Chapter 7

Reproduction, Broodstock Management, and Spawning in Captive Atlantic Bluefin Tuna

Y. Zohar¹, C.C. Mylonas², H. Rosenfeld³, Fernando de la Gándara⁴ and Aldo Corriero⁵

¹Department of Marine Biotechnology, Institute of Marine and Environmental Technology, University of Maryland Baltimore County, Baltimore, MD, USA, ²Institute of Marine Biology, Biotechnology and Aquaculture, Hellenic Center for Marine Research, Aqualabs, Iraklion, Crete, Greece, ³National Center for Mariculture, Israel Oceanographic and Limonological Research Institute, Eilat, Israel, ⁴Centro Oceanográfico de Murcia, Spanish Institute of Oceanography, Murcia, Spain, ⁵Department of Emergency and Organ Transplantation, Section of Veterinary Medicine and Animal Production, University of Bari Aldo Moro, Bari, Italy

7.1 INTRODUCTION

The Atlantic (or Northern) bluefin tuna (Thunnus thynnus, ABFT) is one of the tuna species with the greatest commercial interest for fisheries (Ottolenghi, 2008). It is one of three species of bluefin tuna (with Pacific bluefin tuna, Thunnus orientalis, and southern bluefin tuna, Thunnus maccocoyii). The ABFT can be found in the Western and Eastern Atlantic Ocean, as well as throughout the Mediterranean Sea (ICCAT, 2008). The ABFT is one of the largest fishes, attaining a body size of up to 700 kg. It can reach swimming speeds of 90 km/h and it is one of the most wide-ranging fish, with long transoceanic migrations (Cort and Liorzou, 1991; Safina, 1995). An event documented from ancient times by the Greek philosopher Aristotle (Aristotle, 1994), the ABFT migrates seasonally over long distances between the temperate waters of the Atlantic Ocean where it feeds and the warmer subtropical waters of the Gulf of Mexico and the Mediterranean Sea where it spawns.

ABFT farming and fattening in the Mediterranean Sea (Mylonas et al., 2010a) started in the mid-1990s in Croatia and spread quickly throughout the region, fueled by the insatiable demand of the Japanese sushi/sashimi market and the very high prices offered for fresh or frozen fish at the Tsukiji Market.
in Tokyo (aka the Tokyo Fish Market). The International Commission for the Conservation of Atlantic Tunas (ICCAT), the international body responsible for the management and allocation of fishing quotas (Total Allowable Catches, TACs) for the ABFT (Block et al., 2005; ICCAT, 2005; Rooker et al., 2007; Schaefer, 2001), reduced significantly their TACs in the last decade, in an effort to prevent the collapse of the wild stocks due to heavy overfishing. In parallel, studies were undertaken to examine the potential to produce ABFT entirely in captivity so that aquaculture can eventually become the main source for the seafood market and gradually replace the overexploited ABFT fishery, leading to the preservation of this important marine species.

As for any fish species, one of the prerequisites for domestication and the establishment of a sustainable aquaculture industry is the capacity to control reproductive processes in captivity, and to acquire high-quality gametes (i.e., eggs and sperm) and seeds (i.e., fertilized eggs and larvae) for grow-out to the marketable product (Mylonas et al., 2010b). This allows (i) the reliable, predictable, and consistent production of seeds to be provided to hatchery and nursery operations; (ii) the manipulation of the reproductive season through photo-thermal manipulations, in order to extend the period of seed production; and (iii) the improvement of desirable traits through selective breeding programs.

Achieving the above spawning-related goals for ABFT necessitates a basic understanding of its reproductive biology and endocrinology in the wild and in captivity, which has been studied during the last decade by several coordinated groups supported primarily by European Union multidisciplinary and multi-institutional efforts (e.g., Corriero et al., 2007; Mylonas et al., 2007; Rosenfeld et al., 2012). This chapter reviews the current knowledge on the reproductive biology and broodstock management of ABFT, and describes the current approaches for the production of high-quality fertilized eggs.

### 7.2 GAMETOGENESIS AND ENDOCRINE CYCLES

#### 7.2.1 Gonad Structure

ABFT gonads are paired, elongated organs located in the abdominal cavity and suspended by a mesogonad (mesovarium or mesotestis) (Figure 7.1). The testes consist of two structurally different regions, an outer proliferative region and an inner spermatozoa storage zone. In the proliferative region, germ cells associated with somatic cells (Sertoli cells) develop in cysts (spermatocysts), which constitute the germinal epithelium lining the seminiferous lobules from the periphery of the testis, beneath the connective membrane (tunica albuginea), to a well-developed inner system of ducts (efferent ducts). In the lumen of the efferent ducts, spermatozoa accumulate and move toward the main sperm duct (or vas deferens).
Ovaries are hollow organs with a round cross section that join caudally in a common oviduct, which opens to the exterior in the urogenital pore. Ovaries of adult individuals consist of a thick muscle wall and ovigerous lamellae, containing oogonia and oocytes at different stages of development, embedded in a mass of connective tissue. A single oocyte, surrounded by a single layer of granulosa cells and a double layer of thecal cells (theca externa and theca interna), constitutes an ovarian follicle.

**7.2.2 Spermatogenesis**

Male germ cells develop within spermatocysts and each spermatocyst is comprised of a clone (group of isogenic cells) derived from a single spermatogonium that is associated with somatic (Sertoli) cells (Figure 7.2). As in other tuna species, the ABFT testis is characterized by the occurrence of spermatogonia along the majority of the seminiferous lobules and it belongs to the unrestricted spermatogonial type according to Grier et al. (1980) classification or the lobular type according to Billard’s (1986) classification.
Abascal et al. (2004) provided an ultrastructural description of ABFT male germ cells at different developmental stages. Spermatogonia A are ovoid cells (~10 μm in diameter), with a nucleus showing diffuse chromatin and a central nucleolus; their cytoplasm contains free ribosomes, mitochondria, endoplasmic reticulum cisternae, and “nuages” (“clouds” in French) of perinuclear electron-dense material that could possibly represent mRNA molecules. Spermatogonia B (~10 μm in diameter) are grouped in small

**FIGURE 7.2** Micrograph of the testes of adult Atlantic bluefin tuna in early (A) and advanced (B) spermatogenesis. Haematoxylin-eosin staining. Magnification bars = 100 μm; bar in inset of (A) = 20 μm. Arrowhead: undifferentiated spermatogonium; arrow: type A spermatogonial cyst; curved arrow: type B spermatogonial cyst; double arrow: spermatocyte cyst; sz: spermatozoa released in the lumen of seminiferous lobules. Reproduced from Zupa et al. (2013).
clusters and show a nucleus containing patchy chromatin. Primary spermato-
cytes (~5 μm in diameter) are clustered cells with a heterochromatic nucleus
and cytoplasm containing polysomes, mitochondria, and the diplosome.
Secondary spermatocytes (3–4 μm in diameter), presumably short-lived
germ cells rarely found in histological samples, have a nucleus with diffuse
chromatin-forming electron-dense patches. In early spermatids (2–3 μm in
diameter), the nucleus shows a dense chromatin and some electron-lucent
areas. The chromatin becomes more homogeneous in mid-spermatids and
condenses in a gross granular pattern in late spermatids (2 μm in diameter).
Spermiogenesis involves flagellum elongation, cytoplasm reduction, and
mitochondria rearrangement around the proximal axonema region. Rotation
of the nucleus does not occur during spermiogenesis (characteristic of type-II
teleost sperm).

7.2.3 Oogenesis

Female ABFT germ cells have been described in terms of morphology
(Corriero et al., 2003), histochemical characteristics (Sarasquete et al., 2002),
and ultrastructure (Abascal and Medina, 2005). The ABFT ovary shows an
asynchronous pattern of oocyte development (i.e., presence of all oocyte
stages in the same specimen during the spawning season) (Figure 7.3), which
leads to the release of mature oocytes in multiple batches. The number of
oocytes released per spawning season (absolute fecundity) reflects the num-
ber of oocytes released per spawning event (batch fecundity) and the number
of spawning events per spawning season, which may vary according to the
size of the fish, and environmental or individual conditions. Studies of wild
ABFT (Medina et al., 2002) estimated that batch fecundity is around 93,000
eggs per kg body weight, which means that a 100 kg female can produce
around 9 million eggs in a single spawn. As far as how many times (i.e.,
days, batches) each female can spawn, either from the wild fishery or from
captive-reared broodstock, is not known at this time. Based on the presence
of post-ovulatory follicles in spawning ABFT in the wild, it has been esti-
imated that the spawning frequency is 1.2 days (Medina et al., 2002). This
means that a large proportion of the population spawns every day, but it is
not known for how many days this can continue.

Although different classification criteria have been used, oogonia and the
following five oocyte developmental stages have been described for ABFT:
early oocytes; primary oocyte growth; lipid stage; vitellogenesis; post-vitello-
genesis; and oocyte maturation. Oogonia (8–15 μm in diameter) are found
in small clusters at the periphery of the ovigerous lamellae. Their cytoplasm
is weakly basophilic and possesses many ribosomes, round mitochondria,
Golgi complexes, and a poorly developed endoplasmic reticulum. The
nuclear envelope shows numerous pores and cloudy material (nuages) can be
observed around it. Early oocytes (15–20 μm in diameter) are
FIGURE 7.3 Micrographs of the gonads from two adult Atlantic bluefin tuna specimens. (A) Ovary from an active non-spawning individual showing both primary and vitellogenic oocytes. (B) Ovary from a spawning specimen showing primary oocytes, migratory nucleus stage oocytes, and postovulatory follicles. Haematoxylin-eosin staining. Magnification bars = 500 μm; bar in inset of (B) = 300 μm. Arrow, postovulatory follicle; α, vitellogenic follicle in early stage of atresia (α-atretic follicle); β, vitellogenic follicle in an advanced stage of atresia (β-atretic follicle); ev, early vitellogenic oocyte; lv, late vitellogenic oocyte; p, perinucleolar stage oocyte; mn, migratory nucleus stage oocyte. Modified from Heinisch et al. (2008).
ultrastructurally similar to oogonia but they are found in association with pre-follicle cells that send thin processes between adjacent oocytes and progressively surround them. Primary oocyte growth, also known as the pre-vitellogenesis stage (20–120 μm in diameter), includes two stages of oocyte development: the chromatin-nucleolus and peri-nucleolus stages. Chromatin-nucleolus oocytes (20–40 μm in diameter) are rarely observed in ovarian sections. This stage is characterized by a basophilic ooplasm (due to ribosome abundance) showing the appearance of Balbiani’s vitellin body and a large nucleus with chromatin strands and a large nucleolus. Oocytes at the peri-nucleolus stage (40–110 μm in diameter) have a basophilic ooplasm and a large euchromatic nucleus containing numerous nucleoli adjoining the nuclear envelope. Mitochondria, Golgi complexes, and nuages are visible in close proximity to the nucleus. Oocytes at this stage are surrounded by a continuous layer of flattened granulosa cells. Membrane processes developed from the oocyte extend toward granulosa cells and vice versa, and the formation of the zona radiata (vitelline envelope) starts as a two-layered structure: a moderately electron-dense outer layer and a more electron-dense inner layer. The two thecal layers (theca externa and theca interna) are identifiable at this stage of follicle development. Lipid stage oocytes (110–220 μm in diameter) exhibit a weak ooplasm basophilia and are characterized by an increasing amount of small lipid droplets. Ribosomes, mitochondria, and dictyosomes are found in the ooplasm. Finely granular chromatin is present in the nucleus and the nuclear envelope contains numerous pores. The zona radiata increases in thickness and becomes visible by light microscopy as PAS+ material. Vitellogenic oocytes (up to 500 μm in diameter) are characterized by vitellogenin uptake and by vitellogenin-derived yolk protein deposited in the ooplasm in the form of yolk granules (platelets). The use of anti-vitellogenin antibodies in immunohistochemical staining (Susca et al., 2001) allows the detection of small amounts of yolk proteins in oocytes with a minimum diameter of 220 μm. Early vitellogenic oocytes are characterized by the presence of spherical acidophilic granules and PAS+ cortical alveoli in the peripheral ooplasm. The zona radiata progressively increases in thickness (up to 12 μm in late vitellogenic oocytes) and the granulosa cells become cubic. In oocytes at advanced stage of vitellogenesis, yolk globules increase both in size and number and progressively fill the entire ooplasm. Oocyte microvilli pass through the zona radiata pore canals and extend into the intercellular spaces of granulosa cells. At this stage of vitellogenesis, transmission electron microscopy data has demonstrated an intense oocyte uptake of exogenous substances. The ooplasm is rich in clathrin-coated vesicles (indicative of receptor-mediated endocytosis) containing electron-dense material, which appears to bud from membrane invaginations. Endocytic vesicles lose their clathrin coat and fuse with each other to form yolk platelets, which increase progressively in size. Large lipid droplets are interspersed among yolk platelets. After the completion of vitellogenesis, oocytes
enter post-vitellogenesis or oocyte maturation, which is characterized by the
resumption of meiosis, including migration of the nucleus (also referred to at
this stage as the Germinal Vesicle, GV) toward the animal pole followed by
the breakdown (BD) of the nuclear envelope. Lipid droplets fuse with each
other (coalescence), eventually forming one large lipid globule that occupies
the center of the oocyte. Vitellogenin-derived yolk proteins that are accumu-
lated within yolk granules undergo a proteolytic cleavage (Pousis et al.,
2011), which increases the concentration of free amino acids and small pep-
tides in the oocyte, thereby providing an osmotic mechanism for water influx
into the oocyte (oocyte hydration) and resulting in both the formation of a
fluid yolk mass and the rapid increase of oocyte size (diameter of ABFT
mature oocytes ≈1 mm). After ovulation, follicular cells constitute convo-
luted post-ovulatory follicles, characterized by hypertrophic granulosa cells
that delimit an irregular lumen. Post-ovulatory follicles are short-lived struc-
tures and degenerate rapidly, becoming indistinguishable from atretic folli-
cles and then from connective stroma. Atretic follicles are normally found in
ovaries in vitellogenic and post-vitellogenic development. Follicles in the
early stage of atresia (α-atretic stage) are easily distinguishable because of
their irregular shape and fragmentation of the zona radiata. The nuclear
envelope of an atretic oocyte disintegrates and yolk granules lose their struc-
tural integrity and are progressively phagocytized by granulosa cells, which
invade the oocyte. After the complete degradation of the oocyte, atretic folli-
cles (now called β-atretic follicles) consist of only disorganized granulosa
and thecal cells showing pyknotic nuclei.

7.2.4 Gonadal Cycle and Sexual Maturity

The description of naturally occurring gametogenesis, gonadal activity, and
reproductive cycles in ABFT, as described in this Section 7.2, is strictly
dependent on the availability of wild fish, which is generally limited to a
few months per year due to both fish accessibility and severe restrictions
imposed by fishery regulations. ABFT has been managed by the ICCAT as
two populations, the eastern and western populations, with known breeding
areas in the Mediterranean Sea and the Gulf of Mexico, respectively. Most
of the available information on the temporal pattern of ABFT gonadal matu-
ration refers to the eastern ABFT stock and, in particular, comes from indivi-
duals captured in the Mediterranean Sea. In this basin, ABFT have been
found to be reproductively inactive from August to March, when only un-
yolked oocytes are present in the ovaries, and mainly spermatogonia and
meiotic cells have been found in the seminiferous epithelium. From mid-
May to mid-June, ovaries are in full vitellogenesis and testes are in advanced
spermatogenesis, showing all spermatogenetic stages. The reproductive
period in the Mediterranean Sea, as evident by the presence of individuals
with ovaries containing oocytes at final maturation stage and/or
postovulatory follicles, extends from May through July, coinciding with a rise ( > 23 °C) in sea surface temperature. According to Heinisch et al. (2008), ABFT shows a characteristic spatial-temporal pattern of gonadal maturation across the Mediterranean Sea with an initial peak of spawning activity during mid-May at eastern locations (Levantine Sea), and during June and July at central (Malta) and western (Balearic Islands) locations, respectively. Data on gonad maturation of the western ABFT stock are limited and indicate that spawning occurs in April–May in the Gulf of Mexico (Baglin, 1982), although the presence of additional, unknown, spawning grounds in the western Atlantic has not been excluded (Lutcavage et al., 1999; Goldstein et al., 2007; Cermeno et al., 2015).

Based on the presence of yolked oocytes as a sign of activation of the reproductive axis and providing substantial confirmation of historical data which is based on macroscopic observations of the gonads, Corriero et al. (2005) reported that 50% of ABFT in the Mediterranean Sea reach sexual maturity at 104 cm Straight Fork Length (SFL), corresponding to an age between 3 and 4 years, and 100% at 130 cm SFL (5 years of age). In the western Atlantic, histological examination of ovaries from females points to delayed maturation schedules, and individuals have been reported to reach sexual maturity between age 7 and 8 (Baglin, 1982; Goldstein et al., 2007).

However, by means of a new molecular approach that uses pituitary ratios of follicle stimulating hormone to luteinizing hormone as markers of sexual maturity, Heinisch et al. (2014) demonstrated that western ABFT mature at a considerably smaller size, similar to that of the eastern Atlantic population.

### 7.3 CAPTIVITY EFFECTS ON GAMETOGENESIS AND ENDOCRINOLOGY

#### 7.3.1 Adult Fish

Studies focused on assessing the reproductive potential of adult ABFT, captured at the Balearic Islands and held in floating cages on the coast of Murcia, demonstrated the onset and progression of gametogenesis and the hormones that control it in captivity (De la Gandara et al., 2009, 2010). Endocrine parameters including the hypo-physiotropic gonadotropin-releasing hormone (GnRH1) and the maturational gonadotropin luteinizing hormone (LH) peak in June, during which the highest gonado-somatic index (GSI) values were recorded (Figure 7.4). Furthermore, the levels of both LH and GnRH1 in captive fish did not vary significantly ($P > 0.05$) from those measured in wild ABFT that were collected during June along the Maltese coastline (Figure 7.5B,C). Altogether, these findings indicate that the reproductive endocrine system in captive ABFT is functional and undergoing physiological adjustments in anticipation of the natural spawning season in this region.
FIGURE 7.4 GSI values (A), pituitary LH content (B) and pituitary GnRH1 levels (C) in adult captive Atlantic bluefin tuna during the species’ natural reproductive season in the Mediterranean Sea (May–July 2003). Levels (mean ± SEM) are expressed as percentage (%), total amount (mg) per pituitary, and ng per pituitary, respectively. Groups with different letters are significantly different ($P < 0.05$).
Nonetheless, although gametogenesis did take place in captivity, the GSI values of the caged fish were relatively low compared to wild fish (Figures 7.5A and 7.6). A series of studies demonstrated that both spermato- genesis (Corriero et al., 2007, 2009; Zupa et al., 2013) and oogenesis

**FIGURE 7.5** GSI values (A), pituitary LH content (B) and pituitary GnRH1 levels (C) in captive-reared versus wild-caught adult Atlantic bluefin tuna during the peak of the gametogenic cycle in the Mediterranean Sea (June 2004). Levels (mean ± SEM) are expressed as percentage (%), total amount (mg) per pituitary, and ng per pituitary, respectively. Groups with different letters are significantly different ($P < 0.05$).

Nonetheless, although gametogenesis did take place in captivity, the GSI values of the caged fish were relatively low compared to wild fish (Figures 7.5A and 7.6). A series of studies demonstrated that both spermato- genesis (Corriero et al., 2007, 2009; Zupa et al., 2013) and oogenesis
(Corriero et al., 2007, 2011; Pousis et al., 2011, 2012) are somewhat impaired in captive conditions. In a histological study carried out by Corriero et al. (2007) on 36 ABFT males reared for 1–3 years under commercial conditions in a tuna farm located in the western Mediterranean and sampled during the 2004 and 2005 reproductive seasons, the vast majority (>72%) of the fish were found to be at early spermatogenesis, while less than 5% were classified as spent. Yet, distinctively from captive fish, the histological appearance of the testis in mature wild males (sampled on the spawning grounds during the reproductive season) showed noticeably enhanced testicular development (large seminiferous lobules entirely filled with spermatozoa).

In a comparative study of male germ cell proliferation and apoptosis in wild and captive-reared ABFT, Zupa et al. (2013) reported that the spermatogonial proliferation rate of captive ABFT sampled in May was higher than in wild individuals, while the number of meiotic cysts was lower (Figure 7.7). In June, the density of proliferating spermatogonia and spermatocytes was higher in captive fish than in wild spawners. The authors concluded that rearing in captivity had resulted in a shift of the spermatogenetic process “with a high spermatogonial division in May and predominance of meiosis in June, when the wild spawners were undergoing their last phase of spermatogenesis (i.e. spermiogenesis), with high prevalence of spermatids and spermatozoa.” In the same study, apoptotic spermatogonia and primary spermatocytes were detected in the testes of both wild and captive-reared individuals. The density of apoptotic germ cells was found to be significantly higher in captive-reared than in wild ABFT throughout both periods (May and June) examined during the reproductive cycle. The increased apoptotic rate in captive ABFT appears to be the result of reduced androgen levels, particularly those of 11-ketotestosterone (11-KT), the

**FIGURE 7.6** Mean (± s.e.m.) gonado-somatic index (GSI) of male ABFT broodfish. Comparison between individuals reared in captivity (pooled controls and GnRHa-treated) and wild spawners. Different letters represent statistically different means (ANOVA, \( P \leq 0.05 \)). *Modified from Corriero et al. (2009).*
principal androgen that stimulates spermatogenesis in fish. In fact, in a previous study Corriero et al. (2009) highlighted the close association between high circulating levels of 11-KT and reduced germ cell apoptosis in captive-reared ABFT treated with GnRHa, compared to untreated control fish. It is likely that the relatively low androgen levels typifying captive ABFT reflect a reduced

FIGURE 7.7  (A) Micrographs of testis sections from two adult Atlantic bluefin tuna specimens. (A) Immunolabeling with antibodies against Proliferating Cell Nuclear Antigen (PCNA) showing nuclei of proliferating cells stained in brown. Magnification bar = 50 μm. Arrowhead: undifferentiated spermatogonium; arrow: type A spermatogonial cyst; dashed arrow: type B spermatogonial cysts; double arrow: spermatocyte cysts. (B) Staining with the terminal deoxynucleotidyl transferase-mediated dUTP Nick End Labelling (TUNEL) method. Apoptotic cells and apoptotic bodies appear as dark blue dots. Magnification bar = 200 μm. Reproduced from Zupa et al. (2013).
secretion of pituitary gonadotropin caused by captive conditions, as was proposed for other species of fish (Zohar, 1989).

The effects of tuna captivity and farm rearing conditions on oogenesis are more pronounced than those observed on spermatogenesis. A thorough analysis of ovaries derived from ABFT reared in the western Mediterranean clearly demonstrated that captivity does not prevent vitellogenesis and oocyte growth, since yolk granule formation and oocyte diameter of fully vitellogenic oocytes were similar to wild fish (Corriero et al., 2007). This finding was confirmed further by Pousis et al. (2011, 2012), who demonstrated an absence of any significant differences in vitellogenin (Vg) and Vg receptor (VgR) gene expression, as well as in oocyte yolk accumulation between wild and captive-reared individuals. However, compared to wild fish (analyzed per kg body weight) farmed females consistently exhibit: (i) a relatively lower gonadal mass, (ii) lower numbers of vitellogenic oocytes, (iii) reduced ability to undergo oocyte maturation, and (iv) a higher proportion of atretic oocytes. In this regard, it is interesting to note that acute stress is an additional effector contributing to impaired vitellogenesis. Thus, farmed ABFT individuals have been shown to undergo a massive degeneration of vitellogenic oocytes (up to 100% α-atresia) and a dramatic loss of ovarian mass 24 h following an exposure to crowding-induced severe acute stress (Corriero et al., 2011).

7.3.2 Puberty

In light of increasing interest in the domestication of ABFT, first sexual maturity in captivity was studied by documenting its occurrence and by characterizing the gonadotropins (FSH and LH), the central regulators of gonadal development and gamete maturation (Berkovich et al., 2013). Histological sections of gonads obtained from 2- and 3-year-old (2Y and 3Y, respectively) farmed ABFT juveniles revealed that females of both age classes were sexually immature, showing the perinucleolar stage as the most advanced oocyte stage in the ovaries. The testes of all 2Y males showed a germinal epithelium composed of only spermatogonia. In contrast, the 3Y males were already sexually mature with testes containing all the spermatogenetic stages including spermatozoa in the lumen of seminiferous lobules. An earlier puberty in males, as compared to females, is a common feature of many fish species (Carrillo et al., 1995; Jakupsstovu and Haug, 1988; Holland et al., 2000; Saillant et al., 2003; Hurvitz et al., 2005; Cao et al., 2009), including the Pacific bluefin tuna (Sawada et al., 2007). Moreover, this phenomenon becomes even more pronounced under intensive culture conditions where ample food availability facilitates faster growth rates compared to those in wild populations (reviewed by Carrillo et al., 2009; Taranger et al., 2010). Thus, it is not yet clear whether the observed puberty in 3Y ABFT males is a general feature also occurring in wild populations or a phenomenon induced by the culture conditions.
The expression and accumulation patterns of LH in the pituitary showed a steady increase of this hormone, concomitant with fish age, reaching higher levels in adult females compared to males of the same age class (Berkovich et al., 2013). Conversely, the pituitary FSH levels were elevated only in 2Y and adult fish. The pituitary FSH to LH ratio was consistently higher (>1) in immature ABFT compared to maturing or pubertal fish, resembling the situation in other fish species and higher vertebrates. Nevertheless, the results suggest that a rise in the LH storage level above a minimum threshold may be an indicator of the onset of puberty in ABFT females. Higher pituitary LH levels in sexually mature females versus males may further support this notion. Future studies on the effects of captivity and hormonal treatments on precocious maturity may allow for improved handling of this species in a controlled environment, which would lead to more cost-effective broodstock management.

7.4 HORMONAL MANIPULATION OF REPRODUCTIVE FUNCTIONS

Like many other fishes reared in captivity, ABFT do not spawn consistently when maintained in cages or tanks, thus necessitating the use of exogenous hormones. Early studies examining the potential of ABFT to mature and complete its reproductive cycle in captivity in the Mediterranean indicated that wild fish caught from the fishery and maintained for at least one year in sea cages underwent complete gametogenesis during the May–July reproductive season (Corriero et al., 2007; Gordoa et al., 2009; Mylonas et al., 2007; Zupa et al., 2013). However, while some males were producing releasable sperm of good quality (Suquet et al., 2010), all females failed to undergo oocyte maturation, and hence ovulation and spawning. This is a typical reproductive dysfunction of fishes in captivity (Mylonas et al., 2011; Zohar, 1989; Zohar and Mylonas, 2001), and in ABFT it can be overcome with the exogenous administration of GnRHa (see Section 7.5.1). It is important, however, to point out that in some instances, after years of acclimatization to the captive conditions, ABFT broodstocks have also been shown to spontaneously complete their reproductive maturation and to produce large amounts of high-quality eggs without the employment of any exogenous hormones (Gordoa et al., 2009), as described later (see Section 7.5.2).

Hormonal manipulation of reproductive function in captive fishes ranges from the complete stimulation of gametogenesis practiced in freshwater eels (Anguilla spp.) (Miura et al., 2002) to simply the synchronization of ovulation to improve in vitro fertilization operations in salmonids (Zohar, 1989, 1996). Nevertheless, the most common application of exogenous hormones in aquaculture includes the enhancement of sperm production (Mylonas et al., 2016) and the induction of oocyte maturation, ovulation, and spawning (Mylonas and Zohar, 2001; Mylonas et al., 2010b). A controlled-release
A delivery system (implant) for GnRHa (Mylonas and Zohar, 2001) was developed specifically for ABFT, releasing the hormone for a period of 2–3 weeks, and was used to induce maturation and spawning (Mylonas et al., 2007). In both males and females, administration of the GnRHa implants resulted in a significant elevation of GnRHa levels in the plasma (Figure 7.8), a decrease of pituitary LH content and a concomitant increase in plasma LH (Rosenfeld et al., 2012). These changes demonstrated that (i) during gametogenesis LH is being produced and stored in high amounts in the pituitary and (ii) the administered GnRHa in the provided dose and mode of release and the ensuing surge of plasma GnRHa were appropriate to induce the release of the stored endogenous LH. The plasma LH profiles paralleled the kinetics of the GnRHa released from the implants (Mylonas et al., 2007). In response to the GnRHa-induced increased plasma LH, there were significant changes in steroidogenesis including increases in plasma 17β-estradiol (E2) and 11-KT in females and males, respectively. Furthermore, a significant peak in the plasma of free 17, 20β-dihydroxy-4-pregnen-3-one (17, 20β-P) levels occurred simultaneously in males and females at day 8 after treatment. Histological sections of the ovaries in the GnRHa-induced females contained oocytes at the final stages of maturation and germinal vesicle migration (Corriero et al., 2007), suggesting the role of 17, 20β-P as a maturation-inducing steroid in ABFT, similar to other perciform species (Nagahama, 1997). Interestingly, the stimulatory effects of sustained-release GnRHa implants on the pituitary—gonad axis elicit reproductive hormone dynamics during final oocyte maturation that display striking similarity between ABFT and gilthead sea bream (Sparus aurata), both of which are asynchronous spawners (seabream: Zohar and Gordin, 1979; Zohar et al.,

**FIGURE 7.8** Mean (± SEM) plasma GnRHa levels of captive-reared Atlantic bluefin tuna males and females (n = 7 fish per group for the control females and males; n = 10 GnRHa-implanted females and 16 males), 2–8 days after treatment with GnRHa implants. In both sexes, there was a statistically significant elevation (two-way ANOVA, P < 0.02) in plasma GnRHa in the implanted fish. Reproduced from Mylonas et al. (2007).
The more noticeable similarities include the LH response to sustained release of GnRHa (Zohar, 1988, 1996; Zohar et al., 1990), and the association of the plasma 17, 20β-P levels and the maturation stage of the oocytes during a daily spawning cycle (Gothilf et al., 1997). Surprisingly, even under sustained GnRHa-induced conditions whereby long-term elevated plasma LH levels appear to override the natural daily fluctuation of plasma LH, in both gilthead sea bream (Zohar et al., 1995) and ABFT (De Metrio, 2010) the females still manage to retain their typical daily spawning cycles.

Due to the relatively late reproductive maturation of ABFT (≥4 years of age), advancing the onset of puberty, via hormonal and/or environmental manipulations, will simplify broodstock management and spawning efforts and, in turn, seed production for the aquaculture of this species. Fueled by our expanded understanding of the regulation of puberty in teleost fish (Zohar et al., 2010), a series of in vivo and in vitro trials were carried out to evaluate the (i) dynamics of the maturational process of the brain—pituitary—gonadal (BPG) axis (Berkovich et al., 2013), and (ii) responsiveness of the BPG to exogenous hormones, in ABFT undergoing pubertal development (Berkovich et al., 2011). Different hormonal treatments, including GnRHa, kispeptin decapeptide (Kiss), and E2 administered through controlled-release implants, were tested as potential inducers of puberty in captive ABFT. In general, no clear effects of the different hormonal treatments were observed at the macroscopic (GSI) or microscopic (oocyte and seminiferous tubule diameter) levels. Nevertheless, further in vitro studies demonstrated the capacity of recombinant FSH (rFSH) and, to a lesser extent, rLH to stimulate cell proliferation in immature ovarian and testicular fragments of ABFT. Both rFSH and rLH have failed to stimulate steroidogenesis, yet the in vivo pretreatment with Kiss-containing implants appeared to potentiate rFSH-stimulated steroidogenesis in immature testes. Future research is required to study the effects of environmental and hormonal manipulations on the timing of puberty, with the goal of inducing precocious sexual maturation and enabling spawning of ABFT at relatively small body size. Working with smaller broodstock is expected to facilitate their husbandry and handling in confined environments, and to greatly improve the cost-effectiveness of ABFT broodstock operations, seed production, and in turn its farming profitability.

### 7.5 SPAWNING OF CAPTIVE-REARED BROODSTOCKS

#### 7.5.1 Induced Spawning

As mentioned above, development of any industrial aquaculture operation requires absolute control of the reproductive cycle of the particular fish of interest. Since the spawning season in captive ABFT is very short (about 1–2 months) and not consistent, predictable or reliable, induced
spawning methods are essential for the early expansion of the industry (Corriero et al., 2007, 2009; Mylonas et al., 2007). In the first efforts to induce spawning in ABFT, sexually mature migrating fish (5–12 years old) were captured in the Mediterranean Sea and reared in floating cages. Around the time of ABFT spawning in the wild (June–July), GnRHa was administered to broodstock fish (both males and females) via polymer-based controlled release delivery systems (implants), administered to the fish underwater, as they were swimming (Mylonas et al., 2007). Typically, 2–3 GnRHa implants (3–4 mm in diameter each) were mounted on the needle tip of a spear gun, along with a visible tag, and shot into the swimming fish by free-divers (Figure 7.9). The implants were designed to release the GnRHa into the fish for a period of 2–3 weeks, at a total dose of 50–75 micrograms of the hormone per kg body weight. For two consecutive years during the natural spawning period (June–July), GnRHa-implanted fish were

**FIGURE 7.9** Spawning induction in ABFT using polymer-based controlled release GnRHa implants. (A) GnRHa implant assemblies with a dart-head and colored Floy tag (dart-heads and tags both from Floy Tag & Mfg. Inc.). (B) An implant/tag assembly mounted on the arrow of the spear-gun. Note the broodstock cage with the curtains (background) surrounding the cage perimeter to retain spawned eggs for collection. (C) A diver with the spear-gun ready to administer the GnRHa implant to the broodstock. (D) An implanted broodstock tuna inside a SELFDOTT cage in Cartagena, Spain. Photo credits: (A) Jorge Gomezjurado; (B) Kali Tuna; (C) Kali Tuna; (D) IOE.
sacrificed at different times after treatment in order to monitor the effect of the hormonal implants on inducing final oocyte maturation and ovulation in females and spermiation in males (Mylonas et al., 2007). In males, the GnRHa implants did not result in any differences in the histological appearance of testes (Figure 7.10) and almost all fish (both GnRHa-treated and controls) contained intra-testicular spermatozoa (Corriero et al., 2007). There was an increase, however, in the proportion of spermiating males; in control males ($n = 17$) it was only 12% compared to 26% for the GnRHa-treated

**FIGURE 7.10** Photomicrographs of histological sections from testes of captive-reared Atlantic bluefin tuna in response to treatment with GnRHa implants at the peak of the reproductive season. (A) Non-implanted control; (B) GnRH-implanted male. Both males contained large numbers of spermatozoa (szoa) in their testes, as well as spermatocysts with gametes at an earlier stage of development (sc).
males \((n = 19)\). A much stronger and definitive effect was observed in female ABFT, where GnRHa implantation induced final oocyte maturation (germinal vesicle migration and coalescence of lipid droplets and yolk globules) 2–8 days after treatment (Figure 7.11) in 63% of the fish, and 88% had post-ovulatory follicles in the ovaries, compared to 0% and 21%, respectively, for control females. In addition, two females were found to have ovulated eggs in the ovaries at the time they were sampled and, after insemination \textit{in vitro} with sperm from spermiating males, viable embryos and larvae were produced for the first time in captivity in ABFT (Mylonas et al., 2007). In the same experiments, fertilized eggs were collected from the cages housing the treated fish (Mylonas et al., 2007), demonstrating that the developed GnRHa implants could induce oocyte maturation, ovulation, spermiation, and spawning in captive-reared ABFT maintained in sea cages. Later, another captive stock of ABFT in Italy was induced to spawn using the same GnRHa therapy, resulting in the production of 20 million eggs over four consecutive daily spawns with a fertilization rate of 80% for the first two spawns (De Metrio et al., 2010). These eggs drove the first larval rearing trials and resulted in the first-ever production of cultured ABFT juvenile up to the size of 8 cm in total length at 60 days post-hatching. In 2009, another captive-reared broodstock in Spain was induced to spawn using the same GnRHa implants, resulting in daily spawning for 17 days (reflecting the fact that ABFT is a batch spawner, see Section 7.2.3) and producing a total of 140 million fertilized eggs (Figure 7.12B; De la Gandara et al., 2010). These eggs were sent to various research hatcheries around the Mediterranean Sea, and larval rearing trials produced a small number of fingerlings, demonstrating the feasibility of the aquaculture production of ABFT. More recently, the GnRHa implants were used to extend the spawning season well beyond its natural duration in the Adriatic Sea (Zohar et al., unpublished results). After initiation of some sporadic spawning during the month of June, broodstock ABFT held in sea cages were treated with GnRHa implants several times, at intervals of a few weeks, from early July through the middle of August. These treatments resulted in multiple massive spawning events with eggs of excellent quality and in a spawning season that lasted until the end of August (a total duration of two and a half months). A few million of these eggs were successfully shipped to the United States (Maryland) and led to the first-ever aquaculture production of bluefin tuna juveniles in North America (Zohar et al., unpublished results).

One question relevant to both induced and spontaneous spawning (see Section 7.5.2) is how many individuals from a group of broodstock actually spawn and contribute to egg production. Using microsatellite-based genotyping of broodstock and eggs SELFDOTT investigators (Cilia Antoniou, HCMR, unpublished data) showed that out of 25 genotyped individual ABFT, 15 individuals contributed to the majority (87.8%) of the eggs produced, while the remaining 10 breeders contributed to only 13.2% of the
FIGURE 7.11 Photomicrographs of histological sections from ovaries of captive-reared Atlantic bluefin tuna in response to treatment with GnRHa implants at the peak of the reproductive season. (A) Non-implanted control female with oocytes at the stage of vitellogenesis (vg), with a centrally located nucleus (germal vesicle, gv) and dispersed lipid droplets (l) and yolk globules (y). (B) GnRHa-implanted female with oocytes at the stage of early oocyte maturation, with the gv migrating to the periphery and the lipid droplets coalescing into larger droplets. (C) Another GnRHa-implanted female with oocytes at the stage of advanced oocyte maturation, with almost completely coalesced lipid (l) droplets and coalescing yolk (y) globules.
eggs. Although it was not known which of the 25 studied individuals were induced to spawn (only 15 fish were implanted with GnRHa in this study), it is interesting to note that the majority of spawning was attributed to 15 individuals of the total population. While this study drew no conclusions about the relative contribution to the spawning of spontaneous or induced fish, similar research is required to support decisions on breeder selection, broodstock management and spawning induction, especially in situations where the size of the broodstock is limited, such as will be the case in future land-based broodstock operations. It is interesting to note that a study using the same genotyping approach in a southern bluefin tuna broodstock, held in a land-based tank, demonstrated that only a few females in the tank participated in spawning, and most of them in response to treatment with GnRHa implants (Knibb et al., in preparation).

Considered together, GnRHa implants were found to be an efficient tool to initiate, synchronize and prolong the duration of ABFT spawning, thus helping broodstock management and providing better predictability of egg supply. Since the early demonstration of their efficiency, GnRHa implants have been used by various commercial and research operations with the objective of inducing massive spawning of ABFT broodstock at the beginning or throughout the spawning season (especially when fish do not spawn spontaneously or spawn very small quantities of eggs), as well as to extend the length of the spawning season.
7.5.2 Spontaneous Spawning

In the framework of a European project (7FP SELFDOITT) coordinated by the Spanish Institute of Oceanography (Instituto Español de Oceanografía, IEO), 38 wild ABFT (~60 kg in bodyweight) were captured in June 2007 in the Balearic Sea using commercial purse-seiners. The fish were transferred to a 25 meter diameter, 20 meter-deep floating cage at El Gorguel Bay (Cartagena-Murcia) on the southeast coast of Spain. This broodstock population was fed to satiation once a day, 6 days per week, with raw fish consisting mainly of mackerel (Scomber scombrus) and Spanish mackerel (S. japonicus). The wild-caught broodstock adapted well to captivity, accepting the feed provided from their arrival at the culture facilities and with a low mortality rate (5%) over the 4 years of rearing. Spawning activity and the amount of eggs produced by these fish were monitored from 2008 to 2011 (Figure 7.12; De la Gandara et al., 2011). In 2008 and 2009, fish were treated with GnRHa implants, as described in Section 7.5.1. In 2008, no spawning events were detected and no eggs were collected (Figure 7.12A), which may reflect the fact that the fish were implanted and egg collectors deployed very late in the season (July 15), at a time when the spawning season at this site is typically over (see Figure 7.12, years 2009–2011; De la Gandara et al., 2009). In 2009, massive spawning started between June 29 and 30, roughly 48–72 h after the administration of GnRHa implants, and lasted 17 consecutive days, with a daily maximum egg collection of 34 million eggs and a total collection of 140 million. During this period, water temperature ranged between 22 and 28 °C on the surface and between 19 and 27 °C at the bottom of the cage (Figure 7.12B).

In the following year (17 June 2010), spontaneous spawning was observed prior to the time GnRHa implantation was planned. From this day forward, and more or less on a daily basis, viable eggs were collected for a period of 26 days, thus GnRHa treatment was not employed for this year. During the 2010 reproductive season, a total of 48 million eggs were collected from this stock. In 2011, eggs were again produced spontaneously without any GnRHa treatment on 9 June 2011, about one week after installing the egg collector. Spontaneous spawning lasted for 37 days and the total number of eggs collected was 162 million (Figure 7.12D). During the 2011 spawning period, the water temperature ranged between 21 and 26 °C. In this ABFT broodstock over the monitoring period, spawning typically occurred before dawn, usually from around 02:00 to 03:00 h, but often continued past dawn. Spawning followed intense courtship behavior that occurred at some depth, which therefore was not possible to fully observe and/or describe in detail.

The above ABFT broodstock was maintained past the end of the SELFDOITT project in November 2011, through funding by various research and development projects to IEO and the Ricardo Fuentes e Hijos Group, in order to establish hatchery/nursery production of ABFT. In the spawning
seasons of 2012, 2013, and 2014, spawning was spontaneous and large numbers of ABFT eggs were produced, as monitored by the husbandry personnel. However, daily egg fecundity was not documented as it was done during the SELFDOTT project, since eggs were collected only when they were required by the hatchery for larval rearing experiments.

Gordoa et al. (2009), Gordoa (2010), and Gordoa and Carrerras (2014) also reported spontaneous spawning events in another wild-caught ABFT broodstock, just after their capture from the wild using a purse seine, and also in an ABFT stock after one year in captivity. These stocks spawned in transport cages as they were being towed from the capture area around the Balearic Islands (Spain, western Mediterranean) to the company fattening facility off the coast of eastern Spain, and again as they were towed back from the fattening site to the capture area during the next year’s spawning season (to allow spontaneously spawned eggs to survive in the wild). Also, spontaneous spawning has been reported in a commercial ABFT fattening operation in Malta (Deguara, 2011), again using wild-caught individuals after some years of acclimation, and in Turkey (Kilic Aquaculture) as well as in cages with broodstock that were initially captured in the wild as ~10 kg fish in the Adriatic Sea (Kali Tuna).

As a result of the spontaneous spawning of these wild-caught ABFT stocks maintained by commercial fattening operations during the last 5 years, larval rearing/nursery trials have finally succeeded in the production of high-quality juveniles for sea cage rearing. Consequently, in November 2014 the first-ever hatchery-produced ABFT were sent to the market after ~3 years of rearing at a size of ~20 kg (De la Gandara, personal communication). Therefore, the feasibility of aquaculture production of ABFT, from captive spawning and hatchery to the market, has been demonstrated, as has been achieved earlier with the Pacific bluefin tuna in Japan (Sawada et al., 2005).

Thus, it is clear that captive ABFT broodstock held at low densities in floating cages can spontaneously spawn large amounts of high-quality eggs. However, in most cases spontaneous spawnings are short in duration (3–4 weeks) and in some situations (i) do not occur consistently, (ii) cease for several days or weeks, and/or (iii) consist of small number of eggs as only a small number of females may be spawning out of the total broodstock. In these situations and until a better environmental control of ABFT broodstocks is achieved, GnRHa implants can be used successfully to initiate massive spawning, extend the duration of the spawning season and improve the consistency of egg supply.

7.6 REPRODUCTIVE BIOLOGY AND SPAWNING IN ABFT—THE FUTURE

As in other commercially important marine fish, understanding the endocrine and reproductive cycles of captive ABFT, and comparing them to those of
wild fish, enabled the development of hormonally induced spawning and opened the egg production bottleneck that had previously inhibited the farming of this fish. Captive breeding is expected to lead, in the very near future, to completely closing the life cycle (eggs to broodstock to eggs) of ABFT in aquaculture. However, it is also clear from the successful spontaneous spawning obtained over the past few years in various captive-reared broodstocks (Spain, Malta, Turkey, Croatia) that ABFT collected from the capture fishery can complete their reproductive cycle naturally in confinement and produce large numbers of high quality eggs if maintained optimally. Successful spawning, however, is subject to the combined effect of proper husbandry, nutrition, and the existence of the appropriate environmental conditions (mainly water temperature), because captive broodstocks are unable to migrate and select the desired temperature that is required for maturation and spawning. Furthermore, successful egg collection also requires the existence of very calm weather, weak currents and/or the deployment of effective egg collection systems, which may be a limiting factor in some locations. This is why spawning of ABFT is very seasonal and lasts for only 4–8 weeks in any given broodstock operation, during which time availability of eggs and their numbers are still quite inconsistent. This makes it difficult to plan larval rearing and/or juvenile production, which is a major hurdle to the development of predictable and dependable hatchery, nursery, and grow-out operations. It is clear that an efficient and cost-effective ABFT aquaculture industry cannot develop without on-demand year-round production of seeds (fertilized eggs and viable offspring). For that to happen, broodstock operations will have to be placed on-land, allowing full control of all environmental parameters to which fish are exposed. Broodstock management protocols in such facilities will enable predictable and consistent egg production all year round. Three to four land-based tanks, exposed to phase-shifted environmental conditions, will result in fish with fully developed gonads throughout most of the year. Simulating the photoperiod, temperature, salinity, and water quality required for spawning will most probably result in some fish spawning spontaneously. Such a land-based facility has been operating for the southern bluefin tuna in Australia since 2008 (Chapter 10 of this book and Knibb et al., in preparation) and will start to operate during late 2015 for ABFT in Europe (IEO, Spain).

However, as is the case in other fish species held in captivity and from indications obtained thus far from ABFT, not all the fish in a given captive broodstock are expected to spawn, which can be confirmed through genotyping the eggs and the broodstock. To increase the efficiency and maximize productivity of land-based operations, spawning induction therapies will continue to be used to ensure that most, if not all, fish held in the tanks are actively spawning and contributing to egg production. Actively spawning fish (as indicated through DNA fingerprinting) should be tagged so they are not hormonally treated unnecessarily or repeatedly. To be efficient, spawning induction therapies should be applied only during the very short window of
time when the broodstock are reproductively competent and responsive to exogenous hormonal stimulation, that is, when fish reach the final stages of spermatogenesis or vitellogenesis and before the resumption of gonadal apoptosis and atresia. Predicting the optimal timing of treatment, as well as the female to male ratio, will necessitate more research toward determining hormonal and gonadal status and competence, through analysis of molecular and/or endocrine indicators in fish mucosal samples obtained through gentle and noninvasive mucosal swabs. New approaches discussed in this chapter to accelerate the onset of puberty will enable the production of tuna eggs from smaller fish, thus reducing the cost and increasing the versatility and efficiency of land-based broodstock operations.

The very significant progress, reviewed in this chapter, in understanding the reproductive biology of ABFT and obtaining large numbers of fertilized eggs, together with future development of advanced land-based broodstock operations, will drive year-round availability of fertilized ABFT eggs to enable and support the establishment of an efficient, cost-effective, and environmentally sustainable aquaculture industry for this species.

REFERENCES


