Available online at www.sciencedirect.com
ScienceDirect
Aquaculture 272 (2007) 675–686
Aquaculture
www.elsevier.com/locate/aqua-online

Histological study of the effects of treatment with gonadotropin-releasing hormone agonist (GnRHa) on the reproductive maturation of captive-reared Atlantic bluefin tuna (*Thunnus thynnus* L.)

A. Corriero [a,*], A. Medina [b], C.C. Mylonas [c], F.J. Abascal [b], M. Deflorio [a], L. Aragón [b], C.R. Bridges [d], N. Santamaria [a], G. Heinisch [e], R. Vassallo-Agius [f], A. Belmonte [g], C. Fauvel [h], A. Garcia [i], H. Gordin [e], G. De Metrio [a]

[a] *University of Bari, Department of Animal Health and Well-being, Str. Prov. per Casamassima Km 3, 70010 Valenzano (BA), Italy*
[b] *University of Cádiz, Department of Biology, Faculty of Marine and Environmental Sciences, Avda República Saharaui, E-11510, Puerto Real, Cádiz, Spain*
[c] *Institute of Aquaculture, Hellenic Center for Marine Research, P.O.Box 2214, Heraklion 71003, Crete, Greece*
[d] *Heinrich-Heine Universität, Institut für Zoophysiologie, D-40225, Düsseldorf, Germany*
[e] *Israel Oceanographic and Limnological Research, National Center for Mariculture, P.O.Box 1212, Eilat 88112, Israel*
[f] *Malta Centre for Fisheries Sciences, Fort San Lucjan, Marsaxlokk BBG06, Malta*
[g] *Tuna Graso, S.A., Carretera de la Palma Km 7, Paraje la Estrella, E-30593 Cartagena (Murcia), Spain*
[h] *Institut Français d'Exploration de la Mer, Station Expérimentale d'Aquaculture, Chemin de Maguelone, 34250 Palavas les Flots, France*
[i] *Instituto Español de Oceanografía, Centro Oceanográfico de Murcia, Ctra De la Azohía s/n, E-30860 Puerto de Mazarrón (Murcia), Spain*

Received 31 May 2007; received in revised form 1 August 2007; accepted 2 August 2007

Abstract

The effects of gonadotropin-releasing hormone agonist (GnRHa) administration on the gonads of 66 Atlantic bluefin tuna (*Thunnus thynnus* L.) reared in floating cages (La Azohía, Murcia, Spain) were evaluated using histological analyses. During two consecutive reproductive seasons (June–July 2004 and 2005), about half of the fish in each cage were administered a GnRHa implant using a spear gun, without being anesthetized or physically restrained, and were sacrificed for sampling 2–8 d later. Rearing for 1–3 years in captivity did not prevent vitellogenesis and oocyte growth, since yolk granule formation and oocyte diameter of fully vitellogenic oocytes were similar to wild fish. However, a lower gonad size and number of vitellogenic oocytes were observed compared to wild spawners. None of the 14 untreated Controls had oocytes at final oocyte maturation (FOM) at the time of sampling and postovulatory follicles (POFs) were observed only in three of them. In addition, a higher volume fraction of atretic oocytes was observed in untreated Controls when compared to the wild or GnRHa-treated fish, indicating a shut-down of reproductive activity at this time. On the contrary, all GnRHa-treated females had oocytes at FOM and/or POFs, with the exception of two fully atretic individuals. In the males, no differences in testicular histology were observed between GnRHa-treated and Control fish, and individuals were at early or late spermatogenesis. The study indicates that

* Corresponding author. Tel.: +39 0805443909; fax: +39 0805443908.
E-mail address: a.corriero@veterinaria.uniba.it (A. Corriero).

0044-8486/$ - see front matter
doi:10.1016/j.aquaculture.2007.08.030

wild-caught Atlantic bluefin tuna reared in captivity undergo vitellogenesis and spermatogenesis, but females have a low capacity for spontaneous FOM and ovulation, whereas GnRHa treatment is effective in inducing multiple cycles of FOM and ovulation.

Keywords: *Thunnus thynnus*; Bluefin tuna; Reproduction; Gametogenesis; GnRHa; Induced spawning

1. Introduction

A "capture-based" fattening industry has developed for Atlantic bluefin tuna (*Thunnus thynnus* L.; ABFT) in the Mediterranean Sea over the past decade. The industry is based on the capture of migrating wild fish and their rearing in floating cages, usually for periods of a few months (Miyake et al., 2003; Directorate General for Fisheries, 2004; Ottolenghi et al., 2004; FAO, 2005). The increasing demand for this unique fish by the sashimi–sushi market (Catarci, 2004) has resulted in over-fishing of the wild stock (Fromentin and Powers, 2005). The study of ABFT reproduction in the wild, and the development of captive broodstock management and spawning methods are essential for the support of a true and sustainable aquaculture industry for this species. This would help reduce the fishing pressure with positive effects on wild populations of ABFT as well as threatened bycatch species like sea turtles and dolphins (Silvani et al., 1999; Deflorio et al., 2005).

The Atlantic bluefin tuna reproductive cycle in the western and central Mediterranean Sea has been recently described on the basis of gonad histology (Corriero et al., 2003; Santamaria et al., 2003; Zubani et al., 2003; Abascal et al., 2004). Fish are reproductively inactive from August to March, when only perinucleolar-stage oocytes are present in the ovaries, while the testes have germinal cysts containing mainly spermatogonia and spermatocytes. Gonadal recrudescence starts in April when oocytes at the lipid stage appear in the ovaries, and all spermatogenic stages become present in the seminiferous lobules of the testes. During May, ovaries are characterized by the presence of vitellogenic oocytes, while the lumen of the seminiferous lobules fills progressively with spermatozoa. Ovaries with hydrated oocytes and/or post-ovulatory follicles, and testes full of spermatozoa—both signs indicative of active spawning—are found from late June to early July. During late July to September, ovaries contain only perinucleolar-stage oocytes, as well as late stages of atresia of vitellogenic follicles. During the same period, only residual spermatozoa are present in the testes. The natural spawning of ABFT occurs throughout the Mediterranean Sea, from the Balearic Islands in the western Mediterranean (Nishida et al., 1998; Susca et al., 2001; Medina et al., 2002; Corriero et al., 2003), to Malta and the South Tyrrhenian Sea in the central Mediterranean (Nishida et al., 1998) and the Levantine Sea in the eastern Mediterranean (Karakulak et al., 2004; Oray and Karakulak, 2005). In the Levantine Sea, reproduction occurs in May, almost one month earlier than in the other spawning areas, possibly because the sea surface temperature suitable for Atlantic bluefin reproduction is reached earlier (Karakulak et al., 2004).

Most cultured fishes exhibit some degree of reproductive dysfunction, ranging from the complete absence of gonadal development observed in the freshwater eel (*Anguilla anguilla*) (Tesch, 2003) to the absence of only spawning observed in trout and salmon (*Salmo* and *Oncorhynchus spp.*) (Bromage et al., 1992). The most common dysfunctions, however, are (a) the failure of females to undergo final oocyte maturation (FOM) once vitellogenesis is completed and (b) the production of low amount of expressible semen in males (Zohar and Mylonas, 2001). Therefore, there is a need to study the reproductive function of wild-caught ABFT reared in captivity and determine whether these fish are able to undergo gametogenesis, ovulation/spermiation and spawning. The failure to undergo FOM and reduction of semen production can be alleviated by treatment with various reproductive hormones (Zohar and Mylonas, 2001). Of the available hormones, agonists of gonadotropin-releasing hormone (GnRHa) have been used extensively in broodstock management due to their high potency, lack of species specificity and the stimulation of endogenous luteinizing hormone (LH) release (Crim and Bettles, 1997; Peter and Yu, 1997), the hormone responsible for FOM and ovulation/spermiation in fish (Nagahama et al., 1994; Schulz and Miura, 2002). Combined with a sustained-release delivery system (*i.e.*, implants), GnRHa induces long-term elevation of plasma LH with only a single treatment (Crim et al., 1988; Mylonas et al., 1998a; Mylonas et al., 1998b) and has proven effective in inducing FOM, ovulation and spawning, as well as enhancing spermiation in many fishes (Mylonas and Zohar, 2001a).

Using gonadal histology and stereology evaluations, the objectives of the present study were to (a) examine the potential of natural maturation of wild-caught ABFT

reared for 1–3 years in cages, and (b) assess the effect of treatment with GnRHa implants (Mylonas et al., 2007) in stimulating FOM in vitellogenic females and in enhancing spermiation in males.

2. Materials and methods

Atlantic bluefin tuna were caught by purse seine from spawning grounds around the Balearic Islands, Spain during the fishing campaigns of 2002 and 2003 (May–June). Fish were then immediately moved into a towing cage and were transported over a period of several weeks to the sea cage facilities of Tuna Graso S.A, near the coast of Mazarron, South–East of Spain. There, about 60 fish each year were herded into a floating cage 50-m in diameter and 20-m in depth (adaptation cage). For the experiments during the 2004 spawning season, fish caught in the 2003 fishing season were moved from the adaptation cage to a 50-m diameter cage (No. 3). For the 2005 season experiments, fish caught in 2002 and 2003 were allocated into two 25-m diameter cages (Nos. 1 and 2). Throughout the maintenance of the fish in the cages, they were fed to satiation once a day (except Sundays) with a variety of raw fish, which included round sardinella (*Sardinella aurita*), Pacific mackerel (*Scomber japonicus*), Atlantic mackerel (*Scomber scombrus*), sardine (*Sardina pilchardus*), herring (*Clupea harengus*) and squid (*Loligo* and *Illex spp.*). Water temperature inside the cages was recorded daily at 6 and 12 m of depth from April 2003 to July 2005. A total of five fish died of unidentified causes during the 3 years of the study and were removed by divers within 24 h of death.

The GnRHa implantation experiments in both years were planned for late June–early July, the second half of the natural spawning season of ABFT in the Mediterranean Sea (Medina et al., 2002; Corriero et al., 2003; Abascal et al., 2004; Karakulak et al., 2004), after the sea surface temperature reached 23 °C. Temperatures above 24 °C are considered essential for the spawning of all tunas (see review by Schaefer, 2001), but spawning in captive stocks of the Pacific bluefin tuna (*Thunnus orientalis*) has also been reported at 22 °C (Lioka et al., 2000; Sawada et al., 2005).

The GnRHa implants were prepared by loading 560 (2004 experiments) and 840 mg (2005 experiments) of the agonist desGly10, dAla6, Pro9-GnRH-NEthylamide (Bachem, Switzerland) into a matrix of poly [Ethylene-Vinyl Acetate] (EVAc, Dow Corning) according to the procedure of Freese (1989), with some modifications. In 2004, the implants were designed to produce effective doses of about 40–80 μg GnRHa kg^{-1} body weight, and the dose was increased in 2005 to about 50–100 μg GnRHa kg^{-1}. The two implants were attached to a polyethylene arrowhead (Floy Tag and Manufacturing Company, USA) using a 0.5 mm nylon monofilament passing through their centre. On the same monofilament, a four-color combination tube (Floy Tag and Manufacturing Company, USA) was added and functioned as an indicator of successful implantation as well as an identifier for each fish. The implants were inserted into the dorsal musculature by a diver using a spear gun fitted with a specially designed spearhead. Further details on the preparation of the GnRHa implants and their administration to the fish are given elsewhere (Mylonas et al., 2007).

In 2004, a total of 26 fish were used, of which nine (six females and three males) were treated with GnRHa (Bachem, Switzerland) and 17 (7 females and 10 males) were left untreated as Controls (Tables 1 and 2). For the 2005 experiments, 40 fish were used, of which 26 (10 females and 16 males) were implanted with GnRHa and 14 (7 females and 7 males) were left untreated as Controls. Sacrificing and sampling of the fish after GnRHa implantation was done at different times in order to have a temporal evaluation of the effect of the treatment on gonadal maturation. Hence, both Control and GnRHa-treated fish were sacrificed after 5–6 d in 2004 or after 2–3 d (afterwards indicated as GnRHa-treated 2–3 d) or 8 d (afterwards indicated as GnRHa-treated 8 d) in 2005. Each fish was lifted onto the deck of a service boat and fork length (L_F) and wet body mass (M_B) were recorded to the nearest cm and kg, respectively. Age was estimated from the first spiniform ray of the first dorsal fin (Cort, 1991; Corriero et al., 2005).

Gonads were dissected onboard, placed on crushed ice and transported to the laboratory onshore within 1 h, where their mass (M_G) was recorded to the nearest g. The relative gonadal mass (gonadosomatic index, *GSI*) was calculated as $GSI = 100\ M_G\ M_B^{-1}$. Gonad slices were fixed in Bouin's fixative or 10% buffered formalin, dehydrated in increasing ethanol concentrations, clarified in xylene and embedded in paraffin wax and 5-μm thick sections were cut and stained with haematoxylin-eosin. For the classification of the reproductive status of females, the most advanced oocyte stage was recorded for each specimen, according to the classification of Corriero et al. (2003), with the exception that the category "final maturation stage" was used to identify oocytes at both the migratory nucleus and germinal-vesicle breakdown stages. The presence of postovulatory and atretic follicles was also recorded and atretic vitellogenic follicles were classified into stages "α" or "β" according to Hunter et al. (1986). Briefly, the identification of α atretic follicles was made on the basis of fragmentation of the *zona radiata*, yolk granule breakdown and reabsorption, as well as nuclear disarrangement. In β atretic follicles, the yolk was completely reabsorbed, germinal-vesicle disappeared and oocytes were invaded by follicular and thecal cells. For the classification of the reproductive status of males, the type of spermatogenic cysts was recorded, and the quantity of spermatozoa in the lumen of seminiferous lobules was evaluated subjectively.

The occurrence of recent spawning in females was determined by the presence of postovulatory follicles (POFs) in the gonad. Unfortunately, the irregular shape of POFs did not allow us to make an accurate stereological estimation of the numerical density (N_V) and, hence, the number of oocytes that had ovulated. Alternatively, the numerical areal density (N_A: number of POF transections per unit area) was calculated in order to carry out a comparative analysis between Control and GnRHa-treated tuna, and between years. In order to verify if normal yolk accumulation and vitellogenic growth occurred in the oocytes of

Table 1
Biometric data, estimated age, most advanced oocyte developmental stage and density of postovulatory follicles of captive-reared, GnRHa-treated Atlantic bluefin tuna and untreated Controls, sampled during the spawning season of two consecutive years

F_L (cm)	M_B (kg)	M_G (g)	Estimated age (years)	Date of sampling (days after treatment)	Treatment	*GSI*	Most advanced oocyte stage	Postovulatory follicles N_A (No of sections cm^{-2})
2004								
158	87	1310	7	28 June (5)	Control	1.51	Late vitellogenesis	0
175	112	2680	8	29 June (6)	Control	2.39	Late vitellogenesis*	0
161	85	2650	7	29 June (6)	Control	3.12	Late vitellogenesis	23.5
139	61	1780	5	29 June (6)	Control	2.92	Late vitellogenesis	0
159	90	2650	7	29 June (6)	Control	2.94	Late vitellogenesis	12.6
155	81	2480	7	29 June (6)	Control	3.06	Late vitellogenesis*	0
153	62	450	6	29 June (6)	Control	0.73	Lipid	0
167	117	990	–	28 June (5)	GnRHa	0.85	Lipid**	0
155	76	1520	7	29 June (6)	GnRHa	2.00	Final maturation	23.7
154	82	1370	7	29 June (6)	GnRHa	1.67	Final maturation	22.2
151	62	1060	6	29 June (6)	GnRHa	1.71	Final maturation	23.8
150	82	2540	6	29 June (6)	GnRHa	3.10	Late vitellogenesis	39.7
138	68	1330	5	29 June (6)	GnRHa	1.96	Final maturation	94.5
2005								
187	102	1700	8	6 July (2)	Control	1.67	Late vitellogenesis*	0
194	112	1280	8	6 July (2)	Control	1.14	Late vitellogenesis*	0
176	89	2430	7	7 July (3)	Control	2.73	Late vitellogenesis	67.9
173	87	1970	7	7 July (3)	Control	2.26	Late vitellogenesis*	0
180	100	1340	7	7 July (3)	Control	1.34	Early vitellogenesis	0
174	76	1730	6	7 July (3)	Control	2.28	Late vitellogenesis	0
202	122	1890	9	7 July (3)	Control	1.55	Late vitellogenesis*	0
200	130	3020	10	6 July (2)	GnRHa	2.32	Final maturation	83.6
194	112	3740	8	6 July (2)	GnRHa	3.34	Final maturation	46.2
165	72	1420	6	6 July (2)	GnRHa	1.97	Final maturation	62.2
195	122	2610	8	7 July (3)	GnRHa	2.14	Late vitellogenesis	56.3
180	89	1220	7	7 July (3)	GnRHa	1.37	Late vitellogenesis*	6.2
183	103	1220	–	7 July (3)	GnRHa	1.18	Late vitellogenesis	80.2
186	120	1270	7	8 July (8)	GnRHa	1.06	Late vitellogenesis	11.5
175	105	2220	7	8 July (8)	GnRHa	2.11	Late vitellogenesis	36.0
185	108	2240	8	8 July (8)	GnRHa	2.07	Late vitellogenesis	46.3
185	106	2160	7	8 July (8)	GnRHa	2.04	Late vitellogenesis	59.5

The stage of final maturation includes the migratory nucleus stage and the stage of germinal-vesicle breakdown (which precedes hydration). F_L, fork length; M_B, wet body mass; M_G, gonad mass; *GSI*, gonadosomatic index ($100\ M_G\ M_B^{-1}$); N_A, numerical areal density. * indicates major α atresia of late vitellogenic oocytes (>50% atresia of late vitellogenic oocytes); ** indicates β atresia of late vitellogenic oocytes.

ABFT reared in captivity, the diameter of fully vitellogenic oocytes, and the diameter and occupied surface of eosinophilic yolk granules were compared between captive-reared fish used in the spawning induction experiments and wild individuals collected from spawning grounds. For this purpose, sections of 30 fully vitellogenic oocytes having a large and centrally located nucleus were selected from six captive-reared fish (3 Controls and 3 treated with GnRHa) and compared with an equal number of oocytes at the same stage from a total of six wild adult fish caught in the same period from the spawning grounds around Malta. Oocyte diameter, oocyte surface occupied by yolk granules and diameter of yolk granules were measured from microphotographs taken with a digital camera (DC 300, Leica, Cambridge, U.K.) connected to a light microscope (DMRBE, Leica, Cambridge, U.K), using an image analysis software (QWIN, Leica, Cambridge, U.K.).

The estimations of the numbers of oocytes at the different maturation stages present in the ovaries were carried out by means of the stereological method of Weibel and Gómez (1962) modified by Medina et al. (2007). Reliable calculations of the numerical density (N_V) of atretic follicles were not possible due to their irregular shape. Hence, for the quantification of atresia an estimate of the volume density (V_V) was used, which represents the partial volume of atretic follicles in the ovarian tissue. Stereological quantification of oocytes was carried out for 29 captive-reared ABFT (13 untreated Controls and 16 GnRHa-treated fish) and, for comparison, for seven wild spawners captured by purse seine during the same period around the Balearic Islands, Spain.

Statistical analyses were done using linear statistic software (StatView, Abacus Concepts, USA; Statistica 7.0, StatSoft, Italy) at a minimum statistical significance level of $p<0.05$, and data are presented as means±SEM. Differences in *GSI*

Table 2
Biometric data, estimated age and stage of testicular development of captive-reared GnRHa-treated Atlantic bluefin tuna and untreated Controls, sampled during the spawning season of two consecutive years

F_L (cm)	M_B (kg)	M_G (g)	Estimated age (years)	Date of sampling (days after treatment)	Treatment	*GSI*	Histological classification	Spermiating*
2004								
185	117	2740	–	28 June (5)	Control	2.34	Late spermatogenesis	No
201	168	1980	10	28 June (5)	Control	1.18	Early spermatogenesis	No
185	124	1390	9	28 June (5)	Control	1.12	Early spermatogenesis	No
165	97	1890	7	28 June (5)	Control	1.95	Late spermatogenesis	Yes
167	85	770	7	28 June (5)	Control	0.91	Late spermatogenesis	No
166	104	4020	7	29 June (6)	Control	3.87	Late spermatogenesis	No
158	90	1170	7	29 June (6)	Control	1.30	Early spermatogenesis	No
178	118	2470	9	29 June (6)	Control	2.09	Late spermatogenesis	Yes
143	65	1280	6	29 June (6)	Control	1.97	Late spermatogenesis	No
153	80	480	6	29 June (6)	Control	0.60	Spent	No
169	118	1780	8	28 June (5)	GnRHa	1.51	Late spermatogenesis	No
164	90	750	7	29 June (6)	GnRHa	0.83	Early spermatogenesis	No
159	90	2430	7	29 June (6)	GnRHa	2.70	Late spermatogenesis	No
2005								
212	165	970	11	6 July (2)	Control	0.59	Spent	No
190	103	710	8	6 July (2)	Control	0.69	Late spermatogenesis	No
193	125	1430	8	6 July (2)	Control	1.14	Late spermatogenesis	No
193	125	1380	9	6 July (2)	Control	1.10	Early spermatogenesis	No
212	180	1370	12	7 July (3)	Control	0.76	Late spermatogenesis	No
185	117	1990	7	8 July (8)	Control	1.70	Late spermatogenesis	No
196	128	1570	8	8 July (8)	Control	1.23	Late spermatogenesis	No
184	101	600	7	6 July (2)	GnRHa	0.59	Early spermatogenesis	No
228	204	930	12	6 July (2)	GnRHa	0.46	Late spermatogenesis	No
178	99	830	–	6 July (2)	GnRHa	0.84	Early spermatogenesis	No
189	123	1580	7	6 July (2)	GnRHa	1.28	Late spermatogenesis	No
185	112	1510	7	6 July (2)	GnRHa	1.35	Late spermatogenesis	No
208	147	920	10	7 July (3)	GnRHa	0.63	Late spermatogenesis	Yes
204	122	1040	9	7 July (3)	GnRHa	0.85	Late spermatogenesis	Yes
179	92	1930	7	7 July (3)	GnRHa	2.10	Late spermatogenesis	No
181	98	1460	7	7 July (3)	GnRHa	1.49	Late spermatogenesis	Yes
190	121	1110	9	7 July (3)	GnRHa	0.92	Late spermatogenesis	Yes
202	158	1900	10	8 July (8)	GnRHa	1.20	Late spermatogenesis	Yes
195	131	1800	8	8 July (8)	GnRHa	1.37	Late spermatogenesis	No
216	167	1550	10	8 July (8)	GnRHa	0.93	Early spermatogenesis	No
203	138	2430	10	8 July (8)	GnRHa	1.76	Late spermatogenesis	No
204	146	1970	9	8 July (8)	GnRHa	1.35	Late spermatogenesis	No
198	136	1700	9	8 July (8)	GnRHa	1.25	Late spermatogenesis	No

The histological classification was based on the types of spermatocysts observed in the germinal epithelium and the abundance of spermatozoa in the lumen of seminiferous lobules * Fish were classified as spermiating if they released semen either spontaneously when on board, or in response to pressure on the abdomen; F_L, fork length; M_B, wet body mass; M_G, gonad mass; *GSI*, gonadosomatic index (100 M_G M_B^{-1}).

between untreated Controls and GnRHa-treated individuals for the 2 years of experiments were analysed by one-way Analysis of Variance (ANOVA). Differences in the numerical areal density (N_A) of post-ovulatory follicles between Controls and GnRHa-treated fish were analyzed using one-way ANOVA. Differences in oocyte diameter, yolk granule diameter and yolk granule area of vitellogenic oocytes, between wild and captive-reared fish as well as differences in stereological parameters among wild and Control or GnRHa-treated fish were analyzed using one-way ANOVA.

3. Results

The water temperature in the experimental cages during the 3 years of the study ranged between 13 °C in March and 29 °C in August (data not shown). During the spawning induction experiments in 2004, temperature ranged between 22 and 25 °C, and in 2005 it ranged between 24 and 26 °C. The size of the ABFT used in this study ranged between 138 and 228 cm in L_F, and 61 and 204 kg in M_B (Tables 1 and 2), and according to the size at first sexual maturity estimated by Corriero et al. (2005) all of them were

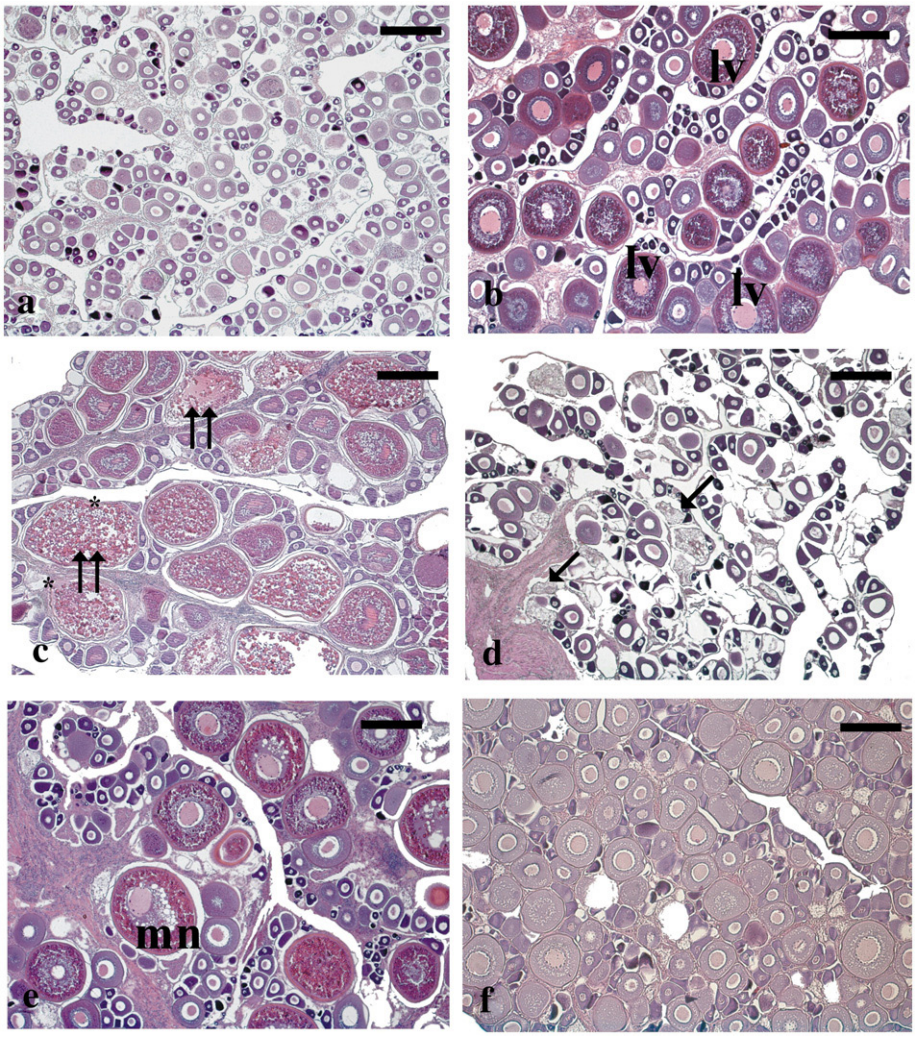

Fig. 1. Micrographs of the ovaries of Atlantic bluefin tuna reared in captivity in sea cages (La Azohía, Murcia, Spain) and sacrificed during the natural spawning season (June–July) after treatment with GnRHa implants. (a) Ovary from an untreated Control having oocytes at the lipid vesicle stage as the most advanced stage of development. (b) Ovary from an untreated Control with oocytes at late vitellogenesis (lv). (c) Ovary from a control fish exhibiting major α atresia of oocytes at late vitellogenesis. d) Ovary from a GnRHa-treated specimen showing advanced (β) atresia of vitellogenic follicles. (e) Oocytes at migratory nucleus stage in a GnRHa-treated spawning individual. (f) Ovary from a GnRHa-treated fish having oocytes at early vitellogenesis. Haematoxylin-eosin staining. Bars=300 μm. Arrows show β atresia of vitellogenic follicles; double arrows show α atresia of vitellogenic follicles; asterisk shows fragmented zona radiata in an atretic vitellogenic follicle; lv, oocyte at late vitellogenesis; mn, migratory nucleus stage oocyte.

adults. For 2004 and 2005, males were found to have a mean age of 7.5±0.4 (n=12) and 8.8±0.3 (n=22) years, respectively, and females had a mean age of 6.5±0.3 (n=12) and 7.6±0.3 years (n=16), respectively. *GSI* mean values did not differed between control and GnRHa-treated males either in 2004 (untreated Controls 1.73±0.30 n=10, GnRHa-treated 1.68±0.55 n=3; ANOVA, P=0.93) or in 2005 (untreated Controls 1.03±0.15 n=7, GnRHa-treated 2–3 d 1.05±0.16 n=10, GnRHa-treated 8 d 1.31±0.11 n=6; ANOVA, P=0.42). Similarly, the hormonal treatment affected significantly the *GSI* mean values of females neither in 2004 (untreated Controls 2.38±0.35 n=7, GnRHa-treated 1.88±0.30 n=6, ANOVA, P=0.31) nor in 2005 (untreated Controls 1.85±0.22 n=7, GnRHa-treated 2–3 d 2.05±0.31 n=6, GnRHa-treated 8 d 1.82±0.25 n=4; ANOVA, P=0.81).

3.1. Histological evaluation of maturation state in females

Captive-reared ABFT females in both years of the study were at different stages of reproductive maturation at the time of sampling (Table 1). Among untreated Controls in 2004, one individual had lipid stage oocytes as the most advanced oocyte population (Fig. 1a), whereas six individuals showed oocytes at

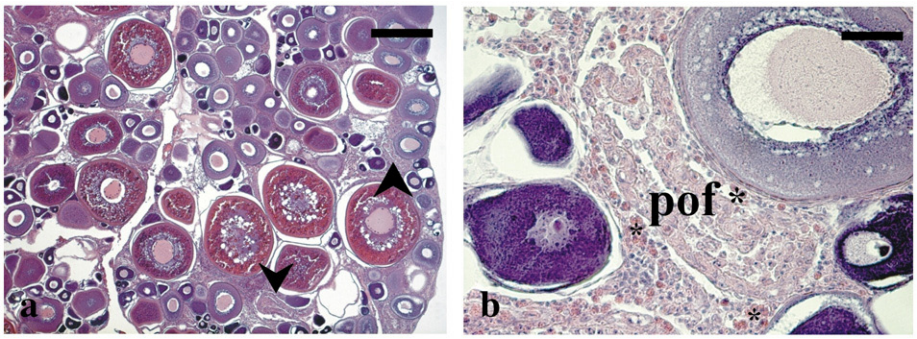

Fig. 2. Micrographs of the ovary of an untreated Control Atlantic bluefin tuna reared in captivity in sea cages (La Azohía, Murcia, Spain) and sacrificed during the natural spawning season (early July) showing oocytes at late vitellogenesis together with postovulatory follicles. (b) Higher magnification of part of (a) showing a postovulatory follicle. Eosinophilic granulocytes are visible inside and around the postovulatory follicles. Bars in (a)=300 μm; in (b)=65 μm. Arrowheads show post-ovulatory follicles and asterisks show eosinophilic granulocytes; pof, postovulatory follicle.

late vitellogenesis (Fig. 1b). Of these six fish, two displayed major α atresia (i.e.>50% α atresia of late vitellogenic oocytes) (Fig. 1c) and two showed POFs. Among GnRHa-treated bluefin tuna in 2004, only in one female the lipid stage was the most advanced oocyte category, displaying also β atresia of vitellogenic oocytes (Fig. 1d). Another individual showed late vitellogenic oocytes, and all the remaining four females possessed oocytes at final maturation (Fig. 1e). Postovulatory follicles were found in all GnRHa-treated fish except the one with lipid stage as the most advanced oocyte population.

In 2005, only one of the seven Controls had oocytes at early vitellogenesis as the most advanced developmental stage (Fig. 1f), whereas all other Controls displayed late vitellogenic oocytes. One of these individuals also had POFs (Fig. 2a, b), and four females displayed major α atresia (Table 1). Among the ten GnRHa-treated individuals, seven had late vitellogenic oocytes as the most advanced oocyte stage and three also showed oocytes at final maturation. Postovulatory follicles were again observed in all GnRHa-treated fish, but in one specimen, whose ovary contained late vitellogenic oocytes and also showed major α atresia, the density of ovulated follicles was low (N_A=6.2 POFs cm^{-2}) (Table 1).

Postovulatory follicle density differed significantly between control and GnRHa-treated females both in 2004 (untreated Controls 5.16±3.54 POFS cm^{-2}, n=7; GnRHa-treated 33.98±13.16 POFS cm^{-2}; n=6; ANOVA, P<0.05) and in 2005 (untreated Controls 9.70±9.70 POFS cm^{-2}, n=7; GnRHa-treated 49.44±8.10 POFS cm^{-2}, n=10; ANOVA, P<0.01). In 2005, no differences in POF density could be attributed to the period intervened between implantation and sampling (GnRHa-treated 2–3 d 38.53±10.14 POFS cm^{-2}, n=4, GnRHa-treated 8 d 55.78±11.49 POFS cm^{-2}, n=6; ANOVA, P=0.33).

3.2. Yolk quantification and stereological analysis

The diameter of fully vitellogenic oocytes, the oocyte surface occupied by eosinophilic yolk and the diameter of yolk granules of captive-reared and wild ABFT did not exhibit any significant differences (Table 3).

No statistical differences were found in the quantification of the different oocyte categories between untreated Controls sampled in 2004 and in 2005 (atretic oocytes: ANOVA, P=0.19; lipid stage oocytes: ANOVA, P=0.08; vitellogenic oocytes: ANOVA, P=0.06; FOM: absent in both groups). Similarly, no statistical differences were found among fish treated with GnRHa in 2004, fish treated with GnRHa in 2005 and sampled 3 d after the implantation, and fish treated with GnRHa in 2005 and sampled 8 d after the implantation (atretic oocytes: ANOVA, P=0.11; lipid stage oocyte: ANOVA, P=0.12; vitellogenic oocytes: ANOVA, P=0.16; FOM: ANOVA, P=0.39). Therefore, the results of the stereological quantification of the different oocyte categories from 2004 and 2005 untreated Controls as well as fish treated with GnRHa in 2004 and 2005 were pooled and compared with wild spawners (Table 4). No statistically significant difference was found in the volume fraction of atretic oocytes between GnRHa-treated and wild fish, whereas a significantly higher quantity of atretic oocytes was observed in Controls. The number of oocytes at the lipid stage did not differ between Control and GnRHa-treated fish, but was lowest in wild spawners. Vitellogenic oocytes did not differ in number among captive bluefin tunas, but wild females had significantly greater numbers than captive ones. No oocytes at FOM were found in the ovaries of untreated Controls, while GnRHa-treated individuals had

Table 3
Diameter of fully vitellogenic oocytes, surface occupied by yolk and diameter of yolk granules in captive-reared and wild Atlantic bluefin tuna

	Oocyte diameter (μm)	Yolk surface (μm^2)	Yolk granule diameter (μm)
Captive-reared	434.1±5.0	63187.0±2767.9	12.5±0.2
Wild	435.1±4.3	60298.9±1374.6	11.5±0.5

Data are means (± SEM) of 30 measurements from 6 different fish per group.
No statistical significance existed in any of the parameters examined, between captive-reared and wild bluefin tuna (ANOVA, P>0.05).

Table 4
Stereological data (means±SEM) of ovaries from wild and captive-reared Atlantic bluefin tuna during the spawning season, and in response to treatment with GnRHa implants

	Wild ($n=7$)	captive (Control) ($n=13$)	captive (GnRHa-treated) ($n=16$)
Atretic oocytes V_V	0.02 ± 0.01^a	0.15 ± 0.04^b	0.04 ± 0.01^a
Lipid stage oocytes (No. g^{-1} W_B)	700.58 ± 90.42^a	1319.88 ± 153.54^b	1161.06 ± 68.64^b
Vitellogenesis stage oocytes (No. g^{-1} W_B)	245.45 ± 54.89^a	139.32 ± 30.76^b	130.68 ± 18.59^b
Final maturation stage oocytes (No. g^{-1} W_B)	8.96 ± 4.32^a	0.00 ± 0.00^a	24.81 ± 6.28^b

Within each row (parameter), means with different superscripts were significantly different (ANOVA, $P<0.05$).

a significantly higher number of oocytes at FOM than wild fish.

3.3. Histological evaluation of maturation state in males

Captive-reared ABFT males in both years of the study were at different stages of spermatogenesis (Table 2). Among the 10 untreated Controls in 2004, three individuals were found to be at early spermatogenesis, as their testes showed that most of the germinal epithelium was occupied by cysts containing spermatocytes and spermatids, but cysts containing spermatozoa and luminal spermatozoa were also observed in most of the testicular lobules and ducts (Fig. 3a). In six individuals the testes were at late spermatogenesis, since their germinal epithelium consisted mainly of cysts containing spermatids and spermatozoa, and the lumen of the seminiferous lobules was partially filled with spermatozoa (Fig. 3b). Two fish of this latter group were in spermiating condition, *i.e.* semen was released from the testes when the fish were brought onboard for sampling, either spontaneously or in response to external pressure. One specimen was considered spent since the germinal epithelium consisted almost exclusively of spermatogonia and spermatocysts containing spermatids or spermatozoa were visible only occasionally; luminal residual spermatozoa could also be observed (Fig. 3c). Of the three individuals treated with GnRHa implants, one was at early spermatogenesis and two at late spermatogenesis.

In 2005, one of the seven untreated Control males was found to be at early spermatogenesis, five at late spermatogenesis and the remaining individual was spent. Of the 16 GnRHa-treated fish, three were at early spermatogenesis and 13 at late spermatogenesis. Five of the GnRHa implanted males with testes in late spermatogenesis released semen when brought onboard (spermiating fish).

4. Discussion

The increasing demand of a supply of ABFT and the urgent need to reduce the fishing pressure on the wild stocks has prompted several attempts to close the life cycle of this fish in captivity (Doumenge, 1996; Lioka et al., 2000). However, most fish reared in captivity fail to attain final oocyte maturation (Zohar, 1989; Peter et al., 1993) and produce low quality-milt (Billard, 1986, 1989). Thus, the control of reproduction represents one of the most crucial steps towards the domestication of ABFT.

Yolk content is an important determinant of egg quality in fish, as it represents the major nutrient for the developing embryo (Brooks et al., 1997). Based on the histological evaluation of the captive and wild females in the present study, no differences attributable to rearing conditions were observed in oocyte growth. For example, no difference in the size of oocytes at the end of vitellogenic growth was found between captive and wild individuals sampled in the same period. In addition, the surface occupied by yolk granules, as well as their size was not different between the two groups. Finally, no differences in terms of morphology and staining affinity of yolk granules were observed between captive and wild ABFT (data not shown). After 1–3 years in captivity, the fish used in the present study reached *GSI* values remarkably lower than wild females captured during the reproductive season in the spawning grounds of the western (*GSI*=4.12±1.65, *n*=24; Medina et al., 2002) as well as the eastern Mediterranean Sea (*GSI*=5.16±0.48, *n*=23; Karakulak et al., 2004). These differences in *GSI* could indicate that although a normal growth occurred at the oocyte level, the rearing conditions were responsible for an impaired ovarian development at a macroscopic level. Interestingly, a similar incapacity in the attainment of high *GSI* values occurred in the Pacific congener when reared in captivity. For example, mean *GSI* values >5 were reported for wild Pacific bluefin tuna during the spawning season (Chen et al., 2006), while most of wild-caught specimens were not able to attain *GSI* value >2 even after 5–12 years in captivity (Miyashita et al., 2000). The presence of a significantly lower number of vitellogenic oocytes in the ABFT reared in captivity compared to wild spawners, suggests that the limitation in gonad development could be related to the low vitellogenin plasma levels observed in captive individuals (unpublished data) as well as to a diminished oocyte recruitment.

In captive fishes, low plasma levels of LH can result in failure to undergo FOM and in the onset of atresia in

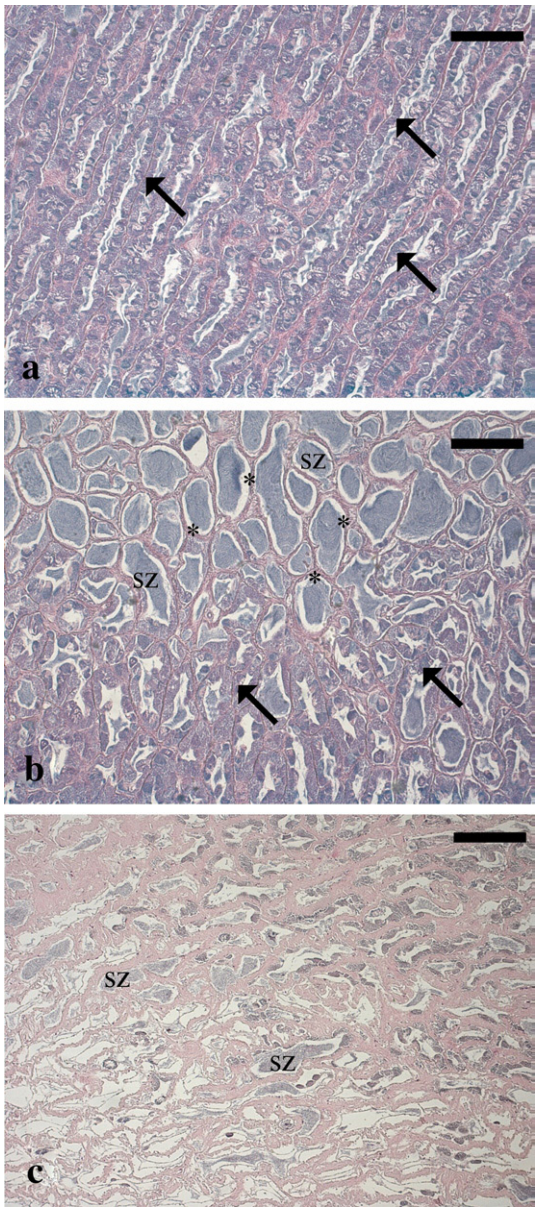

Fig. 3. Micrographs of the testes of Atlantic bluefin tuna reared in captivity in sea cages (La Azohía, Murcia, Spain) and sacrificed during the natural spawning season (June–July) after treatment with GnRHa implants. (a) Testis from an untreated Control classified at early spermatogenic stage showing germinal epithelium occupied by cysts containing spermatocytes and spermatids; luminal spermatozoa can also be observed. (b) Testis from an untreated Control classified at late spermatogenesis. The germinal epithelium consists mainly of cysts containing spermatids and spermatozoa, and the lumen of seminiferous lobules is filled with spermatozoa. (c) Testis from a spent, untreated Control fish. The wall of seminiferous lobules is often devoid of spermatocysts and residual spermatozoa can be observed in their lumina. Haematoxylin-eosin staining. Bars=300 μm. Arrows show germinal epithelium; asterisks indicate wall of seminiferous lobules devoid of spermatocysts; sz, spermatozoa.

vitellogenic oocytes (see review by Mylonas and Zohar, 2001a). In the present study, all but two Control females had oocytes at late vitellogenesis, but 50% of them showed major α atresia of vitellogenic follicles, which may be indicative of a recent cessation of reproductive activity (Hunter et al., 1986; Schaefer, 1998). This could have been caused by the stress of the implantation procedure, which involved raising the bottom of the cage and crowding the fish into a limited space for a few hours, while implanting some of the fish with GnRHa implants (Mylonas et al., 2007). Although the Control fish were not implanted, the procedure involved a certain degree of disturbance to them as well, and in the absence of an exogenous hormonal stimulus, as provided to the GnRHa implanted fish, the ovaries of a larger percentage of them underwent atresia. In agreement with this finding, oocytes at FOM were not found in any of the Control females, whereas maturing oocytes were present in seven of the GnRHa-treated ABFT. However, two Control females in 2004 and one in 2005 contained POFs in their gonads, suggesting that these fish had released some eggs shortly before sampling, as POFs in tunas can be distinguished for only up to 24 h after ovulation (Hunter et al., 1986; Schaefer, 1996). Coupled with the evidence of a normal vitellogenic oocyte growth, the occurrence of POFs in even a very small number of untreated Control females, demonstrates that it is feasible for captive-reared ABFT to complete their reproductive cycle in sea cages, as it has been reported for Pacific bluefin tuna (Miyashita et al., 2000; Sawada et al., 2005; Masuma et al., 2006) and yellowfin tuna (*Thunnus albacares*) (Wexler et al., 2003; Margulies et al., 2007).

An accurately programmed timing of an external hormonal treatment is essential for successful induction of ovulation in cultured fishes. Thus, in experiments of GnRHa-induced ovulation the hormone should be administered when the fish have completed vitellogenin uptake; if the hormonal stimulation is delayed, GnRHa treatment can be unsuccessful due to atretic degeneration of the vitellogenic oocytes (Mylonas et al., 1997). The GnRHa implantation experiments in 2004 and 2005 were carried out during the natural spawning period of the species in the central-western Mediterranean (Mylonas et al., 2007). In particular, the hormonal treatment in 2005 was given when the sea surface temperature was >23 °C, which is the spawning temperature threshold in several tuna species (Schaefer, 2001). The finding of β atretic follicles and no vitellogenic oocytes in one GnRHa-treated bluefin tuna indicates that oocyte degeneration started several days before sampling and presumptively before the hormonal treatment. Another specimen had ovaries with major α atresia of vitellogenic follicles. All other implanted fish showed FOM and/or

POFs, thus indicating that at the time of sampling they were ready to ovulate or had just ovulated. Aside from the β atretic individual, the hormonal treatment was effective in 14 out of 15 female ABFT, which represents 93% successful ovulation induction. Oocytes at FOM occurred only in fish sacrificed 2–6 d after hormonal administration, but not in fish sampled 8 d after implantation. The fact that these latter females also contained POFs in their ovaries may suggest that they have just completed a cycle of FOM and have not yet started the next one. Alternatively, this observation can be explained by the reduction in GnRHa release 7 d after administration of the GnRHa implants, as shown by the in vitro and in vivo studies (Mylonas et al., 2007).

As in other teleost fishes, the ABFT ovary consists of ovigerous lamellae with numerous follicles at different stages of development (Corriero et al., 2003). The simultaneous presence in a single individual of all oocyte development stages in the ABFT during the spawning period (Medina et al., 2002; Corriero et al., 2003) indicates that this species has an asynchronous oocyte development and is a multiple spawner (Tyler and Sumpter, 1996), as are all tunas (reviewed by Schaefer, 2001). Considering that POFs persist in the ovary for about 24 h, the spawning fraction of GnRHa implanted fish (93%) represents an average spawning frequency of 0.93 spawns d^{-1}, which corresponds to an interspawning interval of 1.1 d. These estimates are comparable to those obtained from samples of wild spawners caught at the peak of the reproductive season (Medina et al., 2002, 2007). As mentioned above, an interesting effect of the GnRHa treatment was the apparent reduction of the incidence of atresia as compared to untreated individuals. The amount of atresia quantified in GnRHa-treated captive fish was, in fact, similar to the levels measured in wild ABFT, but significantly lower than those found in the cage-reared Controls. This finding suggests that the hormonal treatment applied during late vitellogenesis, not only induced FOM and ovulation, but maintained the viability of subsequent batches of vitellogenic oocytes. This effect of sustained administration of GnRHa in maintaining viability of the vitellogenic oocytes has been reported to be similar in other fish species (Hodson and Sullivan, 1993; Mylonas and Zohar, 2001b; Fauvel et al., in review).

In the present study, according to the subjective evaluation of the reproductive stage of males based on the presence of different types of cysts and the quantity of spermatozoa, no differences were found between GnRHa-treated and Control fish. For example, individuals at early and late spermatogenesis were present in both the GnRHa and Control groups. On the contrary, none of the GnRHa implanted males were spent, and five of them in 2005 were spermiating compared to none of the controls. The absence of evident effects of GnRHa treatment on testicular histology is in agreement with the absence of statistically significant effects of GnRHa implantation on most general semen characteristics evaluated in ABFT (Mylonas et al., 2007). This observation may be due to the fact that apart from a rapid increase in seminal fluid volume effected by the GnRHa-induced plasma LH elevation (Clemens and Grant, 1965; Mylonas and Zohar, 2001b), the effect of the hormonal treatment on spermatogenesis may require a much longer period to be expressed, compared to its effects on oogenesis. This is due to the fact that each cycle of cell division in the developing spermatocysts usually lasts for many days, and some weeks may be required for the process to be completed with the release of the flagellate spermatozoa in the seminiferous lobules (Schulz and Miura, 2002). In a study of the effects of GnRHa treatment on testicular histology, no differences were observed between Controls and GnRHa implanted male European sea bass after 7 d (Rainis et al., 2003). On the other hand, quite evident differences were observed 21 d after hormonal treatment, with the testes of Control fish having hypertrophied somatic cells and almost no spermatozoa in their testes, whereas GnRHa implanted males had testes with lobules still containing large numbers of spermatozoa. Therefore, a longer GnRHa treatment period could be necessary to determine a more pronounced effect on bluefin tuna spermiation.

In conclusion, the histological evidence presented here indicates that wild-caught female ABFT reared for 1–3 years in captivity do undergo vitellogenesis, although a lower number of oocytes is produced compared to wild spawners. In addition, captive-reared fish have a reduced capacity to undergo FOM and ovulation spontaneously. However, the stage of maturity reached by females is adequate for the induction of FOM, ovulation and spawning (Mylonas et al., 2007) using exogenous hormonal therapies. Similarly in males, it seems that spermatogenesis proceeds normally in captivity, and apart from an increase in the percentage of spermiating males, there was no clear positive effect of GnRHa treatment in testicular function. These results provide hope that under more appropriate conditions (*e.g.*, feeding, stocking density, water quality and longer residence and acclimation to captivity) spontaneous spawning and production of viable embryos may be expected from wild-caught ABFT maintained in sea cages, as has been reported for Pacific bluefin tuna (Lioka et al., 2000; Sawada et al., 2005; Masuma et al., 2006) and yellowfin tuna (Wexler et al., 2003).

Acknowledgements

This work was undertaken as part of the research program «Reproduction of the bluefin tuna in captivity—a feasibility study for the domestication of *Thunnus thynnus*» (REPRODOTT), and was supported by a research grant from the European Community under the Quality of Life and Management of Living Resources program (contract Q5RS-2002-01355). The authors would like to thank Drs. K. M. Schaefer and B. J. Macewicz for valuable assistance in the identification of POFs in some specimens. Thanks are also due to the excellent technical assistance provided by the boat crew and divers of Tuna Graso, S.A.

References

Abascal, F.J., Megina, C., Medina, A., 2004. Testicular development in migrant and spawning bluefin tuna (*Thunnus thynnus* (L.)) from the eastern Atlantic and Mediterranean. Fish. Bull. 102, 407–417.

Billard, R., 1986. Spermatogenesis and spermatology of some teleost fish species. Reprod. Nutr. Dev. 26, 877–920.

Billard, R., 1989. Endocrinology and fish culture. Fish Physiol. Biochem. 7, 49–58.

Bromage, N., Jones, J., Randall, C., Thrush, M., Springate, J., Duston, J., Barker, G., 1992. Broodstock management, fecundity, egg quality and the timing of egg production in the rainbow trout (*Oncorhynchus mykiss*). Aquaculture 100, 141–166.

Brooks, S., Tyler, C.R., Sumpter, J.P., 1997. Egg quality in fish: what makes a good egg? Rev. Fish Biol. Fisher. 7, 387–416.

Chen, K.S., Crone, P., Hsu, C.C., 2006. Reproductive biology of female Pacific bluefin tuna *Thunnus orientalis* from south-western North Pacific Ocean. Fish. Sci. 72, 985–994.

Catarci, C., 2004. World Tuna Markets. FAO, Rome. 135 pp.

Clemens, H.P., Grant, F.B., 1965. The seminal thinning response in carp (*Cyprinus carpio*) and rainbow trout (*Salmo gairdneri*) after injections of pituitary extracts. Copeia 1965, 174–177.

Corriero, A., Desantis, S., Deflorio, M., Acone, F., Bridges, C.R., De la Serna, J.M., Megalofonou, P., De Metrio, G., 2003. Histological investigation on the ovarian cycle of the bluefin tuna in the western and central Mediterranean. J. Fish Biol. 63, 108–119.

Corriero, A., Karakulak, S., Santamaria, N., Deflorio, M., Spedicato, D., Addis, P., Desantis, S., Cirillo, F., Fenech-Farrugia, A., Vassallo-Agius, R., De la Serna, J.M., Oray, Y., Cau, A., Megalofonou, P., De Metrio, G., 2005. Size and age at sexual maturity of female bluefin tuna (*Thunnus thynnus* L. 1758) from the Mediterranean Sea. J. Appl. Ichthyol. 21, 483–486.

Cort, J.L., 1991. Age and growth of the bluefin tuna, *Thunnus thynnus* (L.) of the Northeastern Atlantic. ICCAT Col. Vol. Sci. Pap. 35, 213–230.

Crim, L.W., Bettles, S., 1997. Use of GnRH analogues in fish culture. In: Fingerman, M., Nagabhushanam, R., Thompson, M.F. (Eds.), Recent Advances in Marine Biotechnology, Vol. 1: Endocrinology and Reproduction. Oxford & IBH Publishing Co., New Delhi, pp. 369–382.

Crim, L.W., Sherwood, N.M., Wilson, C.E., 1988. Sustained hormone release. II. Effectiveness of LHRH analog (LHRHa) administration by either single time injection or cholesterol pellet implantation on plasma gonadotropin levels in a bioassay model fish, the juvenile rainbow trout. Aquaculture 74, 87–95.

Deflorio, M., Aprea, A., Corriero, A., Santamaria, N., De Metrio, G., 2005. Incidental captures of sea turtles by swordfish and albacore longlines in the Ionian Sea. Fish. Sci. 71, 1010–1018.

Directorate General for Fisheries, E.U., 2004. Tuna: a global fishing activity. Fish. Eur. 23, 1–12.

Doumenge, F., 1996. Aquaculture of bluefin tuna. Biol. Mar. Mediterr. 3, 258–288.

FAO, 2005. Report of the third meeting of the Ad Hoc GFCM/ICCAT working group on sustainable bluefin tuna farming/fattening practices in the Mediterranean. Rome, 16–18 March 2005. FAO Fisheries Report No. 779. FAO, Rome. 108 p.

Fauvel, C., Suquet, M., Severe, A., Mylonas, C.C., Papandroulakis, N., in review. Slow-release GnRHa therapy prevented atresia during vitellogenesis and induced ovulation of captive wreckfish (*Polyprion americanus*). Cybium.

Freese, A., Sabel, B.A., Saltzman, W.A., During, M.J., Langer, R., 1989. Controlled release of dopamine from a polymeric brain implant: in vitro characterization. Exp. Neurol. 103, 234–238.

Fromentin, J.M., Powers, J.E., 2005. Atlantic bluefin tuna: population dynamics, ecology, fisheries and management. Fish. Fish. 6, 281–306.

Hodson, R., Sullivan, C.V., 1993. Induced maturation and spawning of domestic and wild striped bass, *Morone saxatilis* (Walbaum), broodstock with implanted GnRH analogue and injected hCG. Aquac. Fish. Manage. 24, 389–398.

Hunter, J.R., Macewicz, B.J., Sibert, J.R., 1986. The spawning frequency of skipjack tuna, *Katsuwonus pelamis*, from the South Pacific. Fish. Bull. 48, 895–903.

Karakulak, S., Oray, I., Corriero, A., Deflorio, M., Santamaria, S., Desantis, S., De Metrio, G., 2004. Evidence of a spawning area for bluefin tuna (*Thunnus thynnus* L.) in the Eastern Mediterranean. J. Appl. Ichthyol. 20, 318–320.

Lioka, C., Kani, K., Nhhala, H., 2000. Present status and prospects of technical development of tuna sea-farming. In: Basurco, B. (Ed.), Mediterranean Marine Aquaculture Finfish Species Diversification. Cah. Options Méditerr., Vol. 47. CIHEAM, Instituto Agronomico de Zaragoza, Zaragoza, Spain, pp. 275–285.

Margulies, D., Suter, J.M., Hunt, R., Olson, R.J., Scholey, V.P., Wexler, J.B., Nakazawa, A., 2007. Spawning and early development of captive yellowfin tuna *Thunnus albacares*. Fish. Bull 105, 249–256.

Masuma, S., Tezuka, N., Koiso, M., Jinbo, T., Takebe, T., Yamazaki, H., Obana, H., Ide, K., Nikaido, H., Imaizumi, H., 2006. Effects of water temperature on bluefin tuna spawning biology in captivity. Bull. Fish. Res. Agency (Suppl. 4), 157–172 (In Japanese, with English abstract).

Medina, A., Abascal, F.J., Megina, C., García, A., 2002. Stereological assessment of the reproductive status of female Atlantic northern bluefin tuna during migration to Mediterranean spawning grounds through the Strait of Gibraltar. J. Fish. Biol. 60, 203–217.

Medina, A., Abascal, F.J., Aragón, L., Mourente, G., Aranda, G., Galaz, T., Belmonte, A., de la Serna, J.M., García, S., 2007. Reproductive performance of bluefin tuna in the western Mediterranean: influence of sampling gears in the assessment of reproductive parameters. Mar. Ecol. Prog. Ser. 337, 221–230.

Miyake, P.M., De la Serna, J.M., Di Natale, A., Farrugia, A., Katavic, I., Miyabe, N., Ticina, V., 2003. General view of bluefin tuna farming in the Mediterranean area. ICCAT Coll. Vol. Sci. Pap. 55, 114–124.

Miyashita, S., Murata, O., Sawada, Y., Okada, T., Kubo, Y., Ishitani, Y., Seoka, M., Kumai, H., 2000. Maturation and spawning of cultured bluefin tuna *Thunnus thynnus*. Suisan Zoshoku 48, 475–488 (In Japanese, with English abstract).

Mylonas, C.C., Bridges, C.R., Gordin, H., Belmonte Ríos, A., García, A., De la Gándara, F., Fauvel, C., Suquet, M., Medina, A., Papadaki, M., Heinisch, G., De Metrio, G., Corriero, A., Vassallo-Agius, R., Guzmán, J.M., Mañanos, E., Zohar, Y., 2007. Preparation and administration of gonadotropin-releasing hormone agonist (GnRHa) implants for the artificial control of reproductive maturation in captive-reared Atlantic bluefin tuna (*Thunnus thynnus thynnus*). Rev. Fish. Sci. 15, 183–210.

Mylonas, C.C., Woods III, L.C., Zohar, Y., 1997. Cyto-histological examination of post-vitellogenesis and final oocyte maturation in captive-reared striped bass. J. Fish. Biol. 50, 34–49.

Mylonas, C.C., Woods III, L.C., Thomas, P., Schulz, R.W., Zohar, Y., 1998a. Hormone profiles of captive striped bass (*Morone saxatilis*) during spermiation, and long-term enhancement of milt production. J. World Aquac. Soc. 29, 379–392.

Mylonas, C.C., Woods III, L.C., Thomas, P., Zohar, Y., 1998b. Endocrine profiles of female striped bass (*Morone saxatilis*) during post-vitellogenesis, and induction of final oocyte maturation via controlled-release GnRHa-delivery systems. Gen. Comp. Endocrinol. 110, 276–289.

Mylonas, C.C., Zohar, Y., 2001a. Use of GnRHa-delivery systems for the control of reproduction in fish. Rev. Fish. Biol. Fish. 10, 463–491.

Mylonas, C.C., Zohar, Y., 2001b. Endocrine regulation and artificial induction of oocyte maturation and spermiation in basses of the genus Morone. Aquaculture 202, 205–220.

Nagahama, Y., Yoshikuni, M., Yamashita, M., Tanaka, M., 1994. Regulation of oocyte maturation in fish. In: Sherwood, N.M., Hew, C.L. (Eds.), Molecular Endocrinology of Fish. Fish Physiology, Vol. XIII. Academic Press, San Diego, California, pp. 393–439.

Nishida, T., Tsuji, S., Segawa, K., 1998. Spatial data analyses of Atlantic bluefin tuna larval surveys in the 1994 ICCAT BYP. ICCAT. Coll. Vol. Sci. Pap. 48, 107–110.

Oray, I.K., Karakulak, F.S., 2005. Further evidence of spawning of bluefin tuna (*Thunnus thynnus* L., 1758) and the tuna species (*Auxis rochei* Ris., 1810*, Euthynnus alletteratus* Raf., 1810) in the eastern Mediterranean Sea: preliminary results of TUNALEV larval survey in 2004. J. Appl. Ichthyol. 21, 236–240.

Ottolenghi, F., Silvestri, C., Giordano, P., Lovatelli, A., New, M.B., 2004. Capture-based Aquaculture. The fattening of eels, groupers, tunas and yellowtails. FAO, Rome. 308 pp.

Peter, R.E., Yu, K.L., 1997. Neuroendocrine regulation of ovulation in fishes: basic and applied aspects. Rev. Fish. Biol. Fish. 7, 173–197.

Peter, R.E., Lin, H.R., van der Kraak, G., Little, M., 1993. Releasing hormones, dopamine antagonists and induced spawning. In: Muir, J.F., Roberts, R.J. (Eds.), Recent Adv. Aquac., Vol. IV. Blackwell Scientific Publications, Oxford, pp. 25–30.

Rainis, S., Mylonas, C.C., Kyriakou, Y., Divanach, P., 2003. Enhancement of spermiation in European sea bass (*Dicentrarchus labrax*) at the end of the reproductive season using GnRHa implants. Aquaculture 219, 873–890.

Santamaria, N., Corriero, A., Desantis, S., Zubani, D., Gentile, R., Sciscioli, V., de la Serna, M., Bridges, C.R., De Metrio, G., 2003. Testicular cycle of the Mediterranean bluefin tuna (*Thunnus thynnus* L.). CIHEAM, Cah. Opt. Méditerr. 60, 183–185.

Sawada, Y., Okada, T., Miyashita, S., Murata, O., Kumai, H., 2005. Completion of the Pacific bluefin tuna *Thunnus orientalis* (Temmich et Schlegel) life cycle. Aquacult. Res. 36, 413–421.

Schaefer, K.M., 1996. Spawning time, frequency, and batch fecundity of yellowfin tuna, *Thunnus albacares*, near Clipperton Atoll in the eastern Pacific Ocean. Fish. Bull. 94, 98–112.

Schaefer, K.M., 1998. Reproductive biology of yellowfin tuna (*Thunnus albacares*) in the eastern Pacific Ocean. Inter-Am. Trop. Tuna Comm., Bull. 21, 489–528.

Schaefer, K.M., 2001. Reproductive biology of tunas. In: Block, B.A., Stevens, E.D. (Eds.), Tuna: Physiology, Ecology, and Evolution. Academic Press, San Diego, pp. 225–270.

Schulz, R.W., Miura, T., 2002. Spermatogenesis and its endocrinology. Fish Physiol. Biochem. 26, 43–56.

Silvani, L., Gazo, M., Aguilar, A., 1999. Spanish driftnet fishing and incidental catches in the western Mediterranean. Biol. Conserv. 90, 75–85.

Susca, V., Corriero, A., Bridges, C.R., De Metrio, G., 2001. Study of the sexual maturity of female bluefin tuna: purification and partial characterization of vitellogenin and its use in an enzyme-linked immunosorbent assay. J. Fish. Biol. 58, 815–831.

Tesch, F.-W., 2003. The eel. Blackwell Science, Oxford. 408 p.

Tyler, C.R., Sumpter, J.P., 1996. Oocyte growth and development in teleosts. Rev. Fish. Sci. 6, 287–318.

Weibel, E.R., Gómez, D.M., 1962. A principle for counting tissue structures on random sections. J. Appl. Physiol. 17, 343–348.

Wexler, J.B., Scholey, V.P., Olson, R.J., Margulies, D., Nakazawa, A., Suter, J.M., 2003. Tank culture of yellowfin tuna, *Thunnus albacares*: developing a spawning population for research purposes. Aquaculture 220, 327–353.

Zohar, Y., 1989. Fish reproduction: its physiology and artificial manipulation. In: Shilo, M., Sarig, S. (Eds.), Fish Culture in Warm Water Systems: Problems and Trends. CRC Press, Boca Raton, pp. 65–119.

Zohar, Y., Mylonas, C.C., 2001. Endocrine manipulations of spawning in cultured fish: from hormones to genes. Aquaculture. 197, 99–136.

Zubani, D., Corriero, A., Desantis, S., Acone, F., de la Serna, M., Eicker, J., Bridges, C.R., Megalofonou, P., De Metrio, G., 2003. Ovarian cycle of the Mediterranean bluefin tuna (*Thunnus thynnus* L.). CIHEAM, Cah. Options Méditerr. 60, 201–203.