of action of Bmp15 were elucidated by functional approaches, highlighting that this protein blocks premature oocyte maturation by suppressing sensitivity to MIH and inhibiting steroid synthesis (Clelland et al., 2007; Peng et al., 2009; Tan et al., 2009; Yadav & Lal, 2019). On the other hand, *gdf9* showed a similar pattern of expression (i.e. previtellogenic peak followed by decreasing levels) in the rainbow trout *Oncorhynchus mykiss* (Lankford & Weber, 2010), European sea bass (García-López et al., 2011), gibel carp (Liu et al., 2012), zebrafish (Liu & Ge, 2007) and the short-finned eel (Lokman et al., 2010), highlighting an important role in this phase.

In zebrafish, treatment with human chorionic gonadotropin (hCG) decreased *gdf9* expression in a dose- and time-dependent manner (Liu & Ge, 2007), but contrasting results were reported in other species (Liu et al., 2012). Interestingly, in recent years the *gonadal soma derived factor* (*gsdf*), in addition to discoveries of its role in sex differentiation, emerged as an important element to control early folliculogenesis according to the severe phenotypes observed in KO studies (Guan et al., 2017; Yan et al., 2017). Therefore, it appears that during the primary growth phase autocrine/paracrine processes play an important role, although the action of circulating hormones other than pituitary gonadotropins along with their autocrine/paracrine systems certainly deserve future investigations. Unveiling major players at this stage will have the potential to fully comprehend their impact on follicle recruitment and therefore, on fecundity.

Transition into secondary growth

The appearance of the cortical alveoli is the event marking the entry into the secondary growth phase. The cortical alveoli are membrane-limited vesicles of variable size containing protein, carbohydrates and calcium deposits known to be involved in the so-called cortical reaction (Golpour et al., 2016; Shibata et al., 2012). It must be noted that in some species such as eels and tunas, concomitantly or shortly after the appearance of the cortical alveoli there is a remarkable accumulation of lipid droplets and therefore this stage can also be found as “lipid stage” (Medina et al., 2002; Unuma et al., 2011). In the coho salmon *Oncorhynchus kisutch* the expression of *fshr*, *amh* and *gsdf* increased from the perinucleolar to the cortical alveoli stage (Luckenbach et al., 2008) and the abundance of cortical alveoli was enhanced by E2 (Forsgren & Young, 2012). Similarly, in the same species, increases in plasma and pituitary FSH, plasma E2 and expression of *star*, the key rate-limiting enzyme of the steroidogenesis, were associated with the appearance of cortical alveoli (Campbell et al., 2006). In zebrafish, the expression of *fshr* dramatically increased with the appearance and accumulation of cortical alveoli (Kwok et al., 2005). Similar results were reported in the European sea bass, with the upregulation of *fshr* during early secondary follicle growth (Rocha et al., 2009). Therefore, it appears that the activation of the HPG axis, particularly the effects mediated by E2, is the primary mechanism driving the formation of the cortical alveoli which precedes the phase of vitellogenesis. For such reasons, it is widely accepted that this early secondary growth stage and the presence of cortical alveoli/lipid droplets can be considered a marker of puberty onset (Okuzawa, 2002).

Oocyte lipidation

In the oocytes, most of the polar lipids (e.g. phosphoglycerides) derives from the vitellogenin (Johnson 2009). However, a substantial amount of neutral lipids (triacylglycerol, cholesterol, steryl esters) can be found in species spawning fatty pelagic eggs, an aspect that contributes to their buoyancy and specific low density (Tocher, 2003, 2010) and is related to their ecology. The uptake and storage of these neutral lipids occur during the oocyte secondary growth as clearly evidenced by the accumulation of lipid droplets in the ooplasm at this phase (Fig. 3). Most of the knowledge on this aspect comes from experiments carried out in anguillid eels (Damsteegt et al., 2015; Divers et al., 2010; Endo et al., 2011) and salmonids (Hiramatsu et al., 2015; Ryu et al., 2014). In the Japanese eel *Anguilla japonica*, upon stimulation with 11-KT, the main source of neutral lipids were shown to be the very low-density lipoprotein (Vldl) and to a far lesser degree the high-density (Hdl) and low-density lipoprotein (Ldl), all serum lipoproteins (Endo et al., 2011) (Fig. 3). The question of how these neutral lipids are endocytosed by the growing oocytes is still unanswered, although some mechanisms were recently investigated. So far, the candidate processes for oocyte lipidation are 1) in the endothelium or somatic compartment of the follicle, circulating VLDL is processed by the ovarian lipoprotein lipase (Lpl) into low-density lipoprotein (Ldl) and the resulting free fatty acids (FFAs) released from triacylglycerol (TAG) moieties are taken up by the oocytes and resynthesized as lipid droplets; 2) Vldl may bind to one or more ovarian lipoprotein receptors belonging to the Ldl receptor (Ldlr) family and be endocytosed into the oocyte before being stripped of FFAs, which are then utilized for lipid droplet formation (reviewed by Hiramatsu et al., 2015; Lubzens et al., 2017). However, the endocrine mechanisms driving oocyte lipidation seems to differ between species since 11-KT was shown to enhance lipid uptake in cultured eel follicles while no effects were observed in cultured trout follicles (Endo et al., 2011). This last result was further supported by recent observations that 11-KT treatments up-regulated several lipidation-related genes but had negligible effects at a morpho-histological level in the sturgeon *Huso huso* (Akhavan et al., 2019). Furthermore, 11-KT treatment had no effects in terms of oocyte size and lipid content in the hapuku *Polyprion oxygeneios* (Kohn et al., 2013).

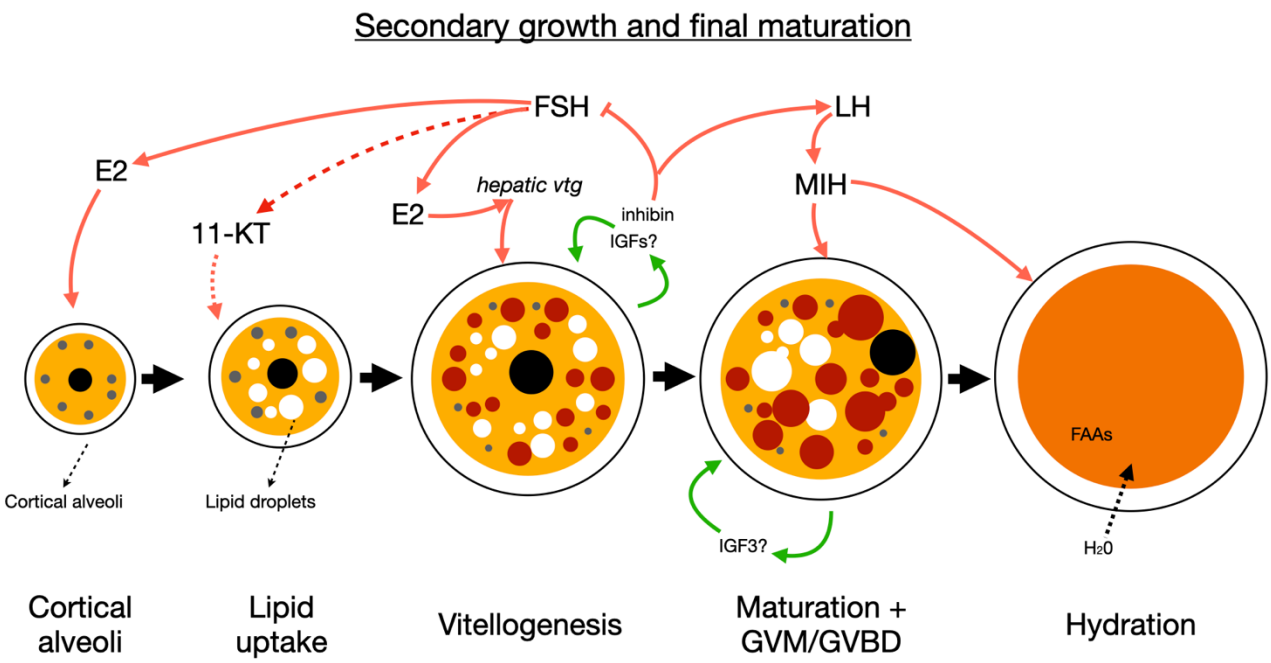

Figure 3. Schematic representation of the secondary growth phase of oogenesis and final oocyte maturation. Red arrows=systemic signals; green arrows=autocrine/paracrine signals; dotted red arrows= putative systemic signals from which an experimental validation is still lacking.

Secondary growth: the vitellogenesis

The classic and widely recognized model of endocrine regulation of the vitellogenesis depicts the main effector as the circulating Fsh which, through its relative receptor Fshr, regulates the expression of a wide array of steroidogenic enzymes such as *star*, *cyp11a1*, *hsd3* and *cyp19a1* resulting in E2 synthesis (Guzmán et al., 2014; Kazeto et al., 2006; Nunez & Evans, 2007; Raghuveer & Senthilkumaran, 2012). Then, E2 triggers the synthesis of vitellogenin in the liver via nuclear estrogen receptors (ERs) and cognate estrogen responsive elements (EREs) in the vitellogenin gene promoter, whose product will then be released in the bloodstream and taken up by the growing oocytes (Fig. 3 and Fig. 4) (Hara et al., 2016; Hiramatsu et al., 2006, 2015; Reading et al., 2011a, 2018). It represents an essential process because, along with yolk proteins (vitellogenin), the oocytes also incorporate vitamins, neutral and polar lipids that will be essential to the future embryo to develop properly and therefore determine the survival of the offspring (Lubzens et al., 2017).

In addition to the endocrine Fsh-mediated control of vitellogenesis, some autocrine and paracrine factors involved in this process emerged over the last two decades (Fig. 3). The ovarian Igf system, mainly through Igf-1 and Igf-2, is among those factors shown to play important roles in enhancing the E2 biosynthetic capacity (Higuchi et al., 2016; Jia et al., 2019; Mukherjee et al., 2017; Nakamura et al., 2016) and oocyte maturation (Das et al., 2017; Picha et al., 2012). However, in this context a distinction between systemic (i.e. hepatic) and local (i.e. ovarian) Igf systems must be drawn because E2 downregulates the Gh/Igf axis at hepatic level while either upregulates or left unchanged that at ovary level (extensively reviewed in Reindl & Sheridan, 2012). In the fish ovary, the *igf-1 receptor*, *igf-1* and *igf-2* were expressed in follicular cells (Baroiller et al., 2014; Berishvili et al., 2006; Higuchi et al., 2016), in agreement with their roles in modulating the steroidogenic cascade. Thus, it appears that secondary oocyte growth and maturation can be also regulated by Igf proteins. Intriguingly, the discovery of a fish- and gonad-specific Igf, namely Igf-3 (Wang et al., 2008), was demonstrated to be strongly steroidogenic in tilapia gonads (Li et al., 2012) and a key mediator of the Lh during oocyte maturation and ovulation in zebrafish (Li et al., 2015, 2018). Other than Igf proteins, the TGF-β member inhibin (*inhibin-specific a subunit*: *inha*) was postulated to modulate Fsh/Lh pituitary production via a negative feedback loop between the ovary and the pituitary in zebrafish, partly by antagonizing activin effects (Poon et al., 2009), with a similar endocrine response as the one observed in mammals (Namwanje & Brown, 2016). The same study revealed that *inha* mRNA levels increased upon Fsh stimulation, although both Lh and hCG were without effects. In support of this, in coho salmon *inha* expression peaked during the vitellogenesis and was correlated with *fshr* and Fsh levels (Guzmán et al., 2014) and similar findings were reported in rainbow trout for *inhibin α* (*inha*) and *inhibin βα* (*inhba*) transcripts (Lankford & Weber, 2010).

The vitellogenin

The vitellogenin is a dimeric phospholipoglycoprotein and a member of the large lipid transfer protein superfamily, which includes also other serum lipoproteins such as the low-density lipoprotein (Ldl) (Babin et al., 1999). In addition to lipids, vitellogenin also incorporates ions, such as calcium, magnesium, iron, zinc, copper, and several minerals and vitamins, such as retinoids and carotenoids (Lubzens et al., 2010). As for many genes in teleosts, the evolutionary history of the vitellogenin led to multiple gene copies, whose duplication and variation in structure were shown to be lineage-specific (Biscotti et al., 2018;

Finn & Kristoffersen, 2007; Reading et al., 2009). Briefly, according to these studies, the ancestral chordate gene of *vtg* duplicated to form *vtgCD* and *vtgAB*, this latter subsequently gave rise to *vtgA* (present in all teleosts) and to *vtgB*, which is extinct in most derived fishes. On the other side, *vtgCD* duplicated into teleost *vtgC* (phosvitinless vtg), which is present in teleosts from different taxa and to *vtgD*, which was lost. Duplication events not involving whole genome duplication (WGD) led to additional paralogue genes (i.e. *vtgAa* & *vtgAb*) and occurred in different lineages virtually covering almost all the ray finned fish such as Acanthopterygii (e.g. Perciformes, Pleuronectiformes, Tetraodontiformes) and Paracanthopterygii (e.g. Gadiformes) (Finn & Kristoffersen, 2007). However, in Protacanthoperygii (i.e. salmonids) the duplication occurred through independent tetraploidization and species belonging to this group usually bears two copies of *vtg* genes, either *vtgAsa* or *vtgAsb* and *vtgC* (Finn & Kristoffersen, 2007). Furthermore, group-specific tandem duplication events, producing higher number of copies, were also observed as in the case of zebrafish in which up to seven *vtg* genes were identified (Wang et al., 2005). It is plausible that tandem duplication events, like the one observed in zebrafish, will be uncovered also in other fish species through improvements of future genome assemblies. To sum up, in its simplest form and excluding species-specific tandem duplications and the salmonids notation, teleosts would bear a total of three *vtg* genes, namely the *vtgAa*, *vtgAb* and *vtgC* (Fig. 4A). The first two give raise to nearly identical Vtg proteins (Vtga, Vtgb) and the latter produces an incomplete Vtg usually lacking phosvitin (Vtgc).

In its complete form the vitellogenin protein is composed by up to five domains arranged in a linear fashion from the amino-terminus: 1) lipovitellin heavy chain (LvH), 2) phosvitin (Pv), 3) lipovitellin light chain (LvL), 4) β'-component (β'-c), and 5) C-terminal peptide (Ct). Among the different domains, the LvH is by far the largest (~114 kDa) and supplies the embryo with phospholipids and amino acids in order to provide substrates for catabolic (i.e. energy production) and anabolic processes (i.e. synthesis of proteins and membranes). Instead, phosvitin is a metalloprotein consisting widely of serines (>50%) to which phosphates are covalently attached during post-translational modifications and, in turn, these negatively charged phosphates attract calcium, magnesium, zinc, and other multivalent metal cations (e.g. ferric iron) via ionic interactions occurring either in the bloodstream or in the oocyte (Reading et al., 2009).

Vitellogenin uptake by the growing oocytes

From the theca capillaries, the vitellogenins reach the oocyte surface through the pore canals of the zona radiata and are endocytosed by receptor mediated endocytosis involving clathrin-coated pits of vesicles (Fig. 4) (Hara et al., 2016). Once vitellogenins-containing coated vesicles move into the ooplasm they fuse with lysosomes forming multivesicular bodies (Wallace and Selman, 1990), where vitellogenins are cleaved by lysosomal enzymes as cathepsins into smaller yolk proteins (Fig. 4) (Carnevali et al., 1999a, 1999b, 2006).
Our understanding of the driving mechanisms of vitellogenins uptake is improving due to a complex receptor-ligand system identified in a few fish species (Hiramatsu et al., 2015). The receptors involved in this process belong to the large family of low density lipoprotein receptor (Ldlr) related proteins (Schneider, 1996). It is current opinion that, at the level of the oolemma, certain vitellogenins bind preferentially to specific receptors, some of which were recently characterized while others are still unknown. For instance, in the white perch *Morone americana*, two receptors, namely Lr8 (Vtgr) and Lrp13 (LR7+1 type), were shown to bind specifically to VtgAb and VtgAa, respectively (Reading et al., 2011b, 2014). Similarly, in the cutthroat trout *Oncorhynchus clarki* two receptors that specifically bind the salmonid VtgAs were identified as Lrp13 (LR13+1 type) and LR8 (Vtgr) (Mizuta et al., 2013; Mushirobira et al., 2014, 2015). In both species how Vtgc enters the oocytes remain poorly understood (Hiramatsu et al., 2015). The idea of a ligand-receptor system and the specificity in terms of how different Vtg forms are taken up by the oocytes is further strengthened by different ratios of circulating and endocytosed vitellogenins observed in a variety of species (Amano et al., 2008, 2010; Williams et al., 2014). Therefore, the emerging picture is that a of ligand-receptor system which allow to exquisitely control over the proportion of vitellogenins in the oocytes by fine tuning the rate at which each form is endocytosed, a mechanism that likely cooperate with the regulation of the expression of *vtg* genes at hepatic level.

Vitellogenesis and reproductive strategies

Life history traits, ecology and reproductive strategies are thought to have shaped the evolution of *vtg* genes. In this respect, fish eggs released in sea water must deal with hypertonic environment and a water reservoir is needed to hydrate the eggs and counteract the osmotic efflux (Bodinier et al., 2010; Cerdà et al., 2007; Fyhn et al., 1999). Indeed, a

physiological compensation is not feasible before cells and organs of the developing larva become fully differentiated and functional. According to this scheme, marine pelagophil (i.e. spawning pelagic eggs) and bentophil (i.e. spawning benthic eggs) fish species evolved

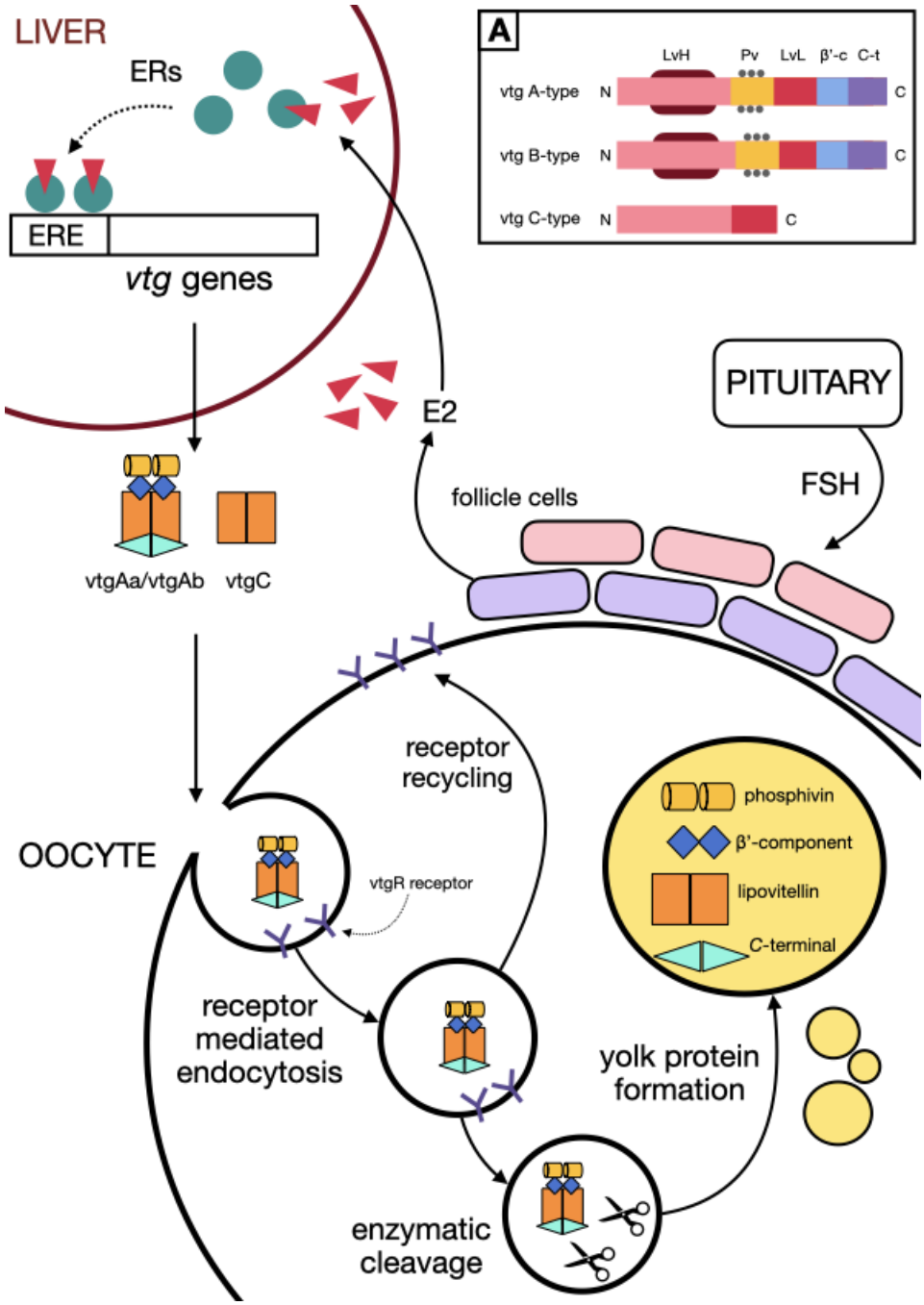

Figure 4. Schematic representation of the vitellogenesis and vitellogenin uptake in fish. The different colours and shapes of the vitellogenin protein represent the structural components that are enzymatically cleaved upon oocyte uptake. The insert A depicts the structure of the three forms of vitellogenins at protein level.

separate strategies to regulate egg water reserves and osmolarity through differential cleavage/hydrolysis of LvH from VtgAa and VtgAb (Finn et al., 2002; Finn & Kristoffersen, 2007; Reith et al., 2001). Interestingly, in teleosts, the pool of free amino acids (FAA), the major driving force of oocyte hydration within the maternal ovary and early source of energy for the developing embryo (Wright & Fyhn, 2001), is generated through preferential hydrolysis of the VtgAa form, a finding corresponding to a neo-functionalization for this gene (Finn & Kristoffersen, 2007). On the other hand, the VtgAb retains its ancestral function in which its LvH domain may be nicked but not hydrolysed as in the case of VtgAa.

Oocyte maturation

At the completion of vitellogenesis, fully-grown oocytes enters the next and last phase that precedes ovulation, when the oocytes become a functional egg ready to be fertilized. The main events associated with the so-called oocyte maturation are 1) a steroidogenic shift from E2 to MIH 2) the resumption of the meiosis and extrusion of the first polar body 3) a coalescence of lipid and vitellogenin globules 4) the final hydration of the oocyte (Fig. 3) (reviewed in Biran & Levavi-Sivan, 2018; Kagawa 2013; Lubzens et al., 2010, 2017; Nagahama & Yamashita, 2008; Yaron & Levavi-Sivan, 2011). At endocrine level, it is well established that the major initial driver of such changes is a surge of plasmatic Lh levels, which do not act directly but mediates its effects through gonadal synthesis of sex steroids following a two-cell type (granulosa+theca) model (Nagahama, 1994, 1997). Indeed, the first event that the LH surge triggers is a shift in the steroidogenic cascade from the synthesis of E2 toward MIH, which is either 17α,20βP or 17,20β,21-trihydroxy-4-pregnen-3-one depending on the species (Nagahama & Yamashita, 2008). However, in order to produce the MIH, there must be a shift from the synthesis of testosterone to 17α-hydroxyprogesterone and a down-regulation of the aromatase activity in favour of the enzyme 20β-Hsd. To achieve this shift, which was well described as multiple waves of key sex steroids in group-synchronous spawners (Asturiano et al., 2002), the P450c17 is the enzyme at a key branch of the steroid production because it possesses both 17,20-lyase (C19 steroids synthesis) and hydroxylase activity (C21 steroids synthesis) and, to massively produce MIH at oocyte maturation, its 17,20-lyase activity needs to be downregulated, although the underlying process was poorly known until the last decade (Senthilkumaran et al., 2004). This was resolved by the discovery of two *p450c17* genes, namely *p450c17-I* and *p450c17-II*, in the genomes of multiple teleost species, with the former having both 17α-

hydroxylase and 17,20-lyase activities while the latter with only 17α-hydroxylase activity without any 17,20-lyase activity (Zhou et al., 2007). A temporally controlled switching was observed in the expression of these two genes during the steroidogenic shift from E2 to MIH according to their specific enzymatic activity in the sex steroid synthesis. Following the identification of this mechanism, efforts were then directed towards understanding of how this temporal switch was controlled. As one might expect, what emerged is that the transcription of several steroidogenic enzymes, including *p450c17-I*/*p450c17-II*, *aromatase* and *20β-hsd*, is regulated by specific transcription factors such as the cAMP response element-binding protein (Creb), the adrenal 4 binding protein/steroidogenic factor 1 (Ad4bp/Sf-1) and the forkhead box L2 (Foxl2) that bind to responsive elements in the promoter region of the target genes (Senthilkumaran, 2011; Wang et al., 2007).

On the oolemma, the MIH binds to membrane-bound progestin receptors (mPRs), classified based on sequence similarity as *mprα*, *mprβ* and *mprγ* (Thomas et al., 2006). The presence of mPRs is crucial for the oocyte to respond to MIH and to induce the so-called maturational competence. Upon MIH-mPR binding at oocyte surface, the inhibition of the adenylate cyclase (Ac), the promotion of the cAMP-dependent phosphodiesterase (Pde) activity and other non-genomic pathways cause a decline of intracellular cAMP levels (Das et al., 2017), a signalling cascade that promotes withdrawal of meiotic arrest through the activation of the maturation-promoting factor (MPF), the final inducer of meiosis resumption and oocyte maturation (Clelland & Peng, 2009; Lessman, 2009). This mechanism of action was elucidated in several teleost species such as goldfish (Tokumoto et al., 2006; Tokumoto, 2012), zebrafish (Hanna & Zhu, 2011), rainbow trout (Mourot et al., 2006) and medaka (Roy et al., 2017). The MPF is a dimeric protein consisting of two subunits, the cell division cycle kinase (Cdc2 or Cdk1) and the cyclin b1 (Ccnb1). In goldfish, immature oocytes contain monomeric 35 kDa Cdc2 but not Ccnb1, which is instead translated by a cytoplasmic pool of masked mRNA after a signal transduction cascade initiated from *mprα* through a signalling pathway that includes Gi, cyclin, cAMP and Pka (Nagahama, 1994; Nagahama & Yamashita, 2008; Yamashita, 2000). Upon phosphorylation, the activation of the MPF triggers the germinal vesicle migration (GVM) and subsequent breakdown (GVB), two processes that mark the resumption of the meiosis (Nagahama & Yamashita, 2008). Concomitantly with oocyte maturation and GVM, the oocyte undergoes ooplasmic clearing and yolk protein proteolysis mediated by cathepsins (Carnevali et al., 2006).

In addition to the macro regulatory effects of the Lh-mediated signalling, autocrine/paracrine mechanisms are at play during oocyte maturation and include both the dual control of E2

and MIH on oocyte maturation and the ovarian GnRH system. Indeed, although the Lh-stimulated MIH-mediated oocyte maturation is a widely accepted model, it is important to highlight that inhibition of E2 synthesis by the ovarian follicle cells caused spontaneous oocyte maturation in zebrafish without any MIH stimulation (Pang & Thomas, 2009), indicating the presence of both MIH-dependent and MIH-independent pathways. In zebrafish follicles, this effect was modulated by the action of the G protein-coupled estrogen receptor (Gper or Gp30), which, upon E2 stimulation, down-regulated the protein expression of Mprα and this action was reversed by MIH treatment, suggesting a dual control of the onset of oocyte maturation mediated by the action of E2 and MIH via their cognate receptors Gper and Mprα (Pang & Thomas, 2010). Similar results were reported in a perciform species, the yellow Atlantic croaker *Micropogonias undulatus* (Pang et al., 2008). It appears that E2 acts through Gpr30 to activate a stimulatory G protein (Gs) with the subsequent stimulation of adenylate cyclase activity and increases in cAMP production, whose high levels help to maintain meiotic arrest, possibly through downstream signalling molecules such as the protein kinase A (Thomas, 2012). In addition to the local interplay between progestins and estrogen, also the ovarian GnRH system has attracted increasing interests over the last years. Indeed, there is unambiguous evidence that GnRH and GnRH receptors are present in the ovary of fish and other vertebrates (Gazourian et al., 1997; Nabissi et al., 2000; Pati & Habibi, 1992, 1993, 1998). Recently, the GnRH-III and gonadotropin inhibiting hormone (GnIH) were demonstrated in vitro to be essential autocrine/paracrine factors for the normal induction of final oocyte maturation in zebrafish (Fallah & Habibi, 2020). The administration of several GnRH peptides promoted meiosis resumption and Histone H1 kinase activity (i.e. a marker of meiotic maturation) in goldfish ovaries in vitro, although heterogeneous results among different GnRH forms were observed using GnRH antagonist, suggesting the possible involvement of different pathways mediating stimulatory/inhibitory effects (Pati & Habibi, 2000). Furthermore, a close relationship between the expression of GnRH variants, their cognate receptors and reproductive status was found in the pituitary, brain and ovary of the ornamental pejerrey *Odontesthes bonariensis* (Guilgur et al., 2009). Thus, it seems that the GnRH system in the ovary is part of a complex multifactorial regulatory system that control both oocyte growth, maturation and resumption of meiosis through autocrine and paracrine mechanisms integrated with circulating gonadotropin hormones. Moreover, this local control might have also important implications for the synchronization of different batches of oocytes released during the spawning season.

Finally, one of the last step of oocyte maturation, the hydration, is differentially regulated according to the ecology of the species as seen in marine pelagophil eggs, which undergo extensive hydrolysis of the LvH (Finn & Kristoffersen, 2007), creating a pool of free amino acids functioning as an osmotic effector and therefore causing extensive hydration of the oocyte via aquaporin-mediated processes (Fabra et al., 2005). The positive buoyancy of marine fish eggs in sea water, achieved with the hydration of the oocyte, is a critical factor for the survival and dispersion in the ocean and for the proper development of the offspring.

Ovulation

Ovulation is a dynamic process which results in follicle rupture and subsequent discharge of the egg from the ovarian follicle into the ovarian cavity or into the abdominal cavity. Follicle wall rupture during ovulation is certainly the core event and is primarily caused by a protease system that digest the extra cellular matrix (ECM) in the follicle (Takahashi et al., 2019). Accordingly, the expression of proteases such as *serine protease 23* (*sp23*) and *disintegrin and metalloproteinase domain 22* (*adam22*) were found to be up-regulated in the periovulatory follicle of rainbow trout (Bobe et al., 2006). Furthermore, a dramatic change in gene expression was observed for the *disintegrin-like and metalloproteinase with thrombospondin type 1 motif 9* (*adamts9*), *adamts8b* and *mmp9* (aka *gelatinase B*) in the periovulatory follicle of zebrafish (Liu et al., 2017). More mechanistic insights were elucidated in medaka, in which two proteolytic enzyme systems (Plau1, Mmp) were differentially controlled in a time dependent manner before ovulation to finally degrade the ECM and release the eggs from the follicle cells (reviewed in Takahashi et al., 2019).
At endocrine level, the Lh was shown to in vitro stimulate ovulation in medaka (Ogiwara et al., 2013), brook trout (Crespo et al., 2013) and zebrafish (Tang et al., 2017) and key roles of gonadotropins with their cognate receptors were also identified through knockout studies in zebrafish (Zhang et al., 2015). In addition to Lh, the MIH was shown to regulate the expression of important genes required for ovulation via genomic mechanisms of action through the *nuclear progestin receptor* (*npr*) (Ogiwara & Takahashi, 2017; Tang et al., 2016).

The Atlantic bluefin tuna

The Eastern and Western stocks

The Atlantic bluefin tuna (*Thunnus thynnus*) fishery is one of the most profitable around the world and often high-quality individuals are sold at the Japanese auctions (Tokyo Tsukiji Market) at extraordinary prices usually hitting the headlines, confirming this country as the number one client of the fishery (de la Gándara et al., 2016). This species has an impressive migratory behaviour and a broad distribution covering the North Atlantic Ocean and its adjacent areas, especially the Mediterranean Sea. The population is composed by two stocks of unequal sizes spawning on different grounds, the smaller reproducing in the Western Atlantic Ocean (Western stock) and the bigger in the Mediterranean Sea (Eastern stock) (Rooker et al., 2007). The migratory behaviour and exceptional swimming capabilities allow the species to cross boundaries and management areas (i.e. exclusive economic zones) which, in addition to complex life-history, population dynamics and shared status of the fishery, challenge the effective management of the population by the body in charge, the International Commission for the Conservation of Atlantic Tunas (ICCAT) (Fromentin & Powers, 2005; Sumaila & Huang, 2012). Based on the commonly accepted division at the 45° meridian which reflects natal homing, the Eastern and Western stock are managed as separate units, although the measure is at the centre of a long-standing debate based on a certain degree of mixing between the two stocks not fully resolved yet (Rooker et al., 2007, 2019). Reproductive dynamics underlying the two spawning grounds are a matter of debate too. As an example, until recently it was accepted that Eastern individuals (Corriero et al., 2005) attained sexual maturity well before Western individuals (Baglin, 1982; Diaz, 2011; Porch & Hanke, 2018) but such a result turned out to be in sharp contrast with their similarity in terms of growth rates (Porch et al., 2019), fecundity and spawning period (Knapp et al., 2014). Therefore, it is likely that the estimates of sexual maturity of one or both stocks may be biased (Medina, 2020).

In the Mediterranean Sea, common fishing methods targeting the Atlantic bluefin tuna schools are the purse-seine, long-line, tuna traps and recreational bait fishing (Mylonas et al., 2010). Briefly, purse-seining consists of casting a vertical net to surround the school of bluefin tunas and close the bottom to confine them. Once captured, they are transported at very low speed (~1 knot), to prevent the collapse of the cage, to farming/fattening site in a journey which can last several weeks and costs up to 3000$/day. The long-line fishing methods consists of casting at sea several hundreds of hooks armed with a bait (e.g.

cephalopods, small pelagic fish) connected by a long line with regularly distanced buoys to keep the hooks at the desired depth. The tuna trap is by far the most traditional and oldest fishing methods adopted for the bluefin tuna in the Mediterranean and, in its simplest explanation, is a fixed system of chambers whose walls consist of vertical nets. This system takes advantage from the migration path of bluefin tunas to reach spawning grounds in the Mediterranean (before the spawning season) or to return at feeding ground in the Atlantic Ocean (following the spawning season). Different quotas are allocated to each method, with the purse-seine obtaining the largest fraction (Ottolenghi, 2008).

A substantial amount of work has been done over the past two decades to fill the gap in the reproductive biology of this species, with the aims of both providing advices for the fishery management and shifting the current capture-fattening system in the Mediterranean to a complete production chain from hatcheries to harvests (Medina, 2020; Mylonas et al., 2010; Zohar et al., 2016). However, despite significant efforts and collaborations since the beginning of the century, some questions still remain unanswered regarding the basic reproductive physiology, recruitment, offspring production and spawning stocks (Porch et al., 2019).

Ovarian structure

The ovary of bluefin tuna is a paired organ, elongated at the centre of the abdominal cavity and with variable amount of perigonadal fat depending on the reproductive condition and period (Fig. 5A-C). Inside, the ovaries are hollow and join caudally in a common oviduct which opens to the exterior in the urogenital pore (Fig. 5A). In sexually mature females the ovary consist of a thick muscle wall and ovigerous lamellae containing oogonia and oocytes at different stages embedded in connective tissue (Fig. 5B), which vary along the reproductive cycle. The perigonadal fat (Fig. 5C) is believed to function as a fat deposit and it is depleted during the migration to spawning grounds to sustain gonadal development (Mourente et al., 2001). Like many fish species reproducing seasonally in temperate waters, throughout the reproductive cycle a substantial change in terms of colour, dimension, vascularity and compactness occurs and these features are adopted by macroscopic methods to classify the reproductive status of females (West, 1990).

Oogenesis

During the reproductive season, the ovary of the Atlantic bluefin tuna exhibits oocytes at all stages of development, releasing multiple batches of eggs and corresponding to an asynchronous ovary with indeterminate fecundity (Aragón et al., 2010; Medina et al., 2002). The different stages of oocyte development have been thoroughly described at ultrastructural (Abascal & Medina, 2005), histological and histochemical (Corriero et al., 2003; Sarasquete et al., 2002) level and can be broadly grouped in five classes: early oocytes, previtellogenic, vitellogenic, maturing and mature oocytes. The following descriptions are based on the aforementioned works.

Figure 5. Pictures of mature ovaries of the Atlantic bluefin tuna showing A) the two paired lobes that join caudally in a common oviduct B) the ovigerous lamellae that are marked by black asterisks C) the perigonadal fat surrounding the ovaries

Oogonia (8-15 μm) are usually grouped in clusters (i.e. oogonial nests) and exhibit a weakly basophilic cytoplasm, many ribosomes, round mitochondria with few cristae, Golgi complexes and a poorly developed endoplasmic reticulum. Early meiotic oocytes (15-20 μm) are similar to oogonia at ultrastructural level, although at this stage start to associate

with pre-follicle cells. Both oogonia and early meiotic oocytes exhibits a high nucleus-cytoplasm ratio and a single nucleolus at the centre of the nucleus. The previtellogenic phase (aka primary growth) includes two stages of oocytes, the chromatin nucleolus (20-40 μm) and the perinucleolar (45-100 μm) stage. The former is seldomly observed and displays for the first time, during the oocyte development, the Balbiani's vitelline body in addition to a strongly basophilic cytoplasm, a single nucleolus and a nucleus with strands of chromatin. The perinucleolar stage consists of polyedric oocytes with a strong ooplasm basophily, numerous nucleoli at the periphery of the nucleus and a single layer of flattened granulosa cells becomes clearly visible. At ultrastructural level, the zona radiata begins to be deposited at this stage and is already identifiable as inner and outer zona radiata. However, this structure is hard to observe at this stage with regular histology. The lipid stage oocytes (110–220 μm), which mark the onset of the secondary growth phase, exhibit a weak ooplasm basophily, small lipid droplets irregularly scattered in the ooplasm, cortical alveoli at the periphery of the cytoplasm and a thickened Pas^{+} zona radiata well discernible with light microscopy. Vitellogenic oocytes exhibit a continuum of features from the so-called early (220–300 μm) to late (300–500 μm) stages and some of the most obvious changes in the oocytes are observed mainly caused by the uptake of exogenous vitellogenin. Indeed, the relevant increase in diameter is due to an accumulation of yolk and lipid globules, which progressively enlarge, until they occupy almost the entire ooplasm. An evident zona radiata (inner+outer) becomes clearly visible (up to 20 μm) and its thickness increases as vitellogenesis proceeds. The hallmarks of the maturing oocytes (500-750 μm) are the migration of the germinal vesicle towards the animal pole, the event which marks the resumption of the meiosis, and the coalescence of yolk and lipid globules. Once migrated, the nuclear envelope breakdowns and the coalescence of yolk and lipid globules produces large amorphous plaques. The last process, which makes the oocyte mature (750–900 μm), consists of the final hydration. At this stage, the yolk proteins are proteolytically cleaved (Pousis et al., 2011), which increases the concentration of the pool of free amino acids and small peptides within the oocyte, therefore providing an osmotic mechanism for water influx into the oocyte (hydration) and resulting in an increase of oocyte size up to 1 mm. Accordingly, the hydrated oocytes are characterized by a homogeneous mass of yolk, a large single oil droplet and the detachment of the follicular layers. After ovulation, the follicular cells surrounding the volume once occupied by the oocyte, become hypertrophied. These structures are temporary and degenerate rapidly, becoming almost indistinguishable from atretic follicles. Degenerating oocytes are normally found in vitellogenic ovaries and

they are named as α and β atretic oocytes. The α atretic oocytes are characterized by the fragmentation of the zona radiata, nuclear envelope breakdown and progressive phagocytosis of yolk granules by the surrounding granulosa cells. At more advanced stages of atresia (β-atresia), once the degradation of the oocyte is complete, only disorganized granulosa and theca cells exhibiting pyknotic nuclei are visible.

According to key histological aspects such as the distribution of the oocytes stages, the presence of post-ovulatory follicles and the extent of follicular atresia, the ovarian classification is based on the following nomenclature: Active nonspawning (ANS), Active spawning (AS), Inactive mature (IM) and Resting (R) (Schaefer, 1996, 1998). The classifications along with their histological features are summarized in Table 1.

Table 1. Classification of the Atlantic bluefin tuna females according to Schaefer (1998). The table was taken from Medina (2016) with some minor modifications. *= Referred to as immature in the original classification of Schaefer (1998).

Stage	Histological features
Resting (R)*	Previtellogenic or early vitellogenic oocytes. No atresia
Active nonspawning (ANS)	Advanced vitellogenic oocytes and no to minor (<50%) α atresia
Active spawning (AS)	Advanced vitellogenic oocytes and no to minor (<50%) α atresia plus POFs and/or migratory-nucleus oocytes
Inactive mature (IM)	Previtellogenic or early vitellogenic oocytes plus α and/or β atresia, or advanced vitellogenic oocytes plus major (>50%) α atresia

Ovarian cycle and sexual maturity in the Mediterranean Sea

The Atlantic bluefin tuna migrates from feeding grounds in the Atlantic Ocean to spawning grounds in the Mediterranean Sea and this is well reflected by the pattern of ovarian development and ANS females found along this migration route, with females from the well-known spawning ground at the Balearic Islands containing five-fold more highly yolked oocytes than females entering the Mediterranean Sea at Barbate (Strait of Gibraltar, Spain) (Medina et al., 2002). Spawning begins in the Levantine Sea in May (Karakulak et al., 2004)

and lasts until the end of July in the central and western Mediterranean (Abascal et al., 2004; Corriero et al., 2003; Susca et al., 2001; Zupa et al., 2009), following a clear relationship with water temperatures and triggered at T≥23°C as in several tuna species (Schaefer, 2001). However, this commonly accepted threshold was challenged by Gordoa and Carreras (2014), who recorded spawning events from temperatures as low as 19-20°C and attributed to day length a more relevant role to influence the timing of spawning. Similarly, Atlantic bluefin tuna was shown to spawn as early as possible after temperatures reached ≥20°C, highlighting how spawning phenology reflects multiple oceanographic conditions such as seasonal productivity and zooplankton availability other than temperature (Reglero et al., 2018). Despite the reproductive season is well established from May to July, in the Mediterranean basin the ovarian development and gonadosomatic index (GSI) exhibited a geographical and temporal gradient (Heinisch et al., 2008). Indeed, the authors observed high GSI values in the Levantine Sea in late May/early June (Fork Length = 151.81 ± 2.20 cm) followed by increasing values in Malta (Fork Length = 220.96 ± 3.77 cm) and Balearic Islands (Fork Length∼207 cm) with a delay of 2 and 4 weeks, respectively, suggesting that the Mediterranean Sea might host young bluefin tuna until they reach a certain size to begin migration toward the Atlantic. Therefore, the existence of more complex population dynamics and additional spawning grounds cannot be ruled out (Cermeño et al., 2015; Piccinetti et al., 2013) and fill such a gap of knowledge currently represent one of the main bottleneck for our understanding of reproductive dynamics of this species in the wild. During the non-reproductive period, the bluefin tuna ovaries contained almost entirely perinucleolar oocytes (i.e. resting stage) while lipid stage oocytes could be observed only from April in the central Mediterranean (Corriero et al., 2003). However, lipid stage oocytes were observed approximately in the same area in October-November in caged individuals at the end of the fattening period (Carnevali et al., 2019).

According to histological examinations of bluefin tuna ovaries in the Mediterranean Sea, Corriero et al. (2005) established the L_{50}=103.6 cm Fork Length (FL) corresponding to 3 years old while 100% of the examined females were mature at ≥ 135 FL (4-5 years old), both findings also supported by sex steroid profiles (Susca et al., 2001). At first, the discrepancy with the Western stock was relevant since several estimates placed the sexual maturity in the area between 8 and 15 years old (Baglin, 1982; Diaz & Turner, 2007; Diaz, 2011; Porch & Hanke, 2018). Yet, Heinisch et al. (2014) applied a novel approach for this field of research based on the pituitary Fsh/Lh ratio and revealed that both males and females in the range 134-185 cm Curved Fork Length (approximately 5-8 years old) were

already sexually mature. This further supported the idea that current estimates might be biased and one key factor to be carefully considered is the geographical locations of sampling activities (Corriero et al., 2020; Medina, 2020).

Captivity effects on the HPG axis and oogenesis

To achieve domestication of the Atlantic bluefin tuna a deep knowledge on its larval development, dietary needs, immune response, growth and reproductive processes must be achieved. The current commercial model is based on wild-caught individuals kept in floating cages for up to 36 months until ready to meet the market demand (Karakulak et al., 2016; Mylonas et al., 2010; Ottolenghi, 2008). This system, albeit heavily dependent on the supply of wild specimens, allows for a certain production of larvae since the bluefin tuna is well-known to spontaneously spawn in cages (Gordoa et al., 2009; Gordoa & Carreras, 2014; Medina et al., 2016). However, in the early days, performances in terms of larval survival and volume of spawned eggs were still miles away from required commercial standard (De Metrio et al., 2010), although extremely valuable for scientific purposes to find out best rearing conditions (Blanco et al., 2017, 2020; Reglero et al., 2014). Therefore, understanding how the HPG axis responds to captivity is today essential to mitigate negative effects (i.e. unbalanced diet, overcrowding, handling stress), obtain adequate amounts of good-quality fertilized eggs and discover dynamics underlying puberty onset.

It is quite clear that captivity does not prevent neither vitellogenesis nor spawning as extensively documented. Indeed, yolk uptake and diameter of fully grown vitellogenic oocytes were observed in 1-3 years captive-reared females to be similar to wild fish (Corriero et al., 2007). The results were further supported by *vtg* (*vtgA* and *vtgB*) gene expression analysis, which revealed comparable levels between wild and captive-reared females, the latter even exhibiting enhanced yolk accumulation (Pousis et al., 2011). The authors suggested how the administration of an enriched diet based on squid, with its higher phospholipid and cholesterol content, lead to increased yolk amount in late vitellogenic oocytes. Notwithstanding captivity conditions does not trigger a complete reproductive dysfunction, some considerations and findings need to be highlighted to understand ongoing and future research directions. Indeed, lower *vtgr* levels were detected in captive-reared compared with wild individuals (Pousis et al., 2012). Furthermore, Corriero et al. (2007) recorded a lower number of vitellogenic oocytes and smaller GSI values in captive-reared than wild individuals. Interestingly, similar observation were reported for the Pacific bluefin

tuna *Thunnus orientalis* as the GSI of individuals reared up to 12 years in captivity was never ≥ 2% while values from wild individuals were ≥ 5% (Chen et al., 2006; Miyashita et al., 2000). To overcome such limitations in the Atlantic bluefin tuna, intense attempts were made in the past using agonist Gonadotropin-Releasing Hormone (GnRHa) implants and first results at histological level confirmed the effectiveness of the treatment in inducing multiple cycles of final oocyte maturation and ovulation (Corriero et al., 2007). Later on, the endocrine background and steroid responses following this treatment were studied, revealing successful maturation and ovulation in addition to an elevation of 17,20β-dihydroxy-4-pregnen-3-one (17,20βP) levels 8 days after the implant and therefore fully activating the HPG axis (Rosenfeld et al., 2012). Circulating levels of Fsh were higher during the non-reproductive season (October-November) in mature caged individuals than during the reproductive season (May-June) in wild-caught individuals, supporting the idea of an early preparation for the next cycle of gametogenesis and suggesting that captivity conditions did not significantly impair the reproductive cycle at endocrinological level (Carnevali et al., 2019). Finally, confinement alone in 1-year captive-reared individuals did not elicit significant levels of follicular atresia, although both starvation and crowding-induced severe panic frenzy triggered a decrease in gonad mass and higher levels of α atresia up to 100% of vitellogenic oocytes (Corriero et al., 2011). To sum up, captivity conditions does not seem to completely impair the reproductive capacities of the Atlantic bluefin tuna but they likely play a role in preventing the full and complete activation of the HPG axis, with hormonal treatments demonstrated to be an effective tool to overcome this drawback. Therefore, the bottleneck is now shifted from obtaining large amounts of fertilized eggs to efficiently raise the larvae and current efforts are indeed attempting to figure out some key aspects of the ontogeny such as the larval development, growth, nutritional and light needs (Betancor et al., 2017, 2019, 2020; Blanco et al., 2017, 2020; de la Gándara et al., 2016).

Larval development

At a commercial level, achieving a good knowledge of the larval development of the Atlantic bluefin tuna is required to develop feeding protocols, design appropriate feeds and establish effective light regimes, all aspects aimed at supporting the industry and turn the sector to be economically sustainable and competitive. In this context, pioneering works on this species took advantage from the successes achieved in the closely related Pacific bluefin tuna *Thunnus orientalis*, for which the completion of the life cycle dates back almost twenty years

ago (Sawada et al. 2005). However, beside commercial purposes, understanding larval biology has also important implications for stock assessment since taking into account certain parameters such as growth rates, feeding patterns and survival while developing models is crucial for a reliable outcome (Catalán et al., 2011; Domingues et al., 2016; Laiz-Carrión et al., 2015). For instance, a good understanding of the aforementioned aspects allows to obtain more precise estimates of population recruitment, that is the number of new individuals entering the population and that contribute to its renewal (Maunder & Thorson, 2019).

According to Yúfera et al. (2014), based on the feeding mode and morphological/histological characteristics of the developing organs, the Atlantic bluefin tuna larval development can be divided into four distinct phases: i) stage 1 (0–1 dph), ii) stage 2 (2–3 dph), iii) stage 3 (4–10 dph) and iv) stage 4 (11 dph-around 30 dph). Certainly, larval development is a highly dynamic process and thus the definition of stages, especially on individual basis, is subject to a certain degree of variability, although some events are essential to reconstruct the ontogeny. One of the most widely and universally used in fish is the flexion of the notochord, an event linked to swimming capabilities and caudal fin formation, which defines three broad periods classified as pre-flexion, flexion and post-flexion (Fig. 6) (Downie et al., 2020). Most of the following descriptions of the Atlantic bluefin tuna stages are taken from Yúfera et al. (2014) because it is the unique and most comprehensive work describing the organogenesis during the larval development of the Atlantic bluefin tuna at a histological level. A comparison with other tuna species and integration with the relative information is performed to offer a broader perspective of the larval ontogeny in the *Thunnus* genera.

At stage 1 (0–1 dph) the yolk sac still represents the main source of nutrients for the growing larva, as evidenced by the fact that both mouth and anus are closed, the digestive and visual systems are still undifferentiated, thus neither predation nor digestion are possible at this early phase. However, the yolk sac is rapidly consumed and around 1-2 dph is greatly

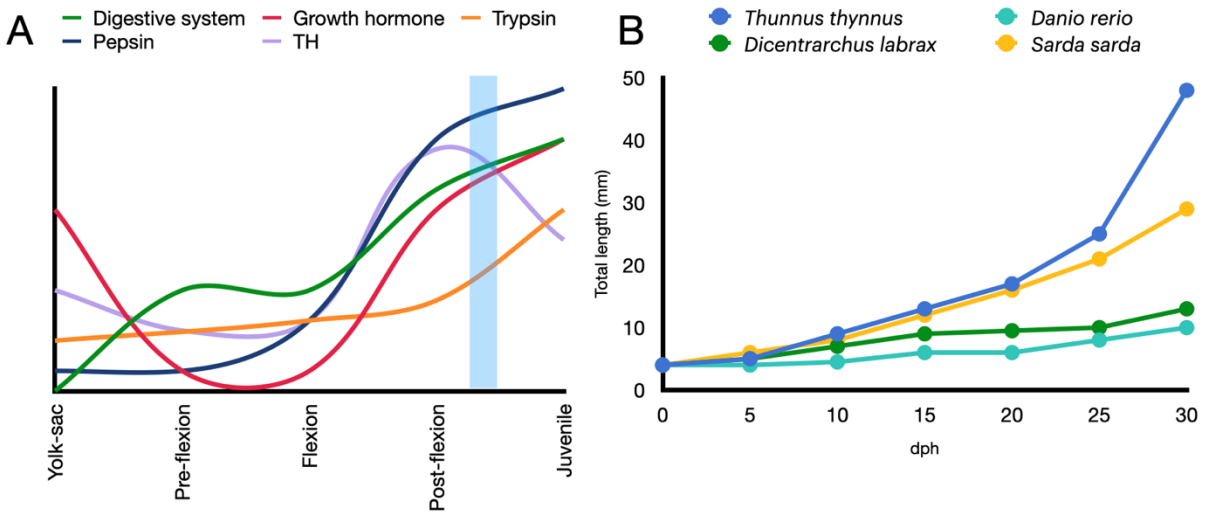

Figure 6. A) Conceptual scheme that shows the main drivers of the larval period with their relative contribution during the different stages. The scheme was inspired by reading the work of Kaji (2003), which was drawn with some minor modifications. B) Growth expressed as gain in total length of different teleost species.

reduced. At stage 2 (2–3 dph), the feeding mode changes to exogenous and it is accompanied by the precocious establishment of a primitive larval type digestive system, as evidenced by the differentiation of the buccopharyngeal cavity, oesophagus, intestine, hepatocytes and exocrine pancreas as well as by mouth and anus that are now opened. Visual system also begins to differentiate and within 3 dph the retinal layers are completely developed and the eye pigmented. Notably, pigmentation of the retinal pigment epithelium was influenced by the thyroid hormone T4 and its cognate receptor thyroid hormone receptor β (TRβ) in 2-3 dph larvae of *T. orientalis* (Kawakami et al., 2008a). This transitional stage, which is critical in any fish larvae (Yúfera & Darias, 2007), coincides with the switch from yolk reserves to exogenous first feeding and, as seen, is accompanied by the development of the early machinery necessary to localize and digest preys. Massive mortalities were reported at this time in a variety of tuna species (Kurata et al., 2011; Sawada et al., 2005; Woolley et al., 2013) To maximize chances of survival during this phase, a positive allometric increase of organs related to predation (e.g. mouth size) and digestion during the first days of feeding is generally observed in fish (Gisbert et al., 2002; Sala et al., 2005). Interestingly, at this early phase of predation, embryonic lateral line was hypothesized to facilitate prey detection before the visual system progressively takes over to become the main prey-localizing organ in the Atlantic bluefin tuna larvae (Ghysen et al., 2012). It is important to

remark that both stage 1 and 2 occur before notochord flexion and thus are referred as pre-flexion stages. At stage 3 (4–10 dph) the thyroid follicles intensively proliferate, the gas gland increases considerably in size along with the swim bladder. In the sibling species *T. orientalis*, this period was characterized by increased expression levels and specific activity (mU/mg protein) of key enzymes indicative of pancreatic proteolysis capacity (trypsin, chymotrypsin), lipid (pancreatic and bile salt-activated lipases) and carbohydrate (amylase) digestion (Murashita et al., 2014). Moreover, in laboratory reared yellowfin tuna *Thunnus albacares* larvae, a high GH cell-mass volume to pituitary volume, which expresses the GH production potential, was detected after the first exogenous feeding (4-6 dph) followed by a sharp decline at 7 and 9 dph and a subsequent large increase from 18 dph onward (Fig. 6A) (Kaji et al., 1999). Although thyroid hormones (T3 and T4) levels were not elevated during this stage, expression of *thyroid hormone receptor αA* (*TrαA*) peaked at 7 dph and then declined at later stages in the Pacific bluefin tuna (Kawakami et al., 2008b). Between stage 3 and stage 4, notochord flexion begins and therefore this developmental phase (~8-13 dph) is referred to flexion stage (Blanco et al., 2019). At stage 4 (11-28 dph), in addition to the post-flexion phase (~13-14 dph), the main and most obvious event is the metamorphosis from larvae to early juvenile at around 20 dph. In the Pacific bluefin tuna, this event was characterized by a peak of thyroid hormones (T3 and T4) (Kawakami et al., 2008b) and by both increasing expression levels and enzymatic activity of pepsin, the main acidic protease indicative of a fully functional stomach (Fig. 6A) (Murashita et al., 2014). During this stage, both Atlantic and Pacific bluefin tuna exhibited enhanced transcription rates, as evidenced by higher RNA:DNA ratios (Blanco et al., 2019; Tanaka et al., 2007) At histological and morphological level the Atlantic bluefin tuna larvae shows a notable development of pronephros and mesonephros, a fully formed heart as well as the swim bladder, the pyloric caeca differentiates and the outer nuclear layer of the retina develops two layers, the cone and rod nuclei. The *N-aminopeptidase* expression, a marker of maturation of the intestine, showed an increase from 10 dph onward, strengthening the idea of precocious development of this organ while the *myosin heavy chain*, indicative of somatic growth, peaked at 20 dph (Mazurais et al., 2014). By the end of the first month, the Atlantic bluefin tuna exhibited one of highest larval growth rate documented so far, reaching a total length of 4-5 cm (Fig. 6B) (Yúfera et al., 2014). In order to sustain high growth and developmental rates a great amount of energy must be invested, an hypothesis that well fits the results of Blanco et al. (2020), in which a high oxygen consumption was found during the piscivorous phase. Furthermore, the jaw length/total length ratio, indicative of mouth and head development, increased

quickly up to 21 dph, a finding that suggests how the ingestion of large prey is facilitated as early as possible therefore balancing the energy expended to prey with the energy gained with its ingestion (Yúfera et al., 2014). All these observations are in line with the fast growth rates found also in other scombrids (e.g. tunas, bonitos) and thought to be related to early piscivory and high swimming capabilities (Buentello et al., 2011; Kaji et al., 2002; Miyashita et al., 2001). To sum up, in about two weeks, the larvae acquires an advanced degree of development in terms of digestive system, sensory and visual structures, thyroid gland, swim bladder, kidney and heart that allows an efficient predation and digestion, being almost fully functional around 17–18 dph, right before it metamorphoses into juvenile (Yúfera et al., 2014). Indeed, this precocious development of digestive, swimming and predatory capabilities lays the ground for the next phases of metamorphosis and early juvenile, in which almost exponential growth rates are observed.

The swordfish

The swordfish *Xiphias gladius* is an iconic species with a rather unique phenotype consisting of an elongated upper jaw forming the rostrum, a structure used by the fish during hunting. This structure was recently discovered to enhance swimming hydrodynamics through an oil-producing gland connected to capillaries that communicate with oil-excreting pores in the skin of the head at the base of the rostrum (Videler et al., 2016). The swordfish is large, epipelagic, highly migratory and capable of long distance movements as documented by both conventional (Neilson et al., 2007) and pop-up satellite tags (Dewar et al., 2011; Sedberry & Loefer, 2001) with a wide geographical distribution which includes temperate, subtropical and tropical waters between the latitudes 45°N and 45°S (Palko et al., 1981). From a taxonomical point of view, it belongs to the broader groups of the so-called billfishes (order: Istiophoriformes) that comprises also marlins and these animals share the rostrum as common distinct physical feature.

Due to its cosmopolitan geographical range, the management of the swordfish fishery is a complex matter and can also be considered even more challenging than that of the Atlantic bluefin tuna, which is distributed only in the Mediterranean sea and Northern Atlantic Ocean. As seen in the previous pages, in this area the ICCAT is the officially recognized international body in charge of the management of large migrating species such as tuna-like species, sharks and the swordfish. In our area, the current geographical subdivision to manage the swordfish fishery follows a two separate stocks model, corresponding to the

Mediterranean and Atlantic, with the boundary in between placed at the strait of Gibraltar, although this measure contrasts with population genetic results that extended such limit at approximately 10°W (Smith et al., 2015).

The Mediterranean stock

For certain aspects, the history of stock management in the Mediterranean between the swordfish and the Atlantic bluefin tuna share common traits. Indeed, both species are heavily targeted by international fleets, the fishery has a long tradition in the area and the two species have complex life history traits (i.e. spawning dynamics, larval recruitment, seasonal migration, long distance movements) that, in conjunction with poorly effective measures, led ultimately to severe overfishing (ICCAT, 2016a). Upon recognizing the overfished status of the Mediterranean swordfish stock over the last 30 years, in 2016 a recovery plan was established including measures such as total allowable catches (TAC), fleet capacity limitations, closed fishing season, maximum number of hooks on longlines and a minimum size (ICCAT, 2016b). This decision was taken after a period of mounting, although not straightforward, evidences that the Mediterranean stock was in a poor status. Back in 1999, it was recorded a decreasing trend of the average weight of catches in an Italian port in the northern Ionian Sea, from 50 to 10 kg (De Metrio et al., 1998). Similar results were reported in the eastern Mediterranean, in which individual swordfish weights decreased from 30 kg in 1998 down to 23 kg in 2004 accompanied by a moderate decline in abundance from 1998 to 2003 with a subsequent rise in 2004 (Damalas et al., 2007). Indeed, a general trend of decreasing average sizes was noted during those years, but the high uncertainty associated with these preliminary results caused by a lack of data undermined the reliability of the outcome, although serious concerns were already raised about the high catch rates of juveniles (ICCAT/SCRS reports, 1995-1999: https://www.iccat.int/en/pubs_biennial.html). At that time, the interpretation of the health status of the Mediterranean stock was further complicated by contrasting results from landings, which instead showed steady average sizes in the period 1986-1999 in the central and eastern Mediterranean (Tserpes et al., 2001) along with stable levels of exploitation and recruitment (Tserpes et al., 2003a), although with large fluctuations of standardized catch-per-unit-effort (CPUE) among years (Tserpes et al., 2003b). A stable mean length of catches from the Moroccan Mediterranean fishery during the period 1999-2006 was also reported (Abid & Idrissi, 2009). In 2004, the ICCAT stock assessment indicated that exploitation levels

were sustainable, albeit the absence of historical data and long-time series limited the accuracy of estimates (ICCAT, 2004). It must be noted that most of these reports and stock assessments suffered from an overall lack of data which might have hampered the reliability of results, as highlighted by almost all scientists in the mentioned technical reports. Although substantial progresses have been made over the years by ICCAT in terms of collaboration among nations and exchange of information, it cannot be excluded that either the lack of data or longer and reliable historical time series might have hampered the interpretation of results in those years. In 2007, evidences of declining stock abundances emerged, along with the continuous large catches of small sized swordfish, gave rise to non-negligible risks of rapid decline of the Mediterranean stock (ICCAT, 2007). In support of this, the overfishing condition of the stock was further confirmed by Tserpes (2008), who found stock biomass levels 10-12% lower than those corresponding to the maximum sustainable yield (MSY) level, a target commonly indicating an optimal level of fishing pressure.

Historically, the catches of Mediterranean swordfish fishery have been mainly made of juveniles and for this reason among the early management measures to protect them there was a period of fishing closure in Autumn (Di Natale et al., 2002; Megalofonou et al., 2001). The aim of this strategy was to allow the fall recruitment of young-of-the-year individuals hatched during summer months according to swordfish growth rates (Megalofonou et al., 1995; Tserpes & Tsimenides, 1995). First evidences highlighted that it could be an effective strategy, with a predicted increase of 6% of total catch and a reduction of 18-23% of the catch number of juvenile fish (Tserpes & Peristeraki, 2007). Moreover, Tserpes et al. (2009) modelled different management measures and scenarios, demonstrating that a four- and six-months closure during the recruitment period (October-January) could have the potential to greatly recover the spawning stock biomass, whereas a one- and two-month closure would produce negligible effects. However, other solutions such as the protection of spawning individuals during summer months were and are not considered by ICCAT, most likely because it coincides with the high fishing season, favourable weather conditions and the peak of market demand. On the other hand, a strategy based on the age-dependent habitat identification of Mediterranean swordfish was also proposed, consisting in the introduction of spatio-temporal closures of fishing activities and the establishment of a network of dynamic marine protected areas (Damalas & Megalofonou, 2014). Following the current recovery plan and management measures, catches have decreased significantly from the levels of the last two decades, catches of undersized swordfish have also decreased more than 50%, although the establishment of a minimum size of 100 cm

produced an impressive increase of discards (up to 600%), raising concerns about unreported catches (ICCAT, 2020). To sum up, the full effects of such management measures will be evaluated only during the next years to understand if the current strategy is effective to recover the Mediterranean swordfish stock.

Ovarian structure

The ovary of swordfish is an elongated paired organ at the centre of the abdominal cavity, always round in section even in immature individuals (Palko et al., 1981). Developing and ripening ovaries reach about ½ and ¾ of the abdominal cavity, respectively (Poisson & Fauvel, 2009). Inside, the ovaries are hollow and join caudally in a common oviduct which opens to the exterior in the urogenital pore. In sexually mature females the ovary consist of a thick muscle wall that surrounds ovigerous lamellae containing oogonia and oocytes at several stages of development embedded in connective tissue. The colour, thickness of ovarian wall, size and vascularization change following the species reproductive cycle.

Oogenesis

The ovary of the swordfish contains oocytes at different stages of development, corresponding to several batches of oocytes that will be spawned. Accordingly, this situation corresponds to that of a multiple spawner with an ovary displaying asynchronous oocyte development (Taylor & Murphy, 1992; Uchiyama & Shomura, 1974). The different stages of oocyte have been described and characterized using regular histology (Abid et al., 2019; Arocha, 2002; Corriero et al., 2004; Farley et al., 2016; Marisaldi et al., 2020; Macías et al., 2005; Poisson & Fauvel, 2009; Young et al., 2003), immunohistochemistry (Corriero et al., 2004; Ortiz-Delgado et al., 2008), histochemistry (Corriero et al., 2004; Macías et al., 2005; Ortiz-Delgado et al., 2008), Fourier Transform Infrared (FTIR) Imaging spectroscopy (Carnevali et al., 2019), transmission (Minniti et al., 2005) and electron (Corriero et al., 2004) microscopy. The following descriptions are based on the aforementioned works.

Oogonia (<15 μm) tend to cluster in so-called nests within the ovigerous folds with no signs of degeneration. They exhibit a globular nucleus with finely dispersed chromatin, a single eccentric nucleolus and small basophilic granules. Then, three broad classes of previtellogenic oocytes can be distinguished. The first is the chromatin nucleus stage (15-120 μm), which is characterized by a large nucleus with chromatin strands, one to few

peripheral nucleoli and an increasing ooplasm basophily. Oocytes at this stage begin to be surrounded by follicular granulosa cells squamous in shape. The second is the perinucleolar stage (100-190 μm), which is the most common and frequently observed, especially in regressing and regenerating ovaries. Oocytes of this class are polyhedric in shape and display a large nucleus with many peripheral nucleoli and a decreasing nucleus-to-cytoplasm ratio. The Balbiani's vitelline body appears at this stage and the oocyte is now surrounded by a flat, continuous layer of granulosa cells. The third and last class of previtellogenic oocytes is termed lipid stage (175-300 μm). The name is ascribed to the deposition of lipid droplets, composed by neutral lipids derived from triglycerides-rich serum lipoproteins, at the inner to peripheral part of the cytoplasm, which increased in size and number as the oocyte grow. At this stage, a thin zona radiata of proteinaceous nature make its first appearance between the oolemma and the follicular cells. The external part of the zona radiata is deposited first and an inner homogenous layer, corresponding to the zona radiata interna, begins to be lightly deposited at this phase, becoming more pronounced at later stages. The nucleus of the lipid stage oocyte is oval shaped and contains several nucleoli located near the outer margin while Pas^{+} and Pas^{-} cortical alveoli appear below the oolemma. Following the lipid stage, the oocyte enters the vitellogenesis, the process underlying the massive uptake of yolk proteins, vitamins and other elements that form the main component of the yolk. Early vitellogenic oocytes (275-500 μm) are characterized by the presence of small eosinophilic vitellogenin granules first and globules later, which are concentrated in the inner part of the cytoplasm. The shape of follicular cells becomes cubic and the zona radiata thickness increases and is now evident, well identifiable with classical histology. As vitellogenesis proceeds, further accumulation of yolk globules occurs along with the increase of zona radiata thickness and this continuum of features makes also the different stages of vitellogenesis difficult to discern, being this a continuous process that is forced into discrete stages for classification purposes. The oocytes at later stages of vitellogenesis have diameters that can reach up to 900 μm, they show a remarkable increase of the zona and the cytoplasm becomes completely filled with both yolk and lipid globules. Once the uptake of vitellogenin is completed, the oocyte enters the post-vitellogenic phase. The migration of the nucleus (germinal vesicle) toward the animal pole, which marks meiosis resumption, and the coalescence of lipid and yolk globules are the two main events that characterize maturing post-vitellogenic oocytes, termed now migratory-nucleus oocytes (1200 μm). Extensive hydration and detachment of follicular cells are the final events that precede ovulation and oocytes at this stage can measure up to 1300 μm.

As seen in the Atlantic bluefin tuna, according to key histological aspects such as the oocytes classes, the presence of POFs and the extent of follicular atresia, the ovarian classification of the swordfish can be based on the following nomenclature: 1) Immature 2) Developing 3) Spawning capable 4) Regressing 5) Regenerating. Although the histological features of the swordfish ovary have been thoroughly described (Arocha, 2002; Corriero et al., 2004; Macías et al., 2005; Marisaldi et al., 2020; Poisson & Fauvel, 2009), a general consensus among the scientific community working on this species is still a matter of debate and ongoing discussions to clarify these aspects are being made (Di Natale et al., 2020). Therefore, the proposed scheme is a honest attempt to uniform the nomenclature of the different phases with a broader effort, began by Brown-Peterson et al. (2011), aimed at standardizing the terminology for describing the reproductive cycle in fish. Importantly, the adopted standardized classification awaits the consensus among scientists working on the swordfish and might be reasonable to accept such a reproductive terminology due to the conceptually universal physiological and histological markers of each phase of the reproductive cycle of fish, as previously indicated (Brown-Peterson et al., 2011; Lowerre-Barbieri et al., 2011a, 2011b). The standardized classification along with their histological features is summarized in Table 2.

Table 2. Classification of the swordfish females according to the standardized terminology of Brown-Peterson et al. (2011), with some minor modifications. *= Referred to as pre-pubertal females (i.e. never spawned).

Stage	Histological features
Immature*	Oogonia and previtellogenic oocytes present. No atresia. Thin ovarian wall, no muscle bundles and compact oocytes.
Developing	Previtellogenic, lipid stage and vitellogenic oocytes and some atresia can be present. No evidence of POFs or fully-grown vitellogenic oocytes.
Spawning capable	Fully-grown vitellogenic oocytes and/or POFs present. Migrating-nucleus oocytes and hydrating oocytes can be present (actively spawning subphase). Some atresia of vitellogenic oocytes present.
Regressing	Vitellogenic oocytes and/or lipid stage oocytes. Extensive atresia (any stage) present (>50% of vitellogenic oocytes).
Regenerating	Oogonia and previtellogenic oocytes present. Muscle bundles and thick ovarian wall.

Ovarian cycle and sexual maturity in the Mediterranean Sea

The reproductive season of the swordfish in the Mediterranean Sea spans from May to August and spawning takes place on discrete breeding grounds throughout the basin identified with both direct (larval sampling) (Alemany et al., 2006; Cavallaro et al., 1991) and indirect (fishery-dependent data) (Tserpes et al., 2008b) methodologies. The peak of the spawning season occurs in June-July according to both macroscopical and histological indicators (Alıçlı et al., 2012; de la Serna et al., 1996; De Metrio et al., 1988; Macías et al., 2005; Marisaldi et al., 2020) and this period match the highest market demand, an aspect that certainly affected the strategy to manage and recover the Mediterranean stock. After the reproductive season, the ovary enters into the so-called regressing phase in which the remaining vitellogenic oocytes that were not spawned undergo follicular atresia and a great fraction of the oocytes at this ovarian stage is previtellogenic (perinucleolar, chromatin-nucleus stages) and, at a macroscopical level, the ovary is flaccid, dark red to greyish in colour and its volume decrease significantly as evidenced by a drop in the gonadic index (Macías et al., 2005; Marisaldi et al., 2020; Tserpes et al., 2001b). Females at this phase are considered to have ceased the reproductive activity for that given year (e.g. post-spawning). Throughout the winter months the ovary is in the regenerating phase, where the most advanced group of oocytes in sexually mature females is the early lipid stage oocyte, with scarce to null follicular atresia and no signs of vitellogenesis. From May on, the ovary begins to develop and this event coincides with the triggering of vitellogenesis. From the pool of previtellogenic oocytes, a certain fraction is recruited to enter vitellogenesis and it represents the first batch of oocytes that will be spawned during the reproductive season.
The importance of having a sound knowledge of reproductive dynamics of this species is intimately linked to our understanding of the size at which sexual maturity (aka puberty) is attained and also the identification of discrete spawning areas and season. In turn, this allows to put effective management measures in place such as the minimum size of catches as well as spatio-temporal fishing closures. In the Mediterranean swordfish, the size at which the 50% of the females reach sexual maturity (L_{50}) was found to be 131.5 cm and 133.3 cm Lower Jaw Fork Length (LJFL) using respectively histology and the gonadic index for classification purposes (Marisaldi et al., 2020). According to established age-length relationships, this size corresponds to ~3 years old individuals (Alıçlı et al., 2012; Akyol & Ceyhan, 2013; Tserpes & Tsimenides, 1995). Recently, a L_{50} of 134.3 cm LJFL calculated according to ovarian histology in the central Mediterranean was found by Saber et al. (2020),

a result that mirrors and corroborate the findings of Marisaldi et al. (2020) in the area. However, other results were also reported, especially towards the Western Mediterranean. Indeed, L_{50} of ~140 cm LJFL and 170 cm LJFL were reported in the southwestern Mediterranean Sea (de la Serna et al., 1996; Macías et al., 2005) and Strait of Gibraltar (Abid et al., 2019), respectively. These discrepancies likely reflect a certain degree of stock mixing between the western part of the Mediterranean Sea and the Atlantic, as also suggested by studies on age-growth patterns (Valerias et al., 2008), reproduction (Abid et al., 2019) and population genetics (Bremer et al., 2007; Viñas et al., 2007). Albeit confirmed with multiple approaches, the exact degree of mixing between the two stocks as well as the horizontal movement dynamics are aspects not fully resolved yet. However, all the reported results related to the size at first maturity are well above the current minimum size of 100 cm LJFL adopted by ICCAT and implemented at national level and this might be a critical point to recover the Mediterranean stock over the next years.

Diving patterns and thermoregulation

The swordfish is a highly migratory species able to cover long distances in a short amount of time. A variety of telemetry, conventional and electronic tagging programs have been used to understand the migratory behaviours and movements of this species in the Atlantic (Abascal et al., 2015; Braun et al., 2019; Carey & Robinson, 1981; Dewar et al., 2011; Neilson & Smith, 2010; Neilson et al., 2009, 2014), Pacific (Abecassis et al., 2012; Carey & Robinson, 1981; Dewar et al., 2011), Indian (West et al., 2012) ocean and in the Mediterranean Sea (Canese et al., 2008; Garibaldi & Lanteri, 2017). The information generated by these approaches is extremely useful to shed light on horizontal and vertical movements and, by integrating other approaches such as population genetic and otolith microchemistry, significantly improves our comprehension of natal homing, spawning and swimming behaviour and spatial-temporal movement dynamics of this species.

What is clear is that the swordfish exhibits a diel vertical migration pattern, spending most of the day at great depths (up to 600 mt) and returning to shallow waters (< 50 mt) at night (Abascal et al., 2015). This behaviour is mainly linked to feeding habits (Carey & Robison, 1981), although performing deep dives requires a considerable amount of energy and a challenge in terms of oxygen availability and thermoregulation, therefore remaining in the shallower mixed layer at night would allow the swordfish to recover from such a physical effort. Shifts in water temperatures during these vertical movements are remarkable,

changing as much as 20°C in a few hours (Stoehr et al., 2018). Such a large and rapid thermal stress would cool the brain which in turn affect the central nervous system in terms of visual and cognitive performances. Interestingly, the swordfish possesses a modified eye muscle to keep eyes and brain warm (Carey, 1982). Indeed, this adaptation is among the best documented example of regional endothermy and consist in a morphological and functional modification of the dorsal rectus muscle, which lost its contractile capacity and acquired a heat-producing function (Carey, 1982). This morpho-physiological novelty is an example of convergent evolution as also other phylogenetically distant species (e.g. lamnid sharks, tunas) achieved too cranial endothermy with the goal of maintaining the head warmer than the surrounding water temperature (Block, 1991; Runcie et al., 2009; Sepulveda et al., 2007; Tubbesing & Block, 2000; Weng & Block, 2004). Although the final result, that is a warm head, is similar among several phylogenetically distant species, the anatomical architecture appears to differ. Indeed, the cranial endothermy in the salmon shark is achieved with a cranial orbital retia which function as heat exchanger between cool oxygenated blood from the gills and venous blood (Tubbesing & Block, 2000). Yet, in the most basal tuna species *Allothunnus fallai*, the anatomical position of the extraocular muscle complex, the surrounding lipid deposits and the loss of contractile function are elements that resemble those of the dorsal rectus muscle in swordfish (Sepulveda et al., 2007). Recently, another potential physiological mechanism to limit heat loss in the body was described in the swordfish. Indeed, Stoher et al. (2018) proposed that, albeit with limited capacity of regional endothermy in the medial red muscle, the swordfish might control rates of heat loss or gain during vertical movements by altering the route of blood flow supplying the medial red muscle. Such a physiological adaptation provide the swordfish with the ability to feed on prey well below the thermocline which would be otherwise out of range. However, despite at least these two morpho-physiological evolutionary novelties, the swordfish doesn't exhibit a true endothermy in the aerobic red muscle such as the one documented in tunas (Block & Finnert, 1994; Graham & Dickson, 2001). Nevertheless, its migratory performances are the largest among fish without red muscle endothermy and follow closely behind those of species with red muscle endothermy (Watanabe et al., 2015).

Investigating reproduction in a fishery management scenario

Understanding the reproductive physiology of any organism allows to control over its reproductive cycle and thus perform selective breeding and domestication for commercial

purposes (Gjedrem et al., 2012; McDougall, 2006), to establish conservation programs of endangered species (Andrabi & Maxwell, 2007) or to better exploit natural resources in the wild (Lowerre-Barbieri, 2009). This latter aspect has gained increasingly interest from our society over the last three decades due to the dramatic effects of overexploitation of natural biological resources at any scale, a well-known issue often triggered by mismanagement (Froese & Quaas, 2012; O'Leary et al., 2011). Competing interests, ineffective measures, scarce collaboration between stakeholders in the decision-making process and poor science-driven governance are among those factors hampering the achievement of a good management of natural resources. A clear example of how all these aspects are at play is represented by the case of fishery management.

By definition, fisheries management is that series of processes which create and enforce the rules needed to prevent overfishing and rebuild overfished stocks. Fisheries are managed by local, national, regional, and global institutions, according to whether fish stock distribution is local, transboundary or highly migratory, using a combination of management measures such as fishing quotas, total allowable catches, spatial planning and temporal fishing closure (Sumaila et al., 2016). In this scenario, different degrees of governance institutions, encompassing international organisations and local agencies have been established to manage the sector. Scientific advice for these organisations rely upon methods for estimating essential parameters such as size at first maturity, reproduction timing, spawning stock biomass, setting up reference points and assessing management strategies (Kell et al., 2016). For instance, the size or age at which sexual maturity is attained represents a key component of population dynamics as it affects the intrinsic rate of population growth and represents a de-facto reference point to meet sustainable harvesting, that is allowing individuals to reproduce at least once in their lifetime (Lowerre-Barbieri et al., 2011a). Maturity data are also a prerequisite to estimate the fraction of spawning individuals within a stock to establish its reproductive potential (Marshall et al., 2006). Furthermore, spawning seasonality, duration and geographical distribution of spawning sites play critical roles in determining reproductive success, with species showing restricted spawning season being more vulnerable to both climate changes and harvesting effects of fishing activities (Wright & Trippel, 2009). High fishing pressure reduce the fraction of older and larger individuals within a spawning stock with direct consequences on the demography and renewal of the population since bigger spawners contribute disproportionately to the number of eggs released (Barneche et al., 2018). Indeed, fishing activities, by altering the proportion of these individuals, is thought to influence the duration and timing of the

spawning season of target fish species (Wright & Trippel, 2009). Therefore, understanding the reproductive strategies and physiology of target species and how this integrates with fishing activities is essential for adequately advising fisheries management to exploit the resource at sustainable levels and thus to pass it on to future generations.

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1. Maturity assignment based on histology-validated macroscopic criteria: tackling the stock decline of the Mediterranean swordfish (*Xiphias gladius*)

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1.1. Introduction

In fish populations the reproductive potential is directly related to the proportion of mature individuals at a given length or age, commonly referred to as maturity ogive. In commercially exploited fish species defining this parental fraction which contributes to the population renewal represents the key to setting sustainable harvesting rates and establish successful conservation measures. In this context, the maturity staging process clearly represents a crucial step and errors at this point might lead to subsequent biased estimations of the spawning stock biomass (Midway & Scharf, 2012; Vitale et al., 2006). The assignment of reproductive status is usually accomplished with macroscopic (i.e. visual) and histological approaches using either species-specific or universal reference scales (Brown-Peterson et al., 2011; Tomkiewicz et al., 2003). However, macroscopic classification suffers from relatively high error rates due to poor intercalibration between operators and overall similarity of whole gonads prior and immediately after the spawning season, which makes the histological approach the most reliable method to assign reproductive stages (Ferreri et al., 2009). Nevertheless, histology requires expensive and time-consuming laboratory procedures which means this methodology is not always suitable for routine surveys, especially if resources are limited. Therefore, reliable and cost-effective alternatives based on macroscopic criteria and routinely collected biological information are needed by management agencies. For instance, Midway et al. (2013) achieved up to >90% success rates in predicting reproductive status in the southern flounder *Paralicthys lethostigma* by including regularly collected biological information such as ovarian morphology, sampling dates and fish length in predictive models. Similarly, using only morphological measures to discriminate sexually mature individuals from immature juveniles achieved a 72-80% correct classification in the American eel *Anguilla rostrata* (Cottrill et al., 2002). Nonetheless, the

power of these models to predict the maturity stage performed well only if information regarding gonadal development such as the gonadosomatic index (GSI) or gonadal index (GI) were included. Usually, the GSI represents a measure of the relative gonadal weight to the fish weight while the GI to the length, and they are extensively applied to investigate the timing of reproduction (Lowerre-Barbieri et al., 2011). In recent years the application of these indices has been extended further and indeed adopted as a main predictor of the reproductive status in order to identify spent, actively spawning and immature fish (Flores et al., 2015; McPherson et al., 2011).

In the present study, the GI was applied as a reliable predictor to discriminate sexually mature from immature females in the Mediterranean swordfish (*Xiphias gladius*) by using a logistic multinomial model that has been successfully used also in other species (Flores et al., 2019; McPherson et al., 2011). The swordfish is a cosmopolitan highly valuable commercial target species with extensive seasonal migrations and this migratory behaviour in addition to a challenging management (i.e. dealing with illegal, unreported and unregulated fishing activities) led to severe overfishing of the Mediterranean stock (ICCAT, 2016b). Indeed, recent estimates of the swordfish stock status in this area indicated a 40% reduction in spawning stock mass over the past 20 years which, combined with high fishing mortality rates, gave rise to “non-negligible” risks of rapid future declines in the stock (ICCAT, 2008, 2011). In 2016, in order to recover the Mediterranean stock, the International Commission for the Conservation of Atlantic Tunas (ICCAT) established a multi-annual plan based on key measures such as Total Allowable Catches (TAC), fishing fleet reduction, closed fishing season and a minimum landing size (ICCAT, 2016a). In this context, a clear understanding of the swordfish reproductive biology, spawning patterns and fecundity is necessary in order to support policy-makers to make improvements in current management strategies and to incorporate such information into future stock assessment modelling (Young et al., 2006). Indeed, Neilson et al. (2013) demonstrated that the integration of physiological responses (i.e. reproduction) with stock assessment modelling achieved the rebuilding of the Atlantic swordfish stock. In the Mediterranean Sea, the swordfish spawning season is limited to the summer months and has one of the highest estimated batch fecundities compared with other areas (de la Serna et al., 1996). Ovaries exhibit ovigerous lamellae, asynchronous oocytes development and spawning occurs in multiple batches. Despite previous studies that investigated swordfish reproduction in the Mediterranean Sea (Alıçlı et al., 2012; Corriero et al., 2004, 2007; de la Serna et al., 1996; Macías et al., 2005;

Megalofonou et al., 1995), comprehensive information on reproductive strategies and spawning areas are far from being firmly established.

To improve our understanding of the Mediterranean swordfish reproductive biology, a multi-annual survey (2016-2018) in the central and western Mediterranean Sea was conducted in collaboration with fishermen. The main goal of the study was to develop a suitable histologically-calibrated method to classify immature, mature-spawning and mature-post-spawning females based on the GI following a thorough analysis of this index as a reliable measure of the reproductive status of this species. Finally, spawning patterns and gonadal development were better defined in addition to identifying specific histological markers of different reproductive stages according to previous studies (Brown-Peterson et al., 2011; Corriero et al., 2004; de la Serna et al., 1996; Macías et al., 2005).

1.2. Material and methods

1.2.1. Sampling activities and laboratory processing

Ovary samples were collected from 148 specimens caught by commercial longliners (n=136) and as by-catch in tuna traps (n=12) during the period 2016-2018 in the central (n=103) and western (Balearic islands, n=45) Mediterranean Sea. The number of individuals caught by month was: May (n=15), June (n=30), July (n=35), August (n=20), September (n=27), October (n=11), December (n=10). Ethical approval was not necessary as samples were collected from individuals caught for commercial purposes. Soon after capture, for each specimen, the Lower Jaw to Fork Length (LJFL), Total Length (TL), Gonad Weight (GW), Total and Gutted Body weight were recorded. Small ovary portions (2 cm^3) were excised and fixed in a formaldehyde/glutaraldehyde solution (NaH_2PO^{4-} H_2O+NaOH+Formaldehyde 36.5%+Glutaraldehyde 25%+H_2O) and kept at +4°C until histological analysis. Briefly, following Gioacchini et al. (2019), samples were dehydrated in a series of increasing ethanol baths, cleared in xylene and embedded in paraffin. Sections of 5 µm wide were cut with a microtome (model RM2125 RTS, Leica Biosystems Wetzlar, Germany), stained with Mayer's haematoxylin/eosin and examined under a microscope (Axio Imager 2, Zeiss, Germany). Assessment of the reproductive status of samples was performed according to the modified scale of Brown-Peterson et al. (2011) and to previous works on the Mediterranean swordfish reproduction (Carnevali et al., 2019; Corriero et al., 2004; Macías et al., 2005; Ortiz-Delgado et al., 2008). The individuals caught during the reproductive season were assigned to five stages: i) immature, ii) developing (vitellogenic),

iii) spawning, iv) post-spawning, and v) regenerating (spent). Briefly, immature individuals shows perinucleolar oocytes as the most advanced stage, without any signs of oil droplets in the cytoplasm surrounded by flat follicular cells forming a single layer around the oocytes, scarce connective tissue and absence or rare follicular atresia. Developing ovaries exhibits oocytes from lipid to late vitellogenesis stage, absence of postovulatory follicles (POFs) and minor follicular atresia. The spawning phase is characterized, in addition to vitellogenic oocytes, by oocytes at the final maturation stages (i.e. germinal vesicle migration, coalescence of yolk globules, ooplasm hydration, detachment of the follicular cells), POFs and higher amount of atresia. Post-spawning females displays a considerably lower number of vitellogenic oocytes undergoing atresia, extensive follicular atresia and occasionally POFs. Regenerating females exhibits ovaries with basophilic perinucleolar and few lipid droplets oocytes, scarce to null follicular atresia and absence of POFs.

1.2.2. Size at first maturity (L_{50})

Size at first maturity was estimated from swordfish females caught only during the reproductive season from May to August (n=100) according to previous authors (de la Serna et al., 1996; De Metrio et al., 1989; Macías et al., 2005). The classification of the maturity stage was based on histological assessment. The proportion of maturity at length (PL) was estimated with the following logistic function:

$$\mathrm{PL} = \frac{1}{1 + \exp(\alpha + \beta * LJFL)}$$

where α (intercept) and β (slope) represents the estimated parameters. The proportion of observed mature individuals was calculated in LJFL intervals of 15 cm. The length at which 50% of the females are mature was computed as L50= - α / β. Significance of the estimated parameters was tested with the Wald test.

1.2.3. Gonadal index (GI)

In order to establish the reproductive status of specimens the GI was calculated according to Hinton et al. (1997) using the LJFL as the measure of length as follows:

$$GI = \frac{\ln(GW)}{\ln(LJFL)}$$

with GW indicating the weight of the gonad in grams.

The relationship between LJFL with the gonad weight was modelled as $GW=a_i LJFL b_i$ with a and b representing the estimated parameters for each reproductive stage (DeVlaming et al., 1982). This relationship was log-transformed as $\log(GW) = \log a_i + b_i \log(LJFL)$ in order to linearize the function and the model was selected by residual analysis. The intercepts of the regression lines (a_i=0) and the isometry of the slope bi (b_i=1) among maturity stages, which reflects the independence of ovarian weight and size, were checked with the t-test. Moreover, the homogeneity of the slopes among maturity stages was assessed using a covariance analysis (deVlaming et al., 1982; Erickson et al., 1985) and homogeneity of variance was checked with the Levene's test. The aforementioned relationship between length and ovarian weight was also checked for $GW= b_i(LJFL)^3+a_i$, corresponding to gonadasomatic index calculated as $GI=10^4*GW/LJFL^3$ according to Kume & Joseph (1969), in order to obtain unbiased estimates of the GI.

Immature, mature active (developing + spawning) and mature post-spawning individuals were discriminated by multinomial logistic regression (MLR) which computes the conditional probability of an individual being in the y_i maturity class using the GI as a single predictor (Flores et al., 2019; McPherson et al., 2011). The mature spawning category was considered the reference and each of the other categories were compared with this baseline. As recommended by Flores et al. (2015), individuals caught towards the end of the spawning season (i.e. September) were not considered for this analysis since the GI of mature post-spawning individuals and that of immature individuals is similar. The method was developed and applied on the same females included in the L_{50} calculation. The method applied the following formula:

$$P(Y = y_j|GI)\frac{\exp(\alpha_j + \beta_j * GI)}{\sum_{h=1}^{J}\exp(\alpha_h + \beta_h * GI)}$$

where αj is the intercept, βj is the slope of the corresponding GI and j= {1, 2, ... , J} represents the histology stages. The statistical significance of estimated parameters was tested with the Wald test. In this context, the $GI_{cut\text{-}off}$ score can be defined as the test score at which an individual is as likely to be in category *j* as in category *j*+1. Hereafter this method of classification is referred to as $GI_{cut\text{-}off}$ method. The Cohen's k coefficient (Cohen, 1960) was

applied to assess the agreement between the histological, considered as the reference baseline, and the GI-based classification (Gerritsen & McGrath, 2006). Goodness of fit was assessed using the McFadden pseudo-R square (R^2MF) (McFadden, 1974). The comparison of the L_{50} between the $GI_{cut\text{-}off}$ and the histology-based method was assessed using the likelihood ratio test. All statistical analyses were performed in the R statistical environment (R Core Team, 2018).

1.3. Results

1.3.1. Histological analysis: classification and patterns

The female reproductive cycle of the Mediterranean swordfish consisted of five ovarian reproductive stages based on the histological evaluation of several markers such as oocyte stages, follicular atresia, POFs, amount of connective tissue, thickness of the ovarian wall (Fig. 1; Supporting information 1) and according to previous authors (Corriero et al., 2004; Macías et al., 2005; Ortiz-Delgado et al., 2008). Ovaries from immature individuals (i.e. never spawned) exhibited compact ovigerous lamellae with groups of oogonia, chromatin-nucleus and perinucleolar oocytes surrounded by connective tissue and muscle bundles, as to create bunches (Fig. 1A). Developing ovaries, which entered the gonadotropin-dependent phase, were characterized by the presence of oocytes at different stages of vitellogenesis as well as previtellogenic oocytes, corresponding to multiple batches of oocytes that will be spawned during the reproductive season (Fig. 1B). Vitellogenic oocytes displayed yolk granules that firstly concentrated in the peripheral cytoplasm and then multiplied and increased in size forming a densely packed zone in the inner part of the cytoplasm. The lipid droplets enlarged and continued to occupy the inner mid-part of the cytoplasm. In this stage, the zona radiata becomes very prominent in the larger oocytes measuring 10–45 μm in thickness and showed a fine striated appearance. The spawning ovaries were characterized by the presence of fully grown vitellogenic oocytes and/or mature oocytes as well as post ovulatory follicles (POFs) (Fig. 1C). During this stage the germinal vesicle of maturing oocytes migrates towards the animal pole and marks the resumption of

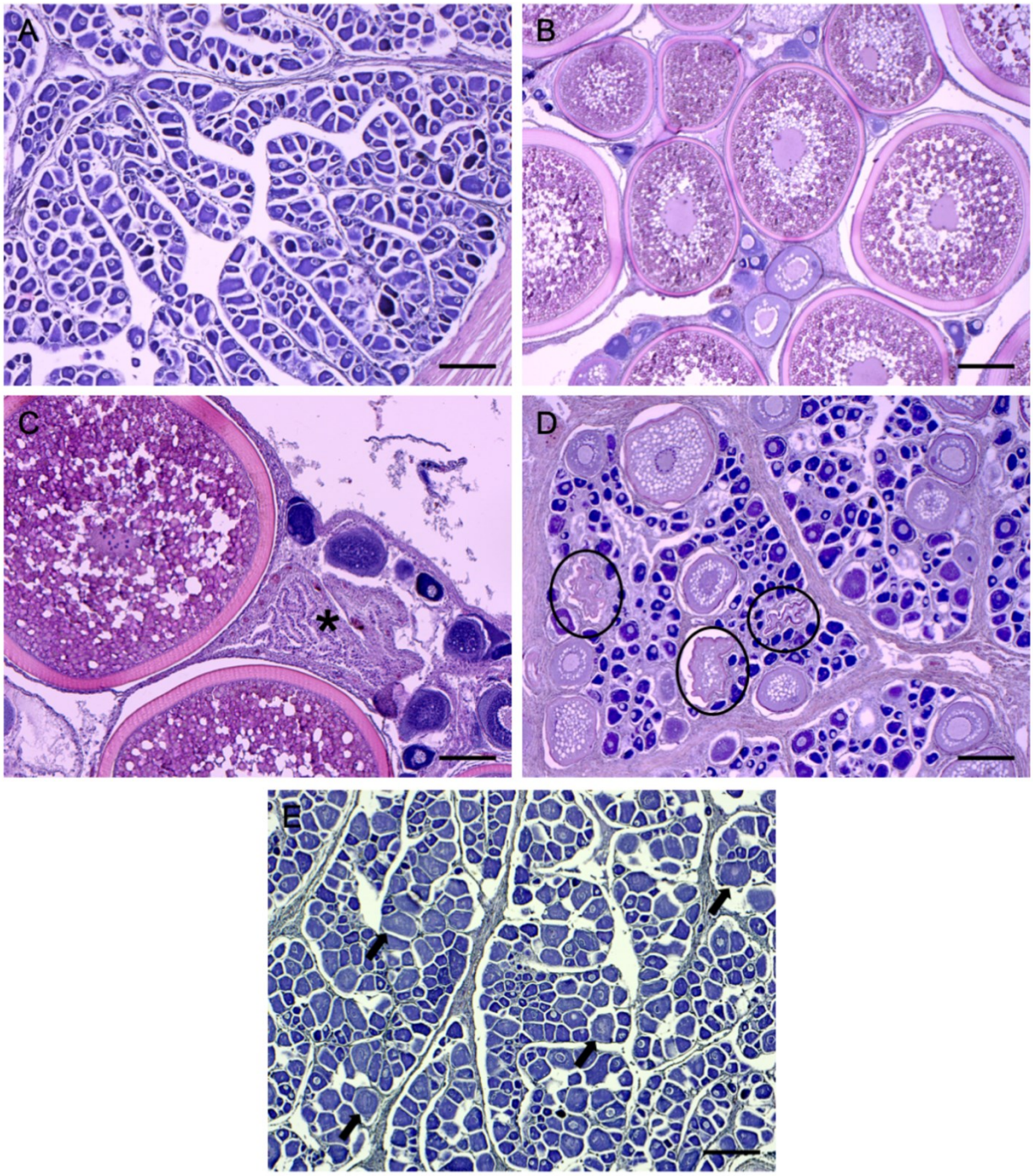

Figure 1. Representative histological images of ovaries for each reproductive stage: (a) immature stage with compact pre-vitellogenic oocytes and little space between ovigerous lamellae; (b) developing stage displaying perinucleolar, lipid, and vitellogenic oocytes; (c) spawning stage with a post-ovulatory follicle (POF, marked by an asterisk) beside fully grown vitellogenic oocytes; (d) post-spawning stage exhibiting a reduced number of vitellogenic oocytes and atretic oocytes (circles); (e) regenerating stage with early lipid stage (arrows) and perinucleolar oocytes. Scale bars: 200 μm

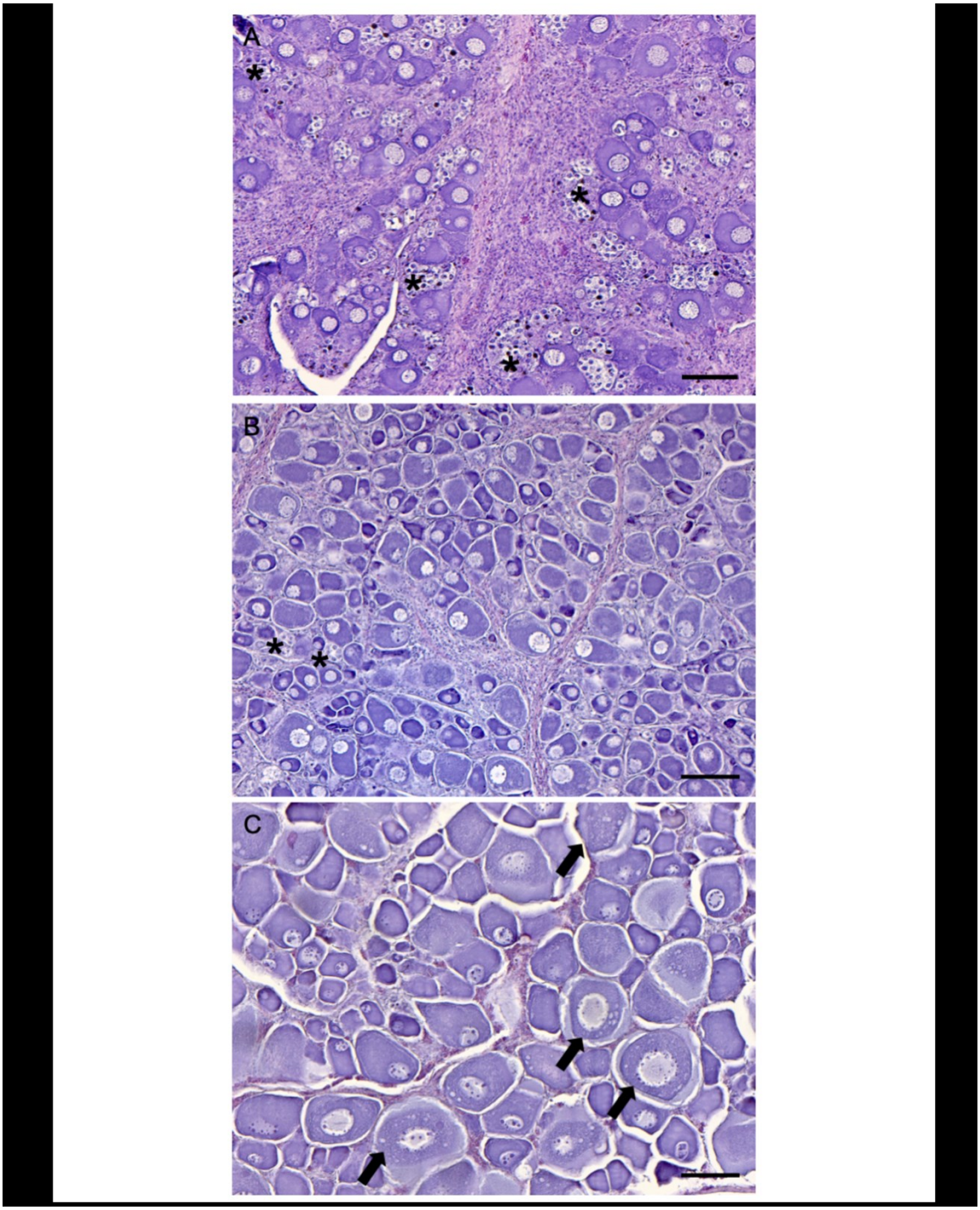

Figure 2. Histological sections of previtellogenic ovaries from sexually immature females (A,B) and sexually mature females (C). Asterisks= oogonial nests, arrows=early lipid stage oocytes. Bars= 100 μm

meiosis. Hydrated oocytes lost their spherical shape as a result of the dramatic hydration process that precedes the spawning event and lipid droplets fused to form a single or few oil droplets while the cytoplasm appeared homogeneous (Supporting information 1).

Extensive atresia, occasionally residual POFs and a greatly reduced number of vitellogenic oocytes undergoing atresia were found in the post-spawning phase according to Macías et al. (2005) (Fig. 1D).

During the winter months, it was possible to discriminate regenerating ovaries (i.e. spent) from those of sexually immature females and a continuum of features was identified (Fig. 1E and Fig. 2). Indeed, ovaries from sexually immature females of the smallest sizes examined in this study (80-90 cm) exhibited multiple evident oogonial nests and the oocytes identified were exclusively at the chromatin nucleus stage (Fig. 2A). Slightly larger females were characterized by more abundant perinucleolar oocytes and a smaller fraction of clearly visible oogonia (Fig. 2B). Lastly, regenerating ovaries exhibited oocytes of more heterogeneous sizes made up of perinucleolar oocytes and few larger early lipid stage oocytes (Fig. 2C). These early lipid stage oocytes in regenerating ovaries were interpreted as a marker of individuals that reached sexual maturity, since the previtellogenic (primary growth) oocytes of immature fish do not display lipid droplets (Berkovich et al., 2013; Chen & Ge, 2013; Grier et al., 2009).

Females at different ovarian maturation stages caught during the entire sampling period were grouped into 10 cm size classes (Fig. 3A). The separation of sexually immature females from those that reached sexual maturity was evident and fitted well with the estimated L50 (see next section). Females above 160 cm LJFL were always found to be mature. Immature individuals were caught over the entire period (Fig. 3B), with the highest numbers in May (n=7, 50%) and August (n=9, 46%). A slight increase of post-spawning females was observed in August (n=3, 15%). During the month of September, in addition to immature females (n=23, 85%), all the sexually mature samples examined were found to be in the post-spawning phase (n=4, 15%). Immature females were caught also during October (n=6, 55%) and December (n=8, 80%). During October (n=5, 45%) and December (n=2, 20%) the regenerating females were found. The relative reproductive stages by geographical area are listed in the Table S1.

1.3.2. Size at first maturity (L_{50})

Size at first maturity based on histological analysis was found to be 131.5 cm (Fig. 4). Estimated parameters of the logistic regression were statistically significant ($p<0.05$) and are summarized in Table 1. The size of individuals included in this analysis were considered representative with LJFL lengths ranging from 80 cm to 240 cm. The value of R^2MF was 0.64.

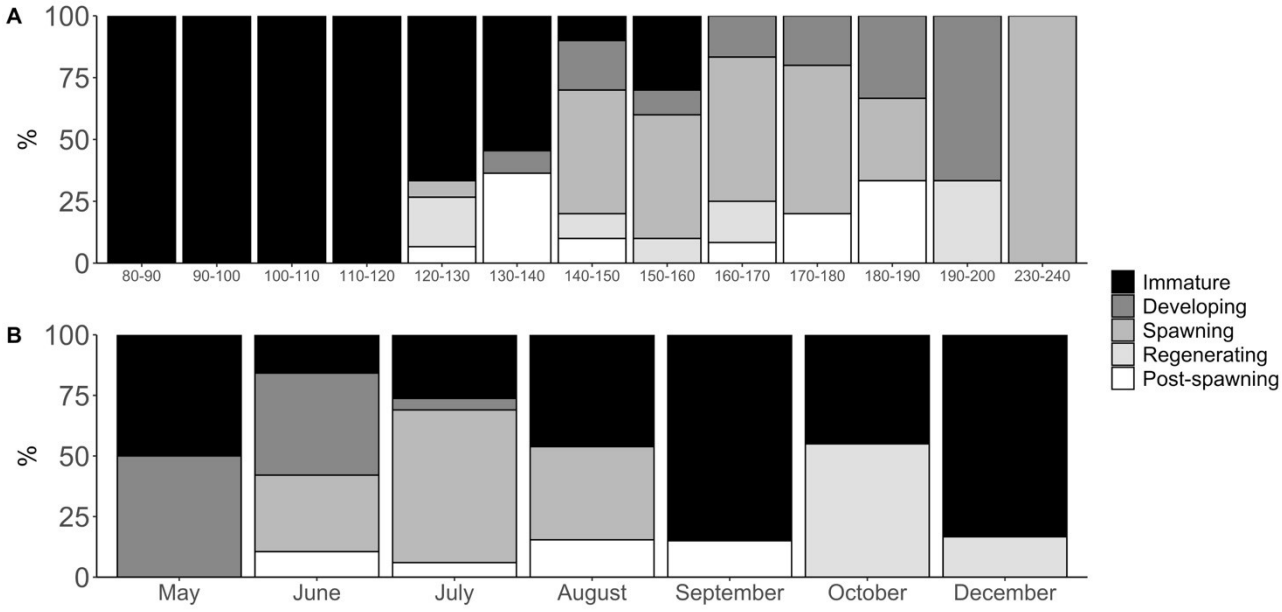

Figure 3. Relative frequency of reproductive stages for each size class (A) and during summer months (B)

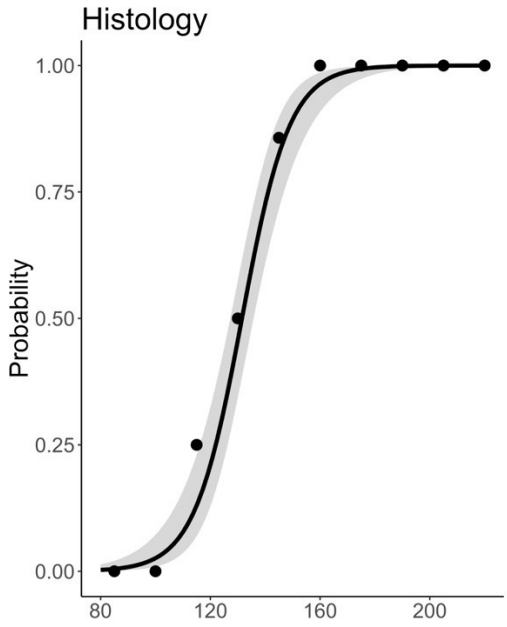

Figure 4. Estimated L50 and observed proportion of mature individuals at each 15-cm length class for histology-based classification. The grey area corresponds to the 95% confidence interval

Table 1. Summary of the L50 estimates based on histological data

L50 Histology				
Coefficient	Estimate	Standard Error	z value	*p* value
α	-15.14	4.28	-3.53	0.00041
β	0.11	0.03	3.69	0.00022

1.3.3. GI

GI exhibited the highest peak between June and July with a subsequent sharp decrease in August and September (Fig. 5). As expected, greater GI values were found in developing and spawning stages while immature and post-spawning stages had a clear overlap of the GI around the value of 1 (Fig. 6).

Isometry (b_i=1) of the log-log regression slope was confirmed for all the maturity stages (Table 2) except for the developing stages in which the p-value of the regression slope was slightly below the threshold of 0.05 (p=0.049). Analysis of covariance showed that regression slopes from the different ovarian stages were homogeneous (p>0.05). Intercepts of the regression slopes were not significantly different from 0 for any of the maturity stages. Moreover, the variance was found to be homogeneous among stages. Therefore, all the assumptions as recommended by deVlamming et al. (1982) were considered as met and the GI= ln(GW)/ln(LJFL) was considered an unbiased estimator of reproductive activity for the Mediterranean swordfish. On the other hand, for the relationship GW = b(LJFL)^3 + a, the regression slopes among maturity stages were not homogeneous according to the analysis of covariance (p<0.05). For each stage the regression slope was significantly different from 1 ($b_i \neq 1$) and the intercepts were not significantly different from 0 (p>0.05) (Table 3).

Estimated parameters of the multinomial model were statistically significant (p<0.05) and the probability of an individual being in each maturity stage is displayed in Fig. 7. The $GI_{cut\text{-}off}$ value to discriminate immature from mature spawning females was found to be 1.14. Using this value as the threshold, the females previously classified through histology were re-classified with the $GI_{cut\text{-}off}$ method and the logistic curve was computed (Fig. 8). The parameters of the logistic model based on the $GI_{cut\text{-}off}$ classification were statistically significant (Table 4) and the size at first maturity calculated was 133.3 cm while the R2MF was 0.66. The L_{50} calculated with histology and the $GI_{cut\text{-}off}$ were not significantly different (p>0.05). Overall, the agreement between $GI_{cut\text{-}off}$ and histological data was 90% and the

Cohen's k was 0.79 (95% confidence interval: 0.63-0.95), which corresponds to a "considerable" level of agreement (Landis & Koch, 1977).

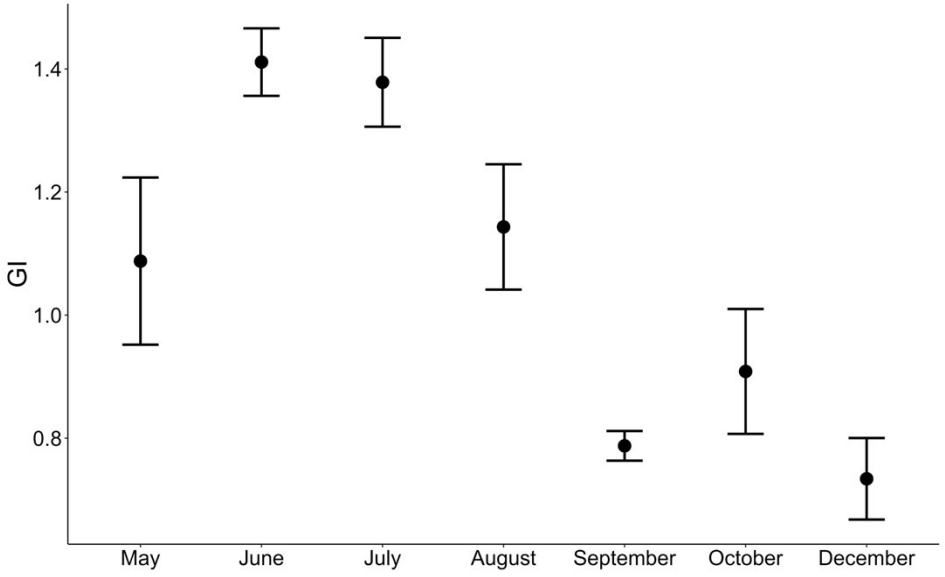

Figure 5. Mean monthly GI values and relative standard errors (vertical bars) of individuals caught in central and western Mediterranean Sea.

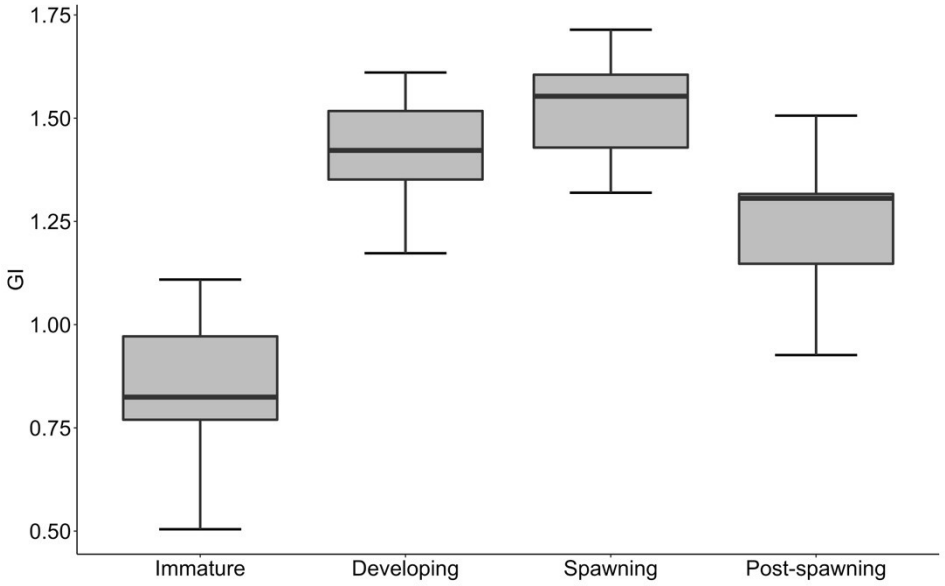

Figure 6. Box plot showing the gonadal index (GI) values for each reproductive stage, classified according to histological observations.

Table 2. Summary of the log-log relationship between length and ovarian weight. Asterisks correspond to the results of t-test: (*)= p<0.05, (**)= p<0.01, (***)= p<0.001

	*log(GW)=b*log(LJFL)+a*	
Stage	b ± s.e.	a ± s.e.
Immature	3.28 ± 1.18	-11.52 ± 5.60
Developing	4.59 ± 1.54*	-16.27 ± 7.94
Spawning	2.72 ± 0.95	-6.06 ± 4.86
Post-spawning	3.68 ± 2.65	-12.4 ± 13.51

Table 3. Summary of the relationship between the cube of the length and ovarian weight. Asterisks correspond to the results of t-test: (*)= p<0.05, (**)= p<0.01, (***)= p<0.001

	$GW=b*(LJFL)^3+a$	
Stage	b ± s.e.	a ($x10^{-2}$) ± s.e.
Immature	0.00005 ± 0.000014 ***	-0.086 ± 0.27
Developing	0.0005 ± 0.0001 ***	-7.66 ± 10.73
Spawning	0.0007 ± 0.0001 ***	-4.22 ± 7.17
Post-spawning	0.00005 ± 0.0001 ***	5.81 ± 7.94

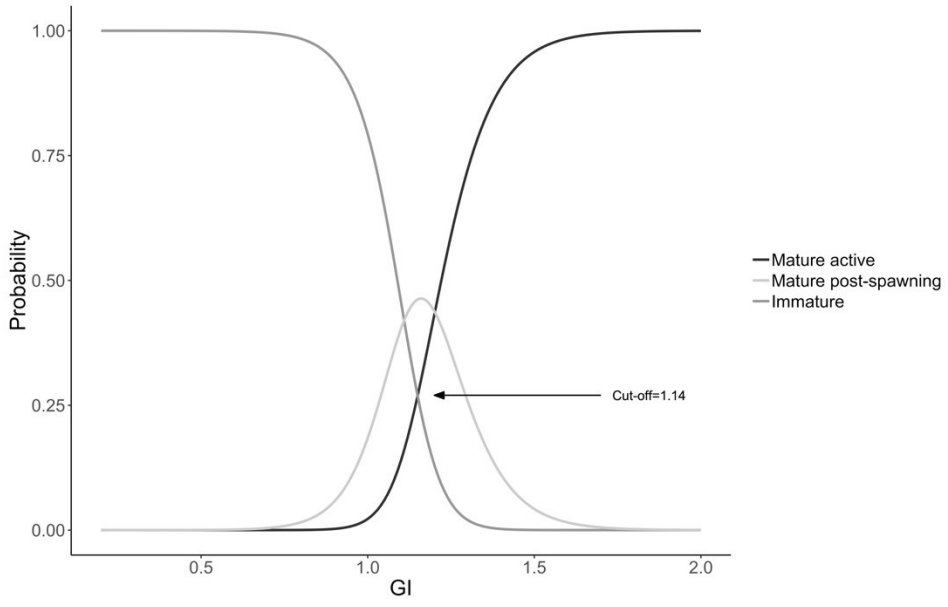

Figure 7. Results of the multinomial regression model representing the probability of individuals being immature, mature active, and mature post-spawning, based on the gonadal index (GI) value. The arrow corresponds to the $GI_{cut\text{-}off}$ value of 1.14.

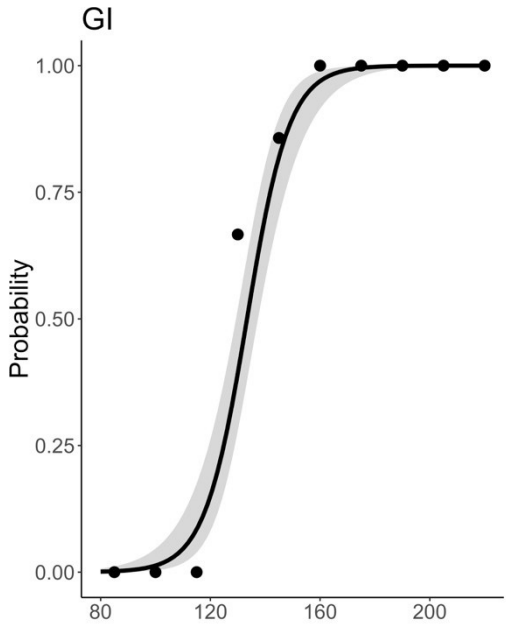

Figure 8. Estimated size at first maturity (L50) and observed proportion of mature individuals at each 15-cm length class for GI-based classification. The grey area corresponds to the 95% confidence interval.

1.4. Discussion

Establishment of reproductive dynamics and the size at first maturity of a species of commercial interest represents one of the keystone points of fishery stock assessment methods and allows policy-makers to define stock management strategies as well as conservation efforts (Powers & Fromentin, 2005; Enberg et al., 2009). Indeed, fisheries management relies on effective scientific advices to reduce the uncertainty of the decision-making process and to achieve sustainable exploitation levels of natural resources.

In this study, we found that sexually immature individuals were extensively caught over the entire spawning season and this represents a significant limitation for the success of the current recovery plan for the Mediterranean swordfish (ICCAT, 2016a). Indeed, it was found that 75-80% of total swordfish catches in the central Mediterranean Sea between May and September were in the range of 100-140 cm (Lombardo et al., 2017; Pignalosa et al., 2019), most below the size at first maturity of females estimated at 131.5 cm in the present study. Within these catches, it is likely that a percentage of about 50% is represented by females according to sex ratio observations in the central Mediterranean Sea (Garibaldi, 2015;

Mariani et al., 2015). Yet, previous authors found greater L_{50} values around 140 cm in the Western Mediterranean (de la Serna et al., 1996; Macías et al., 2005). This finding might be explained by the presence of larger migrators from the Atlantic Ocean able to enter the Western Mediterranean through the Strait of Gibraltar to reproduce (Abid et al., 2019) as well as by differences in sea surface temperature (SST) within the Mediterranean basin during the summer months, a well-known factor influencing the reproduction (Karakulak et al., 2004; Pastor et al., 2019; Poisson & Fauvel, 2009; Reglero et al., 2012). Nevertheless, all the L_{50} calculations are well above the current ICCAT minimum size of 100 cm and this key point should be revaluated soon to achieve a successful recovery of the Mediterranean swordfish stock.

The peak of the spawning season was between June and July as confirmed by the highest GI values and by an increase of spawning individuals, a finding in agreement with previous studies (Corriero et al., 2004; Macías et al., 2005; Tserpes et al., 2008). Therefore, this period represents a critical time window for the reproduction of the Mediterranean swordfish while since only post-spawning females were found in September displaying a dramatic drop in the GI values, this month seems to be confirmed as a non-reproductive period (de la Serna et al., 1996; Macías et al., 2005). Furthermore, the existence of potential spawning grounds was suggested around the Balearic Islands, the Tyrrhenian, Ionian and Levantine Seas (Cavallaro et al., 1991; Damalas & Megalofonou, 2014; Rey & Alot, 1988; Romeo et al., 2011; Tserpes et al., 2008). Thus, a broader and comprehensive sampling effort, with a specific focus on the young-of-the-year, would be required to properly identify and confirm these spawning areas as well as to fully unveil the reproductive dynamics underlying these grounds. Filling such a gap of knowledge in the Mediterranean Sea will have the potential to define the proper timing for fishing closure on spawning grounds and revaluate the current fishing closure adopted by ICCAT with a sound scientific background.

Although the gonadal index is extensively applied to measure fish reproductive activity, its validity for comparing individuals at different maturity stages has been questioned and their use is recommended only upon thorough validation (DeVlaming et al., 1982; Somarakis et al., 2004). In this study, the suitability of the gonadal index GI= ln(GW)/ln(LJFL) as a measure of the reproductive activity among maturity stages in the Mediterranean swordfish was confirmed. The relationship between ovarian weight and body length displayed isometry among all maturity stages suggesting that ovarian growth is independent of fish size irrespective of the reproductive stages, a condition that in fish is met only at advanced reproductive stages (Flores et al., 2015; McPherson et al., 2011). Nevertheless, calculating

the gonadosomatic index as $GI=10^{4*}GW/(LJFL)^3$ is not be recommended for the Mediterranean swordfish due to the dependence of ovarian weight with both body size and reproductive stages and therefore representing a biased index to compare individuals of different length or maturity condition. The initial validation of the GI is a necessary step in order to subsequently test and apply the $GI_{cut\text{-}off}$ method (Flores et al., 2019). In this study, the application of the $GI_{cut\text{-}off}$ method yielded reliable and consistent results with remarkable rates of agreement with the histological classification as reported by the relatively high Cohen's k coefficient. Similar results using the GSI as a histology-calibrated macroscopic method were achieved also in other species and this would confirm the suitability of this approach as a predictor of fish maturity stage (Flores et al., 2019; McPherson et al., 2011; Somarakis et al., 2004). Interestingly, the Mediterranean swordfish GI threshold of 1.14 was smaller than that of 1.37 for the Pacific swordfish (Hinton et al., 1997) and this phenotypic variation likely reflects a different balance between growth and reproduction as well as distinct environmental condition of the two areas in terms of biotic (i.e. food availability) and abiotic (i.e. temperature) factors (Sala-Bozano & Mariani, 2011). The application of macroscopic staging of gonads to assess fish reproductive status is extensively applied because represents a practical and fast approach needed in the field during non-optimal sampling activities (West, 1990). Nonetheless, macroscopic approaches suffer from major drawbacks such as assessors variability, misclassification and misestimation of mature individuals with considerable bias in the evaluation of the spawning biomass and maturity ogive (Gerritsen & McGrath, 2006; McPherson et al., 2011). For instance, Vitale et al. (2006) found that macroscopic staging overestimated the female spawning stock biomass by up to 35 % for Atlantic cod *Gadus morhua* in the Kattegat (Baltic Sea) while Midway & Scharf (2012) revealed that visual staging overestimated the size at first maturity of the southern flounder *Paralichthys lethostigma* with a misclassification of about 40% of developing (vitellogenic) ovaries. For these reasons histological validation is strongly encouraged and suggested as a routine quality check to ensure a proper calibration between visual (i.e. macroscopic) and microscopic staging (Ferreri et al., 2009; Tomkiewicz et al., 2003). Indeed, in this study, the thorough histological assessment of the samples served as the reference baseline for the initial model development phase. Therefore, following initial calibration between histology and the $GI_{cut\text{-}off}$ method and subsequent testing, only periodic re-calibration would be required to adjust for changes in the relationship between GI and maturity. This would be especially needed for those stages which exhibited higher error classification rates such as developing and post-spawning ovaries.

Maturity-based indicators represent a foundation for conservation measures and can be considered as a reference point for practical assessment of fisheries status and trends (Froese, 2004). The histology-calibrated macroscopic $GI_{cut\text{-}off}$ method depicted here is inexpensive, accurate and easy to calculate, therefore has the potential to be widely applied in the swordfish Mediterranean fishery. The application of the method will help the refinement of future spawning stock biomass estimates in the Mediterranean basin as well as defining the GI threshold that discriminates immature from spawning individuals also from other areas such as the Levantine Sea. Moreover, following proper validation, the method could be extended further and applied to other swordfish stocks such as those under the management of different regional agencies to enhance future monitoring programs. The increasing application of methods using gonadal indices as a predictor of maturity also for other species would confirm its suitability as well-performing predictive macroscopic method and this is especially needed in data-lacking fisheries (Flores et al., 2019; Hossain et al., 2012). Field sampling would benefit from this method due to the simple requirements of this approach which is only based on the ovarian weight and length of the specimens, two parameters that could be routinely collected during fish sampling surveys.

Taken together, the reproduction timing, the L_{50} estimations, the high percentage of immature females examined and the developed $GI_{cut\text{-}off}$ method developed by the current study, represent a valuable information for a science-based decision-making process to establish suitable management measures aimed at tackling the ongoing stock decline and to accomplish the rebuilding of the Mediterranean swordfish stock.

1.5. Data availability

All the Supplementary material can be found at the online version of the article at https://doi.org/10.1002/aqc.3248.

1.6. References

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2. A *de novo* transcriptome assembly approach elucidates the dynamics of ovarian maturation in the swordfish (*Xiphias gladius*)

Giorgia Gioacchini, Luca Marisaldi, Danilo Basili, Michela Candelma, Paolo Pignalosa, Riccardo Aiese Cigliano, Walter Sanseverino, Gary Hardiman & Oliana Carnevali (2019). Scientific Reports, 9, 7375. doi: https://doi.org/10.1038/s41598-019-43872-6

2.1. Introduction

The swordfish (*Xiphias gladius* Linnaeus, 1758) is a large, solitary, fast-swimming and highly migratory species with a worldwide distribution. The unique phenotype of this species, in addition to its peculiar heat and lubricating organs (De Metrio et al., 1997; Videler et al., 2016), is thought to contribute to its exceptional predatory behaviour and swimming capacities, which make the swordfish one of the fastest swimmers in the pelagic realm. Owing to the fact that the swordfish is a species of strong commercial interest, valuable fisheries were established from the late 1950s. However, since that time, robust management plans have been lacking due to the scarcity of information on reproduction, growth, sexual maturity and migratory behaviour. Such a lack of knowledge is also caused by clear logistic constraints associated with collecting samples in pelagic areas which often require collaboration with fishermen and ad-hoc sampling surveys. According to a recent stock assessment report by the International Commission for the Conservation of the Atlantic Tunas (ICCAT)(ICCAT, 2016a), the Mediterranean swordfish stock was classified as “overfished and currently suffering overfishing” and a recovery plan was subsequently established including measures such as Total Allowable Catches (TAC), fishing fleet capacity limitations, closed fishing season and a minimum size (ICCAT, 2016b). Nonetheless, the current size of minimum catch of 100 cm (Lower Jaw to Fork Length, LJFL) set is far below the 140 cm (LJFL) size of first maturity (L_{50}) for the Mediterranean swordfish as found by de la Serna et al. (1996). Previous studies provided insights into swordfish reproductive biology in the north-western Atlantic (Arocha, 2002) and in the Mediterranean area (Abid et al., 2019; Macías et al., 2005), however, they do not provide comprehensive details on gonadal development and puberty onset necessary to determine the reproductive potential and to assess the status of the stock. Accordingly, deeper knowledge on swordfish

gonad development is required to establish the duration of the spawning season, spawning pattern and reproductive dynamics as well as to deliver scientific data to policy makers in order to improve current stock management models.

In fish, puberty occurs following gonadal sex differentiation and is characterized by the capacity of fish to produce for the first time in its life, mature gametes (Patiño & Sullivan, 2002). It is well known that the hypothalamus-pituitary-gonadal (HPG) axis plays a key role in regulating puberty in vertebrates (Plant, 2015). Gonadotropin releasing hormone (GnRH) from the hypothalamus stimulates the synthesis and release of the gonadotropins follicle stimulating hormone (Fsh) and luteinizing hormone (Lh) from the anterior pituitary, which in turn act on the gonads inducing oogenesis and spermatogenesis mainly through the stimulation of gonadal steroidogenesis. Although this cascade has been well characterized in many fish species (Nagahama et al., 2008; Zohar et al., 2009), the mechanisms underlying the onset of puberty are not fully understood. Since gonadal development is not entirely modulated by upstream signals, it might be postulated that key factors involved in puberty onset should be investigated at the gonad level possibly acting via autocrine/paracrine mechanisms. Here, we generated a high-quality transcriptome assembly using an RNA-sequencing approach in order to investigate local molecular dynamics driving puberty onset in the swordfish. As a major focus of this study, a pathway-based analysis was employed to specifically depict the ovarian steroidogenesis and vitellogenin synthesis and uptake molecular networks. In addition, by exploring the phylogenetic relationship of the Low-Density Lipoprotein Receptor (Ldlr) superfamily a candidate vitellogenin receptor was identified. This work represents the first swordfish de novo transcriptome assembly and such findings provide the additional knowledge needed to refine current ICCAT recovery plan towards a successful conservation of the Mediterranean swordfish.

2.2. Materials and Methods

2.2.1. Sample collection and experimental design

Swordfish were captured in July 2017 in the central Mediterranean Sea (38°22'66"N; 12°27'30"E) by longliners. Ovary, stomach, intestine, and liver samples were collected from a total of 10 individuals, placed in RNAlater (Ambion, Austin, TX, USA), stored at 4°C for 16 h and then transferred to -20°C until RNA extraction. Small ovary pieces (2 cm^3) were fixed in formaldehyde-glutaraldehyde and stored at 4°C until histological analyses in order to

confirm the reproductive status of each specimen. The fish were caught for commercial purpose and ovaries samples were collected according to ICCAT guidelines for biological sampling. The procedures did not include animal experimentation, and ethics approval is not necessary in accordance with the Italian legislation (D.L. 4 of Mars 2014, n. 26, art. 2).

2.2.2. Histological assay

Ovaries were processed as described in Forner-Piquer et al. (2018). Briefly, after fixation in formaldehyde-glutaraldehyde, samples were dehydrated in a series of alcohol baths, cleared in Xylene and finally embedded in paraffin. Sections 5 µm thick were cut with a microtome and stained with Mayer's haematoxylin–eosin. The histological slides were observed under a Zeiss Aixio Imager M2 microscope and microphotographed with a high-resolution camera Zeiss Axiocam 105 color. Female samples were classified as mature or immature depending on the most abundant oocyte developmental stage (see Supplementary Fig. S2). Immature female size and gutted weight were 111 ± 3.4 cm (LJFL) and 14.8 ± 2.1 Kg, respectively, while size and gutted weight of mature female were 154.6 ± 9.6 cm (LJFL) and 49.2 ± 6.3 Kg, respectively.

2.2.3. RNA isolation, library preparation and sequencing

Total RNA was isolated from the following tissues: 3 ovaries from immature females and 3 ovaries from mature females, 3 livers from immature females and 3 livers from mature females, 3 livers from mature males, 1 stomach and 1 intestine both from mature males. Isolation of total RNA was carried out by RNeasy Plus Mini Kit (Qiagen, Dusseldorf, Germany) according to the manufacturer's instructions. The RNA integrity and concentration were determined by Nanodrop-2000 spectrophotometer (Thermo Fisher Scientific, USA) and Agilent 2100 Bioanalyzer (Agilent, Santa Clara, CA). Genomic DNA was removed by applying RNase-free DNase I (Qiagen) after a treatment of 30 min at 37°C. Libraries were created with the Illumina TruSeq Stranded mRNA Library Prep Kit and then sequenced with an HiSeq2500.

2.2.4. *De novo* transcriptome assembly and annotation

Illumina paired-end 150 bp reads from 17 *X. gladius* samples were processed to produce the transcriptome assembly. Raw reads were trimmed and clipped with BBDuk (DOE Joint Genome Institute, n.d.) setting a minimum Phred-like quality of 35 and a minimum length of 35 nucleotides. The quality of the reads before and after trimming was checked with the software FASTQC (Andrews, 2010). Possible contaminant reads were removed using the GAIA metagenomics suite (www.metagenomics.cloud) using a database of bacteria, archaea, virus, protists and fungi as reference. High quality reads were then normalized with Trinity (Grabherr et al., 2011) using the following options: --SS_lib_type RF --pairs_together --max_cov 50. De novo transcriptome assembly was then performed with Trinity using the options: --SS_lib_type RF --no_normalize_reads --min_kmer_cov 2 --KMER_SIZE 32 --min_per_id_same_path 95. The longest isoform for each gene was extracted with Trinity and then redundancy was removed with CD-HIT-EST (Li & Godzik, 2006) using the following options: -r 0 -g 1. Kallisto (Bray et al., 2016) was used to calculate transcripts expression using the normalized reads and only the transcripts with more than 1 TPM (transcripts per million reads) were retained. Transrate (Smith-Unna et al., 2016) was used to extract the quality statistics about the assembly, whereas the BUSCO (v3)(Simão et al., 2015) pipeline was used to check the presence of Eukaryotic and Metazoan conserved genes. In silico transcriptome translation was performed with TransDecoder.LongOrfs (Haas et al., n.d.), the obtained peptides were blasted (BLASTp, minimum evalue 0.00001) against the NCBI NR (downloaded on January 2017) and the UniProt databases (downloaded on January 2017) and the results were given to TransDecoder.Predict to obtain the final dataset of predicted proteins.

Functional annotation of the transcriptome was performed following the AHRD pipeline (Hallab et al. n.d.). First, the predicted proteins were queried (BLASTp, evalue 0.00001) against the UniProt database of Metazoa and the TrEMBL database of Osteoglossocephalai (both downloaded on January 2017). The resulting files were used as input for the AHRD script including the Gene Ontology annotation of the UniProt database. KEGG annotation was performed using the KEGG Automatic Annotation Service (KAAS)(Moriya et al. 2007).

2.2.5. Transcriptome expression quantification and differential expression analysis

Transcript expression quantification was performed using Kallisto with the Trinity final assembly and the reads from the 17 samples as input. The raw estimated counts were normalized with two approaches: TPM (Transcripts Per Million) and TMM (Trimmed Mean

Normalization of M-values). The TPM were used for plotting purposes. The TMM normalization was used specifically for the differential expression analysis. To assess the reproducibility of the technical replicates and to look for potential outliers a PCA analysis was performed on the TMM normalized counts using the “prcomp” function within the R statistical environment. Differential expression analysis between mature and immature ovaries and livers was carried out within the R statistical environment using the NOISeq package (Tarazona et al., 2011). For the differential expression analysis (DEA), lowly expressed transcripts were removed and transcripts with a probability of being differentially expressed higher than 0.99 (corresponding to FDR <= 1%) were kept. A Gene Ontology Enrichment Analysis (GOEA) on the differentially expressed transcripts was performed with an in-house script based on the method described in Du et al. (2010) and applying a 1% FDR threshold. To facilitate the biological interpretation, a KEGG enrichment analysis to identify the most enriched pathways across the up- and down-regulated genes was performed using the “phyper” function within the R statistical environment (1% FDR). Pathways enriched for genes found to be differentially expressed between both the GIF and the GMF and the LIF and LMF groups were then further investigated using the search&color pathway KEGG web tool (Kanehisa et al., 2017). Given the objective of our study and in order to perform a more comprehensive analysis, the ovarian steroidogenesis and vitellogenin synthesis and uptake pathways were investigated considering genes differentially expressed at 5% FDR. Our rationale was that the FDR threshold must be considered in the context and releasing the stringency to 5% was to guide perform systems level analyses and avoid false negatives, where crucial data is lost. Heatmaps of differentially expressed genes involved in the selected pathways were drawn employing the “heatmap.2” function within the R statistical environment and using “spearman” and “average” as distance measure and agglomeration method, respectively.

2.2.6. Teleost Low-density lipoprotein receptor (Ldlr) orthologs

Following Mushirobira et al. (2015), multiple sequences belonging to the Ldlr superfamily from several species were downloaded from NCBI. Moreover, from the swordfish transcriptome, transcripts annotated as Ldlr which displayed full-length Open Reading Frame (ORF) were selected and conserved domains as well as structural features were assessed by SMART (Letunic & Bork, 2017) and InterProScan (Jones et al., 2014). This strategy allowed us to identify 5 full-length sequences from the swordfish transcriptome and

44 orthologue sequences in other species. Deduced amino acids sequences were first aligned with Muscle (Edgar, 2004) and then the least informative portions of the alignment were trimmed with TrimAl (Capella-Gutiérrez et al., 2009). Finally, a maximum likelihood tree was created with the IQ-Tree suite (Trifinopoulos et al., 2016) applying 1000 bootstrap replicates as phylogeny test and WAG as substitution model (Whelan & Goldman, 2001).

2.2.7. Experimental validation

Validation of key genes involved into steroidogenesis (*fshr*, *lhr*, *srb1* and *star*) and vitellogenin synthesis and uptake (*cathepsin B, D, L i1* and the *vtg receptor*) was performed by means of qPCR. Briefly, from samples selected for transcriptomics analysis, a total amount of 1 µg of RNA was used for cDNA synthesis, employing the iScript cDNA Synthesis Kit (Bio-Rad). PCRs were performed with the SYBR green method in a CFX96 Real-Time PCR system (Bio-Rad) following Gioacchini et al. (2017). Four replicates per condition were used. *Acidic ribosomal phosphoprotein P0* (*arp*) and *ribosomal protein L7* (rpl7) were used as internal standards in order to standardize the results by eliminating variation in mRNA and cDNA quantity and quality. No amplification products were observed in negative controls and no primer-dimer formations were observed in the control templates as indicated by the melting curve analysis. The data obtained were analysed using the CFX Manager Software version 3.1 (Bio-Rad), including GeneEx Macro Conversion and GenEx Macro files and results represented by bar-plots along with the standard error. Statistical significance was attained using a t-test. Specific primer pairs for target genes (see Supplementary Table S7) were designed with Primer-Blast (Ye et al., 2012).

2.2.8. Database

The web server uses NodeJS (version 8.9.4) together with the Express framework (version 4.16) in the back-end. Requested data is saved into files, which are read by the back-end and provided to the front-end, which mainly consists of HTML/CSS and JavaScript, in the corresponding format (e.g. tables or images). The site also contains a BLAST section, in which the user can query any sequence against the transcriptome with blastn (version 2.2.30+)(Altschul et al., 1990). A freely available JBrowse (version 1.12.3)(Skinner et al. 2009) was also integrated to the site, thus allowing visual inspection of the new

transcriptome assembly, the UniProt/TrEMBL matches against the predicted proteins, and the RNA-seq expression.

2.3. Results

2.3.1. Transcriptome assembly

RNA sequencing of samples on Illumina HiSeq 2500 platform generated 606,560,486 millions of raw reads of which 531,938,284 million were maintained after trimming and low-quality filtering steps (see Supplementary Table S1). The raw assembly produced 185.901 transcripts ranging from 201 to 18.293 nt with an N50 value of 2.492 nt and an average size of 1.325 nt while the average GC content was 46%. Further cleaning of raw transcripts resulted in a final assembly of 100.869 transcripts with an N50 value of 2.037 nt, an average size of 937 nt and an average GC content of 44% (see Supplementary Table S2). Furthermore, 83.13% of the reads were successfully mapped back to the assembled transcriptome of which 96% mapped uniquely while 99% and 98.2% matched sets of single copy eukaryotic and Metazoa genes, respectively. The percentage of duplicated and fragmented transcripts accounted for 9.5%.

2.3.2. Transcripts annotation

The SwissProt, TrEMBL, GO and KEGG databases were employed for annotation of the 100,869 sequences. A total of 31,704 (31.4%) sequences were successfully associated to a gene name. More specifically, 3,584 (3.5%) and 28,120 (27.9%) sequences matched the SwissProt and the TrEMBL databases, respectively. These database queries found X. gladius sequences to closely match sequences of *Fundulus heteroclitus* (20.2%), *Oreochromis niloticus* (13%), *Larimychthys crocea* (11%), teleostei (10.7%) and *Danio rerio* (5.7%). In the final transcriptome assembly 30,398 (30.1%) sequences had a significant match against the GO database, of which 37.7% representing biological processes, 27% associated with cellular components and 35.2% matching molecular functions. Sequences matching the KEGG database accounted for up to 18,158 (18%).

2.3.3. Drivers of ovary maturation

Since the overarching goal of our study was to investigate the molecular dynamics underlying ovarian maturation, we focused our effort in the identification of differentially expressed genes between mature and immature ovaries and livers. Before running DEA, a PCA was employed to assess the reproducibility and general quality of the analysis performed (see Supplementary Fig. S1). The overall variance explained by the first two principal components accounted for up to 97.8% and the first principal component best discriminated mature and immature ovaries with 92.5% of explained variance. DEA identified 6,501 transcripts, of which 4,211 functionally annotated, to be differentially expressed between immature and mature ovaries at 1% FDR. Gene Ontology Biological Processes (BP) known to drive and regulate ovarian maturation such as steroid biosynthetic process (GO:0006694), endosomal vesicle fusion (GO:0034058), lipoprotein metabolism (GO:0042157) and cholesterol transporter activity (GO:0017127) were significantly up-regulated while mRNA processing (GO:0006397), protein folding (GO:0006457), ribosome (GO:0005840), fatty acid biosynthetic process (GO:0006633) and clathrin adaptor complex (GO:0030131) were down regulated in mature ovaries (Fig. 1a). These results showed consistent agreement with the KEGG Pathways Analysis, in which ribosome biogenesis (ko03008), RNA transport (ko03013), mRNA surveillance (ko03015), oocyte meiosis (ko04114), cell cycle (ko04110) and fatty acid elongation (ko00062) were significantly down-regulated while endocytosis (ko04144) and cholesterol metabolism (ko04979) were up regulated in mature ovaries (see Supplementary Table S3 and Table S4). Interestingly, ovarian steroidogenesis, a key pathway involved in the progression of ovarian maturation, was found to be enriched when considering genes differentially expressed at 5% FDR.

A total of 2,914 transcripts, of which 1,834 functionally annotated, were found to be differentially expressed between immature and mature livers at 1% FDR. Gene Ontology Biological Processes (BP) known to characterize a mature liver such as lipid transport (GO:0006869), estrogen receptor activity (GO:0030284), steroid binding (GO:0005496), metabolic process (GO:0008152) and endoplasmic reticulum (GO:0005783) were significantly up-regulated while glycolytic process (GO:0006096) and fatty acid biosynthetic process (GO:0006633) were down regulated in mature livers (Fig. 1b). These findings were in agreement with the KEGG Pathways Analysis as estrogen signalling pathway (Ko04915) and protein processing in endoplasmic reticulum (ko04141) were significantly up-regulated while glycolysis/gluconeogenesis (ko00010) and pyruvate metabolism (ko00620) were down regulated in mature livers (see Supplementary Table S5 and Table S6).

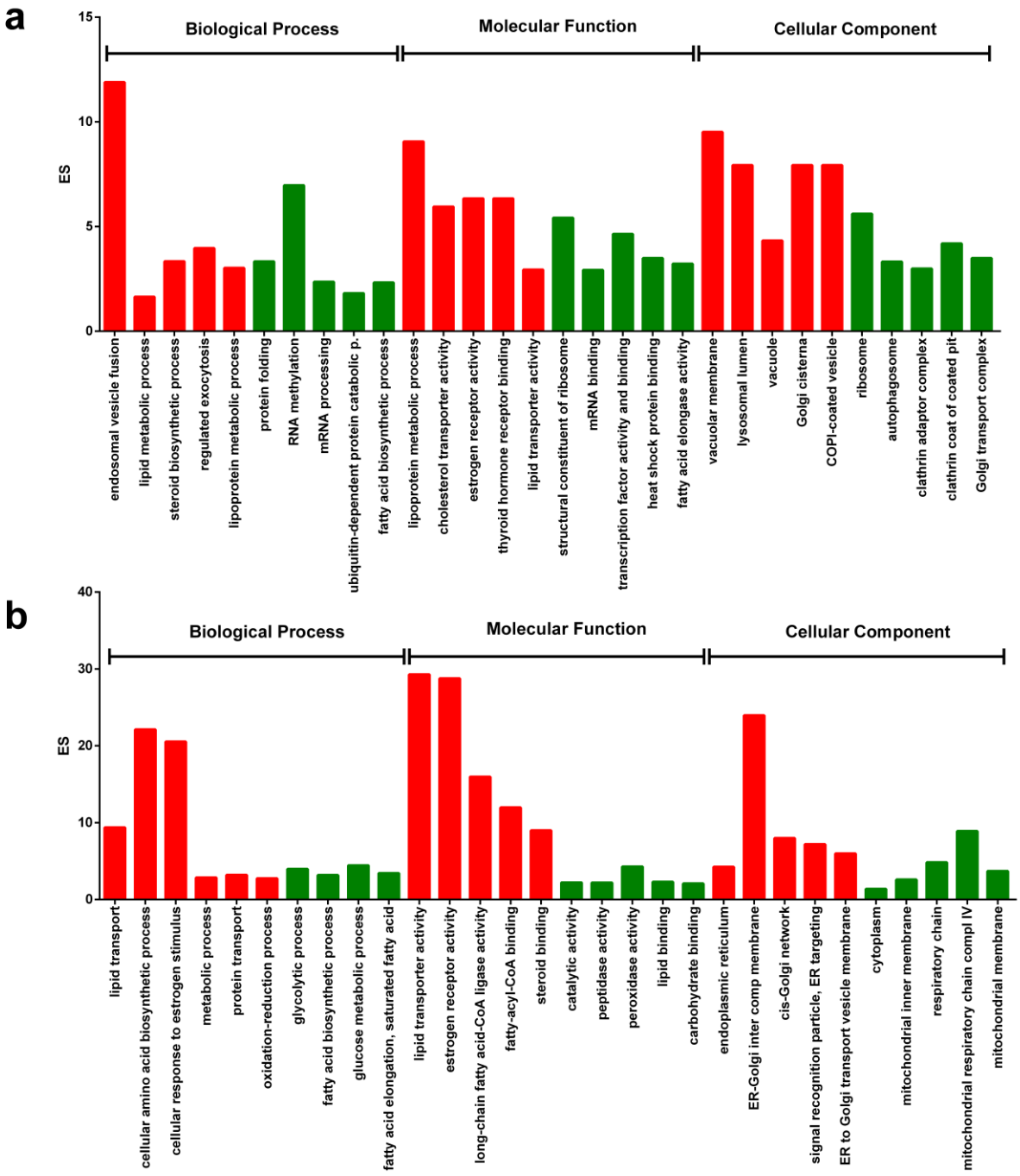

Figure 1. Gene Ontology differences underlying immature and mature ovaries (a) and livers (b). The bar plots show the Gene Ontology terms enriched of genes differentially expressed between mature and immature ovaries and livers. The y-axis represents the enrichment score (ES) and gene ontology terms have been organized according to the three main categories of Biological Process, Molecular Functions and Cellular Components. Red and green bars stand for terms up-regulated and down-regulated in the mature tissues compared with the immature ones, respectively.

A major focus on the steroid biosynthesis pathway revealed that the entire enzymatic cascade producing key sex steroids (i.e. E2, 17a,20b-DP, 11-KT) as well as the *fsh* and *lh receptors* were up-regulated in mature ovaries (Fig. 2-3). Interestingly, the *low-density lipoprotein leceptor* (*ldlr*) and the *scavenger receptor class B member 1* (*srb1*) displayed down regulation during vitellogenesis. Moreover, the *bone morphogenetic protein 15* (*bmp15*) and the *growth and differentiation factor 9* (*gdf9*) were found to be down-regulated in mature ovaries. A representation of the vitellogenin (*vtg*) synthesis and uptake pathway in the swordfish ovary was then established (Fig. 4) and represented along with the gene's expression profile (Fig. 5).

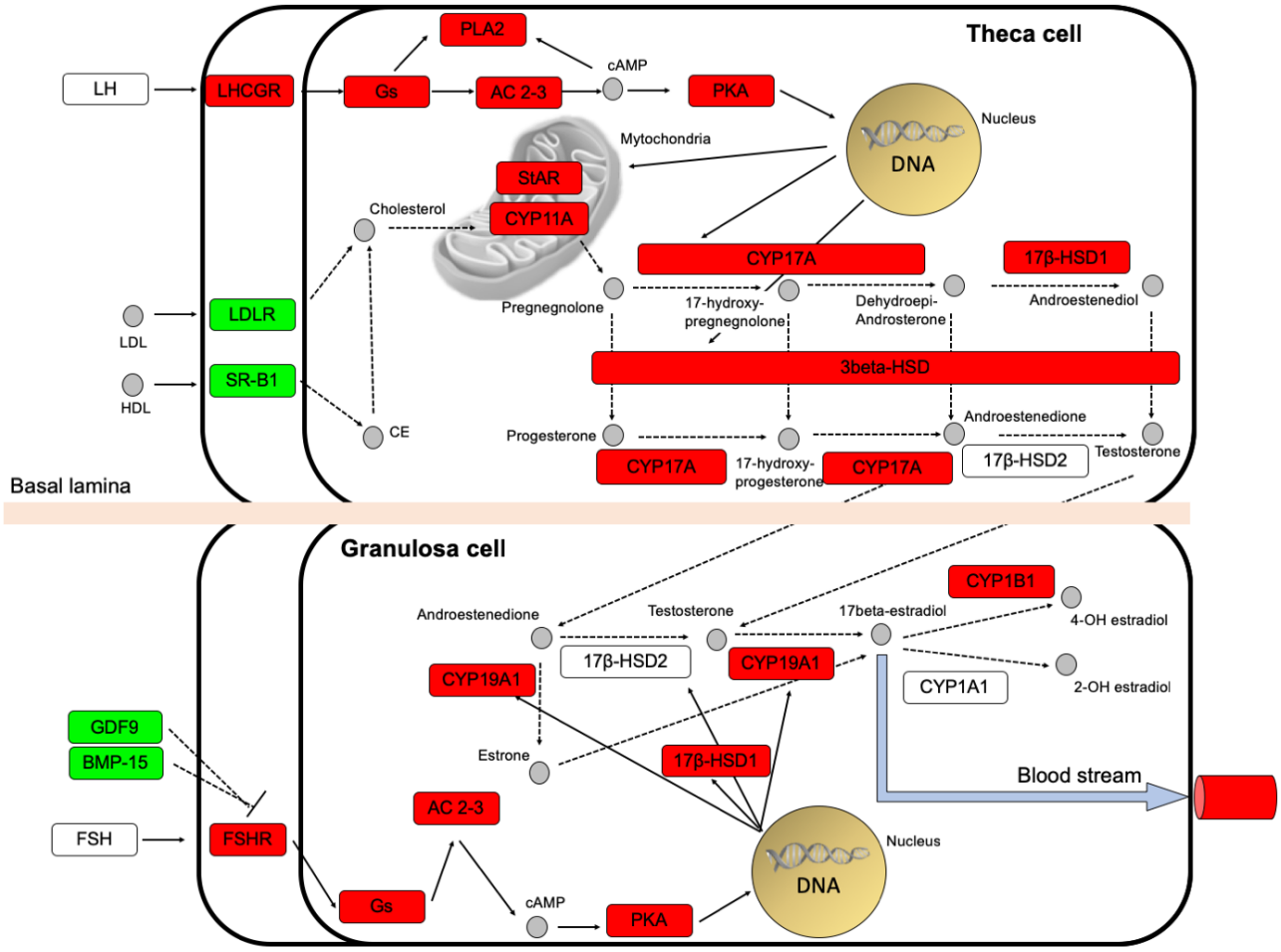

Figure 2. Molecular network of ovarian steroidogenesis adapted from Kegg. Genes up-regulated and down-regulated in the mature ovaries are shown in red and green, respectively, while genes not identified as differentially expressed are displayed in white. Solid lines represent direct interaction while dotted lines stand for indirect interaction.

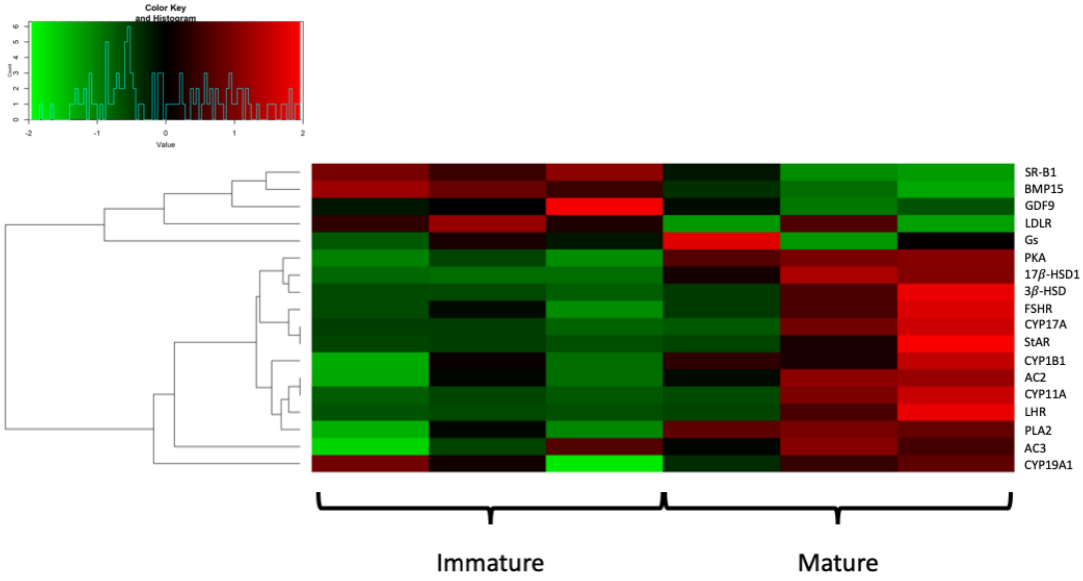

Figure 3. Heatmap of genes involved in the ovarian steroidogenesis pathway. The heatmap shows the behaviour of genes involved in the ovarian steroidogenesis pathway. Red and green are assigned to high and low values of expression, respectively, according to the reference plot.

In the liver, the organ where vitellogenin is mainly synthesised, we identified the *estrogen receptor α* (*esrα*) to be up-regulated in livers of mature individuals. When investigating vitellogenin, three different forms, *vtgAa*, *vtgAb* and *vtgC*, were identified to be up-regulated in livers of mature individuals. Based on experimental approaches aimed at the identification of *vtg* receptors in other teleost species in combination with the analysis of LDLR phylogenetic relationship in the present work, we identified a candidate *vtg* receptor likely to play a role in the vitellogenin uptake which exhibited higher expression in pre-vitellogenic ovaries. Accordingly, clathrin coated pits related transcripts such as *clathrin heavy chain*, *endophilin B1* and *dynamin* also displayed higher expression in pre-vitellogenic ovaries. Moreover, *lysosomal cathepsins D* and *L isoform 1* were downregulated while *cathepsins B* and *L isoform 2* were upregulated in mature ovaries. Key Rab proteins *rab11a*, involved into the slow endocytic recycling towards cell surface, and *rab35*, involved into the fast recycling, were upregulated in vitellogenic ovaries.

Gene expression profiles of a few genes were not always consistent between replicates due to the swordfish being an asynchronous spawner where oocytes at different developmental stages are found. In addition, some degree of variability was expected being the swordfish

a wild species. Some of the key genes identified in the aforementioned pathways were quantified by qPCR and differences were validated (Fig. 6).

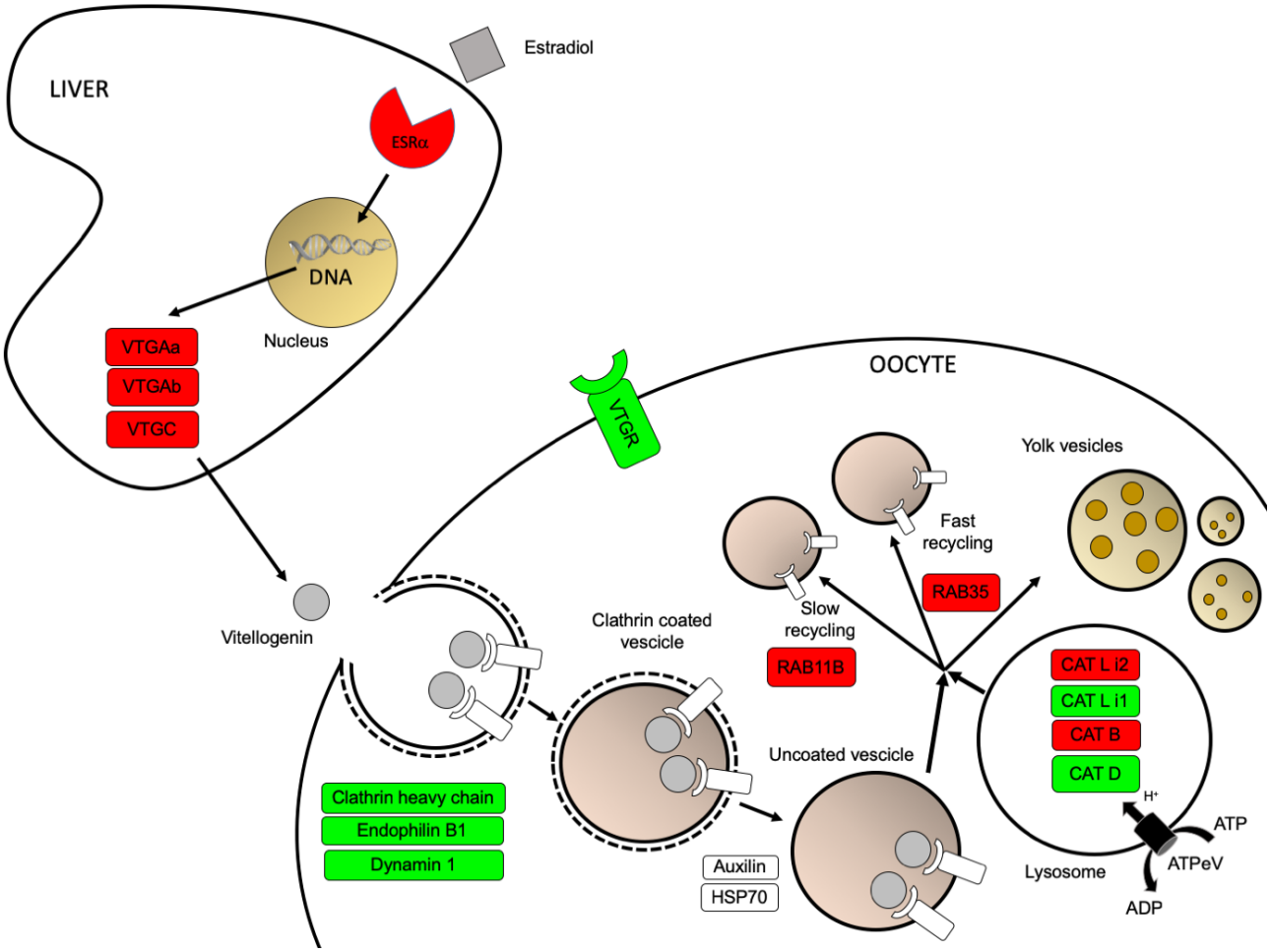

Figure 4. Molecular network of vitellogenin synthesis and uptake. Genes up-regulated and down-regulated are shown in red and green, respectively, while genes not identified as differentially expressed are displayed in white.

2.3.4. Low-Density Lipoprotein Receptor superfamily

The phylogenetic tree showing relationship between *ldlr* orthologs species displayed conserved clusters for each receptor type belonging to this superfamily (Fig. 7). Two well separated clusters supported by high bootstrap values were made up by two spliced variants *vldlr*$^{+}$ and *vldlr*$^{-}$, the latter being the vitellogenin receptor in teleost fishes. Taking advantage of this phylogenetic approach, we were successfully able to identify a potential vitellogenin receptor candidate in the swordfish clustering with *vldlr*$^{-}$ sequences and the amino acid

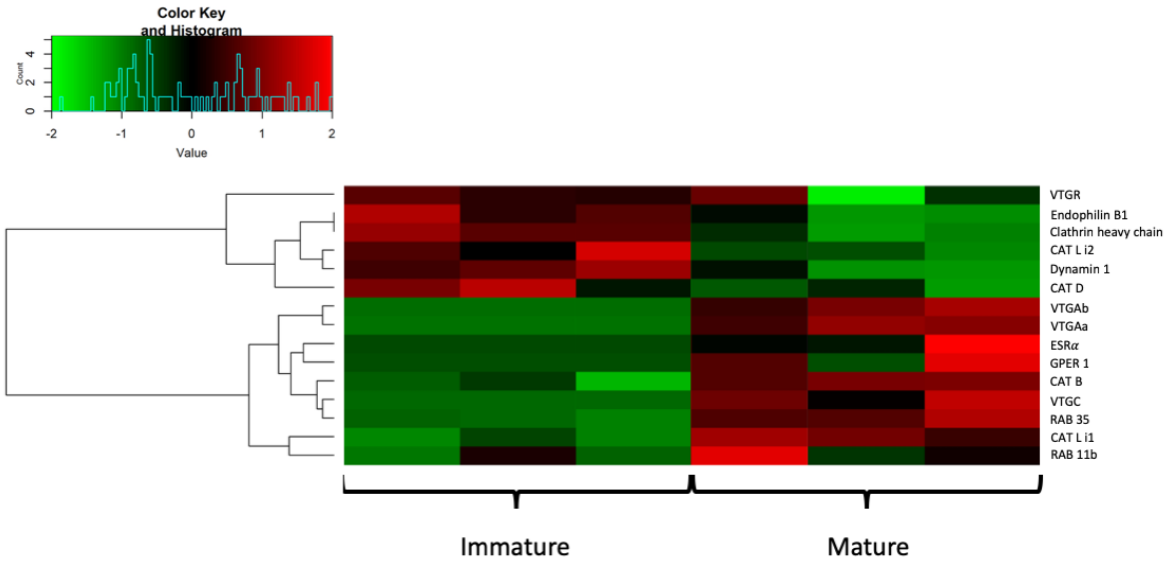

Figure 5. Heatmap of genes known to be involved in both the synthesis and uptake of vitellogenin. Red and green are assigned to high and low values of expression, respectively, according to the reference plot.

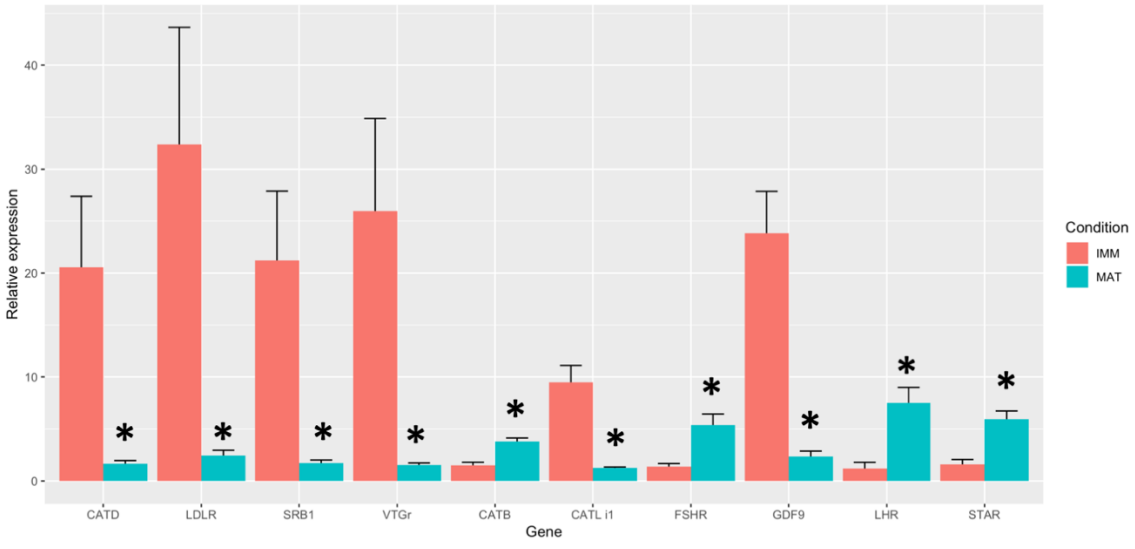

Figure 6. qPCR validation. Bar plot shows gene expression levels of key genes, measured by qPCR, involved in either the ovarian steroidogenesis or the vitellogenin uptake pathways. Significance between mature and immature ovaries for each gene is shown by an asterisk and standard error reported with error bars

sequence included a signal peptide region, eight low-density lipoprotein receptor domain class A (LDLa), a calcium-binding epidermal growth factor-like domain (EGF_CA), four low-density lipoprotein-receptor YWTD domain (LY) and a transmembrane domain. However, in the swordfish transcriptome sequences matching the *lrp13*, a novel receptor which has been recently suggested as an additional vitellogenin receptor in a few teleost species, were absent.

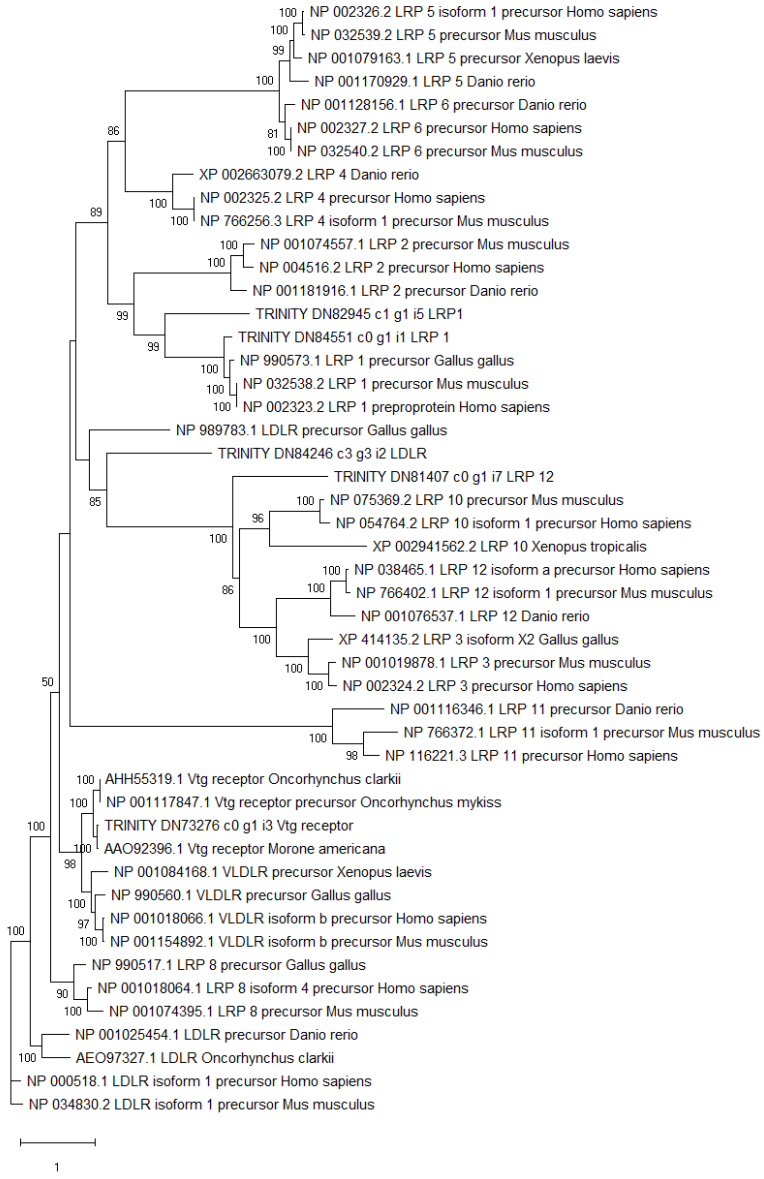

Figure 7. Phylogenetic tree of low-density lipoprotein receptors. The phylogenetic tree shows relationships, based on sequence similarity, between low-density lipoprotein receptors found in swordfish (labelled as TRINITY probes) and in other species. Bootstrap values are shown at each node.

2.4. Discussion

A wealth of studies applying High-Throughput technology has steadily increased in recent years with enhanced insights into reproduction, growth, metabolism and toxicological responses in non-model species in the wild (Basili et al., 2018; Renaud et al., 2019; Richards et al., 2018; Xu et al., 2017). To serve as a resource for future studies of swordfish biology, we have successfully sequenced, assembled and annotated the transcriptome of the swordfish *Xiphias gladius*, starting from several tissues such as female gonads, liver, intestine and stomach. As first extent, we specifically focused on data obtained from mature and immature ovaries in order to improve our understanding of specific molecular pathways driving sexual maturation in this species and to enhance both conservation and management of this resource for the Mediterranean fisheries.

Teleost sexual maturation is triggered by a complex interplay between environmental stimuli (i.e. photoperiod and temperature), energy balance, social interactions, physiological state and reproduction strategy (Wootton & Smith, 2014). Female maturation relies on the production of viable eggs achieved by the loading of maternal molecular cargo in oocytes which underlies the survival and proper development of embryos. Such maternal investment mainly consists of VTG-derived yolk proteins, polar (i.e. phosphoglycerides) and neutral (i.e. triacylglycerol) lipids, vitamins and minerals (Lubzens et al., 2010). Internalization of this bulk is one of the main outputs of the HPG reproductive endocrine axis that chemically links brain centres, able to convey environmental information, with the ovary through systemic signals in addition to autocrine/paracrine factors operating at the gonadal level. Indeed, the general overview provided by the Gene Ontology and KEGG pathways approaches showed up-regulation in mature ovaries of processes related to steroid biosynthesis, lipoprotein metabolism and vesicle trafficking. Nevertheless, such a molecular bulk is not restricted only to nutrients and structural components but also include a plethora of maternal transcripts with key functions during early stages of developing embryos. Accordingly, occurrence of RNA methylation as well as mRNA maturation processes were found to be higher in immature ovaries supporting their pivotal role in early stage of ovary development. Intriguingly, RNA methylation is a recently discovered process and is involved into direct regulation of protein expression, RNA stability and mRNA translation (Fu et al., 2014), thus it is likely to play a significant role in the molecular tuning needed throughout the oogenesis. It is well known that E2 synthesis in follicular cells with its subsequent release in the blood stream is the main response upon circulating Fsh stimulation and leads to hepatic

vitellogenin synthesis (Lubzens et al., 2017; Mosconi et al., 2002). Nevertheless, at the ovary level, synthesis of E2 and 17*α*,20*β* DHP was respectively linked also to oogonial proliferation and initiation of the first meiotic division in the common carp *Cyprinus carpio* and Japanese huchen *Hucho perryi* (Miura et al., 2007). In the present study, the two gonadotropin receptors *lhr* and *fshr* were up-regulated in mature ovaries, in which vitellogenic oocytes were present, and so did the entire downstream cascade of enzymes (i.e. *20β-HSD*, *17α-hydroxylase*, *aromatase P450*, *star*) leading to sex steroids synthesis in the follicular layer. On the other hand, the receptors *ldlr* and *srb1*, thought to be involved into the internalization of Lld and Hdl and thus representing the starting point for cholesterol uptake and steroid biosynthesis (Chang et al., 2017; Temel et al., 1997), were down-regulated in vitellogenic ovaries, suggesting an accumulation of transcripts during early stages of oogenesis prior to active nutrient uptake with subsequent translation and recycling of the protein products during lipidic and vitellogenic stages as found in the cutthroat trout *Oncorhynchus clarkii* (Luo et al., 2013). Increasing levels of *lhr* transcripts in mature ovaries might also be indicative of vitellogenesis completion of a certain batch of oocytes and its subsequent entry into final maturational stages. Indeed, the final maturation of fully-grown follicles is unambiguously driven by a surge of systemic Lh which leads to completion of meiosis, switch of the steroidogenic pathway from E2 to progestin synthesis, oocyte progestin responsiveness and finally hydration (Nagahama et al., 2008). However, oocytes and follicular cells are not just passively regulated by endocrine signals but they play an active role to orchestrate follicle development through autocrine/paracrine signals such as modulation of gonadotropin signals by means of locally produced factors belonging to the transforming growth factor beta (TGF-β) superfamily (Ge, 2005; Hussein et al., 2006). In addition to their well-known role in gametogenesis and follicle development, these factors regulate steroidogenesis and final oocyte maturation via paracrine mechanisms from mammals to fish (Peng et al., 2009; Shimasaki et al., 2004). Indeed, the oocyte-derived Bmp15 was able to regulate ovarian steroidogenesis by suppressing *fshr* expression in rat granulosa cells (Edwards et al., 2008; Moore et al., 2003). Direct in vitro evidences of such BMP-mediated paracrine modulation in teleost was found in the zebrafish *Danio rerio*, in which both Nmp2b and Bmp4 suppressed *fshr* expression and up-regulated *lhr* in co-cultured oocytes and follicle cells (Li et al., 2012). Moreover, in the European sea bass *Dicentrachus labrax*, a reciprocal and positively correlated pattern of gene expression was found between *bmp15* and *gdf9*, with highest expression in pre-vitellogenic oocytes followed by a sharp decline at the onset of vitellogenesis, suggesting a transcriptional control of

gonadotropin receptor expression by Bmp15 and Gdf9 and/or vice versa (Halm et al., 2008). Consistent with such a view, human chorionic gonadotropin (hCG), known to act through the Lhr in zebrafish, was able to down-regulate *gdf9* expression in fully-grown oocytes in vitro (Liu & Ge, 2007). Here, *bmp15* and *gdf9* were down-regulated in vitellogenic ovaries, highlighting the importance of the local autocrine/paracrine control of follicle development and, due to their differential expression, suggesting their possible involvement into regulation of steroid biosynthesis and follicle gonadotropin responsiveness in the swordfish. Vitellogenin is synthesized in the liver under E2 control, released into the blood and endocyted by growing vitellogenic oocytes in the ovary by means of specific receptors (Wallace, 1985). Multiple vitellogenin forms are found in teleost species due to teleost specific whole genome duplication which dates back about 320 million years ago (Finn & Kristoffersen, 2007). A direct consequence of such duplication is the evolution of a complex ligand-receptor system composed by several *vtg* receptors with specific ligand binding affinity (Hiramatsu et al., 2015; Mizuta et al., 2013; Mushirobira et al., 2015) and by yet to discover mechanisms of regulation which lead to a different ratio of plasmatic and endocytosed vtg forms into the oocyte (Amano et al., 2008; Williams et al., 2014). Here, three forms of vitellogenins were identified and found to be up-regulated in the livers of sexually mature females. This would fit well with the evolutionary history of this molecule since three functional forms are found in other fish species (Finn & Kristoffersen, 2007; Hara et al., 2016). It is clear that hepatic vitellogenin synthesis is up-regulated by estrogens and that the main receptor involved into this response is the *esrα* (Nelson & Habibi, 2013). According to these previous findings, the swordfish *esrα* was up-regulated in the livers of sexually mature females caught during the reproductive season, supporting the role of *esrα* as the main estrogen receptor isoform involved into E2-induced vitellogenin production in the liver. One of the aims of the present study was the identification and characterization of the receptor involved in such uptake by analysing the pattern of expression and exploring phylogenetic relationship. The candidate *vtg* receptor (*vldlr*) exhibited higher expression in pre-vitellogenic ovaries, a trend observed also in the cutthroat trout *Oncorhynchus clarkii* and *Morone* species (Mizuta et al., 2013; Reading et al., 2014). Such expression would be related to an intense transcription prior to vitellogenesis followed by both synthesis and recycling of the protein with subsequent depletion of the mRNA pool. Accordingly, the molecular machinery of clathrin-coated pits was down-regulated in mature ovaries, consistent with the idea of pre-vitellogenic transcription and both slow and fast recycling of protein products throughout vitellogenenesis (Mizuta et al., 2017). The phylogenetic tree

showed well conserved clusters of Ldlr family members across vertebrates even if from distantly related species. The candidate vitellogenin receptor formed a cluster with respective orthologues and such cluster was closely related to *vldlr*$^{+}$. The close relationship between *vldlr*$^{-}$ and *vldlr*$^{+}$ was investigated with multiple approaches which revealed the two receptor as spliced variants, the former lacking the O-linked sugar domain and directly linked to vitellogenin uptake (Mizuta et al., 2013). The deduced structure of swordfish *vtg* receptor showed typical Ldlr family domains with high homology to *vtg* receptors of many species (Davail et al., 1998; Dominguez et al., 2012; Pousis et al., 2012). Functional roles of the core receptors architecture include LDLa consecutive domains thought to interact with the receptor-binding domains of the vitellogenin (Jeon & Blacklow, 2005) and the six-bladed β -propeller structure made up by EGF domains and YWTD repeats which mediates the pH dependent ligand release (Rudenko et al., 2002). Interestingly, there were no sequences significantly matching the *lrp13*, a novel vitellogenin receptor recently found in a few teleost species (Mushirobira et al., 2015; Reading et al., 2014). Nonetheless, different reproductive strategies, ecological traits and phylogenetic lineages characterizing teleosts might reflect multiple selective models of vitellogenin uptake, processing and accumulation. For instance, it has been shown that the ligand-binding domain of the vitellogenin receptor interacts also with Apoliprotein B/E in the *Oreochromis aureus* suggesting a coevolution of the two systems and indicating off-target binding of such receptors (Li et al., 2003). Establishing comprehensive principles of how multiple vitellogenin forms (*VtgAa*, *VtgAb*, *VtgC*) are taken up by growing oocytes across species still remains a challenge and major differences likely exists in terms of ligand specificity and subsequent accumulation of derived protein products (Hiramatsu et al., 2015). Overall, the question if other receptors, other than *vldlr*$^{-}$, are responsible for vtg internalization in the swordfish still remain to be answered and will require further investigation at the protein level. Once vtg is internalized into growing oocytes it is cleaved by cathepsins into yolk protein components (YP) (lipovitellins, phosvitin, β'-component and C-terminal component). A well-established pathway in fish oocytes begins with the *cathepsin D*, responsible for the first proteolytic cleavage of vitellogenin, then a second step occurs with *cathepsins L* or *B* depending on the species (Carnevali et al., 1999a, 1999b; Tingaud-Sequeira et al., 2011). Indeed, species spawning pelagophil and benthophil eggs undergo high and low level of final hydration, respectively. Water uptake in pelagic eggs is mainly driven by a proteolytic process occurring at around the time of germinal vesicle breakdown which generates a pool of free amino acids. Intriguingly, a teleost-specific aquaporin (*aqp1ab*) mediating water uptake during the final maturation

processes has been recently characterized at genomic, phylogenetic and molecular level in marine species and in some freshwater species spawning partially hydrated eggs (Cerdà et al., 2013). Such pronounced difference in eggs water content between pelagophil and benthophil species underlies divergent proteolytic processing of the YPs as well as species-specific ecological traits and habitat usage. In the zebrafish *Danio rerio*, a freshwater species spawning benthic eggs, Cathepsin B activates Cathepsin L since Cathepsin L activity was suppressed by a cathepsin B inhibitor (Carnevali et al., 2006). Important differences between species exist even though eggs with similar water content are spawned. Indeed, Fabra and Cerdà found that in the killifish *Fundulus heteroclitus*, a benthophil species, the mRNA levels of *cathepsin L* transiently accumulated in maturing oocytes while those of *cathepsin B* remained stable throughout the whole oogenesis (Fabra & Cerdà, 2004). Nonetheless, Cathepsin B enzyme activity was higher during oocyte maturation and it is considered the major protease involved into YPs hydrolysis in this species (LaFleur et al., 2005). On the other hand, in the sea bream *Sparus aurata*, a pelagophil species, the expression of *cathepsins B*, *D*, *L* decreased from early vitellogenic oocytes through hydrated eggs but the enzyme activity well correlated with transcript levels only for *cathepsin B* (Carnevali et al., 2008). Interestingly, the coho salmon *Oncorhynchus kisutch* showed respectively decreasing and stable mRNA levels of *cathepsin B* and *D* in oocytes entering the cortical alveoli stage (Luckenbach et al., 2008). However, expression of *cathepsin D* and *L* increased in oocytes acquiring maturational competence in another salmonid species, the rainbow trout *Oncorhynchus mykiss* (Bobe et al., 2004). In the swordfish, we found the *cathepsin D*, *B* and two *cathepsin L* isoforms to be differentially expressed between vitellogenic and pre-vitellogenic ovaries. Previously, three different *cathepsin L isoforms* were identified in the genome of zebrafish with different pattern of expression in adult tissues and during embryogenesis (Tingaud-Sequeira & Cerdà, 2007). In the swordfish transcriptome, *cathepsin L isoform 1*, whose expression decreased in vitellogenic ovaries, is likely to play a role in YPs processing since exhibited nearly 20 to 25-fold higher expression than the other isoform. Furthermore, swordfish *cathepsin D* and *B* exhibited down-regulation and up-regulation in vitellogenic ovaries, respectively. According to the findings of Carnevali and co-workers, in the sea bream, the pattern of expression of *cathepsin D* showed decreasing mRNA levels through the vitellogenesis (Carnevali et al., 2008) while *cathepsin B* displayed an opposite trend. These result might underlie several features typical of such enzymes across teleost species: (i) cathepsins are first synthetized as zymogen, pre-pro enzymes which are then activated by a drop in lysosomal pH (Turk et

al., 2000, 2001) and this would reflect the scarce correlation found between mRNA levels and enzyme activities; (ii) different expression patterns were found also in species spawning eggs with similar characteristics in term of final water content. To sum up, it is hard to clearly establish an overall pattern of cathepsin expression across species during the oogenesis since such differences likely underlie different reproductive strategies and ecological traits.
In conclusion, according to the ICCAT plan, recovery of the Mediterranean swordfish stock has at least a 60% probability of success. Despite this percentage represents an optimistic goal, filling the gap of knowledge about swordfish biology of reproduction has the potential to significantly improve the predicted scenario. In this context, application of highly sensitive molecular technologies with the ability to generate “big omics data” offers the potential to drive new understanding of molecular functions. More specifically, RNA sequencing approaches provide a powerful tool to investigate the molecular mechanisms of key biological processes. In this context, we employed the power of *de novo* transcriptome assembly to shed light on the swordfish molecular dynamics occurring when vitellogenesis starts. We successfully depicted the ovarian steroidogenesis and vitellogenin uptake molecular pathways and identified a potential candidate for vitellogenin receptor in this species. Although biological processes driving reproduction are well conserved, especially between fish, differences in molecules driving a given biological pathway may arise. As a consequence, further effort investigating isoforms could potentially increase our understanding of the swordfish reproductive biology. Moreover, we set up a dedicated database (www.swordfishomics.com) that is publicly available where all the information about the swordfish transcriptome can be directly accessed by the scientific community. This offers a powerful tool and the integration of our findings with other approaches as stock assessment methodologies has the potential to refine the current ICCAT recovery plan for the conservation of the Mediterranean swordfish.

2.5. Data availability

All the Supplementary Material can be found at the online version of the article at https://doi.org/10.1038/s41598-019-43872-6. Furthermore, the transcriptome annotations, statistics and results of the differential expression analysis are freely available at www.swordfishomics.com.

2.6. References

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3. Some like it hot: the heater tissue transcriptome of the swordfish *Xiphias gladius*

3.1. Introduction

The successful adaptation of a species to a specific ecological niche comprehends the evolution of specific traits. Multiple examples include the emergence of innovative anatomical, developmental, behavioural and physiological features which entail a selective advantage (Dukas, 2013; Heard & Hauser, 1995). Thus, these biological characters become typical of specific lineages since they were ancestrally established. Yet, selective forces and chance can also generate convergent solutions in response to similar ecological challenges. The endogenous control of body temperature is an illustrative example of such a trait and represents a major evolutionary transition in the history of vertebrates (Nowack et al., 2017). Yet, birds and mammals for example are able to defend their body temperature within the thermoneutral zone (i.e. basal metabolism), albeit with different anatomical solutions and physiological processes (Nowack et al., 2017; Rowland et al., 2015). Therefore, endothermy has probably evolved twice in the amniotes and also other non-amniote lineages display variable forms of endothermy (Clarke & Pörtner, 2010; Legendre & Davesne, 2020). In effect, this phylogenetic dispersal of the ability to regulate, even if partially, the body temperature with respect to the environmental surrounds strongly indicates that endothermy evolved independently (Block et al., 1993). The results of this phylogenetic diversity indicates that there is not a unique evolutionary trajectory leading to endothermy and fish provide an interesting testing ground for this concept. Indeed, as one of the most successful and diverse group of animals on Earth, fish evolved different strategies to achieve endothermy in terms of morphological, anatomical and physiological novelties and further support the idea of how this trait evolved independently in several lineages (Legendre & Davesne, 2020). A cranial endothermy is present in the billfishes (Block, 1986, 1991; Block et al., 1994; Carey, 1982), the butterfly mackerel (Tullis et al., 1991) and the slender tuna (*Allothunnus fallai*) (Sepulveda et al., 2007) while tunas (Graham & Dickson, 2004), lamnid sharks (Goldman et al., 2004; Tubbesing & Block, 2000) and the opah (*Lampris guttatus*) (Wegner et al., 2015) exhibit a more systemic endothermy. Billfishes warm eyes and brain to enhance vision and to maintain performing cognitive functions in deep cold waters during hunting activities (Fritsches et al., 2005), yet they possesses little capacity for whole-body endothermy (Stoehr et al., 2018) as opposed to tunas which exhibit a more widespread red

muscle, cranial and visceral endothermy (Bernal et al., 2001; Dickson & Graham, 2004; Graham & Dickson, 2004).

Among billfishes, the swordfish *Xiphias gladius* (Linnaeus, 1758) is the most performing species in terms of vertical diving behaviour and thermal plasticity (Bernal et al., 2010), therefore it provides a great example of the variety of temperature-body control mechanisms in fish. This large solitary pelagic fish species is globally distributed between the 45°N and 45°S, displays seasonal migrations and has unique morphologies to enhance swimming efficiency such as an oil-producing gland at the base of the rostrum (Videler et al., 2016) and gills fusion to cope with a continuous ventilatory stream (Wegner et al., 2013). The swordfish is an exceptional predator, feeding preferably on cephalopods and pelagic fish (Chancollon et al., 2006) and this foraging strategy is critically marked by daily movements between the surface and deeper waters up to more than 1000 m (Braun et al., 2019). In essence, these activities involve transitions between water bodies with very different temperatures (Fig. 1). Thus, the very unique phenotype and swimming performance of this iconic species is accompanied by an exceptional organ, the so-called heater tissue (Fig. 1). The morphology, anatomy and physiology of this tissue have been previously extensively characterized by several authors with a wide variety of approaches that shed light on its structure, cellular organization and processes (Block 1986, 1987, 1991, 1994; Block & Franzini-Armstrong, 1988; Carey, 1982; De Metrio et al., 1997; O'Brien & Block, 1996; Morrissette et al., 2003; Tullis et al., 1991). Among them, a special mention goes to Barbara Block who, with her pioneering works on the heater tissue of billfishes, paved the way to the broader exploration of evolutionary novelties that allowed endothermy in fish (Block et al., 1993, 1994). This heater tissue is a modification of the extra-ocular muscle dorsal rectus and inserts into the dorsal-caudal area of the eyeball in close contact with ptero/basisphenoid sheet-like bone below the brain (Block, 1986). Functionally, the heater tissue operates as a differentiated structure for heat production, which has lost the sarcomeric organization typical of skeletal muscles (Carey, 1982; De Metrio et al., 1997). Noteworthy, along with the brown adipose tissue (BAT) of mammals, the heater tissue is the only other vertebrate tissue that specifically evolved to produce heat and the other examples of endothermy in the animal kingdom are achieved through a combination of anatomical and physiological novelties but not via the evolution of specific body parts devoted to heating (Dickson & Graham, 2004). Importantly, various studies have addressed the molecular mechanisms underlying endothermy in tunas (Ciezarek et al., 2016, 2019, 2020) and recently the genome assembly of the swordfish identified some genes with key roles in

endothermy as positively selected at conserved sites (Wu et al., 2021). Here, we expand the molecular toolbox available to investigate the evolution of endothermy mechanisms in vertebrates. We provide a high-coverage transcriptome of the heater tissue of the Mediterranean swordfish, as a mean to gain deeper insights into the molecular network responsible for heat production in this highly specialized tissue and expand our understanding of the concept of evolutionary convergence of endothermy among vertebrates.

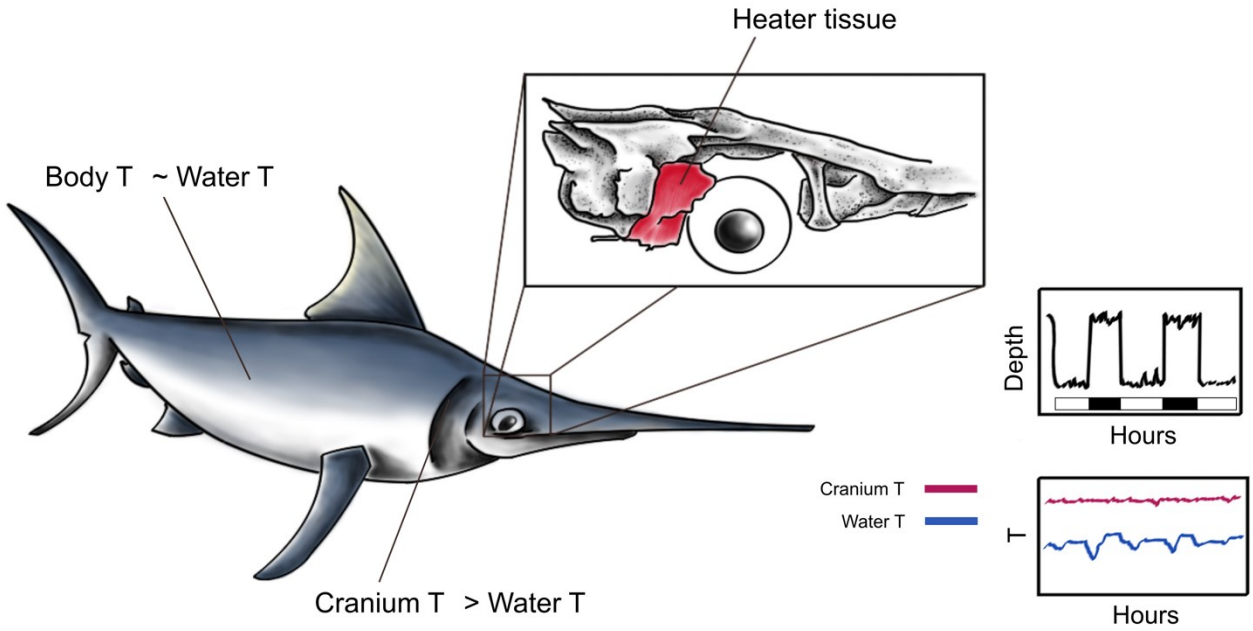


Figure 1. Graphical representation of the heater tissue of the swordfish. The tissue is a modification of the extra-ocular muscle dorsal rectus and its main function is to generate heat, keeping warm brain and eyes during vertical deep dives (cranium T > water T). Indeed, during day time the swordfish exhibits marked vertical migrations to feed on the deep scattered layer, returning to shallow waters at night. This behaviour is marked by dramatic changes of water temperatures. However, the body temperature is in line with that of the surrounding water (body T ~ water T). T= temperature. Scientific drawings credit: Matteo Zarantoniello.

3.2. Materials and methods

3.2.1. Sampling, laboratory processing and sequencing

The samples of the swordfish heater tissue (*n*=4) were collected during sampling activities on board of a longliner off the coasts of Sicilia and at a tuna trap in Carloforte (Sardinia, Italy) (Table 1). Upon sampling, the samples for RNA sequencing were placed into the RNAlater solution (Ambion, Austin, TX, USA) and kept at -20°C until RNA extraction while samples for histological analysis were fixed in a formaldehyde/glutaraldehyde solution ($NaH_2PO^{4-}H_2O$ + NaOH + formaldehyde 36.5% + glutaraldehyde 25% + H_2O) and kept at +4°C. For histological processing, samples were dehydrated in a series of increasing ethanol baths, cleared in xylene and embedded in paraffin. Then, sections of 5 µm were cut with a microtome (model RM2125 RTS; Leica Biosystems, Wetzlar, Germany), stained with Mayer's haematoxylin/eosin and Masson's trichrome and examined under a microscope (Axio Imager 2; Zeiss, Oberkochen, Germany). For RNA sequencing, RNA was extracted with RNAzol (Sigma-Aldrich, St. Louis, MO), the quality of the RNA in terms of 260/230 and 260/280 ratios and yield (µg/µl) was checked with the Nanodrop P330 (Implen GmbH, Münich, Germany). Then, samples were run on 1% agarose gel to check for integrity and finally shipped to Macrogen (Seoul, South Korea) for 100 bp PE sequencing on the Illumina NovaSeq 6000 platform.

Table 1. Sampling and biometric data of the samples used in the present study. * = Total weight was calculated from LJFL length according to the length-weight relationship for the Mediterranean swordfish provided in Pignalosa et al. (2019); Length= from lower jaw to fork; weight= total weight.

Sample ID	Sampling date	Length (cm)	Weight (kg)*	Sex	Sampling coordinates
SA13	29/05/2019	217	98	F	39°11'30"N 8°17'33"E
SA21	01/06/2019	180	55	n.d.	39°11'30"N 8°17'33"E
AD55	05/06/2019	164	41	F	39°00'21"N 15°25'50"E
AD60	07/06/2019	112	12	M	38°50'47"N 15°43'48"E

3.2.2. Raw data clean up and quality control

The sequencing datasets were cleaned following the protocol used in Machado et al. (2020). The FastQC (v.0.11.8) (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/) was used with default parameters to have a global first overview of the data. This tool was applied to check the quality of the raw datasets, then three different tools were sequentially used to proceed with the initial filtering steps. First, Trimmomatic (v.0.38)(Bolger et al., 2014) was used to remove the low-quality reads and those containing ambiguous "N" bases (Parameters: LEADING:5 TRAILING:5 SLIDINGWINDOW:5:15 MINLEN:36), then Rcorrector (v.1.0.3)(Song & Florea 2015) was applied to correct for sequencing errors using default parameters, finally, Centrifuge (v.1.0.3-beta)(Kim et al., 2016) was applied to remove exogenous sequences in a two-step process 1) by scanning all the reads against the nucleotide database of NCBI (ftp://ftp.ccb.jhu.edu/pub/infphilo/centrifuge/data/; v.nt_2018_3_3); 2) by gathering of all the reads without hits or matching the Actinopterygii superclass (NCBI taxonomy Id: 7898). At the end of the process, FastQC was applied once again to assess the quality of the final dataset and the effectiveness of the tools used (Fig. 2).

3.2.3. *De novo* transcriptome assembly

The heater tissue transcriptome was assembled with the Trinity *de novo* assembler (v2.4.0) (Grabherr et al., 2011; Haas et al., 2013) using default parameters. To detect possible sources of contaminations (i.e. biological, vectors, adapters) not detected in the first steps of raw data clean up, both nucleotide NCBI (nt-NCBI) (v.27/10/2019) and UniVec (v.02/04/2019) databases were searched using the Blast-n tool (v.2.9.0) with parameters set according to Machado et al. (2020). At the end of the decontamination process, only highly confident transcripts (i.e. transcripts with the best hit against the Actinopterygii taxon and/or without any hits in UniVec or nt-NCBI database) were considered for further analyses. This corresponded to the initial assembly.

To reduce the dataset complexity, two further filtering steps were added to the pipeline, namely the Open Reading Frame (ORF) prediction and transcript coverage (Fig. 2). First, partial and complete open reading frames (ORFs) with a minimum length of 100 amino acids (aa) were initially predicted using the TransDecoder software (v.5.3.0) (https://transdecoder.github.io/). Initially predicted ORFs were then scanned for homology

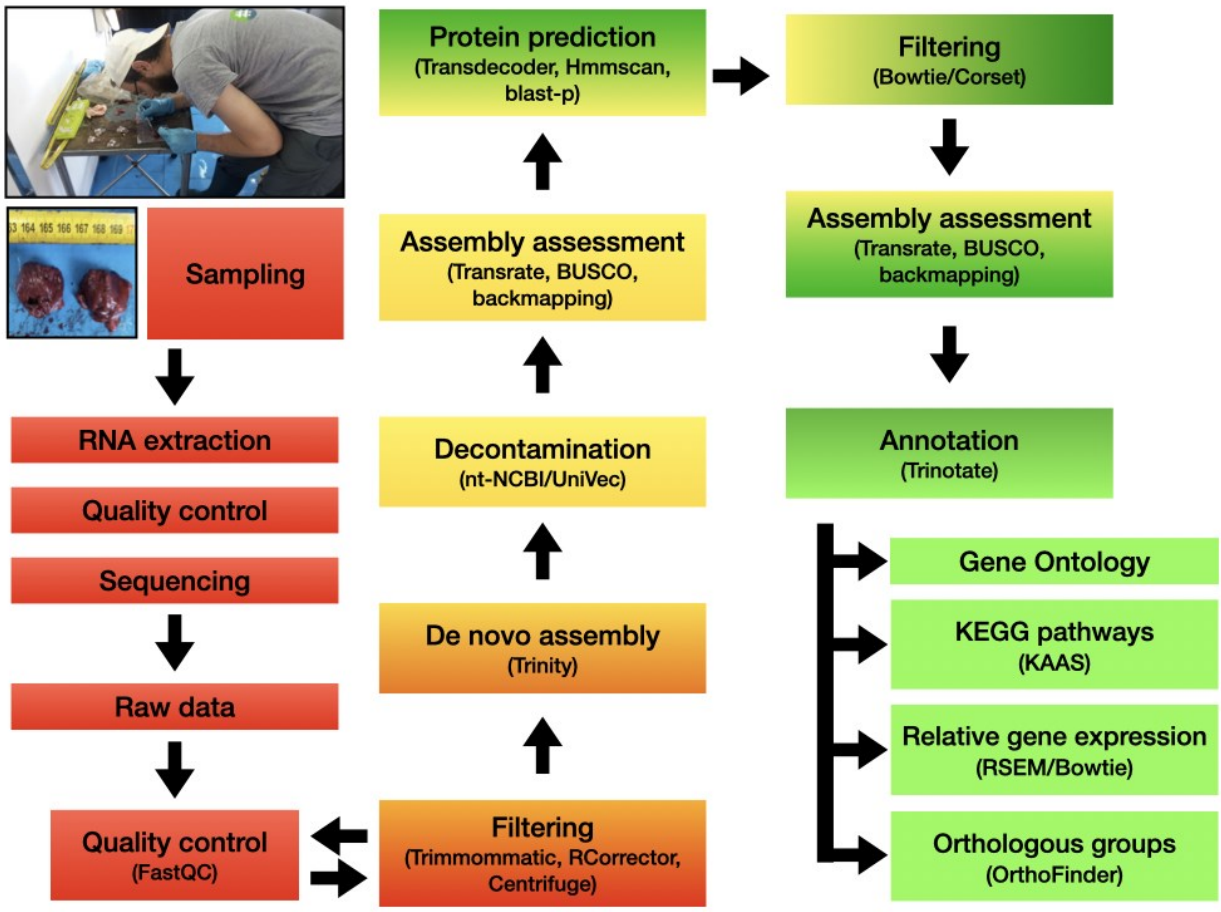


Figure 2. Workflow of the steps required from the sampling, sequencing and raw data cleaning/filtering to *de novo* assembly and annotation. The software used at each step are indicated in brackets.

via blast-p using an e-value cut-off of 1e-5 against the SwissProt database (v.18/02/2020)(Bateman et al., 2017) and via the hmmscan of hmmer2 (v. 2.4i) package against the protein profiles in Pfam database (v. 18/02/2020)(Punta et al., 2012). At the end of these matches, all results were integrated into the TranDecoder pipeline and the final ORF prediction was performed. Due to its features, the ORF prediction step allowed to exclude all the non-coding transcripts. Second, the protein-coding sequences with an ORF were analysed with Corset (v.1.0.9)(Davidson & Oshlack, 2014) to exclude transcripts with less than 10 mapping reads. This was defined as the final assembly. Then, to obtain a non-redundant set of transcripts only one transcript per “Trinity Unigene” with the longest protein coding transcript was collected, a strategy similar to Neuparth et al. (2020). Finally, to assess and compare the initial and final versions of the transcriptome assembly, three methods were used: 1) Benchmarking Universal Single-Copy Orthologs (BUSCO v.3.0.2)(Simão et

al., 2015) with default parameters and the following databases: Metazoa, Eukaryota, Vertebrates and Actinopterygii, 2) read back mapping to the transcriptome (RBMT) and 3) Transrate (v.1.0.3)(Smith-Unna et al., 2016). In the RBMT method, Bowtie2 (v.2.3.5)(Langmead & Salzberg, 2012) was applied to map clean reads against the transcriptome and to evaluate the percentage of reads that mapped back. The complete workflow can be found in Figure 2.

3.2.4. Functional annotation

The functional annotation was performed using the Trinotate pipeline (v.3.1.1) (Bryant et al., 2017). The ORFs and nucleotide sequences of the final assembly were searched in the following gold-standard databases: the Non-Redundant database of NCBI (NCBI-nr) (v.20/01/2020), NCBI-nt (v. 27/10/2019), Swiss-Prot (v.18/02/2020)(Bateman et al., 2017), Uniref90 (v.14/09/2019)(Suzek et al., 2007), Pfam (v.18/02/2020)(Punta et al., 2012) and eggNog (Powell et al., 2012). The blast-n tool (v.2.9.0) was used to search against the NCBI-nt database while the blast-x/p tools of DIAMOND (v.0.9.24)(Buchfink et al., 2014) were applied to search for protein databases. At the end of the annotation process, a SQLite database of Trinotate was created with the pre-generated outputs as well as the nucleotide and protein sequences to generate the final annotation report with an e-value cut-off of 1e-5. The Trinotate pipeline does not allow the automatic integration of NCBI-nt/nr outputs, therefore these results were added to the pre-generated report using built in-house shell scripts.

3.2.5. Transcripts quantification

The transcripts quantification was performed with the abundance_estimates_to_matrix.pl script of the Trinity pipeline. Briefly, all the clean reads were mapped against the final transcriptome assembly using Bowtie2 (v.2.3.5)(Langmead & Salzberg, 2012) with defaults parameters and RSEM (v.1.3.0)(Li & Dewey, 2011) to estimate transcript abundance. In this approach two matrices of counts were generated, one at unigenes level (i.e. groups of transcripts clustered and based on shared sequence content, generated by Trinity pipeline) and the other at isoform level. Next, the values at the unigenes level were normalized with the trimmed mean of M-values (TMM) method and the average counts of each unigene per sample analysed.

3.2.6. Gene Ontology

The Trinotate report was initially used to retrieve the gene ontology terms (GO) and transcripts associated. Importantly, only GO based on blast hits of SwissProt database were collected and used to quantify the number of transcripts per gene ontology term. At the end, the top 10 GO terms per category, namely molecular function (MF), cellular component (CC) and biological process BP, with a higher number of transcripts associated were screened.

3.2.7. Comparative transcriptomics

To perform the comparative analyses, the OrthoFinder (v.2.3.3) (Emms & Kelly, 2015) software was used with the default settings. In this analysis three datasets were used: the final transcriptome of the swordfish heater tissue produced in this study, the RNA-seq data of the swordfish heart published in Marra et al. (2017) and the RNA-seq data of Pacific bluefin tuna red muscle (Ciezarek et al., 2019, 2020). To make uniform the three datasets, several parts of our pipeline were applied to the swordfish heart and Pacific bluefin tuna red muscle datasets, with the settings previously used. As the Pacific bluefin tuna had the transcriptome online available (GenBank Id: GIUO00000000.1), only the filtering step of Corset and the ORF predictions of TransDecoder were applied. Importantly, the transcriptome was composed of several tissues. To guarantee the collection of red muscle transcripts, only red muscle raw datasets were mapped onto the global transcriptome. This strategy allowed to obtain a red muscle transcriptome of the Pacific bluefin tuna. On the other hand, in the case of swordfish heart, several analyses were needed. Accordingly, as the transcriptome was not available online almost all the pipeline of swordfish heater tissue until the TransDecoder step was required. At the end of these processes, three amino acids datasets were used into OrthoFinder.

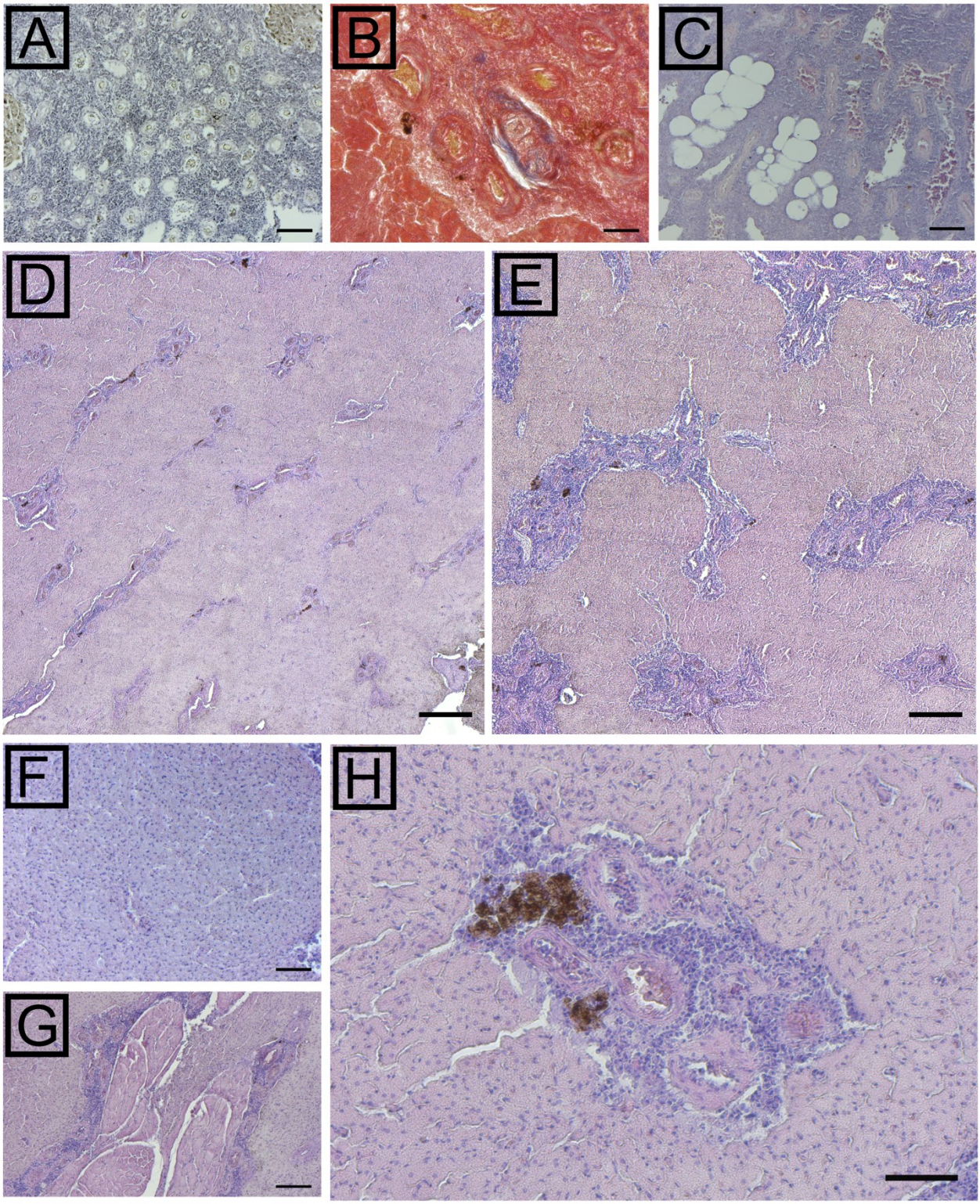
3.2.8. KEGG pathways

To identify the KEGG pathways present heater tissue, several searches were conducted on KAAS webserver following the author's guidance (Moriya et al., 2007). The searches were performed through blast-p using the protein-coding transcripts of *X. gladius* heater tissue and the single-directional best hit (SBH) method. The Blast was performed against 40 species manually selected, for a total of 1,074,327 protein sequences.

3.3. Results and Discussion

3.3.1. Histological description

The superior rectus muscle of the swordfish had a deep red colour (Fig. 2) indicative of high oxidative capacity and was surrounded by abundant poorly vascularized fat, which might fuel its thermogenic capacity as well as buffer heat loss (Block, 1986). Toward the ventral part of the tissue, originating from the internal carotid artery (De Metrio et al., 1997), a rete mirabile consisting of arteries and veins in close contact was observed (Fig. 3A), representing a counter current heat exchanging system that helps to conserve the heat produced in the tissue. The blood vessels were associated with bundles composed of pigmented tissue with a scarce connective component (Fig. 3B). Discrete large vacuoles were seldomly detected in proximity of blood vessels (Fig. 3C). Moving dorsally, the vascular bundles become progressively surrounded by the heater cells (Fig. 3D,E), which were rather irregular in shape, supplied by numerous capillaries and showing a finely granular appearance lacking the typical myofibrillar organization (Fig. 3F). Therefore, the heater cells from the superior rectus muscle acquired the thermogenic capacity at the expense of contractile elements typical of muscles (Block, 1987, 1991; Block & Franzini-Armstrong, 1988; Morrissette et al., 2003), an aspect also highlighted by a cytoplasm composed of more than 60% of mitochondria (Tullis et al., 1991). Toward the dorsal and more rostral part of the tissue a smooth transition was observed, in which heater and regular muscle cells could be observed (Fig. 3G), in a position where muscle cells changes along their length into heater cells (Block, 1986). Throughout the tissue, pigment-rich melanomacrophage centres were frequently observed in close contact with blood vessels (Fig. 3H), although at this stage we could not distinguish yet neither the lysosomal enzymes nor the pigments contained. It is interesting to note that melanomacrophages are not typical of muscles, they are usually found in haemolymphopoietic organs (e.g. liver, spleen, kidney) and they are involved into a variety of physiological responses, including immune response and elimination of exhausted cells (Steinel & Bolnick, 2017). However, it cannot be ruled out that melanomacrophages also represent a defence physiological process linked to the exceptionally high oxidative metabolism and temperature of this tissue, which likely have the potential for increased production of mitochondrial reactive oxygen species (Wiens et al., 2017). Future histological analysis will be aimed at defining the lysosomal and pigment content of the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◄ **Figure 3.** Histological sections of the heater tissue that highlight its main characteristics. A) Rete mirabile with veins and arteries arranged as a counter-current exchange system B) The heater tissue and the vascular bundles are characterized by scarce connective tissue (blue dye) C) Vacuoles D-E) Vascular bundles surround the heater cells that completely lack the sarcomeric organization. F) Heaters cells G) Transition between muscle and heater cells H) Melanomacrophage centers in proximity of blood vessels. Scale bars: A) 100 μm B) 50 μm C) 100 μm D-E) 200 μm E) 50 μm F) 50 μm G) 100 μm H) 50 μm

melanomacrophages centers in the heater tissue and at comparing their morphology with that of other tissues such as the liver to elucidate their roles.

3.3.2. Heater tissue transcriptome assembly

To produce the transcriptome assembly of heater tissue, 177 millions of paired-end reads from four samples were generated. Before performing the transcriptome assembly, three approaches were used to clean up the datasets. Briefly, Trimmomatic was used to remove about 3 millions of raw reads containing adapters or low quality reads (Phred<20), 2 millions of exogenous raw reads were eliminated with Centrifuge while Rcorrector corrected 18 million bases, reaching a final raw dataset of 172 millions of reads. Although the conservative strategy and the removal of 5 millions of raw reads, this value corresponds only to the 3.11 % of the raw datasets, which would suggest a high quality of the sequencing datasets.

The assembly of the heater tissue transcriptome was performed with the de novo assembler Trinity. During the normalization step about of 82 of the 172 millions of reads were selected to perform the assembly. Subsequently, this assembly version was re-decontaminated to exclude possible sources of contamination. Thus, about 7 and 7463 transcripts matched the UniVec and nt-NCBI databases, respectively. Importantly, during the decontamination process, 20,040 hits were found against *Danio rerio*. After screening these *Danio rerio* hits, about 15,058 had the percentage of identity above 98%, 16,850 hits with 0 or 1 gaps, 16,596 hits with the query coverage above 98%, and 17,029 hits with e-values < 9.82E-100. Due the high portion of hits and the associated metrics, these results were considered cross-species contaminations and removed from the transcriptome assembly. The general filter for non-Actinopterygii species was also applied to the *Danio rerio* hits. At the end, about

27,510 hits were removed from the raw transcriptome. This strategy allowed to obtain the initial assembly with 445,976 transcripts with a N50 of 2602, GC content of 44% and transcriptome length of 528 Mb (Table 2).

Table 2. Metrics and statistics of the initial and final assembly transcriptome.

	Initial assembly	Final assembly
Number of transcripts	445,976	28,220
N50	2602	3113
N90	404	1071
GC (%)	44	48
Backmapping rate (%)	93.25%	78%

To guarantee the coding status and minimum of read coverage per transcript, the TransDecoder and Corset software were applied and the longest ORF per “Trinity Unigene” was selected. Initially, TransDecoder predicted 96,710 coding transcripts with an open reading frame (ORF) of more than 100 amino acids. Then, Corset used the number of mapping reads to detect low coverage transcripts and the multi-mapping strategy to detect sequence similarity between contigs. Therefore, the low coverage transcripts (< 10 reads) were removed. Although indirectly, this approach ensured a minimum of gene expression in the final transcriptome. Importantly, both assembly and filtering strategies have been widely used in other transcriptomic projects of teleost species, with similar results (Ciezarek et al., 2019, 2020; Gioacchini et al., 2019). Then, by keeping the longest protein coding transcript per Unigene, the number of transcripts decreased to 28,220, representing the final assembly.

To assess the initial, intermedium and final assemblies, several metrics and stats were used to compare the benefits gained from the cleaning and filtering steps. Accordingly, the Transrate metrics showed an increase of 511 bp in N50 transcript length from the initial (N50=2602) to the final (N50=3113) version (Table 2). On the other hand, the final number of transcripts decreased by more than 90% (Table 2) but, despite this, the gene content did not seem to be affected. Indeed, the BUSCO showed a high amount of complete (single+multi) orthologs found in the final assembly of 96.7 %, 96.7%, 87.6%, and 80.4% in Metazoa, Eukaryota, Vertebrates and Actinopterygii library profiles, respectively (Fig. 4). The backmapping rate highlighted that 15.2% of the reads were lost during the filtering

process, which, albeit being a relevant reduction, is not proportional to the number of redundant transcripts that were removed (Table 2).

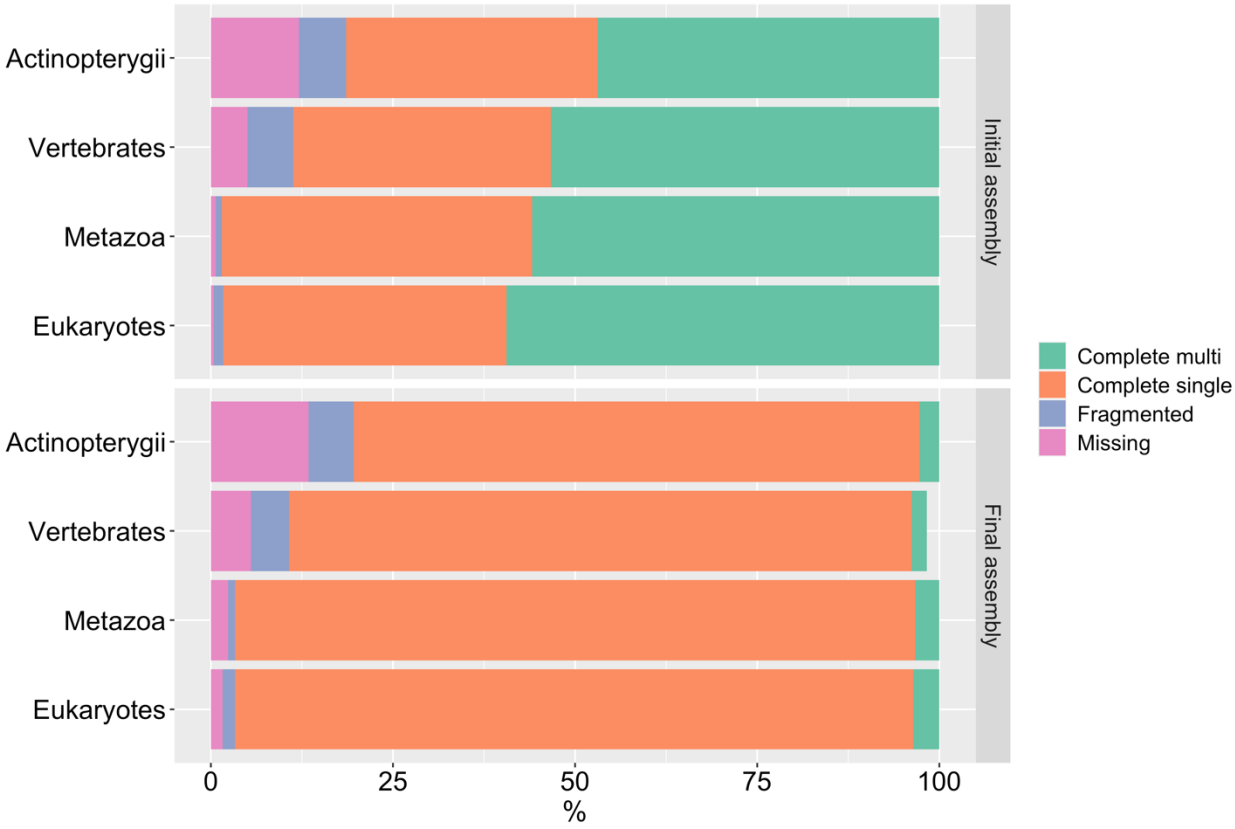

Figure 4. Quality assessment between the initial and final version of the transcriptome. Coloured bars represent the proportion of BUSCOs matched in the following databases Actinopterygii, Vertebrates, Metazoa and Eurkaryotes.

3.3.3. Exploring the functional annotation

The functional annotation of the final assembly was performed with the Trinotate pipeline (https://github.com/Trinotate/Trinotate.github.io/wiki) (Grabherr et al., 2011). Trinotate leverages several software and databases to assign characteristics to the transcriptome, such as homology, protein domains, gene ontology and metabolic pathways. In the heater tissue transcriptome, a total of 27,472 (93.3%) transcripts were found to have correspondence with at least one database. Of these, 16,901 had a match in the KEGG Pathway database, 16,507 in eggNOG and 17,682 contained at least a protein domain. Considering the homology searches, 22,690, 27,304, 19,167 and 22,279 transcripts matched the NCBI-nr, NCBI-nt, Swiss-Prot, Uniref90 databases, respectively. Importantly,

these searches were performed with several algorithms (blast-n/megablast, blast-n/dc-megablast, blast-x, blast-p), which allowed to increase the number and robustness of the annotated transcripts.

Despite the complementarity of these searches, in terms of functional annotation, the remaining analyses were mainly based on the blast results. Thus, to have a first insight in phylogeny of swordfish transcriptome, the blast-x results against NCBI-nr database were analysed in greater detail. From a total of 22,573 hits against this database, 69.95% were found in the top 10 species (Fig. 5). As expected, the top three species with more hits are were *Lates calcarifer*, *Seriola dumerili* and *Seriola lalandi dorsalis*, all belonging to the same clade of *X. gladius* species, that is Carangaria. Interestingly, this clade has nine genomes properly annotated in NCBI database (*Scophthalmus maximus*, *Lates calcarifer*, *Seriola lalandi, Hippoglossus stenolepis*, *Hippoglossus hippoglossus*, *Echeneis naucrates*, *Cynoglossus semilaevis*, *Paralichthys olivaceus*, *Seriola dumerili*) and nine species without

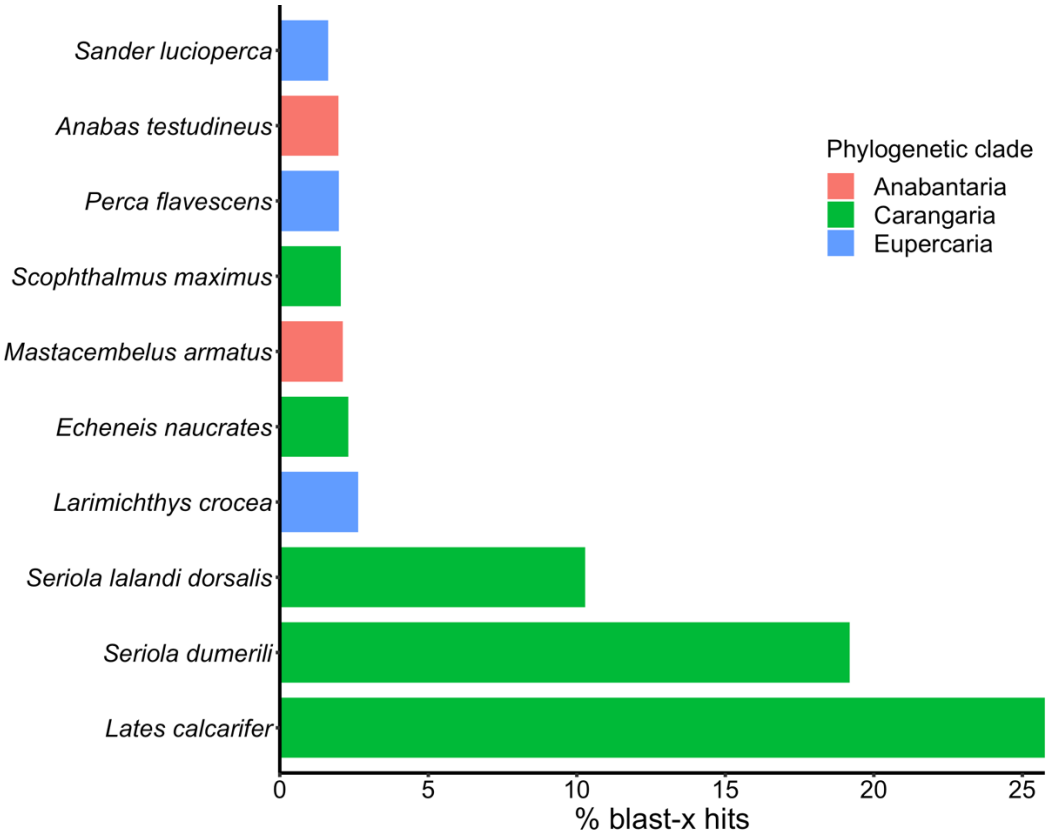

Figure 5. Top 10 species with the highest number of blast-x hits.

annotated genomes (genomes not used in functional annotation). In addition, these analyses still revealed another three Carangaria species in the top 20 species, reaching a total of 61.12% of the swordfish transcripts mapped against Carangaria species. The percentage of identity distribution in top three species showed high levels of identity between the swordfish and other species. Indeed, more than 80% of the transcripts mapping to the three top species had more than 80% of sequence identity. These results were especially relevant for the phylogenetic analyses, because they allow to select suitable genes for more focused analyses.

3.3.4. Relative gene expression and gene ontology (GO) analyses

To gain biological insights into the heater tissue, relative gene expression and gene ontology analyses were performed. These analyses allowed to identify 22,921 unigenes with relative gene expression above of one TMM normalized count.

The Gene Ontology (GO) screening was performed using the blast hits of SwissProt gene ontology results of the Trinotate pipeline. The GO database contains three main categories, namely molecular function (MF), cellular component (CC) and biological process (BP), each composed by set of classes, usually called terms, with relations and operations between them. In the heater tissue transcriptome, the transcripts matched 15,755 GO terms, of which 1,634 GO annotations in CC, 3,713 in MF and 10,408 in BP. In Fig. 6 the top 10 GO terms, per category, with the highest number of mapped transcripts are represented. On average, the CC category (2809 transcripts) had the highest number of transcripts mapped per class, followed by MF (1162 transcripts) and BP (584 transcripts). In addition, the CC showed the cytoplasm, nucleus and, cytosol terms with more than 4,000 mappings, followed by a gradual decrease of transcripts mapping to the remaining classes. On the other hand, the metal ion binding and ATP biding terms of the MF category had more the double of the mapping transcripts than the remaining classes (Fig. 6). Finally, the BP category exhibited a more similar number of transcripts mapped across all terms. Interestingly, cellular components terms GO such as mitochondrion (GO:0005739), plasma membrane (GO:0005886), integral component of membrane (GO:0016021) and membrane (GO:0016020) well reflected the structure of the membrane system and the exceptionally high density of mitochondria (55-70% of cytoplasm volume) typical of the heater tissue (Block & Franzini-Armstrong, 1988). Indeed, most of the cell volume of this tissue is occupied by mitochondria and stacked smooth membranes belonging to the sarcoplasmic

reticulum with a massive presence of Ca^{2+}-ATPase on its cytoplasmic side, characteristics that highlight the exceptional oxidative capacity, heating output and Ca^{2+}cycling of this tissue (Ballantyne et al., 1992; Block & Franzini-Armstrong, 1988; O'Brien & Block, 1996; Tullis et al., 1991). These aspects can be also found among the molecular function terms as indicated by metal ion binding (GO:0046872), ATP binding (GO:0005524) and calcium ion binding (GO:0005509), further confirming the idea that ATP-fuelled calcium cycling is one of the most prominent process driving the physiology of the heater cells that ultimately lead to heat production (Block 1987; Morrissette et al. 2003). Finally, among the biological process, both positive and negative regulation of transcription were well represented, which, along with some molecular functions such as DNA binding (GO:0003677) and DNA-binding transcription factor activity (GO:0003700), would suggest an intense transcriptional activity in the heater tissue (Fig. 6).

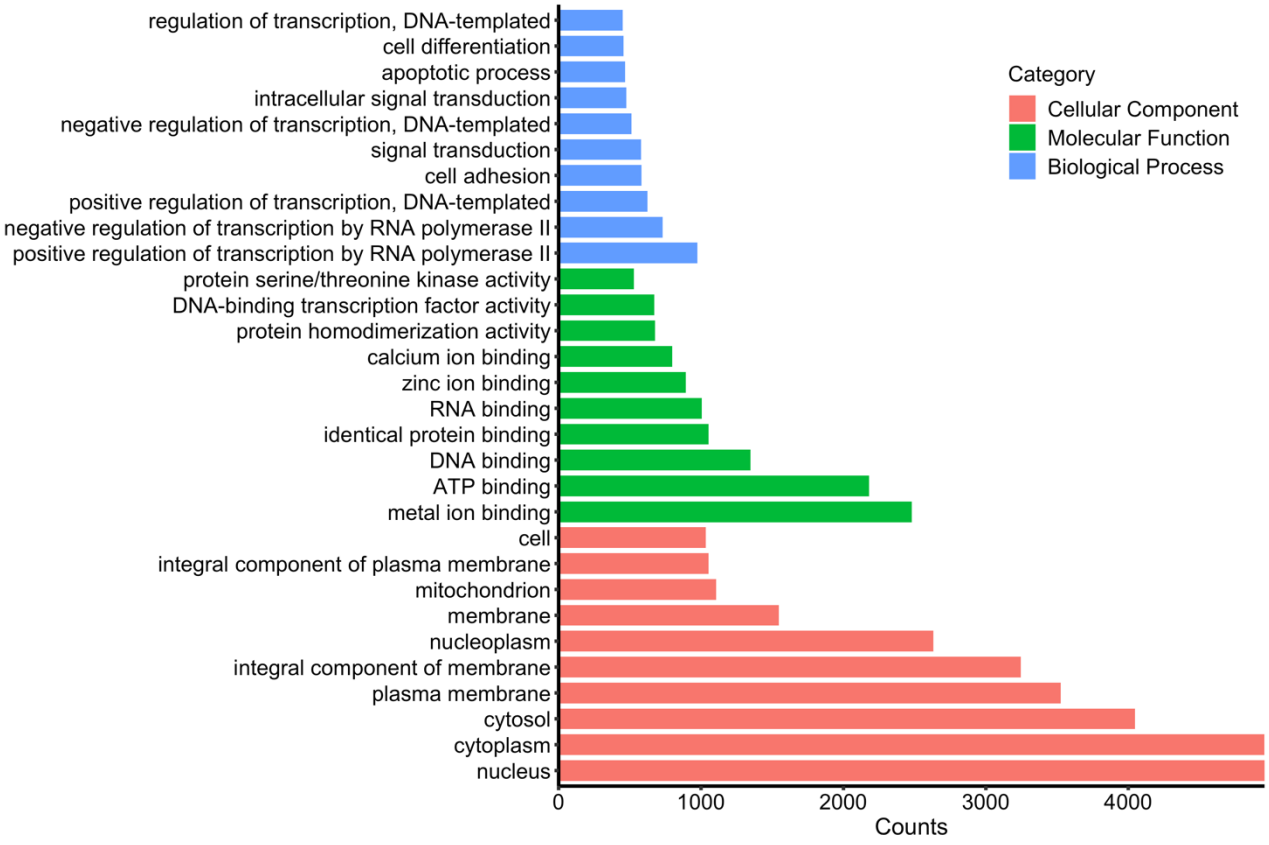

Figure 6. Top 10 GO terms per category (Biological Process, BP; Molecular Function, MF; Cellular Component, CC) with the highest number of mapped transcripts.

3.3.5. Comparative analyses

In the comparative analyses three protein-coding transcriptomes were analysed, the swordfish heater tissue organ and heart and the Pacific bluefin tuna red muscle. While both heater and heart tissues were assembled on this study (raw data of heart tissue was originally published in Marra et al., 2017), the Pacific bluefin tuna red muscle transcriptome was collected from the multi-tissue transcriptome generated in Ciezarek et al. (2020). The protein datasets arising from swordfish datasets showed much lower number of protein-coding transcripts than the Pacific bluefin tuna dataset. Indeed, the swordfish heater tissue transcriptome comprised 28,220 transcripts, the swordfish heart transcriptome 41,011 transcripts while the Pacific bluefin tuna red muscle transcriptome 43,068 transcripts. These different values are expected, as the methodologies used in both studies are quite different. Indeed, while in this study a single-assembler transcriptome strategy, Ciezarek et al. (2020) applied a multi-assembler methodology, followed by an exhaustive redundancy removal. Despite the initial differences in the number of sequences analysed per dataset, these values didn't impact the results and the overall goals of this analysis, that is the identification of assembly-specific transcripts in the swordfish heater tissue and the assessment of common transcripts.

From a total of 112,229 transcript, 93,595 (83.3%) transcripts were assigned to 22,326 orthogroups, of which 12,049 were shared across all datasets and 1488 to single-copy orthogroups. Of these, 20,781 and 35,915 transcripts derived from the swordfish heater tissue and heart transcriptomes, respectively, while 36,899 are from the Pacific bluefin tuna transcriptome. Regarding the assembly-specific orthogroups and corresponding transcripts, the swordfish heater tissue showed 747 orthogroups containing 1847 transcripts, the heart transcriptome showed 2081 orthogroups containing 6537 transcripts while the Pacific bluefin tuna showed 1,739 orthogroups containing 6006 transcripts.

3.3.6. KEGG pathways

The KEEG pathways analyses were performed to detect the global metabolic mechanisms in heater tissue (Fig. 7). From a total of 28,220 protein coding transcripts blasted against

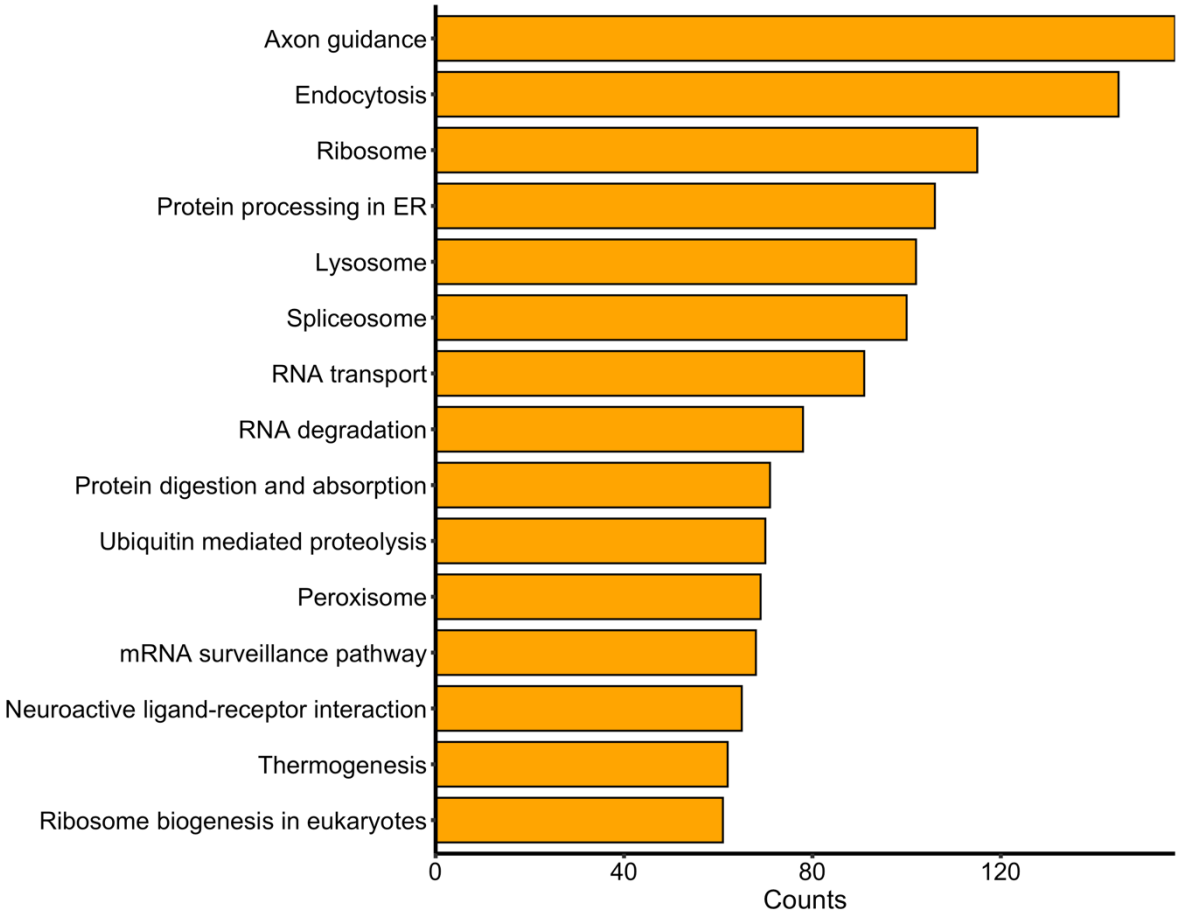

Figure 7. Top 15 pathway with the highest number of mapped transcripts. Abbreviations: ER= Endoplasmic Reticulum

40 species, 8325 were mapped to KEGG ortholog terms (KOs) in 354 pathways maps. Although the general character of the first and second categories of the KEGG database, both were important to set out the path for the subsequent analyses. In category three, several pathways linked to specific biological functions of the heater tissue were found such as the thermogenesis (ko04714).

3.4. Conclusion and future perspectives

Although presented as preliminary data chapter, this work represents the first step toward a better understanding of the molecular pathways and candidate key genes involved into the heat production in the swordfish heater tissue. The future completion of this work will shed light on how phylogenetically distant species such as lamnid shark, tunas and billfishes achieved the regional endothermy and explore in details the process of the so-called convergent evolution.

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4. *De novo* transcriptome assembly, functional annotation and characterization of the Atlantic bluefin tuna (*Thunnus thynnus*) larval stage

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4.1. Introduction

The Atlantic bluefin tuna *Thunnus thynnus* is a well-known iconic species and one of the largest fish in the pelagic realm. Due to a wide geographical distribution range, exceptional migratory behaviour and high commercial interest, the management of the fishing activities requires coordinated international cooperation and networks, a task managed by International Commission for the conservation of Atlantic Tunas (ICCAT). As a result of this international conservation effort, the Atlantic population is divided into two management units, an Eastern and Western stock. However, an ever-increasing demand over recent years, mainly driven by the sushi and sashimi Japanese market, has led the Eastern stock to the brink of collapse (MacKenzie et al., 2009). The strict restrictive measures (ICCAT, 2008) were effective to recover the stock but the risk taken also suggested that the time was ripe to develop an aquaculture-based market.

Any large-scale aquaculture operation requires full control of the production cycle of the species of interest, a key point not yet accomplished for the Atlantic bluefin tuna (Betancor et al., 2019; De Metrio et al., 2010;), whose production today is based on a ranching system (Mylonas et al., 2010). Such an industrial model is heavily dependent on the fishing activities and as a consequence, on natural population fluctuations.

To achieve domestication, a thorough knowledge of the reproductive cycle and the captivity effects on gametogenesis and endocrine axis is necessary (Carnevali et al., 2019; Medina et al., 2016; Zohar et al., 2016). Recently, more attempts to manipulate the life cycle (i.e. induce spawning) and to grow the larvae were carried out with promising achievements (Betancor et al., 2017, 2019, 2020; Blanco et al., 2017; De Metrio et al., 2010; Mylonas et al., 2007; Reglero et al., 2014; Yúfera et al., 2014). In this context, understanding nutritional requirements, growth pattern, responses to external stimuli, morphological and physiological changes and discovering stage-specific markers during larval development are key issues

(Gisbert et al., 2008; Rønnestad et al., 2013). Thus, a deep knowledge of the genes underlying these processes is necessary to obtain a more complete picture of the larval development. However, one of the constrains to figure out requirements and best farming conditions for the Atlantic bluefin tuna is a surprising lack of genomic and transcriptomic resources. This gap also impairs genome evolution approaches and our comprehension of the processes driving unique biological traits observed in this species such as the exceptional growth and early piscivorous habits (Kaji, 2003), migratory behaviour (Block et al., 2001), thermogenesis (Ciezarek et al., 2016, 2019), body size and swimming physiology (Graham & Dickson, 2004). All these aspects make the Atlantic bluefin tuna an interesting model for understanding its physiological novelties (Madigan et al., 2015; Shiels et al., 2011; Wegner et al., 2013).

In the Atlantic bluefin tuna, the first study dealing with omics technologies dates back more than a decade ago with the application of expressed sequence tags (EST) to investigate tissue-specific expression libraries in ovary, testis and liver (Chini et al., 2008). Then, Trumbić et al. (2015) applied RNA-seq on a mixed-tissue library from adult Atlantic bluefin tunas with the main purpose of developing a DNA microarray. Therefore not even a single assembled and ready-to-use transcriptome, along with the relative annotations, was publicly available for this species. It follows that this de novo larval transcriptome assembly represents a crucial step towards a better understanding of the larval development and a significant progress of the transcriptomic resources available for this species. For these reasons, by taking advantage of publicly available databases, the aim of the present study was to generate, annotate and characterize a *de novo* Atlantic bluefin tuna larval transcriptome assembly from a comprehensive pool of several larval stages. Orthologous groups with other fish species as well as assembly-specific orthogroups were established using a comparative approach and, along with the analysis of codon bias and expanding protein families, allowed for the identification of important processes during larval ontogeny that may contribute to shape the unique physical traits observed in this species. The information generated by this study represents a significant advance of the transcriptomic resources available for the Atlantic bluefin tuna and will support future studies aimed at investigating the molecular physiology in different larval stages.

4.2. Material and methods

4.2.1. Data availability, pre-processing steps and *de novo* assembly

The experimental dataset (100 bp paired end; Illumina HiSeq 2000) from a pool of larvae at different developmental stages (0 days post hatch - dph, 2dph, 10dph, 15dhp and 20dph) was downloaded from the NCBI Sequence Read Archive (SRA Accession number: SRR1536893; BioProject PRJNA252566) and analysed in a rigorous bioinformatic workflow (Fig. 1). For this raw data, neither a publication nor any report and/or related information was found at the time of the download (December 2019). The quality of the raw reads was first assessed with FastQC (https://www.bioinformatics.babraham.ac.uk/projects/fastqc/). Along the workflow, FastQC was used before and after each step to compare the benefits derived from each tool applied (Fig. 1). First, ribosomal RNA was filtered out with SortMeRna (Kopylova et al., 2012) by screening the raw reads against the provided representative rRNA databases. Then, potential bacterial, archaeal and viral contamination was eliminated with Kraken2 (Wood et al., 2019) using the standard Kraken database. Decontaminated reads were further processed with Prinseq (Schmieder & Edwards, 2011) to remove low complexity sequences and trim long poly-A/T tails. As a last step before the assembly process, gentle trimming was applied with Trimmomatic (Bolger et al., 2014) with parameters adjusted according to MacManes (2014). The resulting cleaned reads were used for the de novo assembly step with Trinity (Haas et al., 2013) without in silico normalization. The level of strand-specificity of the library was checked with the Trinity script examine_strand_specificity.pl. The complete list of specific parameters set for each software with the relative version is available in Supplementary material S1.

4.2.2. Assembly optimisation and functional annotation

Cleaned reads were mapped back to the raw transcriptome assembly by applying Bowtie2 (Langmead & Salzberg, 2012) and the overall metrics were calculated with Transrate (Smith-Unna et al. 2016). Then, the completeness of the assembly was assessed with BUSCO (Simão et al., 2015) using the Actinopterygii odb9 database and gVolante (Nishimura et al., 2017) using a set of Core Vertebrate Genes (CVG) as identified by Hara et al. (2015). The percentage of full-length reconstructed transcripts was calculated with the analyze_blastPlus_topHit_coverage.pl script of the Trinity package by blasting the translated transcriptome against the proteome (Sedor1 version) of the yellowtail amberjack (*Seriola lalandi dorsalis*) downloaded from Ensembl release 98 (www.ensembl.org). The rationale behind that is the availability of the complete genome for this species and the relatively close phylogenetic relationship of the Carangidae (order Perciformes) with tunas

(Hughes et al., 2018). As an additional assessment, the N50 metric was integrated with the expression data computed with the Trinity tool suite (align_and_estimate_abundance.pl; abundance_estimates_to_matrix.pl) using Kallisto (Bray et al., 2016) as quantification

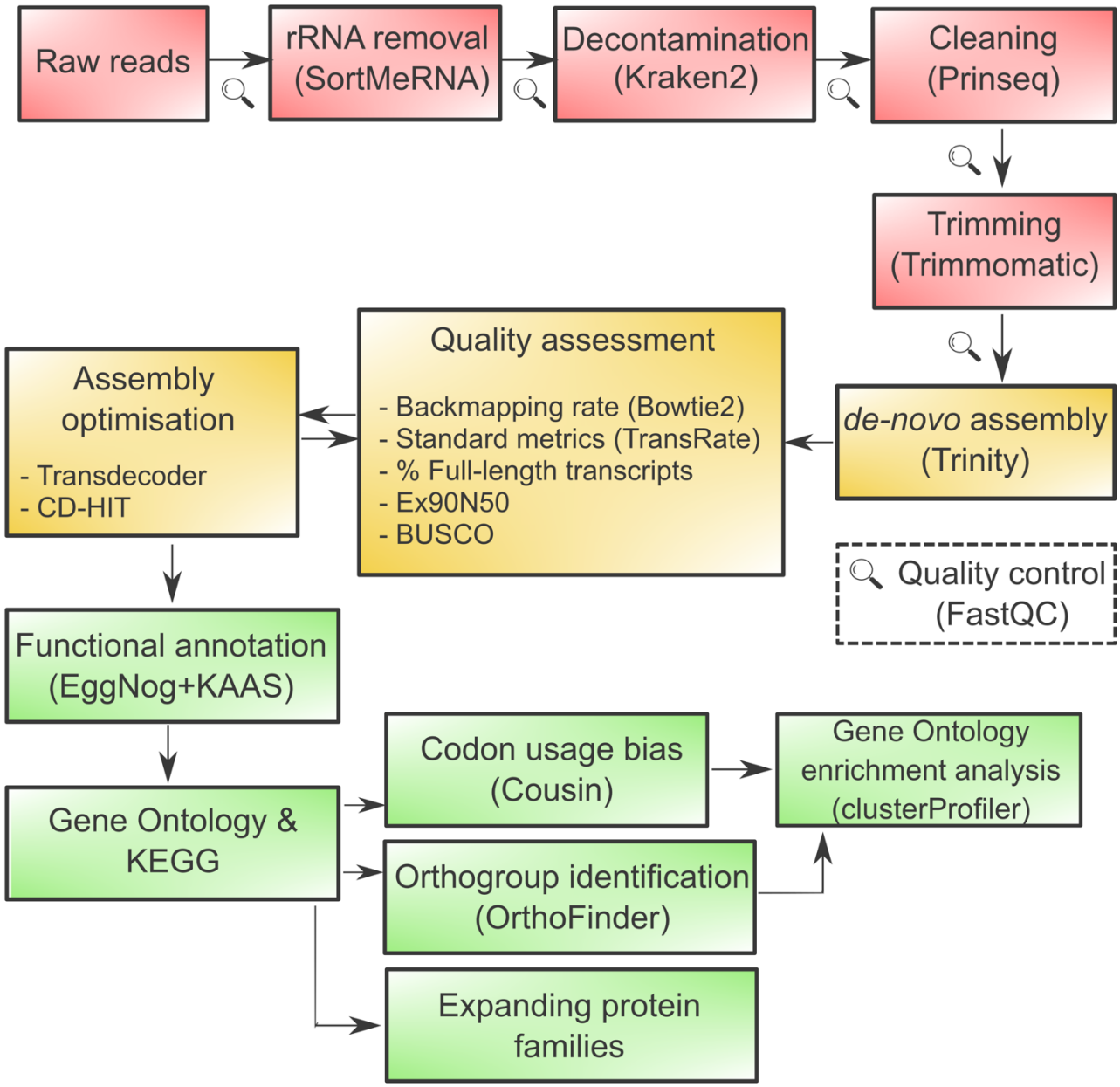


Figure 1. Schematic workflow of the analysis carried out to generate and annotate the de novo transcriptome assembly of the Atlantic bluefin tuna larvae. The tools used at each step are reported in brackets

method. This produced the Ex90N50 metric, that is the N50 statistic limited to the highly expressed transcripts that represent 90% of the total normalized expression data (for

detailed methods see the Trinity GitHub page at https://github.com/trinityrnaseq/trinityrnaseq/wiki/Transcriptome-Contig-Nx-and-ExN50-stats) .

The raw transcriptome was then optimised with TransDecoder (Haas et al., 2013) by keeping the single best Open Reading Frame (ORF) per transcript and with CD-HIT (Fu et al., 2012) to cluster transcripts with 100% amino acid identity. To assess the quality gained with the optimisation procedure, the same checkpoints adopted for the raw transcriptome were re-applied and the corresponding results compared between the two versions (Fig. 1). Most of the steps required up to the quality assessment were performed on the public Galaxy-Europe platform (Afgan et al., 2018). Once optimised, the transcriptome was annotated with EggNog-mapper v2 (Huerta-Cepas et al., 2017) and the Gene Ontology terms analysed with CateGorizer using the GO slim terms as reference (Zhi-Liang et al., 2008). Furthermore, the transcriptome was also annotated with the KEGG Automatic Annotation Server (KAAS) using the Blast bi-directional best hit (BBH) method (Moriya et al., 2007). The results of this step were then analysed with the “KEGG mapper- search and colour pathways” using “reference” as search mode. The information and number of genes/pathway were retrieved using KEGGREST (Tenenbaum, 2018).

4.2.3. Codon usage bias analysis

Codon usage bias was assessed by calculating the Effective Number of Codons (ENCs), a measure of how much codon usage preferences are distant from an equal usage of synonymous codons (Wright, 1990). According to the Transdecoder output, only the coding sequences from transcripts with a complete open reading frame were retained for this analysis. The calculation of the ENCs was performed with COUSIN (Bourret et al., 2019). The transcripts with an ENCs value falling below the first quartile of the distribution were considered as having stronger codon usage bias and therefore isolated for downstream analysis. Then, the Gene Ontology enrichment analysis was performed for these transcripts with clusterProfiler (Yu et al., 2012) in the RStudio environment. The *p*-values (cut-off 0.05) calculated by the hypergeometric distribution were adjusted with the Benjamini-Hochberg method (Benjamini & Hochberg, 1995). Then, Revigo (Supek et al., 2011) was used to obtain representative terms and help to reduce redundancy.

4.2.4. Comparative analysis

The optimised transcriptome was compared with the proteome of other fish species, for which an annotated genome was available on Ensembl release 98 (www.ensembl.org), by using OrthoFinder to identify phylogenetic relationships and orthologous groups, also called orthogroups (Emms & Kelly, 2015, 2019). As explained by the authors, an orthogroup is the extension of the notion of orthology to groups of species, meaning that an orthogroup is the group of genes descended from a single gene in the last common ancestor of a group of species. For this analysis, the chosen species were the zebrafish (*Danio rerio*), the spotted gar (*Lepisosteus oculatus*), the Atlantic salmon (*Salmo salar*), the yellowtail amberjack (*Seriola lalandi*), the Nile tilapia (*Oreochromis niloticus*), the gilthead sea bream (*Sparus aurata*), the pufferfish (*Tetraodon nigroviridis*) and the Atlantic cod (*Gadus morhua*). The species were chosen to be representative of broad taxonomical groups of fish. To visualise intersections between groups, the R package UpSetR (Conway et al., 2017) was chosen instead of the Venn diagrams due to the high number of intersections. The species tree was produced using STAG, a novel algorithm that infers species tree from sets of multi-copy gene trees and displayed comparable to higher accuracy than conventional approaches (for details see Emms & Kelly, 2018). The species tree contains support values that correspond to the proportion of species trees derived from gene trees with that specific bipartition, a more stringent measure than standard bootstrap support from a multiple sequence alignment (Emms & Kelly, 2018). This algorithm is fully implemented in OrthoFinder and run as default (Emms & Kelly, 2015, 2019). The resulting tree from the OrthoFinder analysis was plotted with the online tool iTOL (Letunic & Bork, 2007). Then, a Gene Ontology enrichment analysis was performed on transcripts included only in Atlantic bluefin tuna orthogroups with the aim to shed light on biological functions associated with this assembly-specific signature. After enrichment, Revigo was used to obtain representative terms and to help reduce redundancy.

In addition to the aforementioned tools, the final plots were created in the RStudio environment with the following packages: ggplot2 (Wickham, 2016) and ggpubr (Kassambara, 2020). The black silhouettes used for the final figures were downloaded from the PhyloPic website (http://phylopic.org/).

4.2.5. Protein family expansion

The MMSeq2 web-server (Steinegger & Söding, 2017) and rpstblstn (Camacho et al., 2009) were used to identify PFAM functional domains (PFAM 32.0) in the *Thunnus thynnus* transcriptome. For the other species considered, PFAM annotations were directly obtained from Ensembl. The following procedure was adopted according to previous studies (Lan et al., 2017; Martínez-Barnetche et al., 2018) and run separately for results obtained from MMSeq2 and rpstblastn. Briefly, repeated functional domains for the same protein were only counted once. Then, a Fisher's exact test followed by Bonferroni correction was applied to assess the difference of domain numbers between *T. thynnus* and the average number of the other species. Protein families with a domain count larger than that of the average of the other species and with a adjusted *p*-value < 0.05 were considered significantly expanded. Only the protein families identified as expanded using results from both rpstblastn and MMSeq2 were considered, corresponding to a rather conservative approach.

4.3. Results

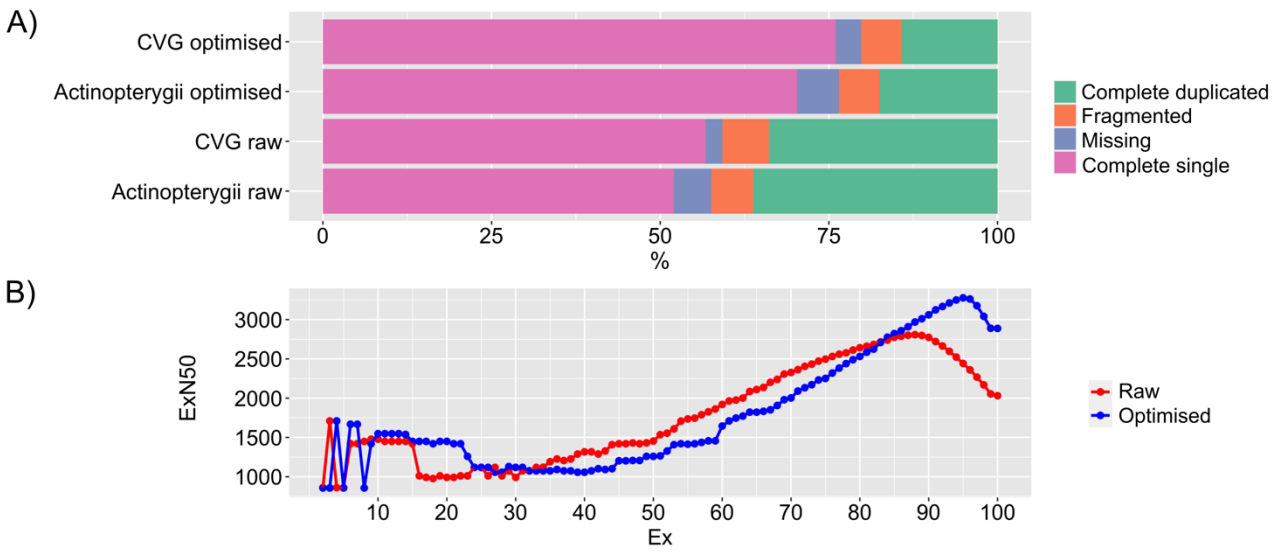
4.3.1. Transcriptome assembly and quality assessment

The raw data consisted of 29,910,574 millions of Illumina paired-end reads which were narrowed to 27,379,333 after the filtering and trimming steps. A comparison between the metrics and quality of the raw and optimised transcriptomes is summarised in Table 1. Up

Table 1. Statistics regarding the quality assessment of the raw and optimised transcriptomes

Statistic	Raw	Optimised
N° sequences	107,465	37,117
GC content	45%	48%
N50	2835 bp	3267 bp
Ex90N50	2774 bp	3061 bp
Backmapping rate	98.30%	83.71%
N° full-length transcripts (>80% coverage)	13,972 (13%)	13,633 (36%)

to 65.5% of the transcripts were dropped in the optimised transcriptome without significantly affecting the overall quality, suggesting that a consistent fraction of the reconstructed transcripts was background noise derived from lowly expressed transcripts. Raw transcriptome benchmarking against the Actinopterygii single-copy orthologues database (odb9, n=4584) found 88.2% complete (Single:52.0%, Duplicated:36.2%), 6.2% fragmented and 5.6% missing BUSCOs while 87.8% complete (Single:70.3%, Duplicated:17.5%), 6% fragmented and 6.2% missing BUSCOs in the optimised transcriptome (Fig. 2A). Similar results were obtained by benchmarking the two transcriptomes against the CVG database (n=233). Indeed, the raw version was found 90.6% complete (Single:56.7%, Duplicated:33.9%), 6.9% fragmented and 2.5% missing BUSCOs while 90.2% complete (Single:76.0%, Duplicated:14.2%), 6.0% fragmented and 3.8% missing BUSCOs for the optimised version (Fig. 2A). The shape of the Ex90N50 curves for the initial and optimised assembly confirmed the efficacy of the post-assembly optimising procedure (Fig. 2B) as outlined by the developers on the Trinity wiki page (https://github.com/trinityrnaseq/trinityrnaseq/wiki). The analysis of the strandness revealed non-strand-specific RNA-Seq data. Since the quality assessment revealed a good improvement following the optimising steps, all the downstream analyses were carried out on the optimised transcriptome. The optimised transcriptome is available as Supplementary material S2.



◄ **Figure 2.** Quality assessment between the raw and optimised transcriptomes A) percentages of BUSCOs identified when searched against the Actinopterygii database (odb9) and the set of Core Vertebrate Genes (CVG); B) ExN50 curves representing the percentage of the total normalized expression data (x-axis) with the relative N50 values (y-axis)

4.3.2. Functional annotation

A total of 34,980 transcripts (94.2% of the total sequences) had a hit against the EggNOG database, of which 25,539 (72.99%), 26,024 (74.38%) and 28,836 (77.6%) were associated with the relative GO terms, KEGG orthology functional annotation and gene symbols, respectively (Supplementary material S3). A few transcripts belonging with high identity (95-100%) to *Artemia spp* and *Brachionus spp*, were detected as contamination and removed for downstream analysis. The KAAS annotation server assigned a KEGG orthology identifier to 14,983 transcripts (Supplementary material S3). The GO slim terms by functional category (biological process, molecular function and cellular component) are summarised in Fig. 3. The most represented biological processes were related to development (GO:0008152), metabolism (GO:0007275), cell organization (GO:0016043), transport (GO:0006810) and cell differentiation (GO:0030154). The terms intracellular (GO:0005623), cytoplasm (GO:0005622) and cell (GO:0005737) were the top represented cellular components. Moreover, among the molecular functions there were catalytic (GO:0003824), hydrolase activity (GO:0016787), transferase activity (GO:0005488) as well as binding (GO:0016740). Among the most interesting KEGG pathways with a relevant number of mapped genes there were metabolism-related pathways (fatty acids elongation: map00062; biosynthesis of unsaturated amino acids: map01040, arachidonic acid metabolism: map00590, PPAR signalling: map03320), phototransduction (map04744), GnRH signalling (map04912), thyroid hormone signalling (map04919) and cell death-related pathways (autophagy-animal: map04140, apoptosis: map04210) (Fig. 3).

4.3.3. Selection of codons during the larval development

The distribution of the ENCs was slightly skewed to lower values with a mean value of 49.88 and the first quartile of 47.31. A total of 3647 transcripts were found below the first quartile of the ENCs distribution (i.e. more biased codon usage) and used for the enrichment analysis. Accordingly, some of the statistically significant over-represented (adjusted p-

value < 0.05) biological processes from the Gene Ontology enrichment analysis were related to translation (GO:0006412), muscle organ development (GO:0007517) and microvillus assembly (GO:0030033). Among the cellular component, there were ribosome-related terms (ribosomal subunit: GO:0044391; large ribosomal subunit: GO:0015934; cytosolic ribosome: GO:0022626), myelin sheath (GO:0043209) and polymeric cytoskeletal fiber (GO:0099513). Instead, among the molecular functions, there were terms linked to ion transmembrane transport (active ion transmembrane transporter activity: GO:0022853,

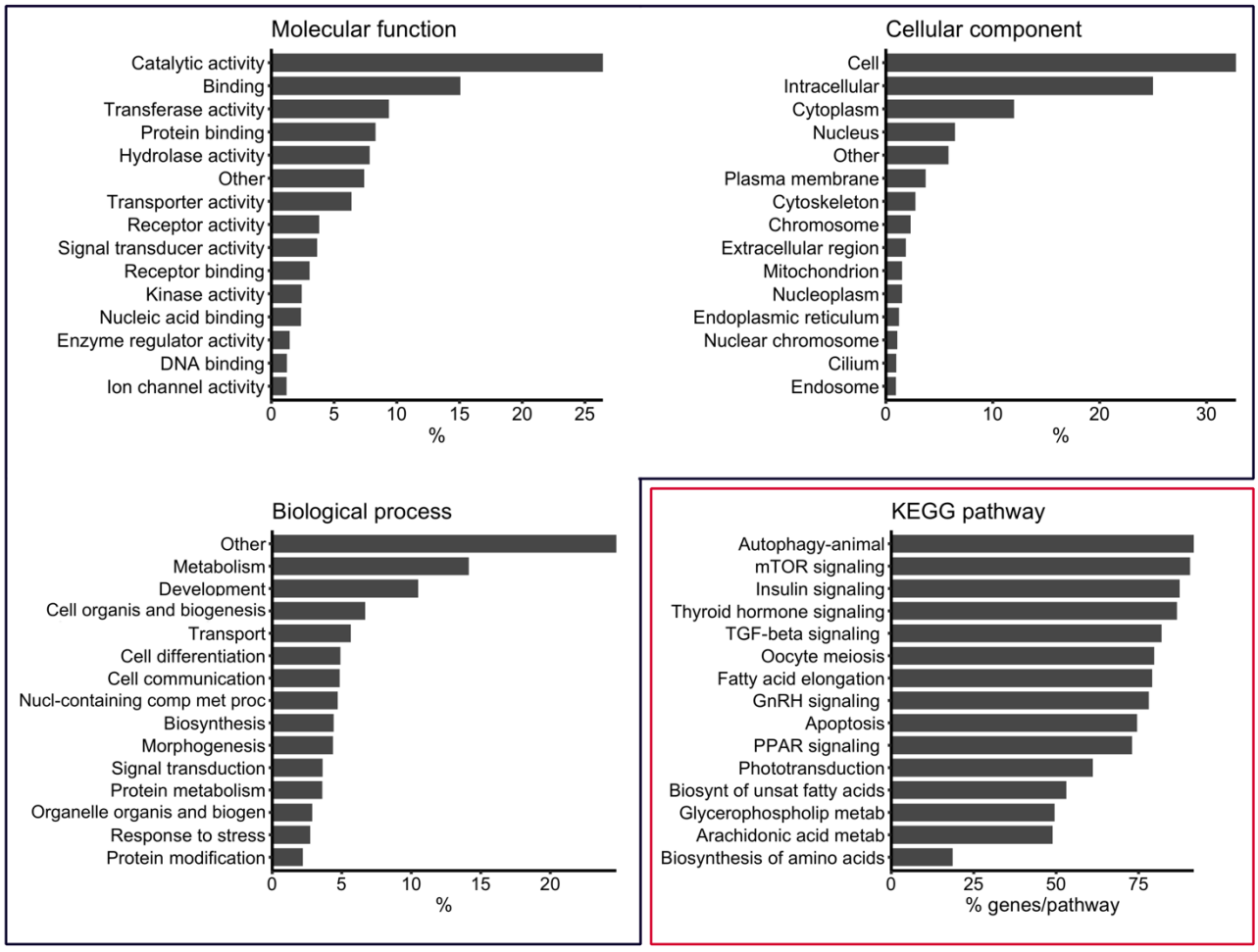
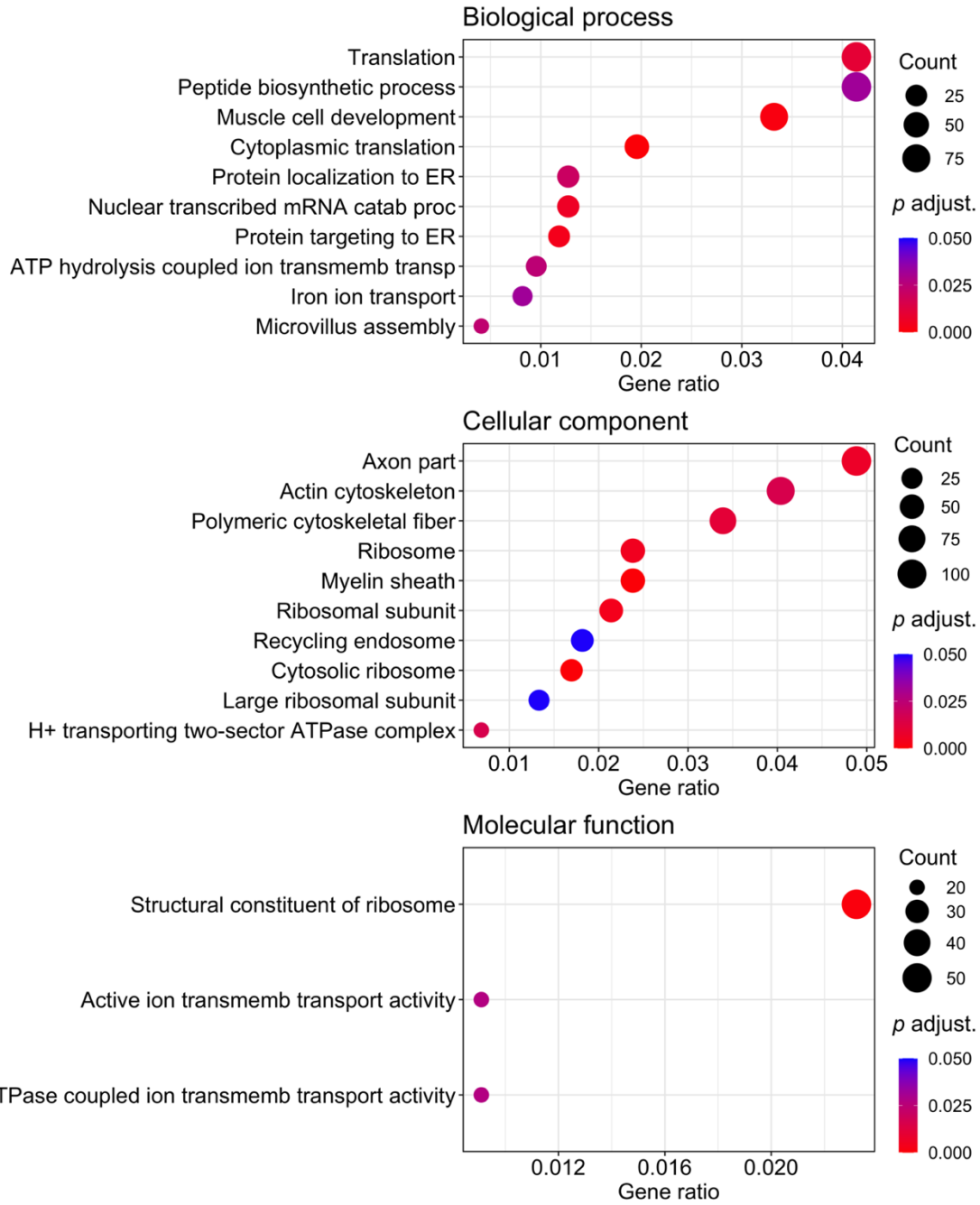


Figure 3. Proportion of Gene Ontology annotations for the three categories corresponding to molecular functions, cellular component and biological process (black box) and the percentage of genes identified in relevant KEGG pathways (red box)

ATPase-coupled ion transmembrane transporter activity: GO:0042625) and structural component of ribosome (GO:0003735). The results of the Gene Ontology enrichment



analysis are reported in Fig. 4. The complete list of enriched terms can be found in the Supplementary material S4.

Figure 4. Gene Ontology enrichment analysis of transcripts falling below the first quartile of the ENCs distribution corresponding to lower values (i.e. more codon usage bias). The gene ratio (x-axis) corresponds to k/n in which k is the number of annotated genes in the gene list of interest for a particular term and n is the size of the list of gene of interest. Abbreviations: ER: endoplasmic reticulum

4.3.4. Orthogroups identification

The OrthoFinder analysis identified 10,196 orthogroups with all the species present (Fig. 5A) and a fraction of genes was in species-specific groups ranging from 1.6 to 12.7% of the total (Fig. 5B) (Supplementary material S5). Between 86.6% and 97% of the genes across

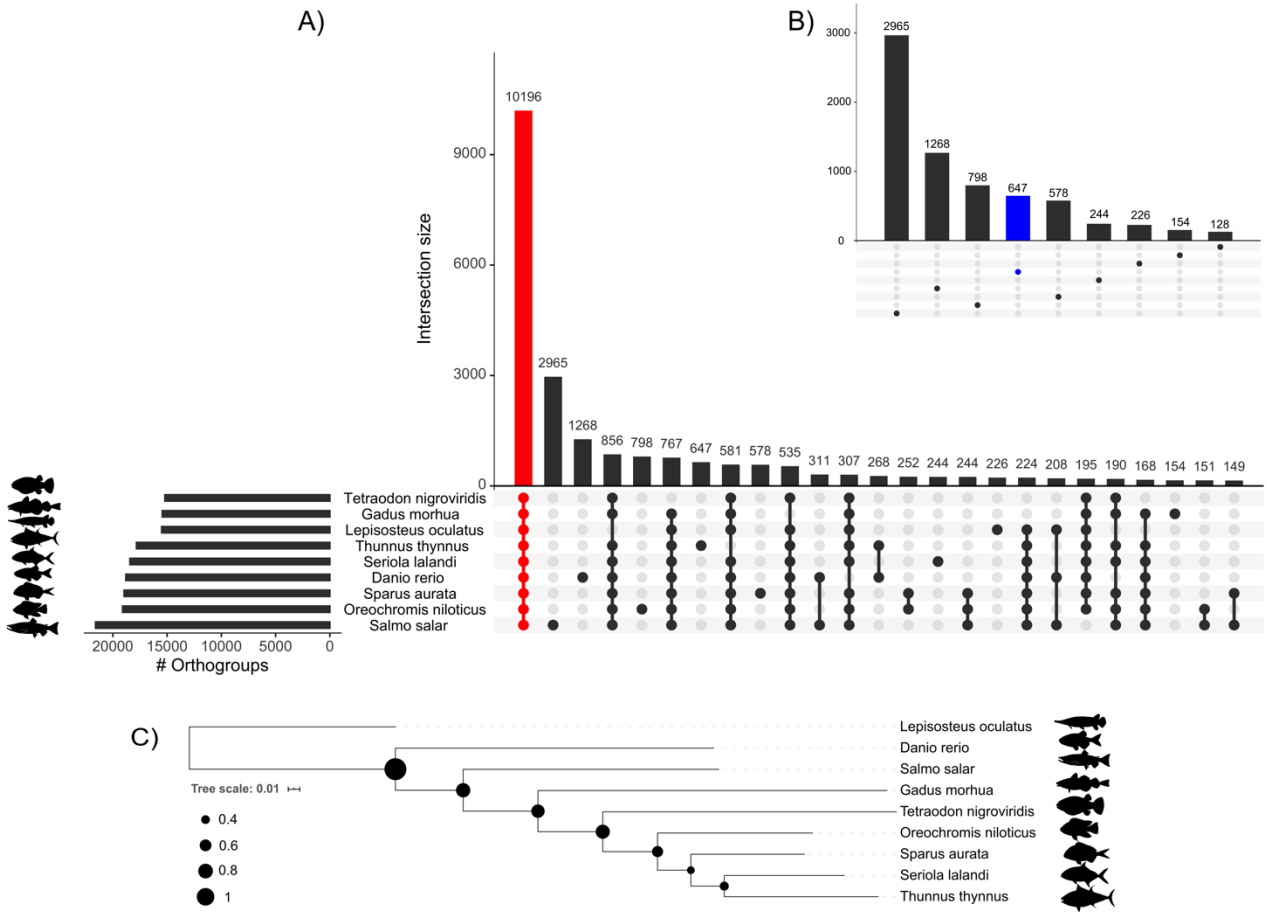


Figure 5. Number of genes in orthogroups in the selected species (A) and in species-specific groups (B). The red bar corresponds to orthogroups shared by all the species considered in the analysis and the blue bar corresponds to the assembly-specific tuna groups. Numbers above each bar represent the number of orthogroups shared in that particular intersection between species. (C) The phylogenetic tree reconstructed by the OrthoFinder analysis. The support values correspond to the proportion of species trees derived from gene trees with that specific bipartition (Emms & Kelly, 2015, 2019).

the seven species were assigned to orthogroups and between 3% and 13.4% of the total was not assigned to any orthogroup (Supplementary material S5). A total of 148 single-copy orthogroups, which correspond to groups containing exactly 1:1 orthologue proteins, were identified. The complete and single-copy list of orthogroups can be retrieved from the Supplementary material S6. The phylogenetic relationships between the selected species, reconstructed by OrthoFinder, are displayed in Fig. 5C. As expected, the spotted gar *Lepisosteus oculatus* was placed at the root of the phylogenetic tree and Atlantic bluefin tuna was more closely related to *Seriola lalandi* (Carangidae) followed by the *Sparus aurata* (Sparidae). The tuna transcriptome resulted in 32,330 (87.1%) and 4777 (12.9%) transcripts assigned and unassigned to orthogroups, respectively. A total of 647 assembly-specific tuna groups, containing 1880 transcripts, were found. Of these transcripts, 986 were annotated with a gene name which were narrowed down to 456 if duplicated annotations were removed. These transcripts were functionally enriched (adjusted *p*-value < 0.05) in terms related to muscle (GO:0006936 muscle contraction; GO:0003012 muscle system process; GO:0030017 sarcomere; GO:0016459 myosin complex), eye (GO:0048592 eye morphogenesis) and bone (GO:0060348 bone development) development, ion transport (GO:0030001 metal ion transport; GO:0022890 inorganic cation transmembrane transporter activity) and other key functions during larval growth such as metamorphosis (GO:0007552) and response to light stimulus (GO:0009416) (Fig. 6). The full results of the gene ontology enrichment analysis as well as the complete list of assembly-specific transcripts can be found in the Supplementary material S7.

4.3.5. Expanding protein families

A total of 40 expanding protein families were identified, including domains of laminin G (PF00054), myosin tail (PF00723), glutathione S-transferase N-terminal (PF13409), immunoglobulin (PF00047, PF13895) and ion channel (PF07885). The complete list of expanding protein families can be found in the Supplementary Material S8.

4.4. Discussion

The larval transcriptome generated in the present study provides extensive molecular information on the Atlantic bluefin tuna, paving the way to future genome evolution approaches and gene discovery. It represents a useful resource for an organism whose

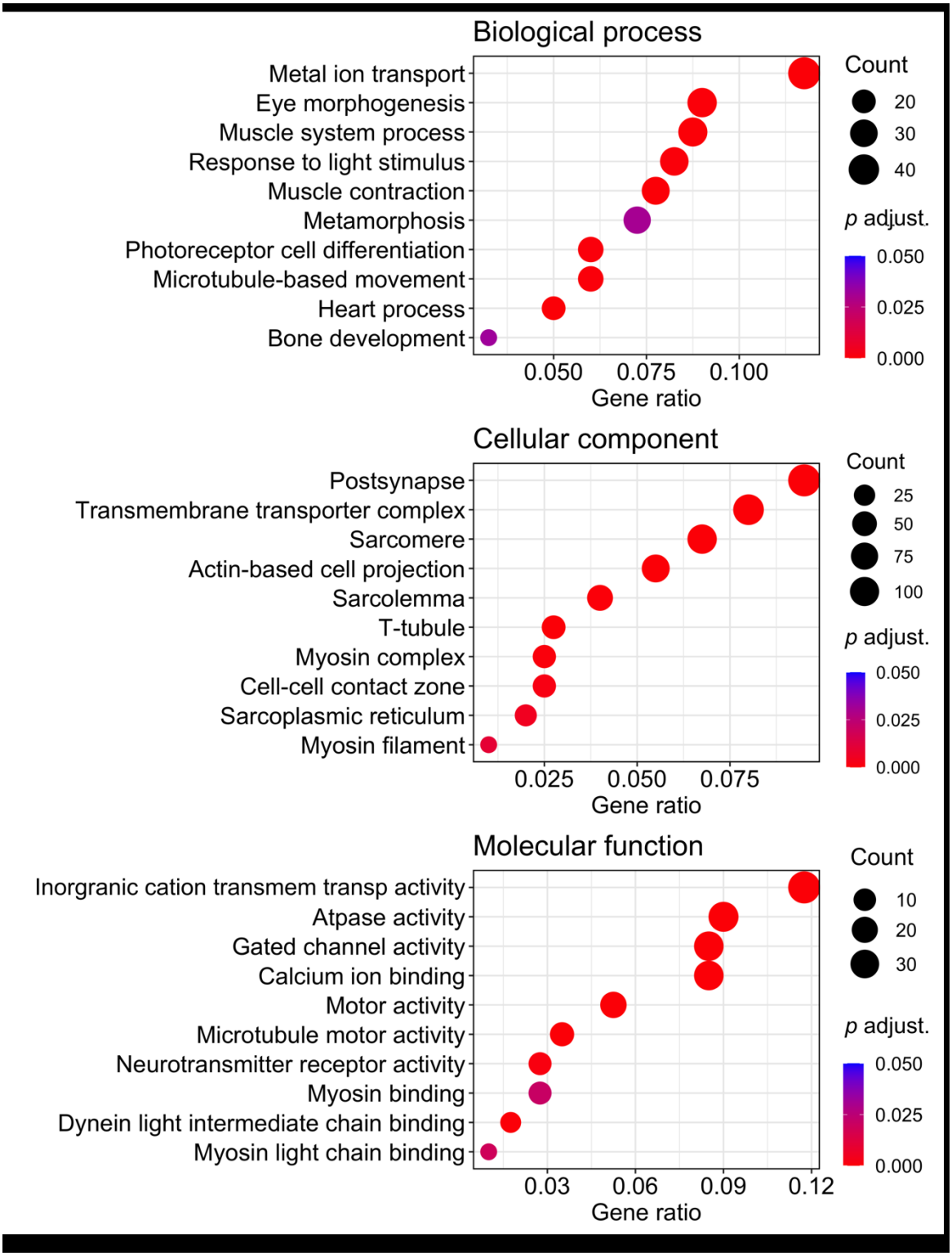


Figure 6. Gene Ontology enrichment analysis of Atlantic bluefin tuna assembly-specific orthogroups. The gene ratio (x-axis) corresponds to k/n in which k is the number of annotated genes in the gene list of interest for a particular term and n is the size of the list of gene of interest.

genome has not been sequenced yet. With more than 30,000 annotated transcripts this transcriptome likely covers a large fraction of protein-coding genes encoded by the *T. thynnus* genome. The present study provides a glimpse into the evolution of a set of genes that might have allowed the Atlantic bluefin tuna to achieve early physical advantages over competing species in the pelagic environment.

According to general principles which have become well established after more than a decade of de novo transcriptomes (Conesa et al., 2016; Moreton et al., 2016), the parameters chosen to assess the tuna transcriptome revealed an overall good quality and significant benefits were gained from optimising the procedure of the raw assembly. Among the parameters, in a transcriptome context, the N50 seems to be inappropriate due to the high variability of the transcriptome (i.e. very lowly vs very highly expressed transcripts) and because longer contigs do not necessarily reflect better assemblies (Salzberg et al., 2012). Accordingly, the N50 classical metric was also integrated with expression values to produce the Ex90N50, a more appropriate and biologically relevant metric for transcriptomes, although in our case the two results were in agreement. Furthermore, the redundancy of the transcriptome, which is expected with this kind of approach, was reduced following the optimisation procedure, as indicated by the decrease of complete and duplicated orthologues. This might be of particular interest in light of the effects that redundant transcripts can have on transcript quantification (Hsieh et al., 2019).

The tendency of some codons to be unevenly used in a transcriptome, guided by codon optimality, is a widespread phenomenon that holds from bacteria to higher eukaryotes (Hanson & Coller, 2018). Codon usage preferences are indeed linked to mRNA stability, protein folding, translation efficiency and elongation, with highly expressed genes showing similar bias in codon content (reviewed in Chaney & Clark, 2015; Hanson & Coller, 2018; Novoa & Ribas de Pouplana, 2012). Genes enriched in optimal codons conserved between frog, fly, mouse and zebrafish and responsible for regulating mRNA stability were enriched in GO terms belonging to housekeeping processes (i.e. translation, protein folding, modulation of actin dynamics) during the maternal-to-zygotic transition (MZT) (Bazzini et al., 2016). Our results showed that more biased transcripts are mainly involved in the translational molecular system and muscle development. Remarkably, two over-represented translation-related biological processes in the present study (see Supplementary material S4) matched exactly conserved processes between the aforementioned species for genes enriched in optimal codons. In European sea bass, muscle development, protein biosynthesis and energy pathways were found to be, among

others, differentially expressed during the larval ontogeny (Darias et al., 2008) and muscle development was specifically up-regulated in Atlantic bonito larvae at 30 dph (Sarropoulou et al., 2014). Similar mechanisms might be at play during the larval development of the Atlantic bluefin tuna, with bias linked to important processes during this key period of development to meet the high demand for protein synthesis. An analogous point was addressed by Vicario et al. (2008), in which the very rapid stage-specific growth of *Drosophila* larvae was attributed to a global fast and accurate translation of expressed genes, which displayed the highest codon usage bias when compared with the embryo, adult and pupae stages. However, to provide a more complete overview of the transcriptional dynamics during the *T. thynnus* ontogeny, this information need to be integrated with gene expression profiling at different larval stages due to the well-known stage-specificity in fish in terms of digestive capacity (Anderson et al., 2018; Murashita et al., 2014;), visual system (Helvik et al., 2001; Taylor et al., 2011) and somatic growth (Miandare et al., 2013). Therefore, investigating these processes during different developmental stages will be essential to understand how they are modulated during the larval growth.

The majority of the protein-coding transcripts were functionally annotated, of which almost 90% were included in orthologous groups with other fish species. The assembly-specific transcripts were involved into key processes such as muscle and bone development along with eye morphogenesis, detection of light stimulus and neurotransmission, all aspects that point out that *T. thynnus*, and most likely also other scombrids, might have evolved specific adaptations to achieve early swimming and predation capabilities. These aspects, together with a precocious development of the digestive system (Mazurais et al., 2015; Yúfera et al., 2014), might have provided the evolutionary basis to sustain the remarkable growth. Indeed, at the end of the first month of life the Atlantic bluefin tuna can attain roughly 40 mm in total length (Yúfera et al., 2014), which is noticeably higher if compared to other teleosts. Therefore, rapid growth and early digestive capabilities would favour the Atlantic bluefin tuna in the pelagic environment by providing physical advantages over other competing species. Effective detection of prey and fine tuning of colour vision in the pelagic ocean was attributed to evolutionary changes in several visual pigment genes in the sibling species Pacific bluefin tuna *Thunnus orientalis*, an adaptation thought to enhance hunting in the pelagic realm (Nakamura et al., 2013). In early juveniles (30 dph) of *Thunnus albacares*, the precocious increase in specific activity of digestive enzymes concurrent with somatic growth were observed, with comparable enzymatic levels found also in other fish species but at

much later developmental stages (Buentello et al., 2011), supporting the concept of an adaptation of scombrid fish larvae to early piscivorous behaviour (Kaji et al., 2002). Accordingly, it is likely that an early development of digestive and hunting capacities (i.e. visual prey detection, buccopharyngeal development, swimming performance) provide the energy substrate to fuel high growth rates.

Domains represent the structural and functional units that form proteins. Domains sharing a common ancestor are grouped together into superfamilies based on multiple evidences at structural, sequence and functional level (Murzin et al., 1995) and assessing the number of domains is a way to evaluate protein family expansion (Vogel & Chothia, 2006). Some of the expanding families, including transcripts coding for the myosin tail and the laminin G domains, were once again related to the organism's structural components. The correct functioning of the skeletal muscle relies on the mechanical linkage between the cytoskeleton of the muscle cells and the surrounding extracellular matrix. In this context, dystroglycan (DG) is an adhesion complex that links the cytoskeleton to the external extracellular matrix in skeletal muscle and other tissues (Hohenester, 2019). The α subunit of this complex (α-DG) is involved in a tight linkage with multiple extracellular proteins and proteoglycans, each typically harbouring tandem arrays of laminin G domains (Dempsey et al., 2019). Collectively, the LG domains establish essential interactions to stabilize the sarcolemma (Han et al., 2009). On the other side, the myosin tail is a functional domain of myosin, the motor protein with a central role in cell motility, best known for its role in muscle contraction (Harrington & Rodgers 1984). Genes encoding for proteins typically required in large amounts for an organism's morphological structure were found to be more subject to lineage specific expansion in eukaryotes (Lespinet et al., 2002). Therefore, the expansion of muscle-related protein families might have provided the raw material for larval/juvenile adaptations to achieve early high growth rates. Expanding families belonging to the immune system and response to environmental stressors (glutathione S-transferase N terminal domain and immunoglobulin domain) were found in the Atlantic bluefin tuna. The expansion of gene families related to immune response and environmental stressors is widespread, occurring in multiple groups of eukaryotes (Lespinet et al., 2002; Vogel & Chothia, 2006) as well as in a lineage-specific fashion in teleosts (Lu et al., 2012; Stein et al., 2007) and invertebrates (Guo et al., 2015; Zhang et al., 2015). This process is also thought to support the colonization of novel environments and to cope with the relative environmental challenges such as the case of the highly invasive goby species *Neogobius melanostomus*, (Adrian-Kalchhauser et al., 2020). Furthermore, in teleosts, the expansion of the family of Toll-like

receptors was found to correlate with latitudinal distribution and maximum water depth (Solbakken et al., 2017). It is therefore possible that families related to the immune response in the Atlantic bluefin tuna might have expanded to adapt to specific challenges posed by the pelagic environment (e.g. thermal gradients).

In conclusion, in years of active interest towards the domestication of the Atlantic bluefin tuna, this work represents the first de novo transcriptome assembly of the larval stages, a key point to close the life cycle of the species. Furthermore, it provides a glimpse into the evolution of set of genes linked to the peculiar characteristics of the larval ontogeny of this species. The expansion of two protein families related to muscle growth and functioning might represent one of the processes through which the Atlantic bluefin tuna attained high growth rates and physical size. The fact that assembly-specific orthogroups were enriched in transcripts linked to body development might help to explain how this species, and likely other scombrids with similar biological traits, have accomplished early physical advantages over competing species in the pelagic environment. Finally, transcripts involved into translation were found having specific codon usage bias, suggesting codon preference in this process as a biological pathway to meet the somatic growth and demand for protein synthesis during the larval stage. More detailed approaches, with an in-depth focus on single protein families, are required to confirm the present findings and investigate the underlying evolutionary processes in addition to understand how different pathways are modulated during the larval ontogeny. Future studies will benefit from our research because of the exploration of physiological responses at a molecular level, an important step to obtain a more complete picture regarding the biology of any species. Overall, this work represents a significant contribution to the transcriptomic resources available for Atlantic bluefin tuna.

4.5. Data availability

All the Supplementary Materials were made freely available through Mendeley Data at https://data.mendeley.com/datasets/kfbcpxgdr9/2.

4.6. References

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5. A comparison of reproductive potential in young and old females: a case study on the Atlantic bluefin tuna in the Mediterranean Sea

5.1. Introduction

At the population level, a variety of animal species undertakes seasonal migration to areas that serve as breeding or natal grounds, whose timing is known to vary according to internal signals and environmental factors (Jachowski & Singh, 2015). Endocrine signals such as sex hormones or glucocorticoids are well-established drivers of observed behaviours in the wild, triggering adaptive responses and shifts in reproductive states (Oliveira et al., 2002; Pankhurst, 2011). In the context of reproduction, the ovary harbours a coordinated network that integrates local and systemic signals, leading to the production of viable eggs and defining the reproductive output (Lubzens et al., 2010, 2017). In addition to circulating endocrine signals as the gonadotropins, the cross-talk between the follicular cells and the growing oocyte is essential for successful reproduction (Charlier et al., 2012) and local factors as small non-coding RNAs are emerging as important signals to regulate folliculogenesis (Armisen et al., 2009; Houwing et al., 2007) and to influence maternal signature of the eggs (Rauwerda et al., 2016).

The miRNAs are a class of small non-coding RNAs that regulate gene expression through post-transcriptional control of target mRNAs (Bartel, 2004) and provide robustness to gene expression networks (Berezikov, 2011). Despite being a relatively recent topic, the miRNAs has transformed our understanding of the mechanisms of gene regulation in almost all cellular functions, biological processes, life histories and response to environmental stressors (Berezikov, 2011; Flynt & Lai, 2008). So far, the mir-202 has been identified as gonad-specific in several teleost species (Bouchareb et al., 2017; Farlora et al., 2015; Juanchich et al., 2013; Zhang et al., 2017) and such a pattern of expression appears to be conserved, with similar results also reported in mouse (Ro et al., 2007) and frog (Armisen et al., 2009). In recent years, new insights have emerged regarding its role in reproduction, highlighting its importance in regulating multiple reproductive processes and relevance as useful molecular marker. Indeed, it was found to deeply affect the fecundity of the medaka *Oryzias latipes* through the regulation of early steps of follicular development (Gay et al., 2018). Furthermore, maternal mir-202-5p was identified as a novel germ plasm-specific

marker in the zebrafish *Danio rerio* (Zhang et al., 2017) and was essential for the proper primordial germ cells migration during embryo development (Jin et al., 2019).

The iconic Atlantic bluefin tuna *Thunnus thynnus* is a highly valuable species characterized by trans-oceanic movements and one of the biggest species inhabiting the pelagic environment (Block et al., 2001; Rooker et al., 2007). In spring, the Atlantic bluefin tuna perform a long migration from feeding grounds in the Northern Atlantic to spawning grounds in the Gulf of Mexico (Western stock) and the Mediterranean Sea (Eastern stock) (Rooker et al., 2007), whose management is based on the commonly accepted division at the 45° meridian which reflects natal homing, although the measure is still debated (Brophy et al., 2020; Rooker et al., 2003, 2008). Indeed, in the Mediterranean Sea, bigger individuals (>100 kg) are able to perform trans-Atlantic migrations as opposed to smaller individuals (<100 kg) which exhibit a more resident behaviour within the basin, moving into the Atlantic Ocean once attained a certain size and therefore suggesting that movement patterns are linked to the size (Cermeño et al., 2015; De Metrio et al., 2005; Rooker et al., 2007, 2014). However, the situation appears more complex since also adults (>100-150 kg) exhibited residency in the Mediterranean Sea (Fromentin & Lopuszanski, 2014; Tudela et al., 2011), consistent with the idea of a substructure in this basin not entirely compatible with the classic two stocks hypothesis (Riccioni et al., 2010). The complex highly migratory behaviour of this species represents a significant physiological challenge because of the need to allocate energy for growth, sexual maturation and reproduction as well as to balance environmental conditions (Chapman et al., 2011). Therefore, the success of this species relies on the delicate trade-off between somatic growth and reproduction before and during the migratory movements to reach spawning grounds.

Certainly, as a species of high commercial interest, a good management of the Atlantic bluefin tuna fishery is needed to conserve the species and the accuracy of stock assessment estimates will benefit if a good understanding of the relative contribution of age classes to the reproductive output is achieved (Medina, 2020). Indeed, mounting evidences are highlighting the importance of old large females in fostering stock productivity, stability and recruitment due to their disproportionally high contribution in terms of reproductive output (Barneche et al., 2018).

In the present study, we sought to test the hypothesis that old females of Atlantic bluefin tuna in the Mediterranean Sea exhibited greater reproductive potential than young females and that the mir-202 could selectively play a role in regulating their reproductive stages, reflecting different dynamics linked to physical condition, habitat usage and migratory

behaviour between the two age classes. Furthermore, reproductively-active individuals were compared with non-reproductive individuals to establish a reproductive baseline. The findings of this work will help to better understand the local control of oogenesis by the gonad-specific mir-202 and to elucidate the age-related reproductive output of this highly migratory species that reproduce seasonally.

5.2. Materials and methods

5.2.1. Experimental design and sampling activities

A total of 26 adult ovary samples were collected in 2017-2018. During the reproductive season (*n*=13, mid-May to late-June) samples were collected in waters south-west of Sardinia in the tuna traps of Carloforte and Portoscuso (Italy), which historically exhibited catches of individuals of age classes from 4 to more than 10 (Addis et al., 2012). Then, during the non-reproductive season (*n*=13, November) additional ovary samples were taken at a Maltese tuna farm to define a reproductive baseline to which compare changes observed in the reproductive season. Furthermore, during sampling procedures, samples of stomach, intestine and liver were also collected. Upon hauling, straight fork length (SFL), curved fork length (CFL) were recorded to the nearest cm and total body weight was registered to the nearest kg. The age was estimated using the length-age conversion key reported by Santamaria et al. (2009). The samples for molecular biology applications were stored in RNAlater® (Ambion, Austin, TX, USA) at 4°C and then placed at -20°C for long-term storage while samples for histological analysis were fixed in a formaldehyde-glutaraldehyde solution (formaldehyde 36.5%, glutaraldehyde 25%, NaH_2PO^{4-}, NaOH) and kept at 4°C. The fish were caught only for commercial purpose and the procedures did not include animal experimentation and ethics approval was not necessary according to the Italian legislation (D.L. 4 of March 2014, n. 26, art. 2).

5.2.2. H&E histological processing and image analysis

Upon fixation, small ovary pieces of about 3-4 mm were dehydrated through graded ethanol of increasing concentration, cleared in xylene and embedded in paraffin. Sections of 5-µm thick were cut with a microtome Leica RM2125 RTS (Biosystems), stained with Mayer's haematoxylin-eosin and mounted on a glass slide with SafeMount® (Bio-Optica, Milan, Italy). Mounted slides were observed under the Zeiss Aixio Imager M2 microscope and

photographed with a high-resolution camera (ZEISS Axiocam 105 colour). The reproductive stages of ABFT females were classified according to Schaefer (1998). This classification consists of 4 stages, based on the observed histological features, as follows: active non-spawning (ANS), active spawning (AS), inactive mature (IM) and resting (R). The frequency of late vitellogenic oocytes and the rate of α-atresia was calculated according to Pousis et al. (2011). The mean diameter of oocytes for each specimen was manually calculated as the mean value of the minor and major axis taken randomly from 30 oocytes cross-sectioned through the nucleus. The image processing and measurements were carried out with Fiji (Schindelin et al. 2012).

5.2.3. RNA extraction and cDNA synthesis

For each sample, small (< 200 bp) and total (> 200 bp) RNA were separately extracted using RNAzol RT (Sigma-Aldrich, USA, St. Louis) according to the manufacturer's instructions with some modifications to enrich the miRNA-containing fraction. The extracted RNA from each sample was diluted in at least 20 µl of RNAse-free water and analysed with the nanophotometer P330 (Implen, Germany) to check concentration, 260/230 and 260/280 ratios. The extracted RNA was then stored at -80°C. Before cDNA synthesis, RNA was treated with the DNase I (Sigma-Aldrich, USA, St. Louis) according to the manufacturer's instructions. To perform an adequate qPCR for the small-RNA fraction (< 200 bp), a total of 2 µg of RNA was polyadenylated with *E.coli* poly(A) polymerase (New England Biolabs) and converted into cDNA with the high capacity cDNA reverse transcription kit (Applied Biosystem) primed with an oligo dT adaptor (Supplementary material 1). For the RNA fraction > 200 bp, upon DNase I treatment, a total of 2 µg of RNA was converted into cDNA using the high capacity cDNA reverse transcription kit (Applied Biosystem) following the manufacturer's protocol.

5.2.4. miRNA identification

Known teleost mature sequences for mir-202 were retrieved from miRbase (http://www.mirbase.org/) and aligned using Blast with the available genomic resources on NCBI for the Atlantic bluefin tuna (BioProject: PRJNA432036). Upon alignment, the corresponding matching region was extended in opposite directions to obtain the sequence containing the precursor. Then, a similarity search was performed on miRBase in an attempt

to identify the precursor sequence, whose overall features (i.e. minimum free energy, hairpin structure, bulges) were evaluated with the RNAfold web server (http://rna.tbi.univie.ac.at/). The two arms (5p and 3p) were experimentally identified within the obtained precursor sequence through qPCR using primers starting at different positions along the hairpin, guided by a heterologous approach, until amplification, melting curves and gel electrophoresis revealed unambiguous and clear signal. Relative 5p/3p length, complementarity along the precursor and loop size were checked for canonical features of bona fide miRNAs (Fromm et al., 2015).

Candidates miRNAs to normalize the expression levels of the mir-202 were screened according to previous studies (Wang et al., 2019; Zhu et al., 2015) and identified using either the aforementioned strategy or retrieved from a set of highly conserved miRNA identified in the *Thunnus orientalis* (Paul et al., 2018). The expression stability of the candidate reference miRNAs was evaluated with NormFinder (Andersen et al., 2004).

5.2.5. Reproductive markers

Several reproductive markers were screened from previous studies and obtained from the available molecular resources. Accordingly, primers of the *vitellogenin receptor* (*vtgR*) were used from Pousis et al. (2012) and primers of *caspase 3* (*casp3*), beclin 1 (*bcn1*), *cytoplasmic polyadenylation element binding protein 2* (*cpeb2*), *steroidogenic acute regulatory protein* (*star*) and *ciclin b1* (*ccnb1*) were designed with PrimerBlast (Ye et al., 2012) using the Atlantic bluefin tuna larval transcriptome (Marisaldi et al., 2020). The primers for the *luteinizing hormone receptor* (*lhr*) were designed on the available sequence on NCBI (GeneBank ID: JX459924.1) using the same methods. The list of primers can be found in the Supplementary material 1.

5.2.6. Quantitative RT-PCR

For each miRNA sequence, a specific forward primer spanning the entire mature sequence and a reverse universal primer complementary to the poly(T) adaptor was designed (Supplementary material 1). Different universal reverse primers were used to adjust for optimal annealing temperatures. For each primer pair, the optimal annealing temperature was assessed by performing a temperature gradient and the best Tm was identified as the temperature at which the most specific amplicon and the lowest Ct value were obtained. For

quantitative expression analysis of miRNAs, each 10 µl reaction mixture consisted of 2 µl of 1:10 diluted cDNA, 5 µl of SYBR® Green PCR Master Mix (Applied Biosystem), 2.6 µl of RNAse-free water and 0.2 µl of each diluted (1:10) forward and reverse primer. For mRNA, 1 µl of 1:10 diluted cDNA was used and the reaction mixture was adjusted accordingly. The qPCR was performed using the CFX Real-Time PCR Detection System (Bio-Rad, USA) with the following cycling conditions: 3' at 95°C, 40 cycles of 10'' at 95°C, 10'' at the optimal primer pair annealing temperature, 30'' at 72°C and 8' at 72°C. The specificity of each primer pairs was assessed by using a dissociation curve performed at the end of the amplification and the qPCR products were run on 2% agarose gel stained with Midori green (Nippon Genetics) with the GeneRuler Low Range DNA Ladder (Thermo Scientific) as reference. All qPCR reactions were performed in duplicate and Ct values were obtained by averaging the values of two technical replicates. The relative expression of mRNA levels was calculated by normalizing target genes with *gapdh*, *β-act* and *18s* according to the principles outlined by Vandesopele et al. (2002).

5.2.7. Fluorescent in situ hybridization (FISH)

Paraffin-embedded ovaries were sectioned (8 µm thickness) with a microtome (HM355, microm). The anti-sense Locked Nucleic Acid (LNA) oligonucleotide was custom designed and produced by Exiqon A/S to label the miR-202-5p form. A LNA Scramble-miR probe (5'-GTGTAACACGTCTATACGCCCA-3') was used as a negative control. All LNA probes were double-DIG labelled at both 5' and 3' ends. FISH was carried out with the microRNA ISH Buffer Set (FFPE) Hybridization Buffer (ref. 90000, Exiqon), according to the manufacturer's instructions with some minor modifications. Permeabilization was performed for 7 min at room temperature using proteinase-K (10 mg/ml, P2308 Sigma). LNA probes were used at 20 nM at 53˚C (30˚C below the RNA Tm) for 2.5 h. Samples were then incubated overnight at 4˚C with a rabbit anti-DIG POD-conjugate antibody (1:500, Roche). Then, the anti-DIG-POD antibody was detected with the TSA-Cy5 substrate (1:50, TSA PLUS Cy5 kit, NEL 745001KT, Perkin Elmer) for 12 min at room temperature in the dark. Nuclei were stained with DAPI (1:500) for 15 min at room temperature in the dark. All pictures were taken under the SP8 confocal microscope. The acquisitions were imported and stitched in Fiji with the Bio-Format plugin and a z projection was carried out to obtain the final composite (DAPI + probe) image.

5.2.8. Statistical analysis

Due to the initial and expected heterogeneity of samples from wild-caught individuals, a data mining strategy to explore the dataset and to cluster similar samples was adopted. Upon initial scaling, the Principal Component Analysis (PCA) was performed using a combination of biometric (CFL, total weight, age), histological (mean follicle size, % atresia, number of late-vitellogenic oocytes) and gene expression (mir-202-3p, mir-202-5p) variables. The expression of the reproductive markers was not included at this initial step instead was evaluated afterwards to check the validity of the PCA.

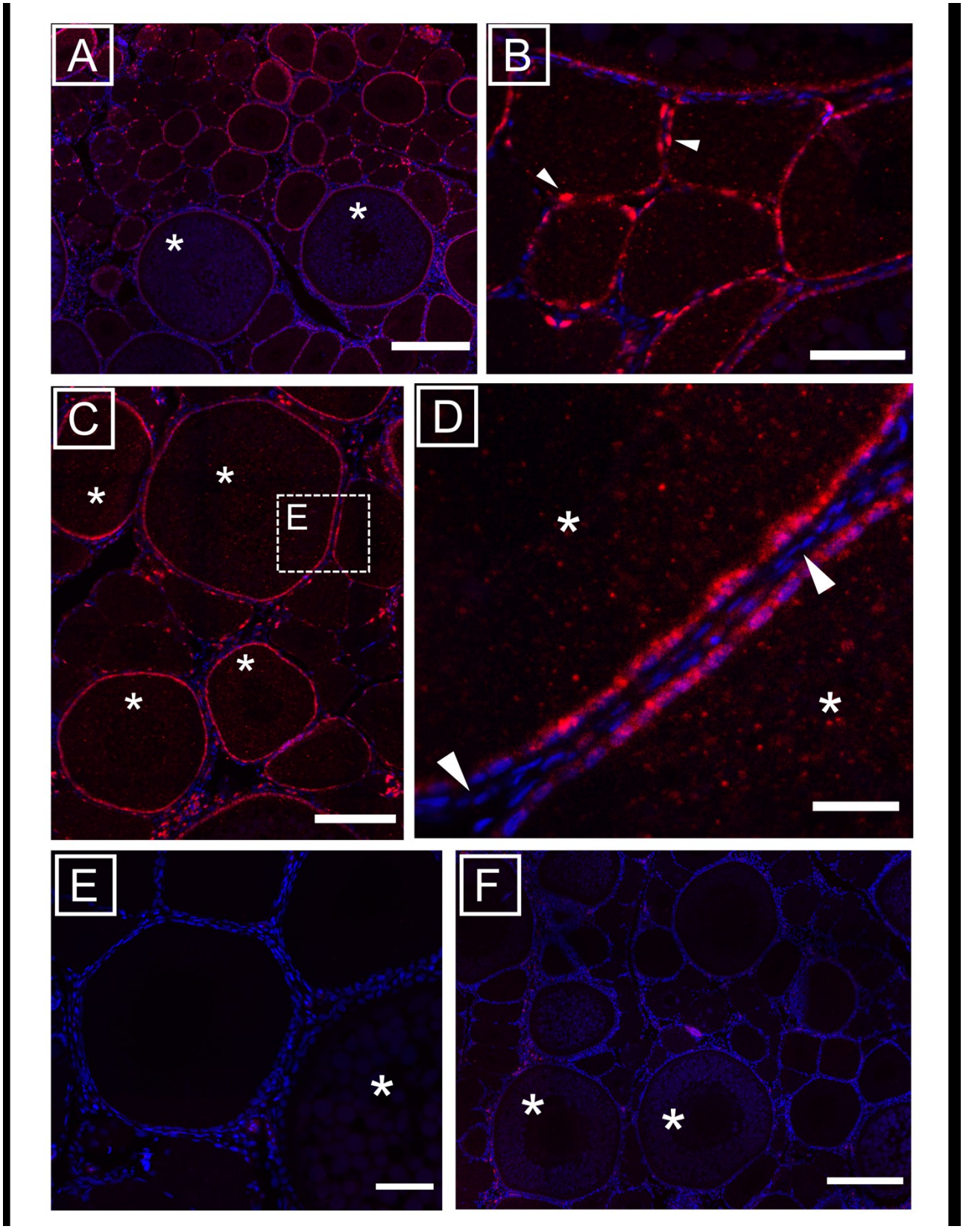
Once the groups were established, the homogeneity of the variance for each variable was checked with the Levene's test. When such a requirement was not met, the data were log-transformed and the test carried out again to confirm the effectiveness of the transformation. Then, the one-way analysis of variance was performed and if statistically significant differences were detected, the Holm procedure for multiple comparisons was chosen as a post-hoc test. When parametric tests could not be used, the Kruskal-Wallis non-parametric test followed by pairwise comparisons using the Wilcoxon rank-sum test was applied. Differences were considered statistically significant if p-value < 0.05. The plots and statistical analysis were performed with JASP (JASP Team, 2020) and RStudio (RStudio team, 2015) using the packages ggplot2 (Wickham, 2016) and MASS (Venables & Ripley, 2002).

5.3. Results

5.3.1. Ovarian localization of mir-202-5p

The localization of the mir-202-5p was primarily detected in the granulosa cells surrounding previtellogenic and vitellogenic oocytes (Fig. 1A, B, C) but not in theca cells (Fig. 1D). A faint rather homogenous signal was localized within the oocytes in the ooplasm (Fig. 1B, D). A progression in the number of granulosa cells could be observed from previtellogenic (perinucleolar, lipid stage) to vitellogenic oocytes and, accordingly, mir-202-5p expression was localized in granulosa cells around previtellogenic oocytes as few spots (Fig. 1B), corresponding to a low density of granulosa cells at this stage.

To validate the hybridization signal observed with the mir-202-5p probe, the FISH was also carried out using a scramble control probe, revealing no detectable signal above background (Fig. 1E, F).


A
B
C
D
E
E
F

◄ **Figure 1**. Localization of mir-202-5p expression (red signal) in the ovary revealed with fluorescent in situ hybridization (FISH). Nuclei are stained with DAPI (blue signal). A) View of the ovarian section used for the experiment containing both vitellogenic and previtellogenic oocytes. The mir-202-5p was detected in granulosa cells of previtellogenic (B, arrowhead) and vitellogenic oocytes (C) but not in theca cells (D, arrowhead). The signal was also detected in the ooplasm (B, D). Scramble control probe revealed no detectable signal above background (E, F). Asterisks= vitellogenic oocytes. Scale bars A) 200 μm B) 50 μm C) 100 μm D) 20 μm E) 50 μm F) 200 μm.

5.3.2. Multivariate classification reveals two subgroups during the reproductive period

According to the PCA, three groups (A1, A2, B) were distinguished, with the first and the second components explaining 56.6% and 19.4% of the variance, respectively (Fig. 2). Along the PC1 the two groups belonging to reproductive (A1, A2) and non-reproductive (B) periods were well discriminated and, along the PC2, two subgroups within the reproductive period were identified. The group A2 was composed of smaller and younger individuals, in contrast with the groups A1 and B which were represented by bigger individuals of similar size and age (Table 1).

Table 1. Biometric data of bluefin tuna specimens for each group. Data are shown as mean ± standard deviation. For each variable, different letters correspond to statistically significant differences ($p < 0.05$). CFL: Curved Fork Length.

Group	CFL (cm)	Total weight (kg)	Age
A1	210.8 ± 23.6 [a]	164.1 ± 61.6 [a]	10.8 ± 2.6 [a]
A2	141.7 ± 18.2 [b]	50.5 ± 26.3 [b]	5 ± 1.41 [b]
B	220.1 ± 29 [a]	204.8 ± 63.4 [a]	11.9 ± 2.9 [a]

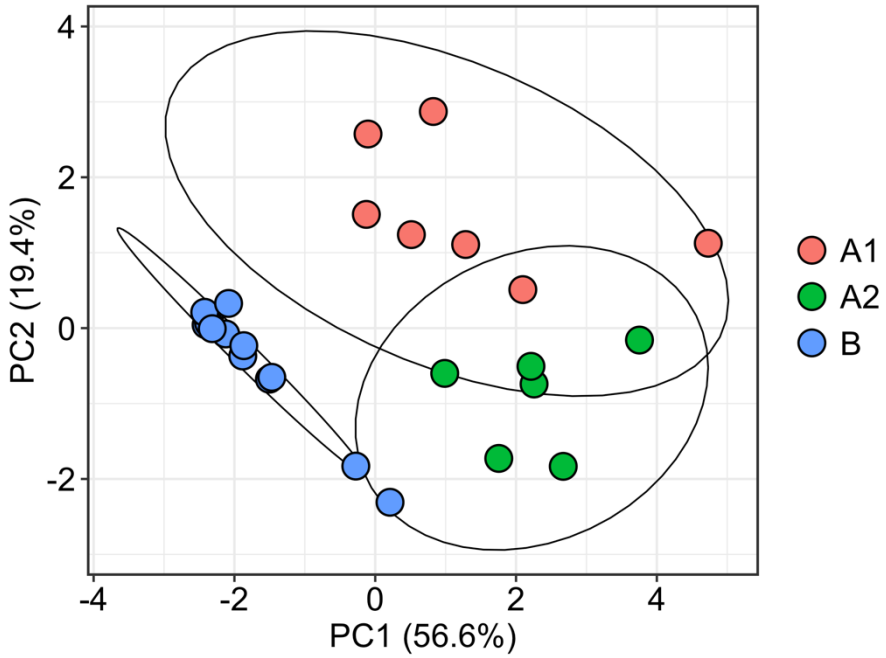
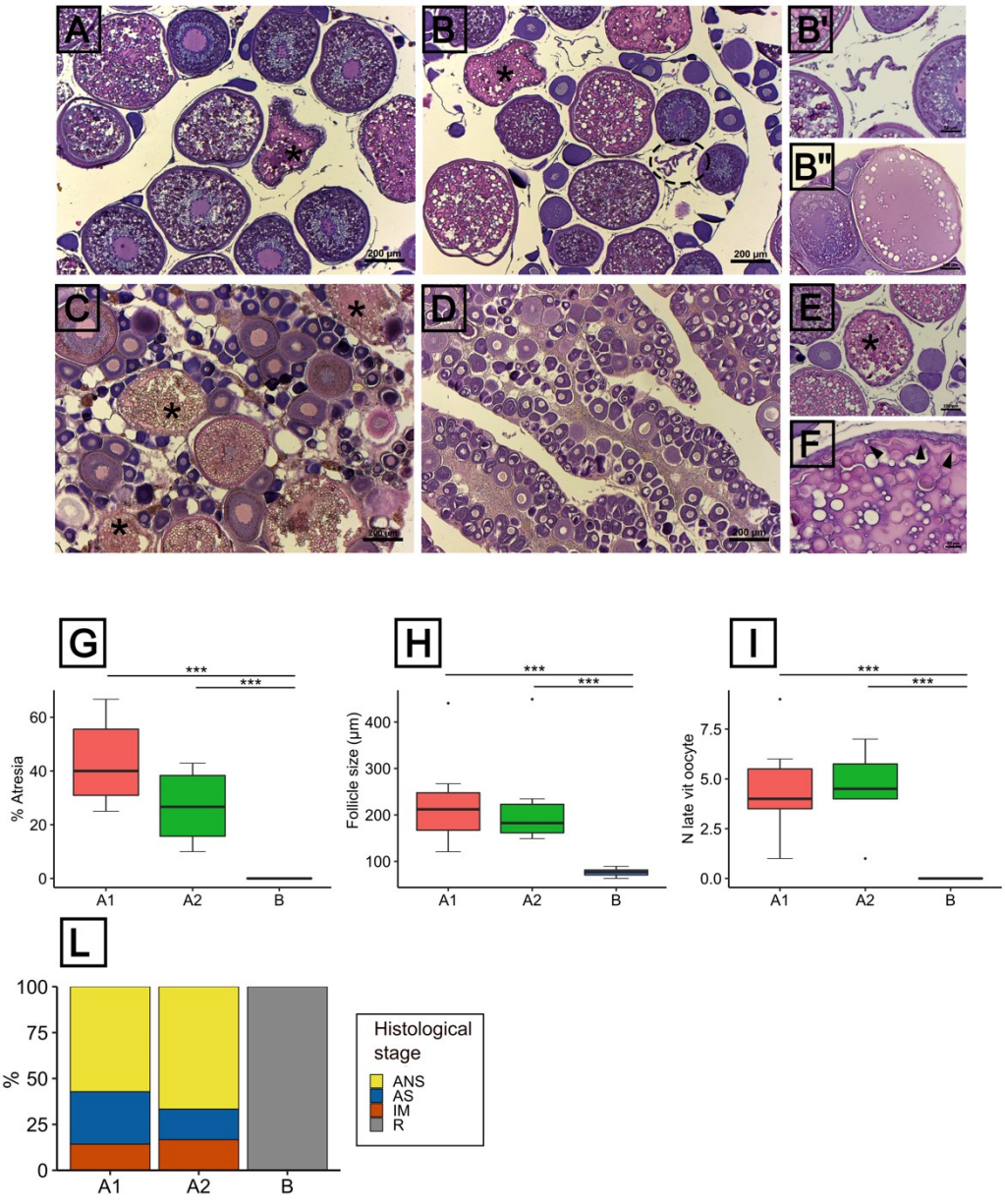


Figure 2. Principal Component Analysis showing the three groups (A1, A2, B) identified in the present study. The two subgroups A1 and A2 correspond to individuals of the reproductive period and group B to individuals of the non-reproductive period. The proportion of explained variance along the axes is reported in brackets.

5.3.3. Histological features of the ovarian condition

According to the histological observations, all the 4 stages currently adopted to classify tuna reproductive cycle could be observed (Fig. 3A, B, C, D). During the reproductive season, the females of the group A2 contained large late-vitellogenic oocytes in addition to lipid stage, previtellogenic and α atretic oocytes (Fig. 3A). The fraction of α atretic oocytes was 26.7% ± 13.8% (Fig. 3G), the mean follicle size was 225.7 µm ± 113.5 µm (Fig. 3H) and the mean number of vitellogenic oocytes was 4.5 ± 2 vitellogenic oocytes/10 mm^2 ovary section (Fig. 3I). Most of the females in this group were classified as active non spawning (ANS) females, with only two samples classified as active spawning (AS) and immature (IM) females (Fig. 3L). The group A1 was characterized by two females showing signs of imminent spawning (i.e. oocytes exhibiting yolk coalescence, germinal vesicle breakdown) or post-ovulatory follicles (POFs) (Fig. 3B, B', B''). The rest of the females were classified as ANS, with only one female classified as IM (Fig. 3L). The fraction of α atretic oocytes was 43.5% ± 17.1% (Fig. 3G), the mean follicle size was 229 µm ± 105.4 µm (Fig. 3H) and the mean number of vitellogenic oocytes was 4.5 ± 2.5 vitellogenic oocytes/10 mm^2 ovary

section (Fig. 3I). Irrespectively of the group, the samples classified as AS were all caught in tuna traps. The ovaries of females caught during the non-reproductive period (group B) had lipid stage oocytes as the most advanced stage (Fig. 3D), no signs of atresia (Fig. 3G) and a mean follicle size of 76.23 µm ± 8.3 µm (Fig. 3H). Yet, a small number of atretic follicles (<1%) was observed in certain, though not all, resting ovaries (Supplementary material 2) but not included in the analysis according to the method adopted for the calculation, which is based on the percentage ratio of α atretic vitellogenic oocytes on the total number of late vitellogenic oocytes. Atretic follicles (Fig. 3E-F) showed typical signs of atresia such as the zona radiata breakdown and yolk resorption (Fig. 3F). More advanced stages of atresia, corresponding to the so-called β atresia, were also observed (Supplementary material 2).



◄ **Figure 3.** Histological features of the ovaries. A-D) Reproductive stages classified according to Schaefer (1998): A) active non-spawning B) active spawning C) inactive mature D) resting; B') Post-ovulatory follicle (POF); B'') On the right: maturing oocyte showing yolk coalescence and breakdown of germinal vesicle, on the left: early-mid vitellogenic oocyte; E-F) α atretic follicles showing signs of zona radiata breakdown and yolk reabsorption; G) Fraction (%) of atretic follicles; H) Size distribution of oocytes; I) Number of late-vitellogenic oocytes; L) Proportion of histological stages. A-F) Asterisks= α atretic follicles; dashed circle= POF; arrowhead= zona radiata breakdown. G-I) Statistical significance: *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$. ANS = active nonspawning; AS= active spawning; R= resting; IM= inactive mature.

5.3.4. mir-202 identification and arm-switching during reproductive and non-reproductive periods

Among the reference miRNAs identified in previous studies, a total of three was successfully isolated in the present study: let-7a-5p, let-7e-5p and mir-26a-5p. To evaluate their suitability as references, NormFinder was chosen to evaluate the stability of expression. According to this analysis, the stability value was 0.36 for let-7a-5p, 0.47 for let-7e-5p and 0.56 for mir-26-5p. The best pair (mir-26a-5p, let-7e-5p) showed a stability value of 0.37 and was therefore chosen to normalize the expression level of the mir-202 according to the principles outlined by Vandesompele et al. (2002). Instead, in extra-gonadal tissues, the mir-26a-5p was used to normalize expression levels with the $2^{-\Delta\Delta ct}$ method (Livak & Schmittgen, 2001) as unspecific amplification was detected for let-7e-5p and let-7a-5p.

The predicted thermodynamic stability of the putative pre-mir-202 was -31.92 kcal/mol and the overall features of the hairpin resembled those typical of pre-miRNAs (Fig. 4A). The expression of mir-202-5p was homogenous ($p>0.05$) among the three groups and, notably, the expression levels during the non-reproductive period were similar to those during the reproductive period (Fig. 4B). On the other side, the opposite arm mir-202-3p showed a significant ($p<0.05$) up-regulation from B to A1 (Fig. 4C), corresponding to a differential arm-usage. By evaluating the expression in extra-gonadal tissues (stomach, intestine, liver) it was confirmed that mir-202 expression was mainly expressed in the gonads (Supplementary material 3). In extra-gonadal tissues, the mir-202-3p expression was below the detection level.

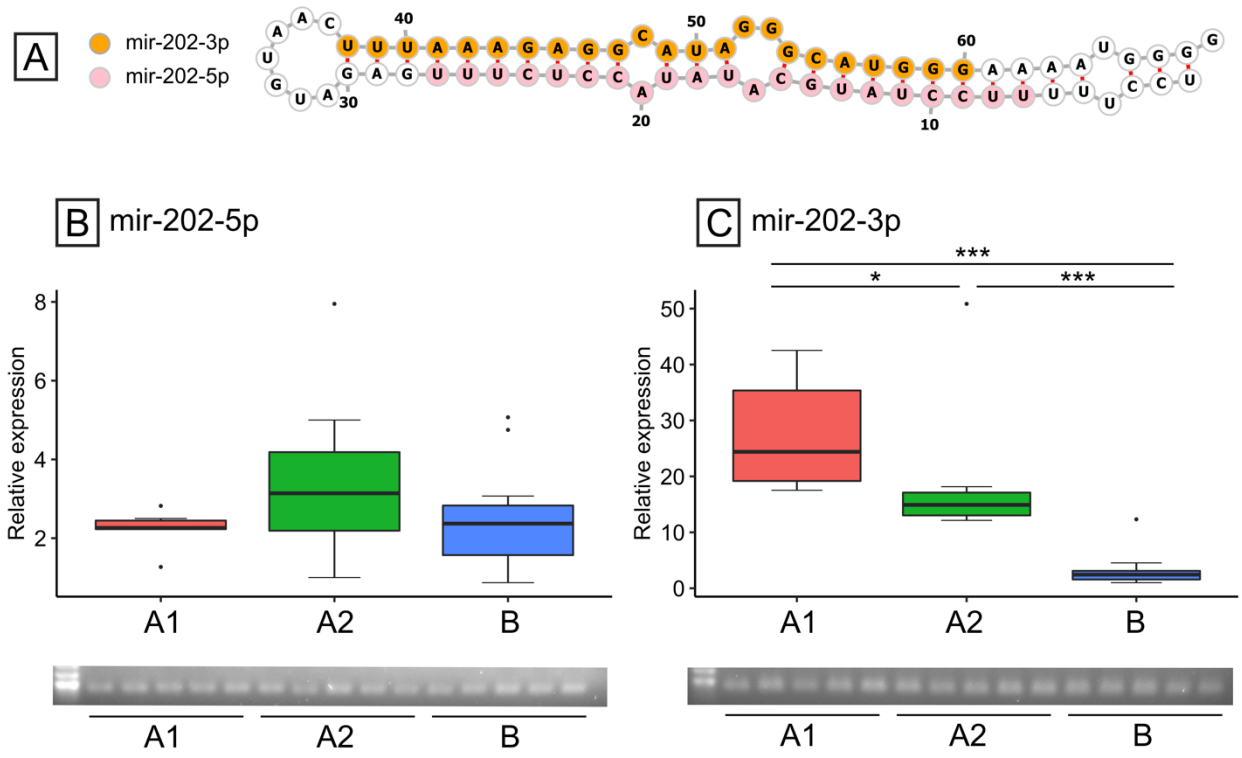


Figure 4. Precursor sequence and expression of mir-202 in the three groups identified. Groups A1 & A2: reproductive period; Group B: non-reproductive period. A) Putative hairpin with 5p and 3p arms. B-C) Expression of mir-202 in the three groups with the relative 2% agarose gels to further confirm the specificity of the amplification. Statistical significance: *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$.

5.3.5. Gene expression analysis of reproductive markers

A general trend, although not statistically significant ($p>0.05$), of decreased levels of *vtgR* expression was observed from group B to A1 (Fig. 5A) while the expression of *becn1* was homogeneous among groups ($p>0.05$), with greater variability in the group A2 (Fig. 5B). On the other side, both *casp3* (Fig. 5C) and *cpeb2* (Fig. 5D) expression levels were higher ($p<0.05$) in both groups during the reproductive season if compared with the non-reproductive group. The levels of star were higher ($p<0.05$) in the group A1 than both A2 and B (Fig. 5E), similarly to the levels of *lhr* although in this latter case the difference was statistically significant only between the reproductive groups (A1, A2) and the group B (Fig. 5G). The levels of *ccnb1* were homogeneous among groups ($p>0.05$) despite an apparent decreasing trend from B to A1 (Fig. 5F).

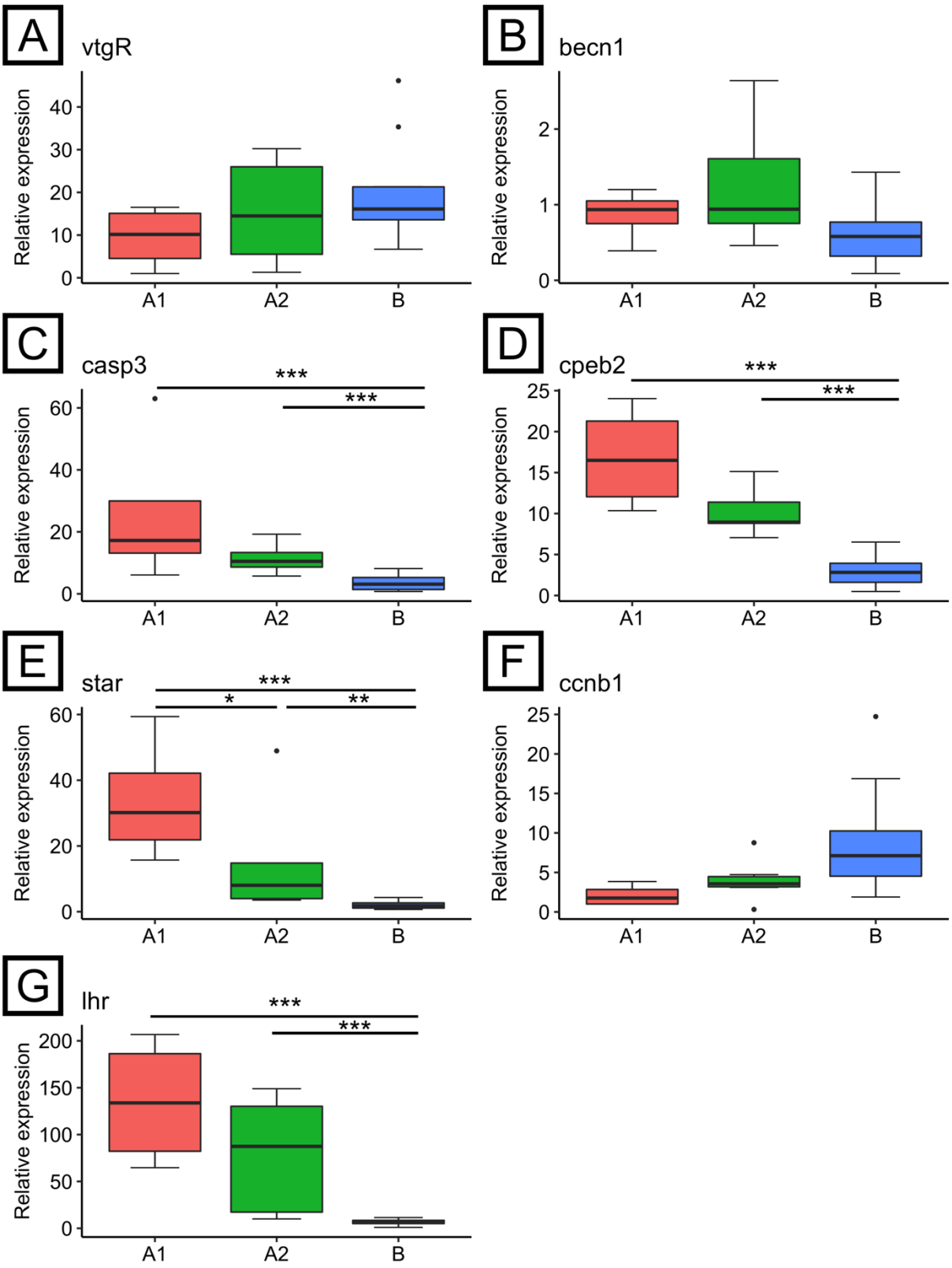


Figure 5. Expression profiles of several reproductive markers in the ovary. The groups A1 and A2 correspond to individuals sampled during the reproductive period while group B during the non-reproductive period. *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$.

5.4. Discussion

In the present study, the majority of females were classified as active non-spawning between mid-May and late-June, in an area corresponding to a migration path to spawning areas (e.g. Balearic Islands, South Tyrrhenian Sea, Sicilian Channel) (García et al., 2005; Giovanardi & Romanelli, 2010; Nishida et al., 1998; Secci et al., 2021). Along the migration route from the Eastern Atlantic Ocean to reach spawning grounds in Western Mediterranean, schools of bluefin tuna exhibit intermediate reproductive traits as found in the Strait of Gibraltar (Corriero et al., 2003; Medina et al., 2002) and waters Southwest of Sardinia (Addis et al., 2016; Corriero et al., 2003), with the latter area exactly overlapping with the present study. This migration and site fidelity to spawning grounds (i.e. natal homing) appears to be well-established for large migrating adults (>100 kg) by taking into account fishery-dependent catches (Idrissi & Noureddine, 2010; Suzuki & Kai, 2012) and tagging results (Abascal et al., 2016; Aranda et al., 2013; Horton et al., 2020; Stokesbury et al., 2007; Walli et al., 2009). However, it is likely that some of the individuals in the present study, especially those from the group A2 (<80 kg; 5 ± 1.41 years old), were resident in the Mediterranean Sea according to the habitat use and migratory behaviour of young/sub-adults found in the area (Cermeño et al. 2015; Fromentin & Lopuszanski, 2014; Tudela et al., 2011). Indeed, this observation is also supported by different spatial-temporal patterns of gonad maturation across the Mediterranean Sea, which reflect a more complex demographic substructure and reproductive dynamics (Heinisch et al., 2008). The presence of POFs and maturing oocytes undoubtedly highlights that the bluefin tuna can spawn also in tuna traps, a well-documented phenomenon in transport cages and farms off the eastern coasts of Spain (Gordoa & Carreras, 2014; Gordoa et al., 2009; A Medina et al., 2016) but irregularly observed in tuna traps in the area investigated (Addis et al., 2016; Carnevali et al., 2019; Corriero et al., 2003). Future efforts with a focus also on abiotic factors (e.g. water temperature) or surface plankton surveys would be suggested to clarify if it represents a sporadic or regular event and eventually define its relevance in the area.

Both the subgroups A1 and A2 were reproductively active, with similar histological features except for levels of follicular atresia which in general remained around or below 50%, a result supported also by *casp3* mRNA levels. Although levels of the other marker of cell death, *becn1*, were homogeneous among groups, the higher variability observed in the group A2 might be related to enhanced autophagy, which, upon oocyte failure to recover the required energy, represent the first step of follicular atresia preceding apoptosis along the sequential

events that lead to yolk resorption and clearance of follicular cells (Morais et al., 2012; Sales et al., 2019). Between the groups A1 and A2, the former was identified as the most reproductively advanced according to the expression profiles of several molecular markers. Indeed, levels of star were higher in A1, similarly to *lhr*, although in this latter case they were not statistically different. *Star*, a well-known marker of final oocyte maturation (Gohin et al., 2010), is the key rate-limiting mediator of the steroidogenic response while *lhr* is the central mediator of the systemic Lh surge that triggers the last steps of oocyte maturation (Senthilkumaran et al., 2004). Furthermore, levels of *ccnb1* and *vtgR* exhibited an apparent decreasing trend from B to A1. Lower levels of *ccnb1* in the A1 group might reflect an ongoing translation of the pool of mRNA stored throughout the oogenesis in preparation for the next phase of meiosis resumption via the activation of the M-phase-promoting factor (Nagahama & Yamashita, 2008). On the other side, the *vtgR* expression in our study reflected the findings of Pousis et al. (2012) in the Atlantic bluefin tuna, with higher levels in unyolked oocytes at an early stage of ovarian development well before the spawning season, indicative of temporally uncoupled expression and translation as well as an early preparation for the next spawning season (Carnevali et al., 2019). Interestingly, *cpeb2* exhibited increased expression levels in group B respect to A1 suggesting a role during the final steps of oocyte maturation to regulate polyadenylation and translation of maternal mRNA, as previously demonstrated for members of the CPEB family (Luong et al., 2020; Stebbins-Boaz et al., 1996). It must be noted that, for several genes, the high variability observed in expression levels likely caused a lack of statistical significance among certain comparisons, something that was expected because individuals were wild-caught and/or not under controlled conditions. In summary, the group A1, composed by older females than the group A2, displayed more advanced reproductive traits at a molecular level, reflecting dynamics that could not yet be observed at a histological scale. Therefore, this reproductive trait highlights greater reproductive potential for older females, suggesting better physical condition, a more protracted duration of the spawning season and/or earlier arrival on breeding grounds for older females, as observed for the southern bluefin tuna *Thunnus maccoyii* (Farley et al., 2015) and albacore tuna *Thunnus alalunga* (Farley et al., 2013). In the Atlantic bluefin tuna, higher fecundity was observed in larger females around the Balearic Islands, although it was linked to their larger ovary size and not affected by the fish length (Aranda et al., 2013) Nevertheless, our and these result once again highlight the importance of preserving old and large females in a fishery management scenario,

confirming their major reproductive contribute at a stock level (Barneche et al., 2018; Hixon et al., 2014).

Nowadays, understanding the roles of miRNAs in biological systems represents one of the biggest opportunities to clarify processes shaping physiological responses. Here, we showed that the mir-202 is a potential good candidate to regulate the oogenesis through stage- or age-dependent processes. This miRNA was mainly expressed in the gonads and consists of two functional arms differentially regulated, of which the dominant arm mir-202-5p is abundantly expressed in the granulosa cells of both vitellogenic and previtellogenic oocytes. A weak expression in the ooplasm would also suggest that this miRNA is maternally accumulated as previously demonstrated in zebrafish developing oocytes (Zhang et al., 2017) and hypothesized in medaka (Gay et al., 2018). This implies that offspring viability and survival might be influenced by such a maternal contribution via the successful growth of developing embryos (Gay et al., 2018; Wang et al., 2020). Interestingly, the mir-202-5p showed similar levels of expression during both the reproductive and non-reproductive period, a result in contrast with other fish species in which greater levels of expression were observed as vitellogenesis proceeded (Juanchich et al., 2013; Zhang et al., 2017). However, in zebrafish follicular cells during the transition from small (stage IIIa) to mid (stage IIIb) vitellogenic oocytes such an increase was also not detected (Zayed et al., 2019), likely due to steady high levels of expression from the cortical alveoli stage onward (Wong et al., 2018). The mir-202-5p was found, among other functions, to control early development of granulosa cells (Gay et al., 2018) and to target the *transforming growth factor β receptor II* (*tgfbr2*) (Juanchich et al., 2013), therefore likely to play a role in the progression of oocyte development. Since in the Atlantic bluefin tuna the expression levels were quite similar between the two reproductive periods, one of which is characterized by almost null levels of atresia, we would exclude that mir-202-5p mediates follicular atresia in this species. Instead, the mir-202-5p might be involved in early folliculogenesis and/or in the maintenance of the pool of previtellogenic follicles before and during the reproductive season. By regulating the recruitment of previtellogenic oocytes into more advanced stages, this miRNA might control the fecundity and therefore affects the reproductive output of the species, as observed in medaka (Gay et al., 2018). On the other hand, the mir-202-3p arm reflected the expression pattern of several genes linked to follicular atresia and oocyte maturation, being more expressed in the most advanced reproductive group. Therefore, it cannot be excluded a role at advanced stages of oogenesis and/or during the progression of follicular atresia. The differential expression of the 5p and 3p arms represents the so-called arm-switching, a well-

known process for miRNAs from which mir-202 makes no exception as observed during zebrafish gonadal development (Presslauer et al., 2017). However, a functional approach is needed to fully address such points and provide mechanistic insights of the differential arm usage.

In conclusion, during the reproductive period, older females exhibited greater reproductive potential than the younger ones, highlighting once again the importance of preserving large and old females in a fishery management scenario. The differences observed between the two age classes can be ascribed to a better condition, a different migratory behaviour, a more prolonged spawning season and/or earlier arrival on spawning grounds for older females. The mir-202 was confirmed as a gonad-specific miRNA and the dominant mir-202-5p arm was abundantly expressed in the somatic granulosa cells and not in theca cells, with a weak expression in the ooplasm indicative of maternal accumulation. The mir-202-3p arm likely plays a role at advanced reproductive stages and/or during follicular atresia while the mir-202-5p arm in the maintenance and recruitment of the pool of previtellogenic oocytes, therefore it represents a potential good candidate to regulate the fecundity of the Atlantic bluefin tuna.

5.5. References

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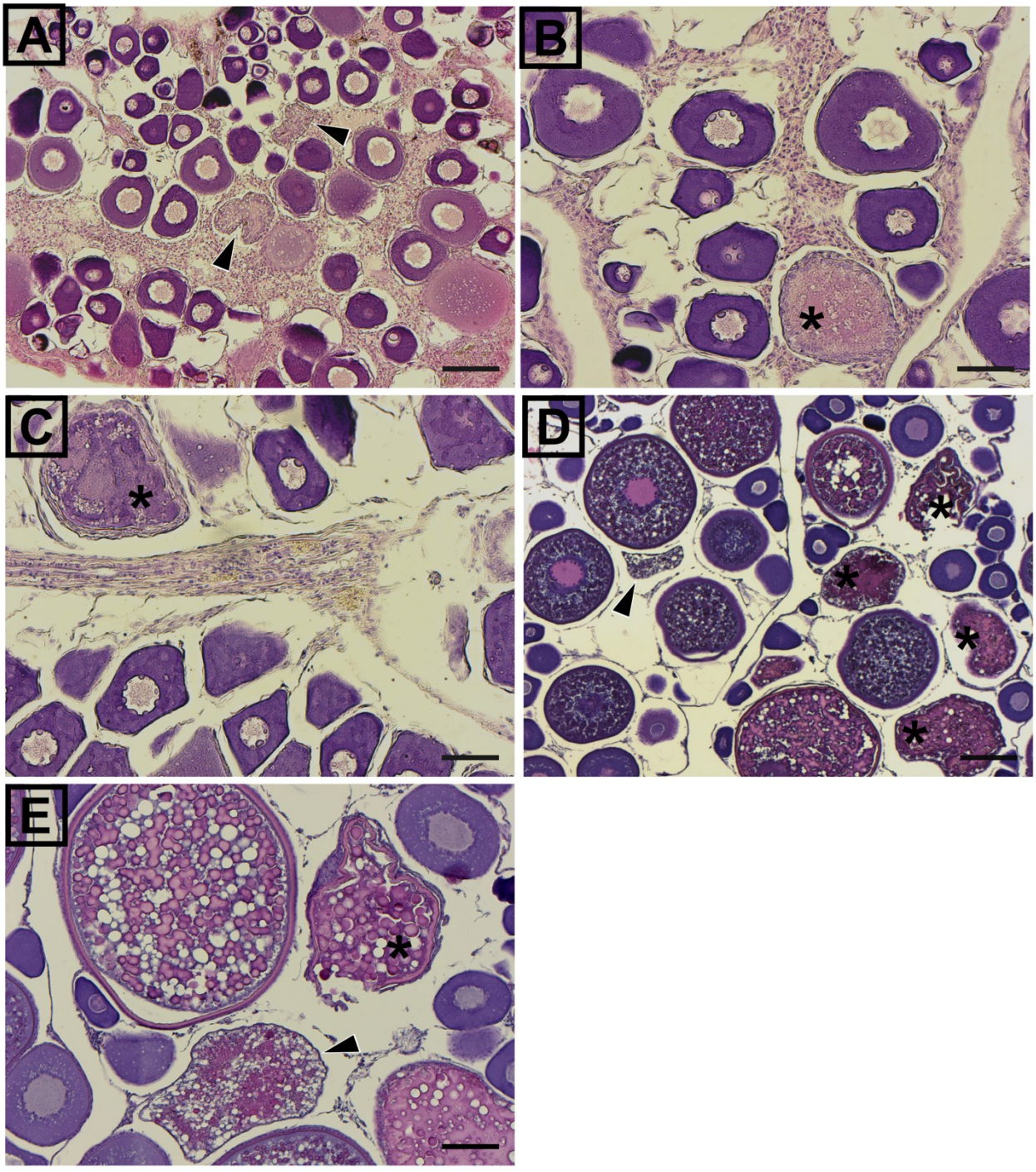
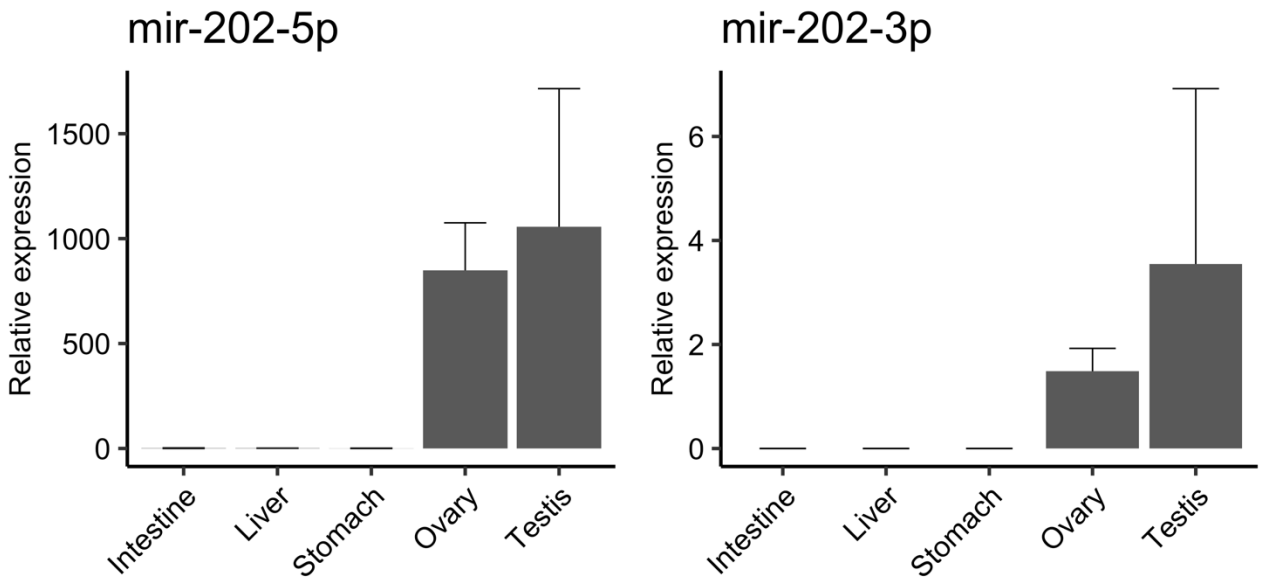
5.6. Supplementary material

Supplementary material 1. List of primers and adapters used in the present study.

Name	Sequence (5'-3')	Length
mir-202-5p	TTCCTATGCATATACCTCTTT	21
mir-202-3p	TTTAAAGAGGCATAGGGCATGGG	23
let-7a-5p	TGAGGTAGTAGGTTGTATAGTT	22
let-7e-5p	TGAGGTAGTAGATTGAATAGTT	22
mir-26a-5p	TTCAAGTAATCCAGGATAGGCT	22
Oligo dT adapter	GCGAGCACAGAATTAATACGACTCACTATAGGTTTTTT TTTTTTVN	46
Univ. reverse #1	GCGAGCACAGAATTAATACGACTC	24
Univ. reverse #2	GCGAGCACAGAATTAATACG	20
Univ. reverse #3	GCGAGCACAGAATTAATACGACTCAC	26
Univ. reverse #4	GCGAGCACAGAATTAATAC	18
casp3 - FW	CTCATGGAGGAACACGCTCA	20
casp3 - RV	TCCAGGTAGGTTGGAGGAGG	20
vtgR - FW	GTGTACCATGTGCTGATCCAG	21
vtgR - RV	TGCTCACATTAGCTTCAGGCC	21
cpeb2 - FW	CAGCAAATCCGGGAAGCAAA	20
cpeb2 - RV	CTGCGTCGGGCAAAAATGAC	20
becn1 - FW	AACCAGATGCGTTACTGCCA	20
becn1 - RV	CACTCCACAGGAACACTGGG	20
lhr - FW	GCTGCCCCTGGTTGGTATAA	20
lhr - RV	ACCGCCAGATAGATCAGCAC	20
star - FW	ATTGTGGCTGCGAATGGAGA	20
star - RV	TGCTGCTCCAACATCACCTC	20
ccnb1 - FW	TGGAGACTTCTGGTTGCGAG	20
ccnb1 - RV	CAACCTCAAGCTGTCGGAGA	20



Supplementary material 2. Histological sections of Atlantic bluefin tuna ovaries during the non-reproductive (A-C) and reproductive (D-E) periods with α (asterisks) and β (arrowheads) atretic follicles. Scale bars: A) 100 μm B-C) 50 μm D) 200 μm E) 100 μm.



Supplementary material 3. Relative gene expression of mir-202 in gonadal and extra-gonadal tissue. Each tissue consists of three biological replicates. Bars and error bars corresponds to group mean and standard deviation, respectively.

6. Conclusions

Chapter 1: Maturity assignment based on histology-validated macroscopic criteria: tackling the stock decline of the Mediterranean swordfish (*Xiphias gladius*)

1. The peak of the swordfish spawning season in the central and western Mediterranean was between June and July according to the histological evaluation of the ovaries and the temporal pattern of the gonadic index. In the context of the current ICCAT recovery plan the fishing closure is either set during the fall (October-November) or winter (January-March) months. Although this measure is intended to preserve the recruitment of juvenile individuals, a fishing closure during June and July to protect the spawning event, eventually spatially restricted to known spawning areas, could provide an alternative solution in the case of poor outcomes from the ongoing management strategy.
2. Sexually immature females represent a significant fraction of catches, a point that *de facto* highlights the fishery being not sustainable on the long run. Indeed, these females never reproduced and therefore did not contribute to the stock renewal, stability and productivity.
3. The gonadic index, in addition to providing useful information on the reproductive seasonality, performed well when calibrated with the histological classification of the ovaries to discriminate between sexually immature and mature females. Therefore, the routine collection of gonad weights and its implementation into the ICCAT observer program is strongly suggested.
4. The L_{50} for the Mediterranean swordfish females was 131.5 cm LJFL and 133.3 cm LJFL according to histological and histology-calibrated gonadic index, respectively. Although other values were reported in the scientific literature, the clear indication is that the L_{50} estimates and the current minimum landing size of 100 cm LJFL are not in line. A further raise of the minimum landing size, however, would not be suitable as management measure alone due its effect on undersized discards and because of the distribution of sizes caught by the Mediterranean fleet targeting the swordfish.
5. Future and regular assessments of the outcomes from the ongoing recovery plan will be critical to provide insights into the biological and economic impacts of the adopted measures.

Chapter 2: A *de novo* transcriptome assembly approach elucidates the dynamics of ovarian maturation in the swordfish (*Xiphias gladius*)

1. A representative mixed-tissue de novo transcriptome assembly from liver, ovary, intestine and stomach was produced for the Mediterranean swordfish, representing the first case of application of RNA-sequencing technologies to this species. This resource has been made freely available through a dedicated online portal that can be found at www.swordfishomics.com.
2. By focusing on sexually immature and mature ovaries, this approach shed light on the molecular dynamics driving ovarian maturation in this species, including important processes such as vitellogenesis and steroidogenesis. In addition to providing information on canonical pathways present in the reference databases, gene expression results were integrated into a molecular network of vitellogenin synthesis and uptake which was drawn in an *ad hoc* pathway.
3. Mature ovaries exhibited increased endocytosis, steroid biosynthesis, lipoprotein and cholesterol metabolism. On the other side, ribosome biogenesis and mRNA maturation processes were down-regulated in mature ovaries, indicative of temporally uncoupled transcription and translation that well fit with the intense transcriptional activity of previtellogenic oocytes followed by translation and eventually recycling of the proteins during vitellogenesis and maturation.
4. The results largely confirmed that molecular processes driving vitellogenesis and ovarian maturation are well conserved in the swordfish. Indeed, relevant markers of primary oocyte growth (*bmp15*, *gdf9*), vitellogenesis (*vtgR*, *fshr*), steroidogenesis (*star*, *srb1*, *cyp19a1*, *20β-HSD*) and final maturation (*lhr*) were consistently expressed as observed in other fish species.
5. A potential candidate of the vitellogenin receptor was identified by analysing its pattern of expression, sequence features and phylogenetic relationships with other members of the Ldlr family in different species. The receptor represented the *vldlr* splicing variant lacking the O-linked sugar domain. According to the complex vitellogenins-receptor system of fish, it is likely that also other receptors are involved into the internalization of the vitellogenin in the oocytes and more focused approaches are required to elucidate their identity in the swordfish.

Chapter 3: Some like it hot: the heater tissue transcriptome of the swordfish *Xiphias gladius*

1. The high-coverage heater tissue transcriptome of the Mediterranean swordfish provides an useful resource to understand the molecular network responsible for heat production that allows the swordfish to keep warm brain and eyes during deep vertical dives. Although presented as a preliminary data chapter, the future completion of this work will help to gain deeper insights into the concept of evolutionary convergence of endothermy among vertebrates.
2. The histology of the tissue highlighted the unique characteristics of the heater cells as highly modified muscle cells that lost the typically organized myofibrils. Indeed, the main function of heater cells is to produce heat. This function mirrors specific anatomical organizations of the tissue to maintain the heat and buffer its loss, represented by a counter-current exchange system and an abundant insulation of cranial fat.
3. A relevant presence of melanomacrophage centres was detected in the heater tissue. Although more typical of haemolymphopoietic organs such as spleen, liver and kidney, the melanomacrophage centres have a role into the adaptive immune response and detoxification, therefore suggesting that local inflammatory processes might be driving this accumulation. Whether the reasons of local inflammation in heater cells are linked to its exceptionally high energetic metabolism is a point that requires future investigations and that can elucidate the evolutionary cost for a tissue that evolved into a furnace.

Chapter 4: *De novo* transcriptome assembly, functional annotation and characterization of the Atlantic bluefin tuna (*Thunnus thynnus*) larval stage

1. The Atlantic bluefin tuna larval transcriptome is a significant molecular resource for the scientific community currently working on the larval rearing efforts to achieve the domestication of this species in the Mediterranean Sea. With over 30,000 annotated transcripts, the transcriptome covers a large fraction of protein-coding genes encoded by the genome, an aspect that is important to consider because of the lack of a sequenced and assembled genome for this species.
2. Transcripts involved into translational processes and muscle development were found having codon usage bias, suggesting that mRNA stability, protein folding, translation efficiency and elongation might be enhanced during the larval period to meet the high demand for protein synthesis and support the high growth rates.
3. The identification of orthologous groups with other fish species allowed for the detection of assembly-specific transcripts, which were mainly related to muscle and bone development, visual system and neurotransmission. Therefore, the Atlantic bluefin tuna might have evolved specific adaptations to achieve early swimming and predatory capabilities. These aspects, along with the known precocious development of its digestive system, provide a conceptual model that explains the processes able to fuel the remarkably high growth rates during the larval period. It is likely that such a model applies also to other scombrids which exhibit similar larval traits.
4. The expansion of protein families related to structural component of muscle, including domains of laminin-G and myosin tail, further strengthen the idea that the muscle development is a key aspect of this species during larval growth.
5. The present study provides a glimpse into the evolution of a set of genes underlying the unique characteristics of the larval ontogeny of this species. Although more detailed approaches are required to confirm these findings, this study helps to explain how the Atlantic bluefin tuna and likely other scombrids with similar biological traits might have accomplished early physical advantages over competing species in the pelagic environment.

Chapter 5: A comparison of reproductive potential in young and old females: a case study on the Atlantic bluefin tuna in the Mediterranean Sea

1. In the Mediterranean Sea, old females of Atlantic bluefin tuna exhibited greater reproductive potential than younger females according to gene expression results of several known reproductive markers, reflecting different reproductive dynamics linked to the physical condition, habitat usage and migratory behaviour. This finding is framed within the context of the fishery management and confirms that old females are key components of the demographic structure of the stock because of their high contribution to the reproductive output.
2. The Atlantic bluefin tuna was able to spawn in tuna traps. This phenomenon has been already described in transport cages off the Eastern coasts of Spain but, although reported by fishermen, not well characterized in tuna traps in waters South-West of Sardinia (Italy). Establishing the relevance in the area will be important to evaluate its contribution to the stock renewal.
3. The two mir-202 arms were differentially regulated. The gene expression of mir-202-5p showed no overall changes, therefore, according to its role in other fish species, might regulates early folliculogenesis before and during the reproductive season. This arm was localized in the granulosa but not theca cells and was faintly detected in the ooplasm, indicative of maternal accumulation. Due to its similar gene expression levels between periods and groups characterized by different levels of follicular atresia, a role in this process would be excluded. On the other hand, the gene expression levels of mir-202-3p reflected those of *lhr*, *star* and *casp3*, suggesting that it might be related to follicular atresia and/or advanced maturational stages.
4. The mir-202 appears as a good candidate to regulate the reproductive output of the Atlantic bluefin tuna in an autocrine/paracrine manner through either stage- or age-dependent processes.

Concluding remarks:

The chapters 1, 2 and 5 contributed to the knowledge of the reproductive biology of the Atlantic bluefin tuna *Thunnus thynnus* and the swordfish *Xiphias gladius*. Due to the high commercial interests on these species and a sound scientific community working on many aspects of their biology, remarkable progress were made towards the sustainable management of the fish stocks in the Mediterranean Sea. Although many gaps and challenges still remain, the lessons learned from past mistakes and the ongoing international efforts represent solid premises for the good management of the fishery in the future.
The chapters 3 and 4 provided a glimpse into the evolutionary trajectories that led to some of the peculiar biological traits of these species. Although future work is needed to confirm and explore further the findings, the path that was set confirmed the two species as valuable models for evolutionary studies.