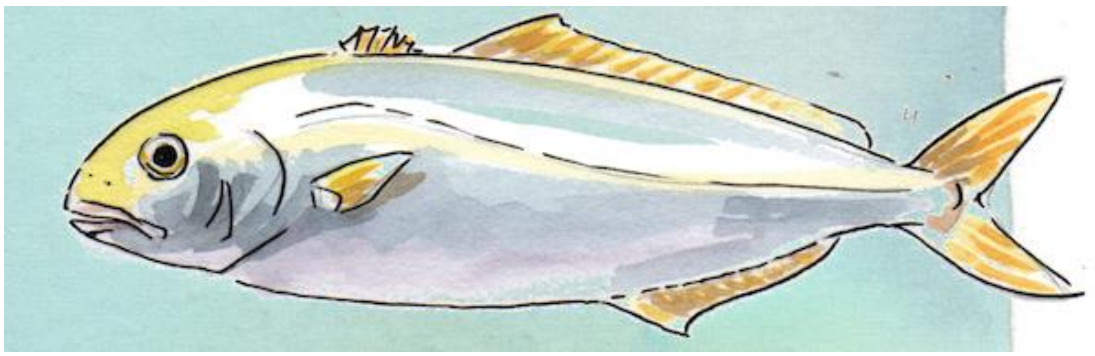
Reproductive biology of the greater amberjack (*Seriola dumerili*) in aquaculture



PhD Thesis

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The illustration of the cover page was made by Clara Cervino

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List of Ph.D.-related published articles

Fakriadis, I., Meiri, I., Bracha, C., Corriero, A., Mylonas, C.C. and Rosenfeld, H. 2021. Gonadotropin expression, and pituitary and plasma levels in the reproductive cycle of wild and captived-reared greater amberjack (*Seriola dumerili*). General and Comparative Endocrinology (in preparation)

Fakriadis, I., Mylonas, C.C., 2021. Sperm quality of greater amberjack *Seriola dumerili* throughout the reproductive season and in response to GnRHa treatment with controlled release implants. Fish Physiol. Biochem. 47, 281-292.

Fakriadis, I., Sigelaki, I., Papadaki, M., Papandroulakis, N., Raftopoulos, A., Tsakoniti, K., Mylonas, C.C., 2020b. Control of reproduction of greater amberjack *Seriola dumerili* reared in aquaculture facilities. Aquaculture. 519, 734880.

Fakriadis, I., Miccoli, A., Karapanagiotis, S., Tsele, N., Mylonas, C.C., 2020a. Optimization of a GnRHa treatment for spawning commercially reared greater amberjack *Seriola dumerili*: Dose response and extent of the reproductive season. Aquaculture. 521, 735011.

Fakriadis, I., Lisi, F., Sigelaki, I., Papadaki, M., Mylonas, C.C., 2019. Spawning kinetics and egg/larval quality of greater amberjack (*Seriola dumerili*) in response to multiple GnRHa injections or implants. Gen. Comp. Endocrinol. 279, 78-87.

Jerez, S., **Fakriadis, I.**, Papadaki, M., Martín, M., Cejas, J., Mylonas, C.C., 2018. Spawning induction of first-generation (F1) greater amberjack *Seriola dumerili* in the Canary Islands, Spain using GnRHa delivery systems. Fishes. 3, 1-22.

Pousis, C., Mylonas, C.C., De Virgilio, C., Gadaleta, G., Santamaria, N., Passantino, L., Zupa, R., Papadaki, M., **Fakriadis, I.**, Ferreri, R., Corriero, A., 2018. The observed oogenesis impairment in greater amberjack *Seriola dumerili* (Risso, 1810) reared in captivity is not related to an insufficient liver transcription or oocyte uptake of vitellogenin. Aquac. Res. 49, 243-252.

Zupa, P., Fauvel, C., Mylonas, C.C., Pousis, C., Santamaría, C.A., Papadaki, M., **Fakriadis, I.**, V., C., 2017. Rearing in captivity affects spermatogenesis and sperm quality in greater amberjack, *Seriola dumerili* (Risso, 1810). J. Anim. Sci. 95, 4085-4100.

Zupa, R., Rodríguez, C., Mylonas, C.C., Rosenfeld, H., **Fakriadis, I.**, Papadaki, M., Pérez, J.A., Pousis, C., Basilone, G., Corriero, A., 2017. Comparative study of reproductive development in wild and captive-reared greater amberjack *Seriola dumerili* (Risso, 1810). PLoS One. 12, e0169645.

List of other published articles

Superio, J., **Fakriadis, I**., Tsigenopoulos, C.S., Lancerotto, S., Rodriguez, A.V., Vervelakis, E., Mylonas, C.C., 2021. Spawning kinetics and parentage contribution of European sea bass (*Dicentrarchus labrax*) broodstocks and influence of GnRHa-induced spawning. Aquaculture Reports. 21, 100766.

De Mello, P.H., Lancerotto, S., **Fakriadis, I.**, Tsoukali, P., M., P., Mylonas, C.C., 2020. The importance of thermoperiod for proper gametogenesis and successful egg and sperm production in meagre (*Argyrosomus regius*). Mediterranean Marine Science. 22, 218-231.

Pérez, E., Linares, F., Rodríguez Villanueva, J.L., Vilar, A., Mylonas, C.C., **Fakriadis, I.**, Papadaki, M., Papandroulakis, N., Papadakis, I., Robles, R., Fauvel, C., Roo, J., Peleteiro, J.B., Lluch, N., Pazos, G., Méndez, B., Sigelaki, I., Gómez, C., Pérez, M., Álvarez-Blázquez, B., 2019. Wreckfish (*Polyprion americanus*). New Knowledge About Reproduction, Larval Husbandry, and Nutrition. Promise as a New Species for Aquaculture. Fishes. 4, 14.

Forner-Piquer, I., **Fakriadis, I.**, Mylonas, C.C., Piscitelli, F., Di Marzo, V., Maradonna, F., Calduch-Giner, J., Pérez-Sánchez, J., Carnevali, O., 2019. Effects of Dietary Bisphenol A on the reproductive function of gilthead sea bream (*Sparus aurata*) testes. International Journal of Molecular Sciences. 20, 5003.

Forner-Piquer, I., Mylonas, C.C., **Fakriadis, I.**, Papadaki, M., Piscitelli, F., Di Marzo, V., Calduch-Giner, J., Pérez-Sánchez, J., Carnevali, O., 2019. Effects of diisononyl phthalate (DiNP) on the endocannabinoid and reproductive systems of male gilthead sea bream (*Sparus aurata*) during the spawning season. Archives of Toxicology. 1-15.

Papadaki, M., Mazzella, D., Santinelli, V., **Fakriadis, I.**, Sigelaki, I., Mylonas, C.C., 2018. Hermaphroditism and reproductive function of hatchery-produced sharpsnout seabream (*Diplodus puntazzo*) under attenuated annual thermal cycles. Aquaculture. 482. 231-240.

Papadaki, M., Peleteiro, J.B., Alvarez-Blázquez, B., Villanueva, J.L.R., Linares, F., Vilar, A., Rial, E.P., Lluch, N., **Fakriadis, I.**, Sigelaki, I., Mylonas, C.C., 2018. Description of the Annual Reproductive Cycle of Wreckfish *Polyprion americanus* in Captivity. Fishes. 3, 1-20.

Mylonas, C.C., Salone, S., Biglino, T., de Mello, P.H., **Fakriadis, I.**, Sigelaki, I., Duncan, N., 2016. Enhancement of oogenesis/spermatogenesis in meagre *Argyrosomus regius* using a combination of temperature control and GnRHa treatments. Aquaculture. 464, 323-330.

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List of abbreviations

11-KT = 11-ketotestosterone

17,20β-P = 17,20β-dihydroxy-4-pregnen-3-one

ANOVA = analysis of variance

AQUALABS = facilities of the IMBBC

ARGO = Argosaronikos Fishfarms SA

AT = atretic follicle

BSA = bovine serum albumin

BW = body weight

CA = cortical alveoli

CASA = computer assisted sperm analysis

D.O.= dissolved oxygen

E_2 = 17β-estradiol

ELISA = enzyme-linked immunosorbent assay

EU = European Union

EVAc = ethyl vinyl acetate copolymer

eVg = early Vg

F1 = first generation

FL = fork length

FORKYS = Forkys Aquacultures SA

FSH = follicle stimulating hormone

FSHβ = β subunit of FSH

fshβ = the gene that encodes the β subunit of FSH

GM = gonad mass

GMF = Galaxidi Marine Farm SA

GnRH = gonadotropin releasing hormone

GnRHa = GnRH agonist

GSI = gonadosomatic index

GtH = gonadotropin

hCG = human chorionic gonadotropin

HCMR = Hellenic Centre for Marine Research

IEO = Centro Oceanográfico de Canarias, Instituto Español de Oceanografía

IMBBC = Institute of Marine Biology, Biotechnology and Aquaculture

LH = luteinizing hormone

LHβ = β subunit of LH

lhβ = the gene that encodes the β subunit of LH

Mct = microtiter plates

OM = oocyte maturation (previously called “final oocyte maturation”)

PIT = passive integrated transponder

PO = primary oocyte

POF = post ovulatory follicle

SOUDA = SOUDA facilities, the pilot sea cage farm of the IMBBC

STR = straightness

T = testosterone

VAP = average path velocity

VCL = curvilinear velocity

Vg = vitellogenic oocyte

VSL = straight line velocity

Γλωσσάρι επιστημονικών όρων

11-KT = 11-κετοτεστοστερόνη

17,20β-P = 17,20β-διυδροξυ-4-πρεγνεν-3-όνη

ANOVA = ανάλυση διακύμανσης

AT = εκφύλιση ωοθυλακίου

BSA = αλβουμίνη βόειου ορού

BW = σωματικό βάρος

CASA = αυτοματοποιημένη ανάλυση ποιότητας σπέρματος

D.O.= διαλυμένο οξυγόνο

E_2 = 17β-οιστραδιόλη

ELISA = ενζυμική ανοσοπροσροφητική δοκιμασία

EVAc = συμπολυμερές οξικού αιθυλεστέρα βινυλίου

eVg = αρχική λεκιθογένεση

F1 = πρώτη γενιά

FSH = ωοθυλακιοτρόπος ορμόνη

FSHβ = β υπομονάδα της FSH

fshβ = το γονίδιο που εκφράζει την β υπομονάδα της FSH

GM = μάζα γονάδας

GnRH = εκλυτική ορμόνη των γοναδοτροπινών

GnRHa = αγωνιστής της GnRH

GSI = γοναδοσωματικός δείκτης

GtH = γοναδοτροπίνη

hCG = ανθρώπινη χοριονική γοναδοτροπίνη

LH = ωχρινοποιητική ορμόνη

LHβ = β υπομονάδα της LH

lhβ = το γονίδιο που εκφράζει την β υπομονάδα της LH

Mct = πλάκες μικροτιτλοδότησης

OM = ωρίμανση ωοκυττάρου

PIT = παθητικός αναμεταδότης

PO = πρωτογενές ωοκύτταρο

POF = ωοθυλάκιο μετά την ωορηξία

STR = ευθύτητα

T = τεστοστερόνη

VAP = μέση ταχύτητα διαδρομής

VCL = ελικοειδής ταχύτητα

Vg = λεκιθογενές ωοκύτταρο

VSL = ευθύγραμμη ταχύτητα

List of scientific names

Atlantic bluefin tuna *Thunnus thynnus*
Atlantic cod *Gadus morhua*
Atlantic halibut *Hippoglossus hippoglossus*
Atlantic herring *Clupea harengus*
Atlantic salmon *Salmo salar*
Ayu *Plecoglossus altivelis*
Bitterling *Acheilognathus rhombea*
Brown trout *Salmo trutta*
Chub mackerel *Scomber japonicus*
Chum salmon *Oncorhynchus keta*
Common dentex *Dentex dentex*
Dusky grouper *Epinephelus marginatus*
European eel *Anguilla anguilla*
European seabass *Dicentrarchus labrax*
Gilthead seabream *Sparus aurata*
Greater amberjack *Seriola dumerili*
Grey mullet *Mugil cephalus*
Greenback flounder *Rhombosolea tapirina*
Hake *Merluccius merluccius*
Jack mackerel *Trachurus japonicus*
Japanese yellowtail *Seriola quinqueradiata*
Longfin yellowtail *Seriola rivoliana*
Mackerel *Scomber colias*
Manipur osteobrama *Osteobrama belangeri*
Meagre *Argyrosomus regius*
Pacific bluefin tuna *Thunnus orientalis*
Pengha *Osteobrama belangeri*
Red gurnard *Chelidonicthys kumu*
Red porgy *Pagrus pagrus*
Red sea bream *Pagrus major*
Senegalese sole *Solea senegalensis*
Sharpsnout seabream *Diplodus puntazzo*
Shi drum *Umbrina cirrosa*

Short-fin eel *Anguilla australis*

Southern flounder *Paralichthys lethostigma*

Spotted rose snapper *Lutjanus guttatus*

Striped bass *Morone saxatilis*

Summer flounder *Paralichthys dentatus*

Two-spotted goby *Gobiusculus flavescens*

Walking catfish *Clarias batrachus*

Winter flounder *Pleuronectes americanus*

Wreckfish *Polyprion americanus*

Yellowfin bream *Acanthopagrus australis*

Yellowtail kingfish *Seriola lalandi*

Abstract

Members of the genus *Seriola* have been notable species for aquaculture production worldwide, with the greater amberjack being one of the most prominent species, due to its cosmopolitan distribution and market acceptability, high growth rates and large size. However, the prerequisite to sustainable aquaculture production -*i.e.* controlling reproduction in captivity- has been lacking for greater amberjack. The **objective of the present doctoral thesis** was to describe the reproductive function of wild and captive reared fish, examine the reproductive maturation potential of fish reared in land-based tanks and sea cages and develop broodstock management methods and spawning induction protocols using gonadotropin releasing hormone agonists (GnRHa). The effectiveness of GnRHa injections versus GnRHa implants of sustained release was examined, and the best method was then optimized by examining (a) optimal dose and (b) time of application. The extent of spermiation period and sperm quality parameters of breeders maintained in different locations and facilities was examined using computer assisted sperm analysis (CASA) to examine any variations over time and after spermiation enhancement trials. Finally, the spawning induction method was also evaluated for the first time on first generation (F1) breeders in Spain to ensure uninterrupted production of fingerlings for genetic breeding programs.

A comparative study of wild and captive greater amberjacks was carried out to compare the endocrinological status of the pituitary-gonad axis related with reproductive developmental stage. A total of 33 (14 males and 19 females) wild and 24 (12 males and 12 females) captive-reared greater amberjack breeders were sampled at three different phases of the reproductive cycle. The sampled wild females were separated in two categories according to the histological analysis of their ovaries, in vitellogenic in early May and spawning capable in late May-late June. Between these two categories a 3 to 4-fold increase of the gonadosomatic index (GSI) was recorded. At the same time a corresponding increase was recorded for pituitary follicle stimulating hormone (FSH) content and plasma testosterone (T), while almost a 10-fold increase was observed in pituitary luteinizing hormone (LH) content. An increasing trend of plasma 17β-estradiol (E_2) and 17,20β-dihydroxy-4-pregnen-3-one (17,20β-P) was also recorded between the two reproductive stages. On the other hand, sampled captive females were separated into four categories of reproductive development, since extended follicular atresia (AT) was observed from late May, with the majority of the fish that had already ceased their reproductive development showing signs of regressed or regenerating ovaries, possibly due to repeated handling. Pituitary content of FSH and LH, *fshβ* and *lhβ* expression and plasma levels of FSH and LH remained unchanged, in contrast with the plasma E_2 and T that were reduced towards the regenerating phase, and 17,20β-P which showed the opposite pattern. No significant hormonal differences, except in 17,20β-P, were recorded between captive and wild vitellogenic females, in contrast to spawning capable females where pituitary LH content, plasma FSH and T were found to be different. Overall, the captive females lagged behind compared

to the wilds on reproductive development and this was probably related with the repetitive handling in sea cages.

Wild male fish were found to be in corresponding reproductive stages with females, as expected. Pituitary LH content, plasma T, 17,20β-P and GSI of wild males exhibited a 3 to 4-fold increase, while an increasing trend of pituitary FSH content, *lhβ* expression levels and plasma 11-ketotestosterone (11-KT) was also observed between the developing and spawning capable reproductive stages, while an opposite trend was observed in plasma LH. On the other hand, sampled captive males fell into three categories of reproductive development, showing significant differences from their wild counterparts even from the developing phase in four out of the ten measured parameters. During spawning capable phase, the situation was even worst, since in almost all the measured parameters reduced levels between wild and captive males were recorded, being statistically significant or showing a strong downward trend. Therefore, the captive males apart from the handling stress that could be related with the worst reproductive development compared to the wilds after the 2nd sampling onwards, showed differences from the first sampling, a more pronounced effect of the captivity compared to the females.

To develop broodstock management and spawning induction methods for greater amberjack, it was examined the possibility of rearing fish either in tanks or sea cages, and evaluated their spawning performance. The advantages of sea cage rearing and spawning include optimal environmental conditions and welfare, and low cost of fish maintenance. The advantages of tank rearing and spawning include biosecurity, ease of management and handling operations, and control of egg collection. Females reared in tanks supplied with well seawater throughout the year exhibited a significant reproductive dysfunction, such as limited gametogenesis, lower oocyte diameter at the peak of the reproductive season, low female eligibility for spawning induction and almost 0% fertilization success. On the contrary, females in three different broodstocks reared in sea cages for three consecutive years underwent vitellogenesis completely, and almost all females were eligible for spawning induction. Males reared under both conditions exhibited reduced sperm production compared to the wild, but unlike the situation in the females, tank rearing did not seem to have any negative effects on sperm quality parameters compared to rearing in sea cages. Spawning performance (eggs collected and fertilization) was best when fish were reared in sea cages during the year and then moved to land-based tanks for spawning after GnRHa treatment, resulting in the production of large numbers of eggs of adequate quality for commercial larval rearing of the species.

The present study examined also two methods for the induction of oocyte maturation/ovulation and spawning, which are based on the induction of endogenous LH release from the pituitary, through the use of GnRHa either in the form of sustained-release delivery systems (implants) or injections (acute release). The stock (n = 28) consisted of wild fish captured in 2011 and each GnRHa administration method was conducted in two replicates with 6 and 8 fish per tank, at a 1:1 sex ratio. Fish were given a GnRHa injection once a week (three administrations), or a GnRHa implant every 2 weeks (two administrations). Mean daily relative

fecundity was significantly higher in the implanted fish compared to the injected fish. Total relative fecundity was also significantly higher in the implanted fish compared to the injected ones, but there were no differences in the quality of eggs in terms of fertilization, 24-h embryo survival, hatching and 5-d larval survival. The number of females with fully vitellogenic oocytes eligible for induction of spawning decreased from the initial to the final sampling, from 7 to 6 females for the GnRHa implant treatment and from 7 to 3 females for the GnRHa injection treatment. The use of GnRHa implants apparently promoted the proper endocrine changes leading to multiple cycles of oocyte maturation, ovulation and spawning, and thus producing larger numbers of eggs. In addition, the use of GnRHa implants may be more appropriate in greater amberjack than multiple injections because (a) it also stimulates vitellogenesis and (b) involves less handling of the fish.

After the successful control of oocyte maturation, ovulation and spawning using hormonal therapies in the form of controlled-release delivery systems of GnRHa, the objective was to optimize the protocol and (a) determine the most efficient GnRHa implant dose and (b) examine the extent of the spawning induction period in greater amberjack reared in sea cages throughout the year, based on the resulting spawning kinetics, egg production and quality. Wild-caught greater amberjack were maintained in two commercial facilities, and were transferred to onshore tanks for spawning after treatment with GnRHa implants (a) of different doses (25 and 75 μg kg^{-1} body weight), or (b) at different times during the expected spawning season (June-July). Both doses were equally effective, and two consecutive implantations in 2 weeks resulted in mean (±SD) total relative fecundity of 185,221± 91,311 to 199,492 ± 16,973 eggs kg^{-1} in 11-18 spawns, without any significant differences in egg quality. Another noteworthy observation from the present study was that “spent” females or with smaller remaining vitellogenic (Vg) oocytes after spawning induction in the onshore tanks, recovered to their initial reproductive stage –in terms of maximum Vg oocyte diameter- after a “resting” period of 14-28 days in sea cages, while their repetitive handling at the time of reproductive stage evaluation didn’t caused any negative effects on their reproductive development. Therefore, it was shown that a protocol where breeders are maintained in sea cages, are taken at different times and are induced to spawn with a GnRHa implant of a recommended dose of 50 μg kg^{-1}, can be used successfully during a period of at least 2 months when seawater temperature is 19-24°C.

In many fish species, the F1 fish face reproductive dysfunctions in captive conditions, such as lower reproductive success, production of unfertilized eggs and lack of spawning, compared to the wild-caught breeding animals. To examine this possibility in greater amberjack, hatchery-produced fish were examined during the expected spawning season and were hormonally induced to spawn. The results showed that hatchery-produced greater amberjack undergo normal gametogenesis and can be induced to undergo maturation, ovulation and spawning after multiple administrations of GnRHa implants, over an extended spawning period lasting from May to September in the Canary Islands, Spain. The use of GnRHa-delivery systems resulted in multiple spawns of fertilized and viable eggs. The developed reproduction control method shows great potential to advance the commercial production of greater amberjack, by enabling the use of

hatchery-produced broodstocks for further breeding selection. However, more research is needed to provide solutions for selective breeding in greater amberjack, due to impossible use of the *in vitro* fertilization protocol in this species.

Finally, sperm quality was evaluated using CASA before and after GnRHa administration and the extent of the spermiation period was determined. Males were in spermiation throughout the monitoring period from the 30th of May until the 18th of July, at temperatures between 19-24ºC. However, lower sperm motility duration, density and survival under cold storage were observed from early July onwards. Sperm quality did not recover after the end of spawning induction experiment in tanks and the return of the fish for 14-28 days to the sea cage in mid-July, which could be related to the high temperatures of this period. An improvement trend was observed in the quality of the milt collected on day 7 after a single GnRHa administration, but a significant decrease was observed on day 21 in sperm density, survival under cold storage and straight line velocity (VSL). On the contrary, a double GnRHa administration spaced 14 days apart maintained the same sperm quality for a longer period of 29 days. Further experimentation needs to be done in greater amberjack to ensure adequate sperm production for high fertilization success.

Considering all the above, the captive greater amberjack is sensitive to handling at the time before the onset of spawning period regarding normal reproductive development. In males, there is a clear effect of confinement on reproductive development. In contrast, when the fish are left unhandled before the spawning period the repetitive handling do not cause any negative effect on their reproductive development. Interestingly, the females recover shortly to their initial reproductive stage after spawning induction after a resting period in sea cages, opposite to the males. A protocol where fish are maintained in sea cages throughout the year, and transferred to onshore tanks after spawning induction with GnRHa implants at a dose of 50 μg GnRHa kg^{-1} body weight (BW) seems to be best in the Mediterranean. Both females and males, at temperatures ranging from 19-24ºC in June-July, can be used to ensure adequate egg production.

Περίληψη

Το μαγιάτικο είναι ένα από τα υποψήφια είδη για τη διαφοροποίηση της παραγωγής της ιχθυοκαλλιέργειας λόγω των συγκριτικών πλεονεκτημάτων του σε σχέση με άλλα είδη, όπως η ευρεία κατανομή του στις εύκρατες περιοχές, ο γρήγορος ρυθμός ανάπτυξης και το μεγάλο μέγεθός του, κατάλληλο για την παραγωγή διαφορετικών προϊόντων από το εδώδιμο τμήμα του. Ωστόσο, δεν έχει επιτευχθεί ακόμη ο έλεγχος της αναπαραγωγής του σε συνθήκες εκτροφής, το οποίο δημιουργεί προβλήματα στην απρόσκοπτη εμπορική παραγωγή του. Αυτό οφείλεται σε αναπαραγωγικές δυσλειτουργίες που παρατηρούνται κατά την διάρκεια της εκτροφής, όπως συμβαίνει και με άλλα νέο-εισερχόμενα είδη. Στόχος της παρούσας έρευνας ήταν να περιγραφεί η αναπαραγωγική λειτουργία εκτρεφόμενων και άγριων ψαριών σε διαφορετικά επίπεδα του άξονα υποθαλάμου – υπόφυσης – γονάδων. Επίσης, να ελεγχθούν οι χερσαίες δεξαμενές και οι ιχθυοκλωβοί ως συστήματα διαχείρισης γεννητόρων για την βέλτιστη γαμετογένεση του μαγιάτικου σε συνθήκες εκτροφής και να γίνει σύγκριση του αναπαραγωγικού δυναμικού των γεννητόρων στις διαφορετικές συνθήκες. Αναπτύχθηκαν μέθοδοι διαχείρισης γεννητόρων, πρωτόκολλα πρόκλησης ωοτοκίας με χρήση αγωνιστών της εκλυτικής ορμόνης των γοναδοτροπινών (GnRHa) και συλλογής αυγών στις υπάρχουσες εγκαταστάσεις της μεσογειακής ιχθυοκαλλιέργειας, με σκοπό να υπερκεραστούν οι αναπαραγωγικές δυσλειτουργίες που εντοπίζονται στο είδος. Σε επόμενο στάδιο, έγινε σύγκριση διαφορετικών μεθόδων χορήγησης GnRHa (ενέσεις για την άμεση εισαγωγή της δραστικής ουσίας στο κυκλοφορικό σύστημα του ψαριού, και εμφυτεύματα για την ελεγχόμενη έκλυση της δραστικής ουσίας, αντίστοιχα) όσον αφορά την ποσότητα και ποιότητα των παραγόμενων αυγών, αλλά και της αναπαραγωγικής κατάστασης των θηλυκών. Στη συνέχεια, η καλύτερη αυτών των μεθόδων ελέγχθηκε για την εξακρίβωση της βέλτιστης δόσης GnRHa, μέσω της αξιολόγησης των ίδιων παραμέτρων, ενώ παράλληλα ελέγχθηκε η βέλτιστη περίοδος εφαρμογής στην εκτιμώμενη περίοδο ωοτοκίας στην Μεσόγειο. Όσον αφορά τους αρσενικούς γεννήτορες, αξιολογήθηκε η παραγωγή και ποιότητα σπέρματος σε διαφορετικές περιόδους κατά την περίοδο σπερμίασης, αλλά και μετά από μονή ή διπλή χορήγηση εμφυτευμάτων ελεγχόμενης έκλυσης GnRHa, με τη χρήση «αυτοματοποιημένης ανάλυσης ποιότητας σπέρματος» (CASA). Τέλος, ελέγχθηκε η αναπαραγωγική λειτουργία μετά από πρόκληση ωοτοκίας σε εκτρεφόμενους γεννήτορες πρώτης γενιάς (F1), ώστε να αποκλειστούν προβλήματα που παρατηρούνται σε νεο-εισερχόμενα είδη στην διαδικασία της εκτροφής, τα οποία περιορίζουν την απρόσκοπτη εφαρμογή προγραμμάτων επιλογής γεννητόρων.

Για να ελεγχθεί η αναπαραγωγική κατάσταση εκτρεφόμενων ψαριών έγινε συγκριτική μελέτη με αλιευμένα ψάρια σε διαφορετικά επίπεδα του αναπαραγωγικού άξονα υποθαλάμου-υπόφυσης-γονάδων. Συνολικά σε 33 αλιευμένα ψάρια (14 αρσενικά και 19 θηλυκά) και 24 εκτρεφόμενα (12 αρσενικά και 12 θηλυκά) έγιναν δειγματοληψίες σε τρεις διαφορετικές φάσεις του αναπαραγωγικού κύκλου. Στα αλιευμένα θηλυκά πραγματοποιήθηκε ιστολογική επεξεργασία των ωοθηκών τους, σύμφωνα με την οποία κατατάχθηκαν σε δύο κατηγορίες ανάλογα με την αναπαραγωγική τους κατάσταση. Συγκεκριμένα, διαχωρίστηκαν σε αυτά που ήταν σε φάση ανάπτυξης των ωοκυττάρων τους, όταν η δειγματοληψία έγινε αρχές Μαΐου και σε αυτά

που ήταν σε φάση ωοτοκίας όταν η δειγματοληψία έγινε τέλη Μαΐου – τέλη Ιουνίου. Μεταξύ των ψαριών των δύο κατηγοριών υπήρξε σημαντική αύξηση του γοναδοσωματικού δείκτη (GSI) (3-4 φορές). Παρόμοια αύξηση παρατηρήθηκε στην συγκέντρωση της ωοθυλακιοτρόπου ορμόνης (FSH) στην υπόφυση και στα επίπεδα της τεστοστερόνης (T) στον ορό του αίματος, ενώ παρατηρήθηκε σημαντική αύξηση (περίπου 10 φορές) της συγκέντρωσης της ωχρινοτρόπου ορμόνης (LH) στην υπόφυση. Καταγράφηκε αυξανόμενη τάση, αλλά όχι στατιστικά σημαντική, της συγκέντρωσης της 17β-οιστραδιόλης (E_2) και της προγεστερόνης 17α,20β-διυδροξυ-4-πρεγνεν-3-όνη (17,20β-P) στον ορό του αίματος μεταξύ των δύο αναπαραγωγικών φάσεων (φάση ανάπτυξης και ωοτοκίας – developing, spawning capable). Η έκφραση των *fshβ* και *lhβ*, αλλά και τα επίπεδα της FSH και LH στον ορό του αίματος δεν διαφοροποιήθηκαν σημαντικά. Από την άλλη, τα εκτρεφόμενα θηλυκά κατατάχθηκαν σε τέσσερις κατηγορίες αναπαραγωγικής ανάπτυξης (developing, spawning capable, regressing, regenerating), δεδομένου ότι παρατηρήθηκε εκτεταμένη εκφύλιση των ωοθυλακίων μετά το τέλος Μαΐου, όπου η πλειοψηφία των ψαριών είχε ολοκληρώσει τον αναπαραγωγικό τους κύκλο, πιθανότατα λόγω των επαναλαμβανόμενων χειρισμών. Η συγκέντρωση της FSH και της LH στην υπόφυση, η έκφραση των *fshβ* και *lhβ* και τα επίπεδα της FSH και LH στον ορό του αίματος παρέμειναν στα ίδια επίπεδα, σε αντίθεση με τα επίπεδα της E_2 και T στον ορό του αίματος, τα οποία μειώθηκαν, ενώ η 17,20β-P έδειξε αντίθετη φορά. Δεν παρατηρήθηκαν σημαντικές διαφορές μεταξύ των αλιευμένων και εκτρεφόμενων ψαριών στις μετρούμενες παραμέτρους, εκτός από την 17,20β-P, κατά τη φάση ανάπτυξης των ωοκυττάρων. Αντίθετα, στη φάση ωοτοκίας η συγκέντρωση της LH στην υπόφυση, αλλά και της FSH και της T στον ορό του αίματος διέφεραν στατιστικά σημαντικά μεταξύ των δύο πληθυσμών. Συνολικά, τα εκτρεφόμενα θηλυκά υστερούσαν σε αναπαραγωγική ανάπτυξη σε σχέση με τα αλιευμένα, γεγονός που πιθανότατα σχετίζεται με τους επαναλαμβανόμενους χειρισμούς στους ιχθυοκλωβούς.

Όσον αφορά τα αλιευμένα αρσενικά, βρέθηκαν σε αντίστοιχες αναπαραγωγικές φάσεις με τα θηλυκά, όπως ήταν αναμενόμενο. Η συγκέντρωση της LH στην υπόφυση, της T και 17,20β-P στον ορό του αίματος και ο GSI αυξήθηκαν σημαντικά (3-4 φορές), ενώ παρατηρήθηκε τάση αύξησης στην συγκέντρωση της FSH στην υπόφυση, την έκφραση του *lhβ* και τη συγκέντρωση της 11-κετο-τεστοστερόνης (11-KT) στον ορό του αίματος μεταξύ των φάσεων της ανάπτυξης (developing) των όρχεων (σπερματογένεση) και σπερμιογένεσης-σπερμίασης (spawning capable), ενώ αντίθετη τάση παρατηρήθηκε στην LH του ορού του αίματος. Δεν παρατηρήθηκε διαφορά μεταξύ των δύο αναπαραγωγικών φάσεων στην έκφραση του *fshβ* και της FSH στον ορό του αίματος. Από την άλλη, τα εκτρεφόμενα αρσενικά χωρίστηκαν σε τρεις κατηγορίες αναπαραγωγικής ανάπτυξης – developing, spawning capable, regressing - σύμφωνα με την ιστολογική αξιολόγηση των όρχεων, έχοντας σημαντικές διαφορές από τα αντίστοιχα αλιευμένα αρσενικά, ακόμη και από την φάση ανάπτυξης των όρχεων σε πέντε (*fshβ*, FSH και LH στον ορό του αίματος, 17,20β-P και GSI) από τις δέκα μετρούμενες παραμέτρους. Κατά την φάση σπερμιογένεσης - σπερμίασης, η κατάσταση έγινε ακόμη χειρότερη στη σύγκριση με τα αλιευμένα αρσενικά, αφού σχεδόν σε όλες τις παραμέτρους παρατηρήθηκαν μειωμένες τιμές των εκτρεφόμενων αρσενικών, είτε στατιστικά σημαντικές ή δείχνοντας τάση μείωσης. Επομένως, τα

εκτρεφόμενα αρσενικά εκτός από την καταπόνηση από τους επαναλαμβανόμενους χειρισμούς στους ιχθυοκλωβούς από τη 2η δειγματοληψία και μετά, που πιθανότατα επηρέασαν αρνητικά την αναπαραγωγική τους ανάπτυξη σε σχέση με τα αλιευμένα, είχαν διαφορές ακόμη και από την 1η δειγματοληψία, δηλαδή η καταπόνηση λόγω αιχμαλωσίας φαίνεται να είναι σημαντικότερη από ότι στα θηλυκά.

Ένας επιπλέον στόχος αυτής της μελέτης ήταν η ανάπτυξη μεθόδων διαχείρισης γεννητόρων, αξιολόγησης της αναπαραγωγικής κατάστασης και πρόκλησης ωοτοκίας σε μαγιάτικα που διατηρούνται τόσο σε δεξαμενές, όσο και σε ιχθυοκλωβούς. Τα πλεονεκτήματα της εκτροφής σε ιχθυοκλωβούς είναι οι βέλτιστες περιβαλλοντικές συνθήκες της θάλασσας, οι συνθήκες ευημερίας των ψαριών και το χαμηλό οικονομικό κόστος διατήρησης των γεννητόρων, ενώ στα πλεονεκτήματα της εκτροφής σε δεξαμενές περιλαμβάνονται η βιοασφάλεια, η ευκολία στη διαχείριση των γεννητόρων και ο έλεγχος στη συλλογή των αυγών. Ωστόσο, στα θηλυκά που παρέμειναν σε δεξαμενές για όλη τη διάρκεια του χρόνου και τους παρεχόταν θαλασσινό νερό γεώτρησης παρατηρήθηκαν αναπαραγωγικές δυσλειτουργίες, όπως η ελλιπής γαμετογένεση, μικρότερη διάμετρος των ωοκυττάρων στα μέσα της αναπαραγωγικής περιόδου, και σχεδόν 0% ποσοστά γονιμοποίησης των αυγών που παρήγαγαν. Αντίθετα, τρεις διαφορετικές ομάδες γεννητόρων που εκτράφηκαν σε ιχθυοκλωβούς για τρία χρόνια ολοκλήρωσαν τη λεκιθογένεση και, σχεδόν, όλα τα θηλυκά ήταν σε κατάλληλη αναπαραγωγική κατάσταση για την επαγωγή της ωρίμανσης, ωορρηξίας και ωοτοκίας. Από την άλλη, τα αρσενικά τόσο στις δεξαμενές όσο και στους ιχθυοκλωβούς είχαν μειωμένη παραγωγή σπέρματος σε σχέση με άγρια ψάρια στη φύση, αν και το περιβάλλον των δεξαμενών δεν φάνηκε να επηρεάζει τα αρσενικά τόσο όσο τα θηλυκά, σε σχέση με τα ψάρια των ιχθυοκλωβών. Ο καλύτερος τρόπος, όσον αφορά τη συλλογή αυγών και ποσοστό γονιμοποίησης αυτών, φάνηκε να είναι ο συνδυασμός των δύο συστημάτων, δηλαδή όταν τα ψάρια διατηρούνταν στους ιχθυοκλωβούς για όλο το χρόνο και μεταφέρονταν στις χερσαίες δεξαμενές μετά από θεραπεία με GnRHa, όπου και παρήγαγαν μεγάλες ποσότητες αυγών καλής ποιότητας.

Η μελέτη εξέτασε επίσης δύο μεθόδους πρόκλησης ωρίμανσης των ωοκυττάρων, ωορρηξίας και ωοτοκίας, οι οποίες βασίζονται στην απελευθέρωση της ενδογενώς παραγόμενης LH, μέσω της χορήγησης GnRHa με μορφή εμφυτευμάτων ελεγχόμενης έκλυσης ή ενέσεις άμεσης έκλυσης. Η ομάδα των 28 γεννητόρων αποτελούνταν από ψάρια που αλιεύθηκαν το 2011, και χωρίστηκαν σε 6 και 8 ψάρια ανά δεξαμενή με 1:1 αναλογία φύλου, σε δύο δεξαμενές για την κάθε μέθοδο. Στα ψάρια χορηγήθηκε μία ένεση κάθε εβδομάδα (3 επαναλήψεις) ή ένα εμφύτευμα κάθε δύο εβδομάδες (2 επαναλήψεις). Η μέση ημερήσια παραγωγικότητα ήταν σημαντικά υψηλότερη στα ψάρια που τους χορηγήθηκαν εμφυτεύματα σε σχέση με αυτά που τους χορηγήθηκαν ενέσεις. Επίσης, η ολική παραγωγικότητα ήταν σημαντικά υψηλότερη, ενώ δεν παρατηρήθηκαν διαφορές στην ποιότητα των αυγών, δηλαδή στο ποσοστό γονιμοποίησης, 24-h επιβίωση εμβρύου, ποσοστό εκκόλαψης και 5-d επιβίωση προνυμφών. Ο αριθμός των θηλυκών με ωοκύτταρα στο τελικό στάδιο λεκιθογένεσης, κατάλληλα για χορήγηση ορμονικής θεραπείας, μειώθηκε από τα 7 στα 6 για τα εμφυτεύματα από την αρχή του πειράματος σε σχέση με το τέλος του πειράματος, ενώ οι αντίστοιχοι αριθμοί για τις ενέσεις ήταν 7 και 3. Τέλος, η χορήγηση εμφυτευμάτων, εκτός από την πρόκληση πολλαπλών κύκλων

ωρίμανσης ωοκυττάρων, ωορρηξίας και ωοτοκίας, πιθανότατα είναι καλύτερη μέθοδος από τις ενέσεις, δεδομένου ότι ενίσχυσε τη λεκιθογένεση στα ψάρια, ενώ περιλάμβανε μικρότερο αριθμό διαχειρίσεων.

Μετά τον επιτυχημένο έλεγχο της ωοτοκίας με τη χρήση εμφυτευμάτων, ο στόχος ήταν να βελτιστοποιηθεί η μέθοδος, δηλαδή να εξακριβωθεί ποια είναι η καλύτερη δόση, αλλά και να εξεταστεί η περίοδος που η παραγωγή και ποιότητα αυγών θα είναι βέλτιστη, μελετώντας την χρονική διάρκεια της περιόδου ωοτοκίας, όταν τα ψάρια διατηρούνταν σε ιχθυοκλωβούς για όλο το χρόνο. Για να επιτευχθεί αυτό, γεννήτορες άγριας προέλευσης που διατηρούνταν σε δύο μονάδες μεταφέρθηκαν σε χερσαίες δεξαμενές μετά από θεραπεία με εμφυτεύματα GnRHa: α) με διαφορετικές δόσεις (25 και 75 μg kg^{-1} BW) και β) σε διαφορετικές περιόδους κατά τη διάρκεια της εκτιμώμενης περιόδου ωοτοκίας (Ιούνιος – Ιούλιος). Και οι δύο δόσεις φάνηκε να είναι το ίδιο αποδοτικές χωρίς σημαντικές διαφορές στην ποιότητα των παραγόμενων αυγών. Μία αξιοσημείωτη παρατήρηση αυτού του πειράματος ήταν ότι τα θηλυκά που θεωρήθηκε ότι είχαν ολοκληρώσει τον αναπαραγωγικό τους κύκλο ή είχαν λεκιθογενή ωοκύτταρα μικρής διαμέτρου μετά την πρόκληση ωοτοκίας στις δεξαμενές, ανέκαμψαν στις αρχικές διαμέτρους ωοκυττάρων μετά από περίοδο παραμονής 14-28 ημερών στους ιχθυοκλωβούς, ενώ οι επαναλαμβανόμενοι χειρισμοί για την αξιολόγηση της αναπαραγωγικής τους κατάστασης δεν είχαν αρνητικές επιπτώσεις στην αναπαραγωγική τους ανάπτυξη. Επομένως, χρησιμοποιήθηκαν επιτυχώς γεννήτορες που διατηρούνταν σε ιχθυοκλωβούς, μεταφέρονταν σε δεξαμενές μετά από θεραπεία με εμφυτεύματα GnRHa με δόση 50 μg GnRHa kg^{-1} BW σε διαφορετικές χρονικές περιόδους για ένα διάστημα δύο μηνών, όταν η θερμοκρασία του νερού ήταν 19-24°C.

Αν και η χρήση ψαριών άγριας προέλευσης φάνηκε να είναι αποδοτική στο μαγιάτικο, δεδομένης της αναπαραγωγικής δυσλειτουργίας που εμφανίζουν τα ψάρια της γενιάς F1 σε πολλά είδη σε συνθήκες εκτροφής, όπως η μειωμένη αναπαραγωγική επιτυχία, η παραγωγή αγονιμοποίητων αυγών και η απουσία ωοτοκίας, σε σύγκριση με ψάρια άγριας προέλευσης, εξετάστηκε η απόκριση τέτοιων ψαριών στη χρήση των παραπάνω πρωτοκόλλων. Τα αποτελέσματα έδειξαν ότι τα ψάρια πρώτης γενιάς μπορούν επιτυχώς να ολοκληρώσουν τη γαμετογένεση, και μετά από επαναλαμβανόμενες ορμονικές θεραπείες να προχωρήσουν στην τελική ωρίμανση των ωοκυττάρων τους, σε ωορρηξία και ωοτοκία για περίοδο μεγάλης διάρκειας (Μάιος – Σεπτέμβριος) στα Κανάρια νησιά, παράγοντας πολλαπλές ωοτοκίες με αυγά καλής ποιότητας. Αυτό είναι ένα χρήσιμο συμπέρασμα για την εφαρμογή μεθόδων επιλογής γεννητόρων, ωστόσο περαιτέρω μελέτη είναι απαραίτητη για την ανεύρεση λύσεων για την επιλεκτική αναπαραγωγή του μαγιάτικου, ενός είδους που η τεχνητή γονιμοποίηση των αυγών του είναι σχεδόν αδύνατη.

Τέλος, εξετάστηκε η ποιότητα σπέρματος με τη μέθοδο CASA πριν και μετά τη χορήγηση θεραπειών GnRHa, όπως και η περίοδος σπερμίασης. Τα αρσενικά ήταν σπερμιάζοντα από τις 30 Μαΐου μέχρι και τις 18 Ιουλίου, σε θερμοκρασίες 19-24ºC. Ωστόσο, χαμηλότερη διάρκεια κινητικότητας σπέρματος, πυκνότητας και επιβίωσης σε ψυχρή συντήρηση παρατηρήθηκε από τις αρχές Ιουλίου. Η ποιότητα του σπέρματος δεν επανήλθε μετά το τέλος του πειράματος και της αντίστοιχης παραμονής των ψαριών στις δεξαμενές, και την επιστροφή τους για διάστημα 14-28 ημερών στους ιχθυοκλωβούς στα μέσα Ιούλη, το οποίο μπορεί να

σχετίζεται με τις υψηλές θερμοκρασίες της περιόδου. Τάση βελτίωσης στην ποιότητα του σπέρματος παρατηρήθηκε την 7η ημέρα μετά τη χορήγηση θεραπείας GnRHa, αλλά επήλθε σημαντική μείωση στην πυκνότητα του σπέρματος, στην επιβίωση σε ψυχρή συντήρηση αλλά και στην ταχύτητα ευθείας γραμμής των σπερματοζωαρίων. Αντίθετα, η διπλή χορήγηση GnRHa σε διάστημα 14 ημερών διατήρησε την ίδια ποιότητα σπέρματος για περίοδο 29 ημερών. Η πραγματοποίηση πειραμάτων ενίσχυσης της σπερμίασης στο μαγιάτικο είναι απαραίτητη ώστε να επιτευχθεί παραγωγή ικανών ποσοτήτων σπέρματος που θα διασφαλίζουν υψηλά ποσοστά γονιμοποίησης.

Συμπερασματικά, τα εκτρεφόμενα μαγιάτικα είναι ευαίσθητα σε χειρισμούς πριν την έναρξη της περιόδου ωοτοκίας όσον αφορά την ομαλή αναπαραγωγική ανάπτυξη. Επιπλέον, στα αρσενικά φαίνεται να υπάρχει αρνητική επίδραση της αιχμαλωσίας στην αναπαραγωγική τους ανάπτυξη ακόμη και όταν δεν γίνονται χειρισμοί. Αντίθετα, όταν δεν γίνουν χειρισμοί στους θηλυκούς γεννήτορες, τα ψάρια έχουν ομαλή αναπαραγωγική ανάπτυξη, και μετά από πρόκληση ωοτοκίας μπορούν να ανακάμψουν στην αρχική τους αναπαραγωγική κατάσταση μετά από μία περίοδο παραμονής στους ιχθυοκλωβούς, κάτι που δε συμβαίνει με τα αρσενικά. Το πρωτόκολλο που θα μπορούσε να χρησιμοποιηθεί με επιτυχία στο μαγιάτικο στη Μεσόγειο, περιλαμβάνει γεννήτορες που διατηρούνται καθ'όλη τη διάρκεια του έτους σε ιχθυοκλωβούς, οι οποίοι μετά από θεραπεία με εμφυτεύματα GnRHa δόσης 50 μg GnRHa kg^{-1} BWμεταφέρονται σε χερσαίες δεξαμενές για ωοτοκία. Τόσο τα θηλυκά όσο και τα αρσενικά μπορούν να χρησιμοποιηθούν επιτυχώς σε θερμοκρασίες 19-24^{o}C την περίοδο Ιουνίου-Ιουλίου για ικανοποιητική παραγωγή αυγών.

Chapter 1 – General introduction

1.1 Aquaculture

Aquaculture is the breeding, rearing and harvesting of fish, shellfish, algae and other organisms in all types of water environment, and it is the aquatic equivalent of agriculture and farming on land (FAO, 1988). There are two main types of aquaculture: marine and freshwater and wherever farming takes place, implies some form of intervention in the rearing process to enhance production, such as regular stocking, feeding, protection of predators, etc. (FAO, 1988). Aquaculture is classified, based on the used productive technology, into extensive, semi-intensive and intensive, with human influence increasing proportionally (Edwards, et al., 1988). One of the first aquaculture attempts may have been made in south-western Victoria, Australia, by the Gunditjmara, the local Aboriginal people, who seems to have farmed short-finned eels 6,600 years ago, aiming to have food all year round (Kemp, 2004). In the Mediterranean, the ancient Egyptians might have farmed gilthead seabream 3,520 years ago (Guy, et al., 2018), while the Romans bred fish in ponds in coastal lagoons almost 2,000 years ago (McCann, 1979). Whenever has happened, aquaculture always had the same purpose, to provide food security.

Control of reproduction, as one of the keystones of the aquaculture production cycle, was realized only 250 years ago when Stephan Ludwig Jacobi wrote an article titled “On the artificial production of trout and salmon”, where he experimented with external fertilization of brown trout and Atlantic salmon (Fish, 1936). The next 200 years passed with the exploitation of wild stocks, but recently overexploitation of some popular fish species and harvest stagnation, pushed up aquaculture production (**Fig 1.1**), which nowadays exceeds the amount produced for human consumption by fisheries (**Fig 1.2**), mainly due to aquaculture in Asia, where the farming production is higher than fisheries. By contrast, the European Union (EU) accounts for only 1,2% of the global aquaculture production. This figure represents about 20% of its own total domestic seafood production. At the same time most fishery stocks are expected to remain maximally sustainably fished or overfished for at least the next decade, so aquaculture must bridge the growing gap between the seafood demand from a growing and wealthier global population and the available supply. Aquaculture has the potential to help countries achieve their economic, social and environmental goals, thus contributing to the 2030 Agenda, especially in the EU, as it is one of the most environmental friendly protein production systems, in terms of land and fresh water use, carbon footprint and feed conversion efficiency, compared to land-based livestock farming and fisheries (Nijdam, et al., 2012).

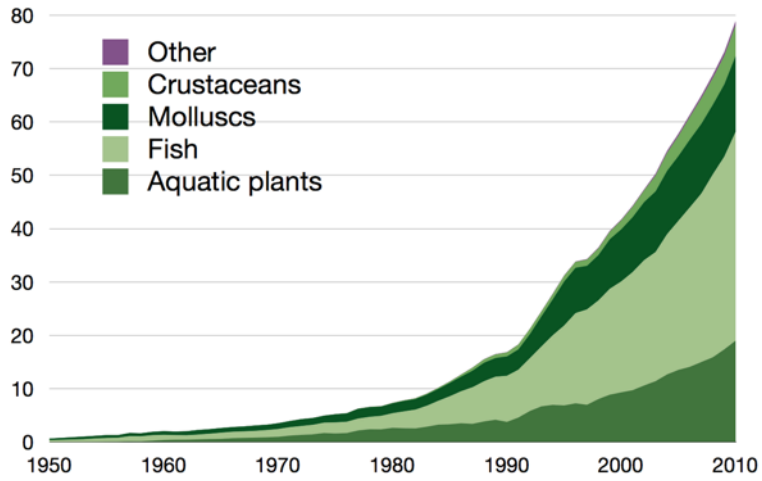

Figure 1.1 Global aquaculture production in million tones, 1950 – 2010 (FAO, 2018)

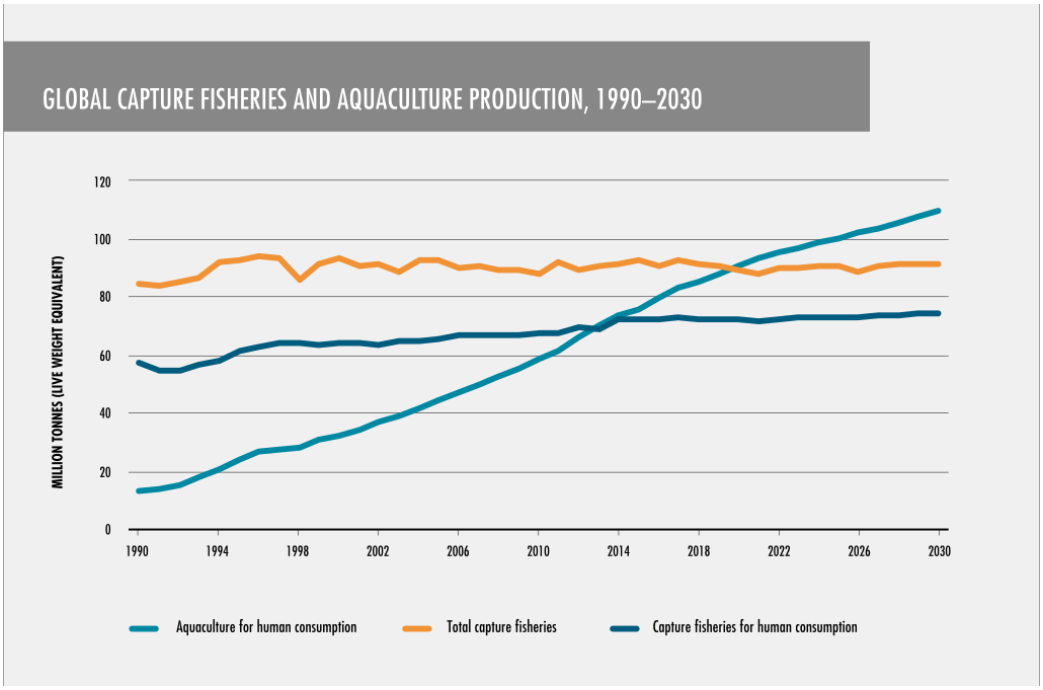

Figure 1.2 Global capture fisheries and aquaculture production, 1990-2030 (FAO, 2018).

1.2 Fish reproduction

Reproduction is the biological process by which a new individual organism is produced or by which genetic material is passed from one generation to the next (Wootton & Smith, 2015). Reproduction is expensive (Parker & Cheung, 2020), especially in egg producing animal and it requires energetic, ecological, physiological, anatomical, biochemical and endocrinological adaptations (Rocha, et al., 2008). And since reproduction is expensive, reproductive physiology is sensitive to an array of endogenous and exogenous

signals, in order to ensure that it takes place at the optimal time for the survival of the produced progeny (Parker & Cheung, 2020).

Reproduction in fish, and specifically in teleosts, display the most striking diversity among vertebrates (Trudeau & Somoza, 2020; Wootton & Smith, 2015). The reproductive modes, sexuality and mating systems of teleosts vary regarding sex determination, gender systems, mode of fertilization, mating system, secondary sexual characteristics, spawning site preparation and parental care (Jalabert, 2005; Wootton & Smith, 2015). Fish reproduction has gained special significance the last decades because of the increasing importance for the aquaculture (Rocha, et al., 2008), as one of the most serious bottlenecks in the development of this sector (Zohar & Mylonas, 2001). In fact, control of reproductive processes of fish in captivity is a prerequisite for the expansion of aquaculture, and a first step to reduce the gap with the livestock and poultry production, which have been practiced for millennia.

1.2.1 Gametogenesis

The ovaries and testes change size and therefore weight during the reproductive season following gametogenesis, which is the process of producing mature haploid gametes, *i.e.* ova and spermatozoa. The ovum is the final product of a complex process called oogenesis (Lubzens, et al., 2010), while the spermatozoon is the final product of the male germ cell development, a process called spermatogenesis (Schulz, et al., 2010; Uribe, et al., 2014). Females are producing a relatively small number of large gametes, while on the opposite males are producing a much larger number of small gametes, which evolved as highly specialized, motile genome vectors (Schulz, et al., 2010). In both processes there are high metabolic requirements for gamete production, but especially so for female fish, as they must provide with the necessary nutrients (yolk) for embryonic development (Parker & Cheung, 2020; Tyler & Sumpter, 1996).

Despite the fact that formation of egg and sperm show common principles, many aspects of gametogenesis differ between sexes and further distinction of various phases varies depending on the physiological, biochemical, morphological and histological criteria used (Lubzens, et al., 2010; Patiño & Sullivan, 2002; Schulz, et al., 2010; Selman, et al., 1993; Tyler & Sumpter, 1996). Considering the above information, oogenesis in general can be distinguished in five different phases: primary oocyte (PO) development, cortical alveolus (CA) phase, vitellogenesis, maturation and ovulation. Briefly, oogonia are transformed into primary oocytes where the organelles of the cytoplasm are formed, while at the same time the cell is covered with a membrane called the zona radiata. At the end of this phase, the oocytes are organized one by one into the follicles. In the cortical alveolus phase, the homonymous particles appear, which during vitellogenesis are displaced to the periphery of the cell and function after ovulation and fertilization in order to inhibit polyspermy. Vitellogenesis is the predominant process of oocyte growth causing a large increase in their size. It refers to the concentration of vitellogenin by the oocytes, a substance that is produced mainly in the liver, but also in the oocytes, and is transported by the blood. The following phase is the maturation during which the production of proteins continues, a process that stops simultaneously with the release of material

after germinal vesicle breakdown, while the phase is completed with the hydration of the oocyte. Once gametogenesis is complete, ovulation follows, during which the eggs are released into the ovarian cavity or into the abdominal cavity of more ancient fishes (**Fig 1.3**) (Goetz & Garczynski, 1997; Kagawa, 2013; Lubzens, et al., 2017; Patiño & Sullivan, 2002; Selman, et al., 1993; Tyler & Sumpter, 1996; Wallace & Selman, 1990).

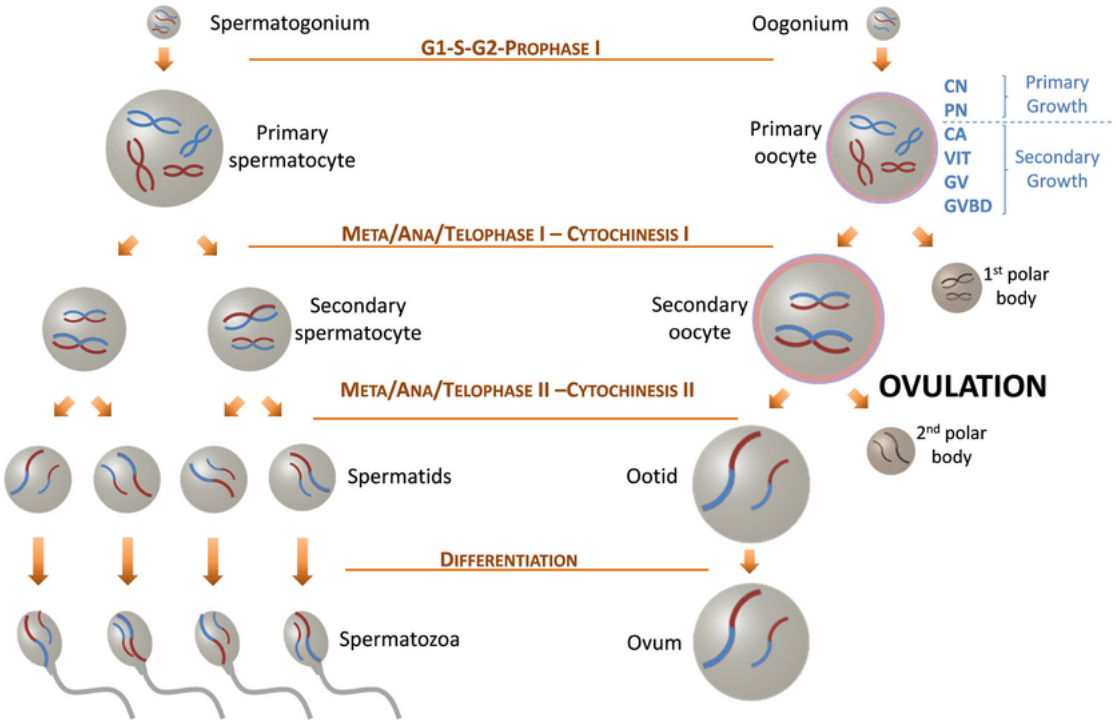

Figure 1.3 The process of gametogenesis (Domínguez-Petit, et al., 2017).

On the other hand, spermatogenesis can be divided in four main phases: mitotic divisions of spermatogonia, meiosis of spermatocytes, spermiogenesis and spermiation. During spermatogenesis, different types of male germ cells are distinguished, such as spermatogonia, spermatocytes, spermatids and spermatozoa. As happens with the females, the predominant cell types contained in a testis are indicative of the reproductive cycle phase of the fish. Initially, spermatogonia result from the differentiation of primordial germ cells. This is followed by the multiplication of spermatogonia through mitotic divisions, the number of which depends on each species. The transformation of spermatogonia into type A spermatocytes takes place during the last mitotic division. The first phase of meiosis results in the production of secondary spermatocytes (type B), where DNA duplication occurs. During the second phase of meiosis, the secondary spermatocytes are transformed into spermatids. The morphological transformation of spermatids into flagellated spermatozoa is the final stage of gametogenesis in male fish and is called spermiogenesis. The release of sperm into the

environment is the corresponding process of ovulation in females and is called spermiation (Schulz & Miura, 2002; Schulz, et al., 2010; Uribe, et al., 2014).

Both sexes share a common process for the programmed degradation of their germ cells, which is the process of apoptosis. In females, this hormonally induced process is called follicular atresia while in males germ cell apoptosis. This process occurs to a greater or lesser extent in all stages of oocyte and sperm development. The intensity of the process seems to increase under stress (Tyler & Sumpter, 1996). It has been shown that stress can result in reduced levels of estrogens, androgens, vitellogenin, LH and GnRH mRNA. Stress was also shown to cause delayed ovulation, reduced egg sizes in females and sperm counts in males, and overall increased gonadal atresia (Karine, et al., 2020).

To avoid misinterpretations and difficulties of comparisons on the developmental phase of the reproductive cycle of each fish, a simple and standardized terminology has been proposed recently (Brown-Peterson, et al., 2011). It distinguishes the reproductive cycle in four phases: a) the developing, which signals entry into the gonadotropin (GtH)-dependent stage of oogenesis and spermatogenesis and ultimately results in gonadal growth; b) the spawning capable, which includes (1) those fish with gamete development that is sufficiently advanced to allow for spawning within the current reproductive cycle and (2) batch-spawning females that show signs of previous spawns (i.e., postovulatory follicle complex) and that are also capable of additional spawns during the current cycle; c) the regressing phase, which indicates completion of the reproductive cycle and, for many fish, completion of the spawning season, and finally d) the regenerating phase, when the fish are sexually mature but reproductively inactive (Brown-Peterson, et al., 2011).

Fish in terms of the number of spawns they can produce during their reproductive season are classified into synchronous, group synchronous and asynchronous (Tyler & Sumpter, 1996). The separation between the latter two has been recently disputed due to the large variation in the number of spawns, the time between them, and the stages of oocytes present in their ovaries (Ganias & Lowerre-Barbieri, 2018). However, it is still necessary in reproductive biology to discriminate species that spawn once in their lifetime, such as the European eel, once every year, such as the Atlantic herring (Ganias & Lowerre-Barbieri, 2018), 1-5 times a year, such as the European sea bass (Superio, et al., 2021) and almost every day in a spawning season, such as the gilthead seabream (García-Fernández, et al., 2018), since different groups of oocyte stages are expected to be observed in their ovaries at different times in their reproductive cycle, as well as different levels of hormones that regulate gametogenesis making it necessary to conduct research at species level.

1.2.2 Endocrine control of fish reproduction

Reproductive function in vertebrates, from puberty and gametogenesis to reproductive behavior, and from seasonal reproduction to sex change in hermaphrodites is regulated endocrinologically, through the brain-pituitary-gonad axis (Munoz-Cueto, et al., 2020; Trudeau & Somoza, 2020; Zohar, et al., 2010). Until recently it was considered that reproduction is modulated indispensably by the GnRH system (Whitlock, et al., 2019). However, it was shown recently from knockout studies of the *gnrh* genes that other neuropeptides, such as

kisspeptin, GtH-inhibitory hormone, neurokinin, gamma-aminobutyric acid, noradrenaline, serotonin etc, may provide compensatory regulatory mechanisms of reproduction (Munoz-Cueto, et al., 2020; Trudeau, 2018). Even so, GnRH is still considered the master molecule in reproduction, when present (Gore, 2002; Munoz-Cueto, et al., 2020), but more studies need to be done to describe the complex function of reproduction and to clearly define the neuropeptides that are involved.

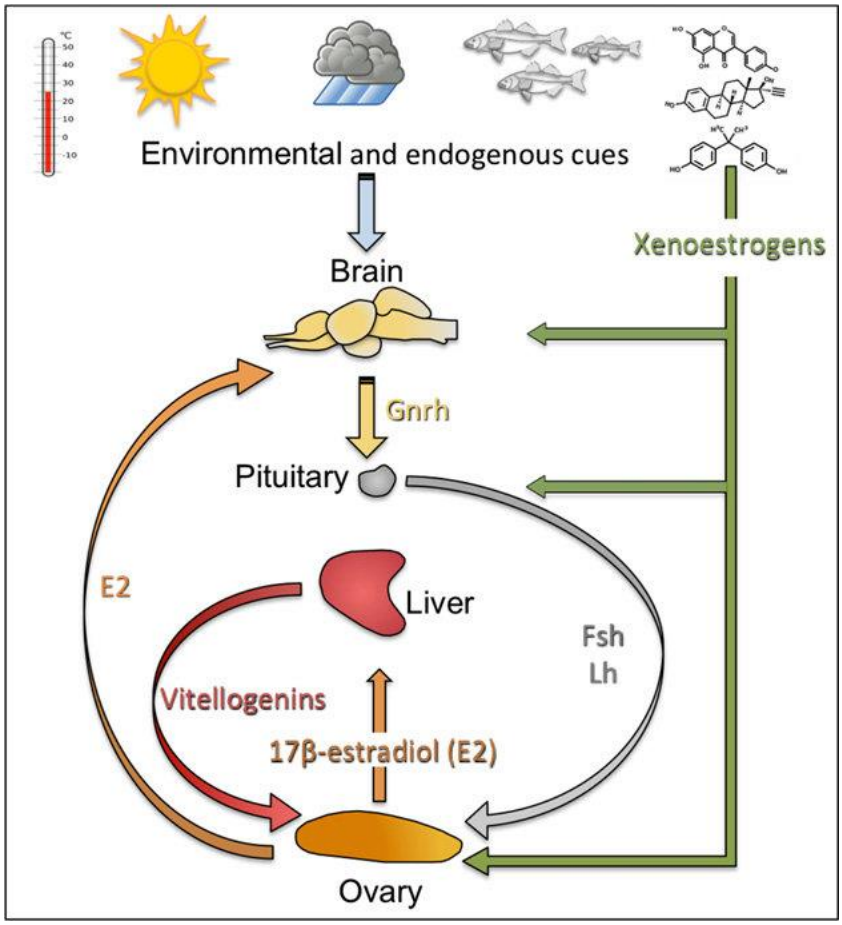

Figure 1.4 The brain-pituitary-gonad axis in fish (Sullivan & Yilmaz, 2018).

The brain integrates endogenous signals, such as developmental stage, size, nutritional status and energy stores, and environmental signals, such as temperature, photoperiod, habitat conditions, presence of partners, which converge into the positive or negative regulation of the activity of GnRH, a 10 amino acid long neuropeptide (**Fig. 1.4**) (Karine, et al., 2020; Munoz-Cueto, et al., 2020; Parker & Cheung, 2020). Then, GnRH binds to the respective receptors expressed by the gonadotropic cells, located in the pituitary, and stimulates the synthesis and release of FSH and LH, which are considered the key factors regulating gonadal maturation (Levavi-Sivan, et al., 2010; Zohar, et al., 2010). The FSH and LH are heterodimers with a common α subunit and differing in their β subunits, and LHβ compared to FSHβ is more conserved (Levavi-Sivan, et al., 2010;

Rosenfeld, et al., 2007). In salmonids, which are synchronous spawning fishes, FSH has a predominant role in early oogenesis/spermatogenesis while LH is mainly involved in the final stages of maturation. However, in asynchronous or group synchronous spawning fishes the situation is more complex and the available studies by far fewer, without any obvious predefined pattern (Rosenfeld, et al., 2007), and species-specific studies needs to be done to discriminate the function of the GtHs. At the gonadal level, E_2 is considered responsible for the oocyte growth while the maturation-inducing hormone is produced during oocyte maturation (Levavi-Sivan, et al., 2010). In males, T and mainly 11-KT are responsible for spermatogenesis and spermiogenesis, while 11-KT have a peak before the onset of spermiation period. The progestins (17, 20β-P or 20βS) induce sperm maturation and release (Vizziano, et al., 2008).

1.2.3 Gamete quality

One of the major objectives of the aquaculture industry is the production of a large number of viable eggs with high survival (Lubzens, et al., 2010). However, one of the limiting factors of the reproductive success, either for aquaculture production or creation of a gene bank, is the quality of male and female gametes (Bobe & Labbé, 2010; Zadmajid, et al., 2019). This relies on optimal broodstock management, both in terms of nutritional and environmental needs of the captive fish (Migaud, et al., 2013). Nevertheless, after some decades of systematic fish farming, survival of larvae at post-weaning stage exceeds 50% in salmonids, being much lower in other farmed species, such as the European sea bass, gilthead seabream, turbot, etc. (Migaud, et al., 2013).

Fish egg quality is defined as the ability of the egg to be fertilized and develop to a normal embryo (Bobe & Labbé, 2010; Migaud, et al., 2013). Although, this definition seems specific and simple, it involves complex processes in an ever-changing environment, where any generalization seems risky and most of the factors affecting egg quality are still unknown. For example, different criteria, such as the appearance of zona pellucida, the shape of the egg, the size of the egg, the distribution of oil globules, the shape of the first embryonic cells, the ability of marine fish eggs to float and the nutrient materials of the egg supplied through broodstock diet, have been suggested as indicators of egg quality for various species (Brooks, et al., 1997). However, for almost every one of the above criteria there is at least a study that challenges them (Avery, et al., 2009; Bromage, et al., 1992; Bromage, et al., 1994; Cerdá, et al., 1994; Chatzifotis, et al., 2020; Ciereszko, et al., 2009; De Mello, et al., 2020). Survival at specific developmental stages of the eggs can be considered as the most common practice for egg quality evaluation. For example, survival at a particular embryonic stage, hatching success or larval survival at yolk-sac absorption, together with recording of mortalities at every developmental stage and distinguishing malformations is a robust method to show differences between experimental treatments or rearing conditions (Bonnet, et al., 2007; De Mello, et al., 2020; Kopeika, et al., 2003).

Consequently, fish sperm quality can be defined as its ability of fertilizing an egg and allowing the development of a normal embryo (Migaud, et al., 2013), after usually being released in a hostile external

medium (Cosson, 2007). Finally, both the intrinsic gamete quality and quantity and the environment where fusion of gametes take place determine fertilization success (Fauvel, et al., 2010). Many factors affect sperm quality, including them related with broodstock management, the time of the sampling and the chemical properties of the activation medium (Alavi, et al., 2007; Cabrita, et al., 2009). Several parameters may determine fertilization capacity and reproductive success, including milt volume, sperm density, motility, seminal plasma pH, osmolality and composition, and have been linked with the ability of sperm to fertilize eggs (Fauvel, et al., 2010; Kowalski & Cejko, 2019). However, among sperm quality parameters, sperm motility (Rurangwa, et al., 2004) and velocity (Gage, et al., 2004) are considered the most important and, in some species, percentage of motile spermatozoa is also directly related to egg fertilization success (Gallego & Asturiano, 2018).

1.3 Reproductive dysfunctions during domestication

Almost 70% of the 250 farmed finfish species in the world are linked to a certain extend to the availability on wild resources and only a few species can be considered fully domesticated, such as the cattle or sheep (Teletchea & Fontaine, 2014). Similar to mammalian species, the prerequisite to domestication procedures is the consistent control of reproduction in successive generations of fish maintained and bred in captivity and acquire high quality seeds (Mylonas, et al., 2010).

Many species, towards their domestication procedure exhibit reproductive dysfunctions, which are more common in females. These can be classified in three types: fish that fail completely to undergo vitellogenesis and spermatogenesis in captivity; fish that do not proceed with oocyte maturation and the fish that cannot release their eggs properly to be fertilized, even if they have concluded maturation and ovulation (Zohar & Mylonas, 2001). However, the main reproductive dysfunction observed in captive fishes is the lack or unreliable oocyte maturation, ovulation and spawning (Mylonas & Zohar, 2001) caused by inadequate pituitary LH synthesis and/or release at the end of vitellogenesis (Zohar & Mylonas, 2001), since most of them conclude vitellogenesis in captivity. Additionally, unpredictable and variable reproductive performance in newly cultured fish species could be due to improper broodstock nutrition (Izquierdo, et al., 2001), while failure of spawning can result from improper environmental conditions or by captivity-induced stress (Mylonas, et al., 2010).

In males, reproductive dysfunctions are less severe, as in most species spermatogenesis is completed resulting in sperm production. Two main types have be distinguished: those that fail to produce any sperm or poorly spermiating fishes (Mylonas, et al., 2017b). The first is related with species that their natural reproductive cycle is difficult to be simulated in captive conditions. The second is the most common reproductive dysfunction in captive males and usually results in reduced amount of produced milt, and sometimes with reduced quality (Mylonas, et al., 2017b).

1.4 Hormonal treatments for females and males

To overcome the above-mentioned reproductive dysfunctions of failure of oocyte maturation and poorly spermiating males, various hormonal therapies and administrations have been applied targeting the brain-pituitary-gonadal axis (Mylonas & Zohar, 2001; Zohar & Mylonas, 2001). Initially, the treatment protocols provided the necessary LH, from homogenized fish pituitaries or pituitary extracts, human chorionic godadotropin (hCG) extracted from the urine of pregnant women, or other purified LHs from mammalian origin (Mañanos, et al., 2009; Mylonas, et al., 2010; Zohar & Mylonas, 2001). However, these substances - other than hCG - were costly to manufacture, often successful only in specific cases, and repeated treatment at different breeding seasons resulted in their inactivation, as the fish produced specific antibodies (Mylonas & Zohar, 2001; Zohar & Mylonas, 2001).

The next generation of hormonal methods for inducing maturation, ovulation, spawning and spermiation enhancement involved the administration of synthetically produced GnRHa. This was because natural GnRHs are catalyzed by enzyme activity, making them inactive over a short period of time. In contrast, the use of synthetic GnRHa increases the residence time in the circulation. The GnRHa is more widely used due to its advantages in relation to lower species-specificity and targeting a higher level in the brain-pituitary-gonad axis, stimulating the release of the endogenous LH (Mylonas, et al., 2010). In addition, GnRHa may be administered in the form of a bolus (liquid injection) or in a sustained-release delivery system (Mylonas & Zohar, 2001), each method having important advantages in different species. Also, GnRHa do not cause a reaction of the fish's immune system as they are small decapeptides, while they are biosafe since they are synthetically prepared (Mylonas & Zohar, 2001; Zohar & Mylonas, 2001).

Recently, the production and use of recombinant FSH and LH have been examined for some fish species and the number of relevant studies has been expanding. However, even if in most cases their effectiveness was proven *in vitro* by stimulating the relevant receptors, *in vivo* effects were not always the same, showing that further research is needed in this field (Mylonas, et al., 2017b).

1.5 Greater amberjack

Members of the genus *Seriola* have been notable species for the aquaculture diversification worldwide (Kagawa, 1989; O'Neill, et al., 2015; Sicuro & Luzzana, 2016; Symonds, et al., 2014), while the greater amberjack is one of the most promising species, due to its cosmopolitan distribution (Paxton, et al., 1989) and acceptability, high growth rates and large size (Crespo, et al., 1994; Grau, et al., 1996; Jover, et al., 1999; Lazzari, 1991; Lazzari, et al., 2000; Mazzola, et al., 2000), and late maturation (Micale, et al., 1999; Zupa, et al., 2017b), which allows for the marketing of the fish before growth is affected by reproductive maturation. Also, as consumers show increasing interest towards value-added seafood products (Asche, et al., 2009), large-sized fish species are more appropriate for the processing and production of a number of products suitable for profitable market niches. Furthermore, greater amberjack is found throughout the temperate zone, which is an

advantage for species diversification of the aquaculture industry, compared to other potential candidate species that have a narrower geographical distribution. Worldwide, greater amberjack spawns naturally from February to April in the Gulf of Mexico (Wells & Rooker, 2004), from April to October in the Canary Islands (Jerez, et al., 2006), from April to June in Japan (Kawabe, et al., 1998; Kawabe, et al., 1996; Nyuji, et al., 2016) and from May to July in the Mediterranean (Marino, et al., 1995) with the optimum spawning temperature supposedly ranging from 21.5°C to 23.4°C (Kawabe, et al., 1998; Kawabe, et al., 1996). However, until now the production of greater amberjack has been faced with major bottlenecks, such as inconsistent and unreliable reproduction and production of juveniles for grow out.

Controlling reproduction of greater amberjack in captivity lags behind its congeners the Japanese yellowtail and yellowtail kingfish, even though some sporadic and unpredictable spawning has been observed in greater amberjack in Japan (Kawabe, et al., 1998; Kawabe, et al., 1996) and in the Canary Islands (Jerez, et al., 2006; Sarih, et al., 2018). However, spontaneous spawning had not been reported so far in the Mediterranean region (Grau, et al., 1996), as is observed with many other newly cultured species. As reported earlier, failure of spawning in captivity can result from improper environmental conditions or by captivity-induced stress (Mylonas, et al., 2010) and the main reproductive dysfunction observed in captive fishes is the lack or unreliable oocyte maturation, ovulation and spawning (Mylonas & Zohar, 2001) caused by inadequate pituitary LH synthesis and/or release at the end of vitellogenesis (Zohar & Mylonas, 2001). This reproductive dysfunction has been reported already in cultured greater amberjack (Kozul, et al., 2001; Micale, et al., 1999; Mylonas, et al., 2004b), both in breeders maintained in tanks (Micale, et al., 1999; Mylonas, et al., 2004b) and in sea cages (Kozul, et al., 2001; Zupa, et al., 2017b), and has so far barred its large-scale production in Europe. Recently, a comparative study of reproductive development in wild and captive-reared greater amberjack has shown the need for minimum handling of greater amberjack during the reproductive season, as this apparently induced significant long-term reductions in plasma T, E_2 and 17,20β-P (Zupa, et al., 2017b) in the females. As a result, the GSI was significantly reduced at the peak of the reproductive season, and extensive follicular atresia was present in the ovaries. Similarly, significant reductions in plasma T, 11-KT and 17,20β-P were observed in the males at the peak of spermatogenesis, again resulting in significant reductions in GSI (Zupa, et al., 2017b), concomitant with elevations in plasma E_2, reduction in spermatogonial mitosis and high level of apoptosis at the beginning of the reproductive season (Zupa, et al., 2017a).

1.6 Aims of the study

The key to incorporating a species into the aquaculture industry is the cost-effective control of its production processes (Asche & Bjorndal, 2011; Asche, et al., 2009). Therefore, research in understanding the reproductive biology of greater amberjack and developing methods to control reproduction in captivity is the essential first step for the production of seed for production. The first objective of this study was to describe the endocrinological aspects of the brain-pituitary-gonad axis function of the wild sampled fish, compared to their captive counterparts, making available also the data published so far from the same specimens (Zupa, et

al., 2017a; Zupa, et al., 2017b). This was planned in three different times of the reproductive period, the same between captive and wild populations. The next objective was to develop broodstock management and spawning induction methods using controlled-release delivery systems loaded with GnRHa for greater amberjack maintained in tanks and sea cages, in typical Mediterranean aquaculture facilities. Due to the large size of greater amberjack breeders, the two methods have specific advantages. The advantages of sea cage rearing include optimal environmental conditions and welfare, and low cost of fish maintenance, while the advantages of tank rearing include biosecurity, ease of management and handling operations, and control of egg collection. In the course of this study, an alternative "hybrid" method was tested, combining rearing the breeders throughout the year in sea cages, and then putting them in tanks for spawning after hormonal induction. The third objective was to examine two methods for the induction of oocyte maturation/ovulation and spawning in greater amberjack. These methods were based on the induction of endogenous LH release from the pituitary, through the use of GnRHa either in the form of implants (sustained release) or injections (acute release). The efficacy of the two methods was evaluated in terms of oocyte development, spawning kinetics, egg production and quality. Utilizing the knowledge gained above, the next goal was to determine the most efficient GnRHa dose, using the preferred method of administration and examine the extent of the spawning induction period based on the resulting spawning kinetics, egg production and quality. Regarding the males, an objective of the present study was to determine the extent of the spermiation period in greater amberjack maintained in sea cages during the year, characterize sperm quality parameters during the spawning season, and examine any variations over time. Secondly, the effect of a single or double administration of GnRHa implants was evaluated on sperm quality parameters. Finally, the spawning induction method was also evaluated for the first time on F1 generations breeders in Spain to ensure uninterrupted production of fingerlings for genetic breeding programs.

Chapter 2 – Materials and methods

This section outlines the materials and methods used in the preparation of the doctoral dissertation. More specific information for each separate experiment is included within the specific chapters that follow.

2.1 Broodstock use and maintenance

Wild greater amberjacks were collected from the Ionian or Aegean Sea, Greece as juveniles (~300 g in 2010 and earlier) and were maintained at different locations. These included two research facilities in Greece: a) AQUALABS facilities of the Institute of Marine Biology, Biotechnology and Aquaculture (IMBBC) of the Hellenic Centre for Marine Research (HCMR), Heraklion, Crete, Greece, and b) SOUDA facilities, the pilot sea cage farm of the IMBBC at Souda Bay, Chania, Crete. Fish were also maintained at commercial aquaculture facilities: Argosaronikos Fishfarms SA (ARGO), Salamina, Greece; Galaxidi Marine Farm SA (GMF), Galaxidi, Greece; and Forkys Aquacultures SA (FORKYS), Siteia, Crete, Greece. Also, a F1 broodstock was used which was reared in the facilities of the Centro Oceanográfico de Canarias, Instituto Español de Oceanografía (IEO), Tenerife, Spain.

The fish were reared either in sea cages (SOUDA, ARGO and GMF) or in land-based tanks (AQUALABS, FORKYS and IEO) (**Table 2.1**). One hundred seventeen fish tagged with passive integrated transponder (PIT) tags (Avid, UK) were utilized in 2014 (68 fish in sea cages and 49 in tanks) of 6.3-23.8 kg BW; 129 fish were used in 2015 (67 fish in sea cages and 62 in tanks) of 8.6-42.9 kg BW; 111 fish were used in 2016 (57 fish in sea cages and 45 fish in tanks) of 9.8-23.6 kg BW; 111 fish were used in 2017 (57 fish in sea cages and 45 fish in tanks) of 9.8-23.6 kg BW; 23 fish were used in 2018 (all of them in sea cages) of 12.5-22.6 kg BW. All fish were older than 4 years old at the start of the study in 2014, and were considered reproductively mature based on their age and size (Marino, et al., 1995). The greater amberjack breeders were fed with live fish, raw frozen fish, squid, moist extruded or dry extruded feed (Skretting Vitalis CAL, 22 mm), or a combination of the above (**Table 2.1**). Over the course of the 5-year study an effort was made to switch all broodstocks feeding on live/raw fish to the same commercial broodstock diet (dry extruded feed). Live/raw fish may not be an appropriate diet for broodstock, mainly for biosecurity reasons, but also for nutritional ones (*e.g.* variable raw fish quality, rancidity, vitamin content, etc.). However, the fish did not accept dry feed easily and we gradually moved from raw fish, to moist extruded feed and then dry feed. Feed was given 3 to 5 times a week to apparent satiation or to 1-2% of their estimated body weight in case of dry pellets.

Table 2.1 Description of the various greater amberjack broodstocks maintained in sea cages or tanks during the five years of the study.

2014

Stock	Rearing Method	Volume (m^3)	Number of individuals (males/females)	Size at sampling (range in kg)	Feeding
ARGO	sea cages	512	12/16	10.0-16.0	live, raw fish
GMF	sea cages	1018	14/14	6.0-16.0	live fish
SOUDA	sea cages	1018	8/4	7.4-14.8	moist pellets
AQUAL	tanks	40	12/15	6.5-23.8	raw fish, squid
FORKYS	tanks	25	13/9	7.7-10.3	raw fish, squid

2015

Stock	Rearing Method	Volume (m^3)	Number of individuals (males/females)	Size at sampling (range in kg)	Feeding
ARGO	sea cages	1018	12/15	10.7-19.5	moist pellets, raw fish
GMF	sea cages	1018	14/14	9.0-18.0	live fish
SOUDA	sea cages	1018	8/4	9.9-18.4	moist pellets
AQUAL	tanks	40	12/15	8.6-23.8	moist pellets, raw fish
FORKYS	tanks	25	13/8	9.4-15.9	raw fish, squid
IEO	tanks	500	7/7	8.9-42.9	raw fish

2016

Stock	Rearing Method	Volume (m^3)	Number of individuals (males/females)	Size at sampling (range in kg)	Feeding
ARGO	sea cages	1018	14/15	10.7-23.6	dry pellets
GMF	sea cages	1018	14/14	11.8-21.5	live fish
AQUAL	tanks	40	12/14	9.8-18.5	dry pellets
FORKYS	tanks	25	11/8	12.6-20.3	raw fish, squid

2017

Stock	Rearing Method	Volume (m^3)	Number of individuals (males/females)	Size at sampling (range in kg)	Feeding
ARGO	sea cages	1018	14/14	11.8-26.0	dry pellets
GMF	sea cages	1018	13/13	11.5-22.1	dry pellets

2018

Stock	Rearing Method	Volume (m^3)	Number of individuals (males/females)	Size at sampling (range in kg)	Feeding
GMF	sea cages	1018	12/11	12.5-22.6	dry pellets

At the SOUDA, ARGO and GMF facilities, greater amberjacks were maintained in the sea, either in rectangular (8x8x8 m) or round cages (40 m perimeter, 8 m depth). Fish were maintained in tanks of 40 m^3 (AQUALABS), 25 m^3 (FORKYS) and 500 m^3 in volume. Fish were maintained under simulated natural

(AQUALABS) or natural (FORKYS, IEO) photoperiod regime. The tanks were supplied with surface seawater (IEO), seawater from a well with an ambient temperature profile (FORKYS) or from a deeper well with constant water temperature (18-19°C), with the natural temperature profile being simulated using a recirculation system (AQUALABS). Monitoring of pH, NH_3-N and NO_2-N in the recirculation system was conducted once a week (pH: 6.79-7.86, NH_3-N: 0.01-1.63 mg l^{-1}, NO_2-N: 0.000-0.150 mg l^{-1}), while temperature and dissolved oxygen (D.O.: 70-99%) in all facilities were measured from 1 to 7 times a week.

In addition to the fish used in the breeding experiments, 33 (14 males and 19 females) wild and 24 (12 males and 12 females) captive-reared greater amberjack breeders were sampled at three different phases of the reproductive cycle in 2014 and 2015, which were predetermined according to the available literature (Mandich, et al., 2004) as was described previously (Pousis, et al., 2018; Pousis, et al., 2019; Zupa, et al., 2017a; Zupa, et al., 2017b). Wild fish were commercially caught around the Pelagie Islands (Sicily, Italy), during the fishing seasons of 2014 and 2015 and sampled on board immediately after death. Captive-reared individuals were captured from the wild in 2011 in the area of Astakos (Ionian Sea, Greece). In September 2013, the fish were transferred to a sea cage in ARGO, where they were reared for two years according to standard farming practices, as was described previously (Zupa, et al., 2017b). Fish were sampled for biometric data (fork length (FL), BW, gonad mass (GM)) and blood, gonads and pituitaries were collected and preserved. The gonado-somatic index was calculated as GSI = 100 GM BM^{-1}.

2.2 Fish handling during samplings

Fish were initially tranquilized after a 2-day starvation period in their tank or in an anesthesia sack (20-40 m^3 in volume) within their sea cage, with the use of either clove oil (0.01ml l^{-1}) or 2-phenoxyethanol (0.15 ml l^{-1}). Then, the fish were transferred to a separate tank for complete sedation with a higher concentration of clove oil (0.03ml l^{-1}) or 2-phenoxyethanol (0.4 ml l^{-1}) (Mylonas, et al., 2005) and ovarian biopsies for the evaluation of oocyte development were obtained by inserting a plastic catheter (Pipelle de Cornier, Laboratorie CCD, France) and applying gentle aspiration. Sperm samples were also obtained by inserting a cannula at the base of the genital pore and applying gentle aspiration, as described above for the females.

Fish that were maintained in tanks during the year, they were returned to the same tank after reproductive evaluation (AQUALABS, FORKYS, IEO). For breeders that were maintained in sea cages during the year, two approaches were examined. In the first approach, the breeders were moved back to a round sea cage after reproductive evaluation (ARGO, GMF, SOUDA), which, in case of spawning induction experiments, was equipped with a passive egg-retaining device (see later for description). In the second approach, immediately the selected breeders after GnRHa administration were transferred to land-based tanks (ARGO, GMF). For transferring to land a service boat was employed, transferring the already anesthetized breeders one-by-one in a 1000-l tank containing seawater with a low dose of anesthetic (0.01ml l^{-1} clove oil or 0.15 ml l^{-1} 2-phenoxyethanol). Regardless of the final destination (tank, cage), care was taken with each fish

to recover from anesthesia. After the cessation of spawning (1-4 weeks after) depending on each experiment, all fish were re-evaluated for reproductive stage and where returned to their original rearing facilities (tanks or sea cages).

2.3 Blood, pituitary and gonad collection

As mentioned above, wild fish were commercially caught around the Pelagie Islands (Sicily, Italy), during the fishing seasons of 2014 and 2015 and sampled on board immediately after death for FL, BW, GM, and blood, pituitary and a part of the gonad were collected. Captive-reared individuals were captured from the wild in 2010-2011 in the area of Astakos (Ionian Sea, Greece). In September 2013, the fish were transferred to a sea cage in ARGO, where they were reared for two years according to standard farming practices, as was described previously (Zupa, et al., 2017b). All the captive-reared fish to be sampled were confined in a small cage area using a PVC sack and anesthetized lightly with about 0.01 ml l^{-1} clove oil. The fish were then gently guided into a PVC stretcher, brought on board of a service vessel and anesthetized deeply with about 0.03 ml l^{-1} clove oil. After blood collection, fish were then placed on crushed ice and transferred to the farm facility where biometric data (FL, BW, GM), gonads and pituitaries were collected and preserved. Plasma was separated from the blood by centrifugation (5000 rpm for 5 min) and stored at -80°C for further analyses. Pituitaries were stored at -80°C for further analyses. A part of the gonad was stored in fixative solution for further histological processing.

2.4 Oocyte evaluation

Ovarian biopsies were obtained by inserting an endometrial catheter (Pipelle de Cornier) into the ovarian cavity applying gentle aspiration. A wet mount of the biopsy was first examined under a compound microscope (40 and 100×) to evaluate the stage of oogenesis and measure the mean diameter of the largest, most advanced vitellogenic oocytes (n = 10), and microphotographs were taken for further evaluation. A portion of the biopsies was fixed in a solution of 4% formaldehyde-1% glutaraldehyde for histological analysis. Females were considered eligible for spawning induction if they contained fully vitellogenic oocytes with a diameter >600 μm.

2.5 Sperm production and quality evaluation

Sperm samples were obtained by inserting a cannula at the base of the genital pore and applying gentle aspiration, as described above for the females. Care was taken to avoid contamination of sperm with urine or feces, and prior to sampling the genital pore was rinsed and blot dried to avoid introducing water into the collected sperm. Milt was kept on ice until evaluation of various quality parameters, which were done within 2-3 hours from collection. Males were considered eligible for spawning induction if milt could be obtained with the cannula.

The quantity of produced sperm was evaluated according to spermiation condition –which is a measure of the available milt in the testes- after gentle abdominal pressure was applied, determined by a subjective scale from 0 to 3, as follows: Spermiation index S0 = no milt released, S1= only a drop of milt released after multiple stripping attempts, S2= milt was released easily after the first stripping attempt and S3= copious amounts of milt released with very little pressure. Sampled sperm was stored in 1.5 ml micro-centrifuge tubes, which were then placed on ice and transferred to a 4°C refrigerator until evaluation. Sperm evaluation was done either using a subjective method or CASA.

The sperm quality parameters that were evaluated using the subjective method included: (a) sperm density (number of spermatozoa ml^{-1} (szoa ml^{-1}) of milt), (b) initial percentage of spermatozoa showing forward motility immediately after activation with seawater (spermatozoa motility, %), (c) duration of forward spermatozoa motility of ≥5% of the spermatozoa in the field of view (motility duration, min) and (d) survival of spermatozoa under cold storage at 4°C (spermatozoa survival, days). Spermatozoa density was estimated after a 2121-fold dilution with 0.9% saline using a Neubauer haemocytometer under 200x magnification (in duplicate) under a compound light microscope. Spermatozoa motility and motility duration were evaluated on a microscope slide (400x magnification) after mixing 1 μl of sperm with a drop of about 50 μl of seawater (in duplicate). Spermatozoa motility (%) was determined subjectively using increments of 10%. Milt was stored at 4°C, and was examined every other day for spermatozoa motility, until less than 5% of the spermatozoa were observed to be motile, in order to estimate spermatozoa survival time (days).

Milt samples that were assessed using CASA (ISAS, Spain) were activated in seawater containing 2% bovine serum albumin (BSA) (1:201 or 1:334) to obtain 200-300 cells in the field and spermatozoa movement was recorded on a disposable counting chamber with a fixed depth (Leja) using the digital camera The Imaging Source DMK 22BUC03 with a resolution of 744x480 pixels at 30 fps attached to a light microscope (Zeiss Primo Star) under 200x magnification, using dark field microscopy, immediately after milt collection. The analyzed parameters were curvilinear velocity (VCL), straight line velocity (VSL), average path velocity (VAP) (μm sec^{-1}), motile cells, progressive cells (>80% straightness (STR)), rapid cells and STR (%). The software settings were adjusted to: 1 to 90 μm for head area; VCL <10 μm sec^{-1} to classify a spermatozoon as immotile; and spermatozoa were considered rapid when VCL was higher than 100 μm sec^{-1}.

2.6 Egg collection and evaluation of egg/larval quality

The egg collection device for the sea cages consisted of a two-section plastic curtain deployed around the inside perimeter of the cage. This is a passive trapping device, which restricts the movements of floating eggs within the cage, as has been used previously for bluefin tuna in Japan and the Mediterranean Sea (De Metrio, et al., 2010; Mylonas, et al., 2007; Sawada, et al., 2005). The “lower” section started at about 30 cm above the water line and goes down to about 3 m in depth, and was secured on the net of the cage throughout its perimeter through portholes in the tarpaulin every 30 cm. The “upper” section was hanging from the rails of the cage using ropes every 30 cm along the perimeter of the cage, and drapes down the cage over the lower

section, overlapping with the top 1.5 m below the water surface. The objective of this two-piece design was to allow wind pressure to be relieved by allowing the upper section on the windward side to lift above the water, while the leeward side is pushed tightly against the net and the lower section, thus preventing the floating eggs from "jumping" over the cage and being lost. The cages were examined for the presence of eggs every day beginning at dawn, and egg collection continued approximately every hour until no more eggs were collected. Tank overflows were fitted with 250-L passive egg collectors and were examined three times a day (8:00 a.m., 3:00 p.m. and 8:00 p.m.). For each spawn, the date, collection time and developmental stage of the eggs (Tachihara, et al., 1993) were recorded, in order to identify different spawns and estimate an approximate spawning time. This allowed calculating the time between hormonal administration and first spawn (latency period). The eggs were collected and transferred into a 10-L bucket. Their number (fecundity) was estimated by counting the total number of eggs in a sub-sample of 10 mL, collected with a pipette after vigorous agitation. Daily relative fecundity was calculated as the number of eggs produced every day in each tank per female biomass, considering the eligible females that had vitellogenic oocytes >600 μm and were expected to spawn after each GnRHa administration. Total relative fecundity was calculated as the number of eggs produced after each hormonal administration per female biomass in each tank, as above. The fertilization percentage was evaluated at the same time by examining each egg in the subsample. After collecting the sub-sample, the eggs were transferred into a 500 L conical tank-incubator fitted with an overflow filter (250 μm mesh size), and supplied with surface seawater (~90% h^{-1} renewal) and mild aeration. The embryonated eggs were then sent to different hatcheries for larval rearing trials (Mylonas, et al., 2016a).

To monitor embryo and larval survival, embryonated eggs from each spawn were collected from the tank incubators and placed individually in 96-well microtiter (mct) plates (in duplicates) according to the procedure of Panini et.al (2001) with some modifications. Briefly, a sample of floating (~100% fertilized) eggs were taken from the tank incubators with a 250 μm mesh sieve, rinsed with seawater and poured in 2 L beakers filled with seawater. Using the sieve, 100-200 floating eggs were scooped from the beaker and placed in a Petri dish. Together with 200 μL of seawater, the fertilized eggs were aspirated with a micropipette one by one and transferred individually to the 96-wells of a mct plate. The plates were checked under a stereoscope and any dead eggs were replaced. Once loaded, the mct plates were covered with a plastic lid, placed in a controlled-temperature incubator and maintained for 7 days, according to the spawning temperature of each batch. Using a stereoscope, embryonic and early larval development was evaluated daily, recording the number of live embryos 24 hours after egg collection (or ~30 hrs after spawning), hatched larvae (examined ~55 hrs after spawning) and viable larvae on day 5 after hatching (near the time of yolk sack absorption). At 21.0-23.5°C, hatching of the greater amberjack eggs took place in 40-55 hrs (39.2 – 48.1 degree-days). The embryo survival was calculated as the number of eggs having live embryos 1 d after egg collection/number of fertilized eggs initially loaded in the mct plates. The hatching success was calculated as the number of hatched larvae/24-hr embryos, and the 5-d larval survival was calculated as the number of live larvae 5 d after hatching.

Estimating survival percentage (%) by using as denominator the number of individuals that survived to the previous developmental stage was considered as a more accurate evaluation of survival within specific developmental stages, without the potential of a distortion effect of the previous stage.

2.7 Hormonal evaluations

Pituitaries were homogenized individually in 200 µl of cold ultra-pure H_20 and used for RNA extraction (60 µl) and GtH measurements (140 µl). The latter fraction was diluted (v:v) with 140 µl of PBS and separated in two aliquots and stored at -80°C for further analyses. Pituitary and plasma LH levels were measured using the heterologous enzyme–linked immunosorbent assay (ELISA) developed for striped bass LH (Mañanos, et al., 1997) and was modified for tuna species (Berkovich, et al., 2013; Rosenfeld, et al., 2012) and validated for the greater amberjack. The related pituitary and plasma FSH levels were measured similarly using a homologous ELISA that was developed in the framework of the DIVERSIFY project. Briefly, the recombinant greater amberjack FSH (*rec-ga*FSH) was produced using the *Pichia pastoris* yeast recombinant DNA expression system (Invitrogen, Carlsbad, CA). Using Ni-Affinity Chromatography (Amersham Biosciences), the rec-gaFSH was purified and used as antigen for both standardization and generation of the specific polyclonal antibodies.

Measurements of sex steroids were the subject of a different study (Zupa, et al., 2017b). However, they are presented also here in order to discuss the differences that occur in wild and captive-reared fish along the reproductive axis in relation with the FSH and LH, and the reproductive dysfunctions that were observed. Briefly, for the quantification of E_2, T, 11-KT and 17,20β -P in the plasma, already established and well-described ELISAs were used with some modifications. For steroid extraction, 200 µl of plasma were extracted twice with 2 ml diethyl ether. Extraction was done by vigorous vortexing (Vibramax 110, Heidolph, Germany) for 3 min. After vortexing, samples were frozen for 10 min at -80ºC and the supernatant organic phase was collected in new tubes and evaporated under a stream of nitrogen (Reacti-vap III, Pierce, Germany). Samples were reconstituted in reaction buffer for running in the ELISA.

2.8 Histological evaluations

Histological evaluation of the gonads of the sacrificed fish was the subject of a different study (Zupa, et al., 2017b). However, they are presented also here in order to discuss the differences that occur in wild and captive-reared fish along the reproductive axis in relation with the FSH and LH, and the reproductive dysfunctions that were observed. Briefly, for the histological analysis of greater amberjack ovaries and testes of the sacrificed fish, 1-cm thick gonad slices were cut and fixed in Bouin's solution, dehydrated in ethanol, clarified in xylene and embedded in paraffin wax. Five-µ m thick sections were then stained with haematoxylin-eosin and Mallory's trichrome.

Ovarian biopsies, before embedding in methacrylate resin (Technovit 7100®, Heraeus Kulzer, Germany) were dehydrated in gradually increasing ethanol solutions (70-96%). Serial sections of 3 µm were

obtained with a microtome (Leica RM 2245, Germany). Sections were stained with Methylene Blue (Sigma, Germany)/Azure II (Sigma, Germany)/Basic Fuchsin (Polysciences, USA) according to Bennett et al. (Bennett, et al., 1976). Sections were examined under a light compound microscope (Nikon, Eclipse 50i) and photographed with a digital camera (Jenoptik progress C12 plus).

Chapter 3 – Reproductive dysfunction of greater amberjack in captivity

This chapter will be submitted as "Fakriadis, I., Meiri, I., Bracha, C., M., Corriero, A., Mylonas, C.C. and Rosenfeld, H. 2021. Gonadotropin expression, pituitary and plasma levels in the reproductive cycle of wild and captived-reared greater amberjack (Seriola dumerili). General and Comparative Endocrinology".

3.1 Introduction

Aquaculture is expected to contribute continually more to the gap between aquatic food demand and supply, as it is more environmental friendly protein production systems -low freshwater use, high feed conversion ratios, low carbon footprints- compared to livestock farming (Nijdam, et al., 2012). Control of reproduction has been recognized as one of the major bottlenecks in the domestication process of fish species (Mylonas, et al., 2010; Zohar & Mylonas, 2001). Almost 70% of the 250 farmed finfish species in the world are dependent to a certain extend to wild resources and only a few species can be considered fully domesticated, and this is mainly due to the lack of control of their reproduction (Teletchea & Fontaine, 2014).

To control reproductive maturation, the brain integrates (a) endogenous signals, such as developmental stage, size, nutritional status and energy stores, and (b) environmental signals, such as photoperiod, temperature, habitat conditions, presence of partners, resulting in the positive or negative regulation of the activity of GnRH, the brain hormone controlling the reproductive axis in vertebrates (Karine, et al., 2020; Munoz-Cueto, et al., 2020; Parker & Cheung, 2020). Then, GnRH binds to the respective receptors expressed by the gonadotropic cells of the pituitary, and stimulates the synthesis and release of FSH and LH, which are considered the key factors regulating gonadal function (Levavi-Sivan, et al., 2010; Zohar, et al., 2010). The FSH and LH are heterodimers with a common α subunit and differing in their β subunits, and LHβ is more conserved compared to FSHβ (Levavi-Sivan, et al., 2010; Rosenfeld, et al., 2007). In salmonids, which are synchronous spawning fishes, FSH has a predominant role in vitellogenesis/spermatogenesis while LH is mainly involved in the final stages of maturation (oocyte maturation and spermiation). However, in asynchronous or group synchronous spawning fishes the situation is more complex and the roles of the two GtHs without a clear pattern (Rosenfeld, et al., 2007). Therefore, species-specific studies are needed to discriminate the specific unction of FSH and LH. At the gonadal level, E_2 is considered responsible for the oocyte growth while the progestins 17, 20β-P and/or 20βS function as maturation-inducing hormone to induce oocyte maturation (Levavi-Sivan, et al., 2010). In males, T and mainly 11-KT are responsible for spermatogenesis and spermiogenesis, while 11-KT have a peak before the onset of spermiation period. The progestins (17, 20β-P or 20βS) induce sperm maturation and release (Vizziano, et al., 2008).

The greater amberjack is a very promising species for aquaculture diversification (Crespo, et al., 1994; Lazzari, et al., 2000; Paxton, et al., 1989). The species exhibits reproductive dysfunction in captivity, since captive-reared breeders in the Mediterranean maintained either in tanks (Micale, et al., 1999; Mylonas, et al.,

2004b) or in sea cages (Kozul, et al., 2001; Zupa, et al., 2017b) failed to reproduce. Recently, a comparative study of reproductive development in wild and captive-reared greater amberjack has shown the need for minimum handling of greater amberjack during the reproductive season, as this apparently induced significant long-term reductions in plasma T, E_2 and 17,20β-P (Zupa, et al., 2017b) in the females. As a result, the GSI was significantly reduced at the peak of the reproductive season, and extensive follicular atresia was present in the ovaries. Similarly, significant reductions in plasma T, 11-KT and 17,20β-P were observed in the males at the peak of spermatogenesis, again resulting in significant reductions in GSI (Zupa, et al., 2017b), concomitant with elevations in plasma E_2, reduction in spermatogonial mitosis and high level of apoptosis at the beginning of the reproductive season (Zupa, et al., 2017a).

The aim of this study was to compare the endocrinological status of the pituitary-gonad axis of captive and wild greater amberjacks during the reproductive season. For this purpose, the FSH and LH levels of greater amberjack in the pituitary and plasma were measured, as an important tool when investigating reproductive vertebrate physiology (Levavi-Sivan, et al., 2010; Weltzien, et al., 2004). The obtained data were examined in relation to the recently published data of GSI, sex steroid levels and gonadal histology (Zupa, et al., 2017a; Zupa, et al., 2017b) and a more comprehensive analysis is provided on the endocrine dysfunction observed when rearing greater amberjack in captivity.

3.2 Materials and methods

3.2.1 Fish maintenance, sampling and welfare

A total of 33 (14 males and 19 females) wild and 24 (12 males and 12 females) captive-reared greater amberjack breeders (**Table 3.1 & 3.2**) were sampled at three different phases of the reproductive cycle in 2014 and 2015, which were predetermined according to the available literature (Mandich, et al., 2004) as was described previously (Pousis, et al., 2018; Pousis, et al., 2019; Zupa, et al., 2017a; Zupa, et al., 2017b): early gametogenesis (EARLY, late April-early May, wild females n=5, wild males n=5, captive females n=4, captive males n=4), advanced gametogenesis (ADVANCED, late May-early June, wild females n=2, wild males n=4, captive females n=4, captive males n=4) and spawning (SPAWNING, late June-July, wild females n=12, wild males n=5, captive females n=4, captive males n=4). Wild fish were commercially caught around the Pelagie Islands (Sicily, Italy), during the fishing seasons of 2014 and 2015 and sampled on board immediately after death. Captive-reared individuals were captured from the wild in 2011 in the area of Astakos (Ionian Sea, Greece). In September 2013, the fish were transferred to a sea cage in ARGO, where they were reared for two years according to standard farming practices, as was described previously (Zupa, et al., 2017b). In 2015, fish were sampled for biometric data (FL, BM, GM) and blood, gonads and pituitaries were collected and preserved. The GSI was calculated as GSI = 100 GM BM^{-1}. After histological examination of the gonads, fish rearranged in different reproductive stages according their reproductive development and the criteria set by

Brown-Peterson et al. (2011) as developing, spawning capable, regressed and regenerating and the statistical analysis that followed was based on this categorization (**Table 3.1 & 3.2**).

Table 3.1 Biometric data (mean±SD) of captive and wild greater amberjack females sampled during the reproductive season in the Mediterranean Sea (modified by (Zupa, et al., 2017b)). Reproductive stage evaluation was done after histological examination of the ovaries following the criteria set by Brown-Peterson et al. (2011).

Fish origin	N	Fork length (cm)	Body mass (kg)	GSI (%)	Reproductive stage
captive	4	95±6	12.9±1.9	1.0±0.2	developing
wild	5	108±6	16.3±3.2	1.1±0.3	developing
captive	2	99±3	12.6±0.4	6.2±1.2	spawning
wild	14	103±7	14.2±3.6	4.8±1.5	spawning
captive	4	99±5	13.3±2.3	1.7±0.7	regressed
captive	2	94±3	10±2.5	1.1±0.0	regenarating

Table 3.2 Biometric data (mean±SD) of captive and wild greater amberjack males sampled during the reproductive season in the Mediterranean Sea (modified by (Zupa, et al., 2017b)). Reproductive stage evaluation was done after histological examination of the testes following the criteria set by Brown-Peterson et al. (2011).

Fish origin	N	Fork length (cm)	Body mass (kg)	GSI (%)	Reproductive stage
captive	4	95±4	13.1±1.5	0.5±0.1	developing
wild	5	113±2	17.3±2.3	2.3±0.4	developing
captive	3	97±7	12.4±2.6	4.4±0.4	spawning
wild	9	105±9	14.7±3.9	6.5±2.4	spawning
captive	5	95±2	11.8±1.6	1.3±0.5	regressed

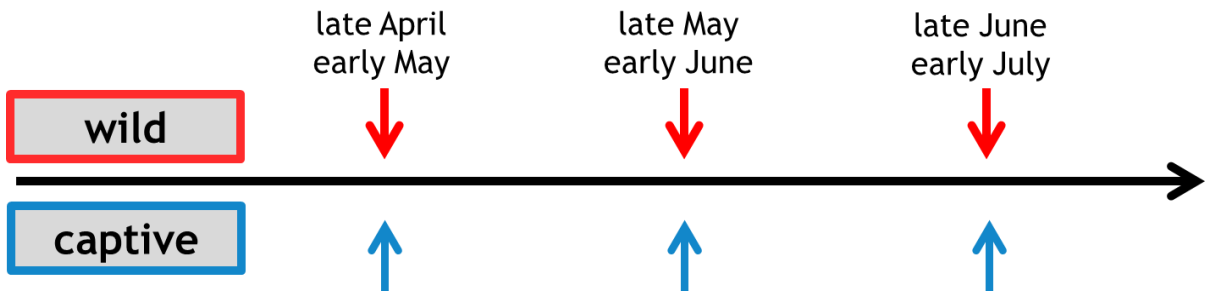

Figure 3.1 Schematic representation of the samplings in wild and captive populations of greater amberjack broodstocks.

For the present study, as described previously (Pousis, et al., 2018; Pousis, et al., 2019; Zupa, et al., 2017a; Zupa, et al., 2017b) ethical approval was not required because this study did not fall within the obligations contained in the Italian decree n. 26 of 04 March 2014 and Greek presidential decree 56/2013

regarding the permission to carry out research studies on experimental animals, as the fish came from a registered aquaculture facility and from commercial catches. The research did not involve any experiments on alive animals. Captive-reared fish originally came from the fishery at 0+ year of age, and were then reared at a registered aquaculture facility for 3 years, according to routine farming practices, before they were recruited for this study, sacrificed and sampled. Each fish was first anaesthetized with clove oil for 10 minutes and then painlessly sacrificed by decapitation. Wild greater amberjack were captured by the commercial purse seine fishing vessel "Graziella" authorized to catch pelagic fish by the port authority of Porto Empedocle (Agrigento, Italy). No specific permission was required because these fish were commercially caught during routine fishing operations, placed on ice by the fishermen and left to die. Immediately after death, those fish considered suitable for the present study were purchased and sampled on board. The greater amberjack is classified as "Least Concern" in the IUCN Red List of Threatened Species (Smith-Vaniz, et al., 2015).

3.2.2 Pituitary and plasma concentrations of gonadotropins

Pituitaries were homogenized individually in 200 µl of cold ultra-pure H_20 and used for RNA extraction (60 µl) and GtH measurements (140 µl). The latter fraction was diluted (v:v) with 140 µl of PBS and separated in two aliquots and stored at -80°C for further analyses. Plasma was separated from the blood by centrifugation (5000 rpm for 5 min) and stored at -80°C for further analyses.

Pituitary and plasma LH levels were measured using the heterologous ELISA developed for striped bass LH (Mañanos, et al., 1997) and was modified for tuna species (Berkovich, et al., 2013; Rosenfeld, et al., 2012) and validated for the greater amberjack. For that purpose, pituitary extract derived from a captive amberjack female was assayed at four serial dilutions (1:50, 1:100, 1:200 and 1:400) and plasma samples of two wild caught fish were assayed at three serial dilutions (1:8, 1:16, 1:32). A clear linearity was obtained in the dilution of the pituitary of the captive amberjack female and the plasma samples derived from wild fish. Moreover, the dilution curves exhibited parallelism with the standard rLH enabling the determination of LH in this species. The sensitivity of the assay was 0.65 ng ml^{-1} and the respective inter- and intra- assay coefficients of variation were 8% and 15%, respectively. Ninety-six well polystyrene plates were coated with recombinant LH (rLH; 2.4 ng $well^{-1}$) and incubated overnight at 4ºC. The plates were then washed with PBST and blocked with BSA (2% in PBST; 100 µl $well^{-1}$) for 0.5 h at 37ºC. The primary antibody (anti-striped bass LH) was diluted 1:80,000 in PBST containing 2% normal goat serum (NGS). Samples and standards were serially diluted in PBST, mixed with the primary antibody solution (v:v in 1.5 ml tubes) and incubated overnight at 4ºC. Then the content in each tube was dispensed into the antigen-coated wells (100 µl $well^{-1}$ in duplicates). Following incubation (overnight at 4ºC) AffiniPure Goat anti-Rabbit IgG (H+L) (Jackson Immunoresearch Laboratories Inc.) in 1% NGS-PBS T was added (100µl $well^{-1}$) for 0.5 h at 37ºC. Then, the wells were washed and SureBlueTM TMB-microwell peroxidase substrate (1-component) (KPL, MD, USA) was added (100 µl $well^{-1}$). The reaction was stopped after 20 to 40 min at room temperature by the addition of 100µl of 1N phosphoric acid and the absorbance was read at 450 nm.

The related pituitary and plasma FSH levels were measured similarly using a homologous ELISA that was developed in the framework of the DIVERSIFY project. Briefly, the recombinant greater amberjack FSH (rec-gaFSH) was produced using the *Pichia pastoris* yeast recombinant DNA expression system (Invitrogen, Carlsbad, CA). Using Ni-Affinity Chromatography (Amersham Biosciences), the rec-gaFSH was purified and used as antigen for both standardization and generation of the specific polyclonal antibodies. The standard curve ranged from 100 ng ml^{-1} to 0.19 ng ml^{-1}. The intra-assay coefficient of variation for standard of 10 ng ml^{-1} in the same plate was 3.0% (n = 10). The inter-assay coefficient of variation for the same plasma sample on different plates was 9.6% (n = 7).

3.2.3 Pituitary gonadotropin gene expression levels

Total RNA was obtained from pituitary using the RNeasy Mini Kit (Qiagen) as described by the manufacturer. The RNA was re-suspended in 50 µl of RNase free water and stored at –80°C. The cDNA was prepared from 1.2 µg total RNA. Random hexamer primers were used for the cDNA synthesis using U SuperScript III Reverse Transcriptase (Invitrogen). Two µg of DNAse treated total RNA were reverse transcribed with random primers using the High Capacity cDNA Reverse Transcriptase kit (Applied Biosystems, Branchburg, NJ) according to manufacturer's protocol. Quantitative real-time polymerase chain reaction (qPCR) was performed in duplicate in 10 µl reaction volumes consisting of Fast SYBR Green Master Mix (Applied Biosystems). Amplification was carried out in a Fast Real Time PCR System (Applied Biosystems). Cycling parameters were as follows: 3s at 95°C, and 40 cycles of 3s at 95°C and 30s at 60°C. The presence of a single amplicon was verified using a melting curve run following the PCR. To normalize the levels of target genes, qPCR for rRNA 18S was also performed with the sample cDNAs. A negative control with sterile water as template was included in order to check for possible reagent contamination. In addition, in order to rule out the presence of contaminating genomic DNA, our qPCR experiments included minus-reverse transcriptase controls (i.e., PCR amplification using DNAse-treated total RNA samples without reverse transcription as a template). The results were analyzed by 7500 Fast Real-Time PCR System software (Applied Biosystems). Gene expression levels were calculated by: relative expression = $2^{-\Delta\Delta Ct}$, Ct – threshold cycle (Livak & Schmittgen, 2001). The gene specific primers (GSPs) designed using the Primer Express 3.0 software (Applied Biosystems).

3.2.4 Sex steroid plasma levels

Measurements of sex steroids were the subject of a different study (Zupa, et al., 2017b). However, they are presented also here in order to discuss the differences that occur in wild and captive-reared fish along the reproductive axis in relation with the FSH and LH, and the reproductive dysfunctions that were observed. Briefly, for the quantification of E_2, T, 11-KT and 17,20β -P in the plasma, already established and well-described ELISAs were used with some modifications. For steroid extraction, 200 µl of plasma were extracted twice with 2 ml diethyl ether. Extraction was done by vigorous vortexing (Vibramax 110, Heidolph, Germany) for 3 min. After vortexing, samples were frozen for 10 min at -80ºC and the supernatant organic phase was

collected in new tubes and evaporated under a stream of nitrogen (Reacti-vap III, Pierce, Germany). Samples were reconstituted in reaction buffer for running in the ELISA.

3.2.5 Histological analysis

Histological evaluation of the gonads of the sacrificed fish was the subject of a different study (Zupa, et al., 2017b). However, they are presented also here in order to discuss the differences that occur in wild and captive-reared fish along the reproductive axis in relation with the FSH and LH, and the reproductive dysfunctions that were observed. Briefly, for the histological analysis of greater amberjack ovaries and testes of the sacrificed fish, 1-cm thick gonad slices were cut and fixed in Bouin's solution, dehydrated in ethanol, clarified in xylene and embedded in paraffin wax. Five μm thick sections were then stained with haematoxylin-eosin and Mallory's trichrome.

3.2.6 Statistical analysis

Differences in the measured parameters were tested using a one-way (analysis of variance) ANOVA followed by Tukey's HSD post hoc test. Data were transformed accordingly to meet the one-way ANOVA assumptions, if not normally distributed. A level of $P \leq 0.05$ was set as minimum statistical significance for the ANOVA tests. Statistical analyses were performed with JMP 12 (SAS Institute Inc., Cary, NC, USA). Results are presented as mean ± standard error (SEM), unless mentioned otherwise.

3.3 Results

Both captive and wild females sampled at late April or early May were found to be in a developing reproductive stage at temperatures between 17.5 to 18.1ºC. All females had mostly POs and CA oocytes (**Fig 3.1**) with few early Vg (eVg) existing. One month later (late May – early June), 100% of wild females were at spawning capable reproductive stage, since late Vg oocytes and post ovulatory follicles (POFs) were observed (Zupa, et al., 2017b). At this time, 50% of the captive females were at the same spawning capable stage and with increased percentage of AT and presence of POFs. The remaining captive females were already regressed with even higher percentage of AT and Vg oocytes of smaller diameters. At temperatures 23.4 - 23.8ºC in late June – early July, all wild females were at spawning capable reproductive stage having late Vg oocytes (**Fig 3.1**) and 17% of them were found to be in maturation phase. No AT was recorded while POFs were visible in almost all fish sampled. Half of the captive females at this time were characterized as regressed with extensive AT concomitantly with a few eVg or midVg oocytes, while the rest of the females were already regenerating their ovaries (**Fig 3.1**).

Both captive and wild males were in a developing reproductive stage in late April – early May. However, one month later 25% of the captive males already ceased their spermatogenic activity and were characterized as regressed. The remaining 75% of the sampled captive males that were characterized as spawning capable had less sz in the lumen of the testicular lobules compared to their wild counterparts, and the area covered by the lobules in the testes was smaller. On the contrary, 100% of the wild males at the same

time were at full spermatogenic activity. Similarly, wild males sampled a month later in late June – early July were categorized as spawning capable, while the testes of captive fish showed clear signs of regression were observed (**Fig 3.1**). Spermatogenesis had ceased and thick somatic tissue was visible among the spermatocysts.

Significant differences in pituitary FSH (one-way ANOVA, P=0.024) and LH (one-way ANOVA, P<0.001) content between spawning and developing wild females was observed, as well as between captive and wild females at spawning capable reproductive stage (one-way ANOVA, P=0.025) (**Fig. 3.2**). However pituitary *fshβ* and *lhβ* expression levels and plasma FSH and LH remained unchanged both for the captive and wild females among the different reproductive stages examined (one-way ANOVA, P>0.05). The only recorded significant difference was in plasma FSH levels between the captive and wild females at spawning capable reproductive stage (one-way ANOVA, P=0.041) (**Fig. 3.2**).

The reported sex steroids in the females exhibited significant differences among different reproductive stages in plasma of E_2 (one-way ANOVA, P=0.002), 17, 20β-P (one-way ANOVA, P<0.001) and T (one-way ANOVA, P=0.011) of captive females, but only in T (one-way ANOVA, P<0.001) of wild females (**Fig. 3.2**). Specifically, plasma E_2 and T of captive females decreased at the degeneration phase, while 17, 20β-P increased in spawning capable femaels and remained unchanged at the later stages. In the wild fish, plasma T increased from developing to spawning capable reproductive stage. Significant differences between the captive and wild females were observed for plasma T at spawning capable (one-way ANOVA, P=0.005) and for 17, 20β-P (one-way ANOVA, P=0.003) at developing stage. Finally, GSI increased significantly both for the captive (one-way ANOVA, P<0.001) and wild (one-way ANOVA, P<0.001) females at spawning capable stage compared to the other stages of reproductive development (**Fig. 3.2**).

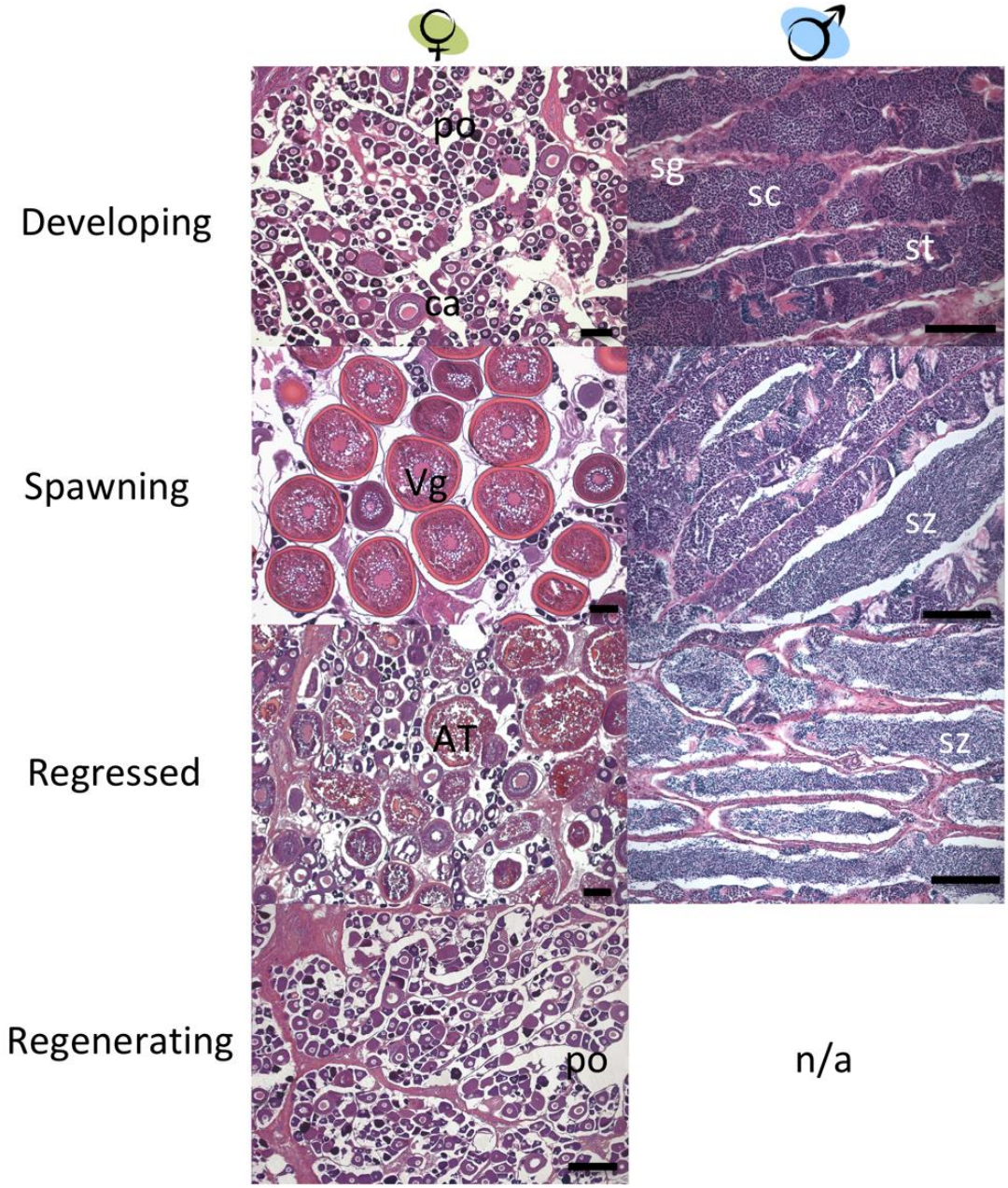

Figure 3.1 Microphotographs of representative ovarian or testicular tissues at different reproductive stages of wild or captive females and males of greater amberjack *Seriola dumerili* after histological processing. po: primary oocyte, ca: oocyte at cortical alveoli stage, Vg: late vitelogenesis oocyte, AT: atretic follicle, sg: spermatogonia, sc: spermatocyte, st: spermatid and sz: spermatozoa. Female bar = 150μm, Male bar = 100μm

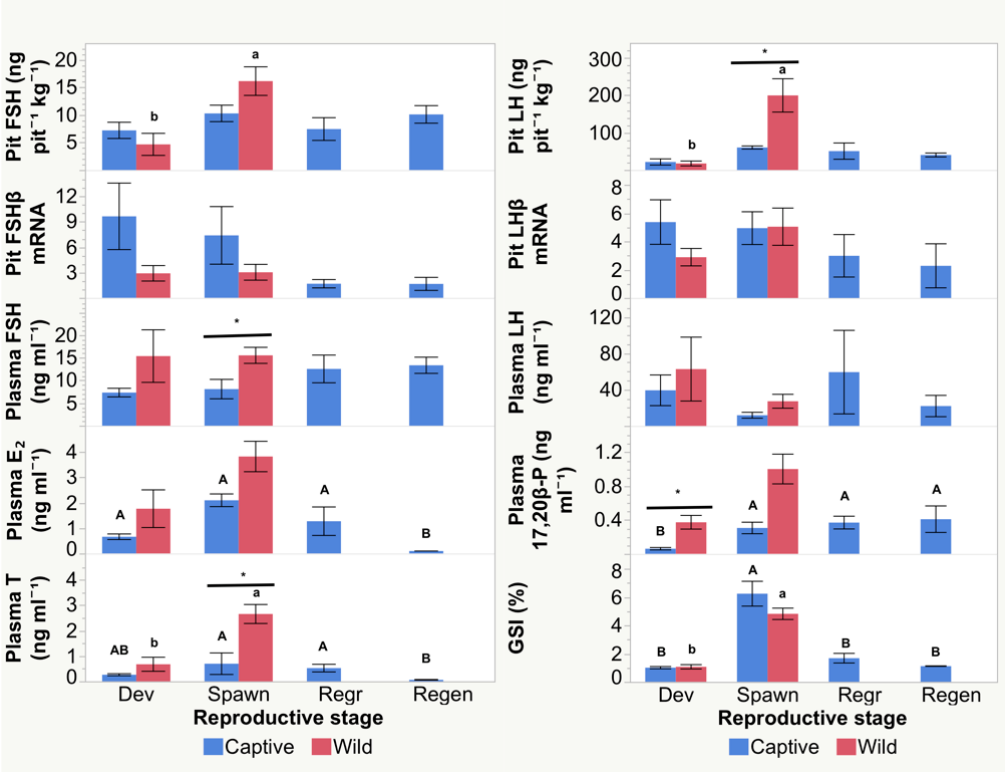

Figure 3.2 Mean (± SEM) pituitary follicle stimulating hormone (FSH)(ng pit^{-1} kg^{-1}), pituitary luteinizing hormone (LH) (ng pit^{-1} kg^{-1}), pituitary *fshβ* mRNA levels, pituitary *lhβ* mRNA levels, plasma FSH (ng ml^{-1}), plasma LH (ng ml^{-1}), plasma 17-β Estradiol (E_2)(ng ml^{-1}), 17,20β-dihydroxy-4-pregnen-3-one (17,20β-P)(ng ml^{-1}), testosterone (T)(ng ml^{-1}) and gonadosomatic index (GSI)(%) of captive (n=12) and wild (n=19) female greater amberjack at different reproductive stages. Capital letters above the means indicate statistically significant differences among reproductive stages of captive females (one-way ANOVA, Tukey's HSD, P≤0.05). Lowercase letters above the means indicate statistically significant differences among reproductive stages of wild females (one-way ANOVA, Tukey's HSD, P≤0.05). Asterisks above the horizontal lines indicate statistically significant differences between captive and wild females at the same reproductive stage (one-way ANOVA, Tukey's HSD, P≤0.05). Reproductive stages: Dev – Developing, Spawn – Spawning capable, Regr – Regressed, Regen – Regenerating.

Regarding the males, no significant differences were observed in pituitary FSH content, pituitary *fshβ* and *lhβ* expression or plasma FSH and LH levels among the reproductive stages of either captive or wild males (one-way ANOVA, P>0.050) (**Fig. 3.3**). However, significantly higher levels of plasma FSH (one-way ANOVA, P=0.045) and LH (one-way ANOVA, P=0.043) were recorded between captive and wild developing and spawning capable males, as well as pituitary LH content (one-way ANOVA, P=0.030) in spawning capable males. The latter increased significantly for the wild males from developing to spawning capable phase (one-way ANOVA, P=0.011) but not for the captive males, where an increase was observed between developing and regressed stages (one-way ANOVA, P=0.026). On the other hand, significantly lower *fshβ* expression levels were recorded in the wild males at developing stage compared to their captive counterparts (one-way ANOVA, P=0.035).

The reported sex steroids exhibited significant differences among different reproductive stages in plasma 11-KT (one-way ANOVA, P=0.001), 17, 20β-P (one-way ANOVA, P=0.027) and T (one-way ANOVA, P=0.010) of captive males, and in 17, 20β-P (one-way ANOVA, P=0.006) and T (one-way ANOVA, P=0.021) of wild males (**Fig. 3.3**). Specifically, plasma 11-KT and T of captive males decreased at the regression phase, while 17, 20β-P increased. In the wild males, plasma 17, 20β-P and T increased from developing to the spawning capable stage. Significant differences between the captive and wild males were observed for plasma 11-KT (one-way ANOVA, P=0.026), 17, 20β-P (one-way ANOVA, P=0.006) and T (one-way ANOVA, P<0.001) in spawning capable fish, and for 17, 20β-P (one-way ANOVA, P=0.049) in males at the developing stage. Finally, GSI increased significantly both for the captive (one-way ANOVA, P<0.001) and wild males (one-way ANOVA, P=0.003) at the spawning capable stage compared to the other stages of reproductive development (**Fig. 3.3**).

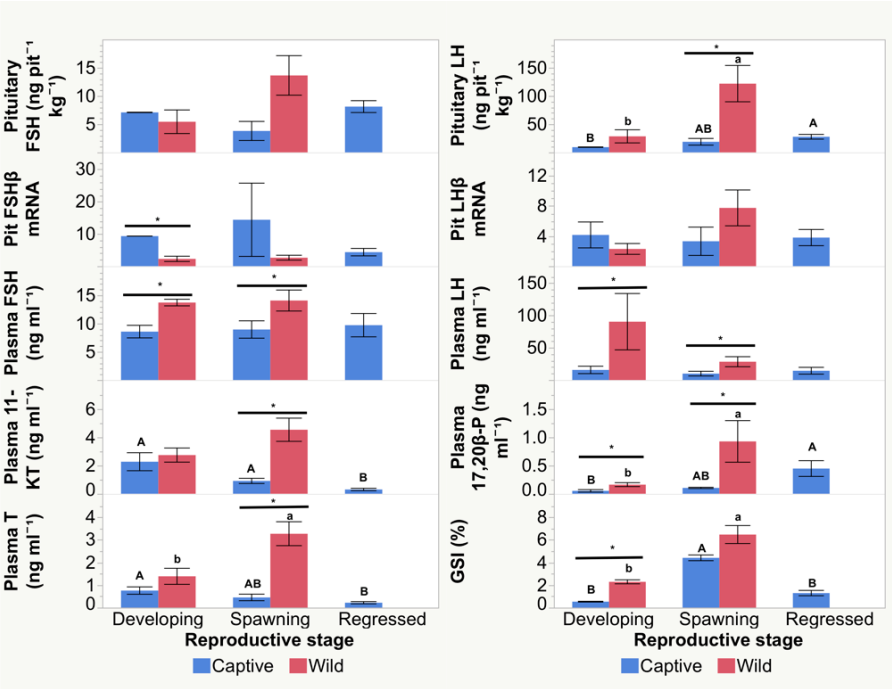

Figure 3.3 Mean (± SEM) pituitary follicle stimulating hormone (FSH)(ng pit^{-1} kg^{-1}), pituitary luteinizing hormone (LH) (ng pit^{-1} kg^{-1}), pituitary *fshβ* mRNA levels, pituitary *lhβ* mRNA levels, plasma FSH (ng ml^{-1}), plasma LH (ng ml^{-1}), plasma 17-β Estradiol (E_2)(ng ml^{-1}), 17,20β-dihydroxy-4-pregnen-3-one (17,20β-P)(ng ml^{-1}), testosterone (T)(ng ml^{-1}) and gonadosomatic index (GSI)(%) of captive (n=12) and wild (n=14) male greater amberjack at different reproductive stages. Capital letters above the means indicate statistically significant differences among reproductive stages of captive males (one-way ANOVA, Tukey's HSD, P≤0.05). Lowercase letters above the means indicate statistically significant differences among reproductive stages of wild males (one-way ANOVA, Tukey's HSD, P≤0.05). Asterisks above the horizontal lines indicate statistically significant differences between captive and wild males at the same reproductive stage (one-way ANOVA, Tukey's HSD, P≤0.05).

3.4 Discussion

Reproductive dysfunctions, which are more common in females, can be classified in three types. These include (a) fish that fail completely to undergo vitellogenesis and spermatogenesis in captivity, (b) fish that cannot proceed with the final oocyte maturation and (c) fish that cannot release their eggs properly to be fertilized, even if they have concluded maturation and ovulation (Zohar & Mylonas, 2001). However, the main reproductive dysfunction observed in captive fishes is the lack or unreliable oocyte maturation, ovulation and spawning (Mylonas & Zohar, 2001) caused by inadequate pituitary LH synthesis and/or release at the end of vitellogenesis (Zohar & Mylonas, 2001), since most of them conclude vitellogenesis in captivity. In males, reproductive dysfunctions are less severe than in females, as in most species spermatogenesis is completed resulting in sperm production, and two types can be distinguished: those that fail to produce any sperm or poorly spermiating fishes (Mylonas, et al., 2017b). The first is related with species that their natural reproductive cycle is difficult to be simulated in captive conditions. The second is the most common reproductive dysfunction in captive males and usually results in reduced amount of produced milt, and sometimes with reduced quality (Mylonas, et al., 2017b).

In this study it was compared the endocrine and gonadal status of wild and captive greater amberjack breeders, sampled at three different times in the reproductive season, in order to highlight the endocrine dysfunctions of captive fish. The categorization and subsequent evaluation was done by grouping the fish in different reproductive stages according to the histological examination of their gonads, in contrast to a recent study that analyzed the same specimens (Zupa, et al., 2017b). This was done in order to describe the endocrinological condition of each fish and the respective reproductive stage, regardless of the time period in which the sampling may have taken place. Here, it is presented the most complete comparative study to date, on the function of the reproductive axis of greater amberjack from the wild and in farming conditions. So far, reported have been limited to the level of the gonad in the wild (Marino, et al., 1995) or only females in captive conditions (Micale, et al., 1999), gonad and plasma sex steroids in the wild (Mandich, et al., 2004) or both conditions (Zupa, et al., 2017a; Zupa, et al., 2017b) and finally elements of the reproductive axis of captive females at questionable levels of maturity (Nyuji, et al., 2016). It is also reported the reproductive dysfunctions observed in captive fish of both sexes in contrast with the normal physiological endocrine processes observed in their wild counterparts, since it is well known that ovarian and testicular development and growth results from the positive balance between gonad development and atresia (Habibi & Andreu-Vieyra, 2007).

Between developing and spawning capable females, a 3 to 4-fold increase of the GSI was recorded. At the same time a corresponding increase was recorded for pituitary FSH content and T, while almost a 10-fold increase was observed in pituitary LH content. In general, higher levels of LH were measured compared to FSH in all the evaluated tissues (pituitary, mRNA of beta units levels, plasma), as was observed also in zebrafish and carp (Hollander-Cohen, et al., 2018). The rest of the analyzed parameters of *fshβ* and *lhβ* expression levels, plasma FSH and LH did not change significantly. Taking all the above into account it is

assumed that FSH and LH plasma levels, as well as *fshβ* and *lhβ* expression levels, do not reflect the levels of the available GtHs in the pituitary. This was also observed in European seabass, where pituitary content of GtHs increased towards the spawning season and showed overlapping profiles for FSH and LH, but their secretion was quite different (Mazón, et al., 2015), and the same was observed also in the short finned eel where *fshβ* and *lhβ* expression levels variations were not reflected in the plasma protein levels (Nguyen, et al., 2019). The absence of any covariation trend of GtH mRNA levels and GtH plasma levels was reported also in previtellogenic greater amberjack females (Nyuji, et al., 2018). In a similar study with the greater amberjack, Gene *fshβ* and *lhβ* expression levels and plasma FSH and LH varied significantly throughout the samplings that were done all year round (Nyuji, et al., 2016), contrary to what was observed here. However, in April and June they did not change significantly, neither a correlation was established between the plasma levels and the gene expression levels (Nyuji, et al., 2016). The absolute values recorded for the FSH and LH plasma levels in the latter study were similar to those of the present one. In contrast, lower levels of E_2 (4-fold) and GSI (2-fold) were recorded, possibly related with the questionable age of maturity of those fish or the captivity induced stress (Nyuji, et al., 2016), as it was observed also here.

On the other hand, our captive females fell into four categories of reproductive development according to their ovarian histological analysis, since extended follicular atresia was observed in late May onwards, with the majority of the fish that had already ceased their reproductive development, showing signs of regressed or regenerating ovaries. However, this dysfunction of captive females was not related to an insufficient liver synthesis or reduced oocyte uptake of vitellogenin (Pousis, et al., 2018). Pituitary content of FSH and LH, *fshβ* and *lhβ* gene expression levels and plasma levels of FSH and LH remained unchanged, in contrast with the plasma E_2 and T which were reduced towards the regenerating phase, and 17,20β-P which showed the opposite pattern. No significant differences, except in 17,20β-P, were recorded between the captive and wild females in developing reproductive stage, in contrast to spawning capable stage where pituitary LH content, plasma FSH and T were found different. Considering all the above, a clear pituitary LH deficiency in the captives compared to the wild females, at the same time with a significantly reduced plasma FSH and T may have caused the extensive follicular atresia and no maturation at all. Additionally, regressed or regenerating ovaries to all captive females sampled in late May-late June, possibly caused by the repeated handling of the fish. This finding also matches with a similar study with greater amberjack where synthesis and secretion of LH and the expression of LH receptors were found to be up regulated at the completion of vitellogenesis (Nyuji, et al., 2016). A significant elevation of plasma LH was also recorded after GnRHa administrations to fish that were at late vitellogenesis stage and ovulated 36-42 h later (Nyuji, et al., 2019). In the latter study, FSH levels have not changed almost 2 days after the GnRHa implantation. It is well known that FSH has an anti-apoptotic effect in trout ovary (Lubzens, et al., 2010) and coho salmon (Luckenbach, et al., 2011), so the 2-fold difference plasma levels of FSH between captive and wild females in the present study may explain at least the follicular atresia. Decreased levels of pituitary and plasma FSH in fish with follicular atresia have been observed also in

European seabass compared to fish at late vitellogenesis stage (Molés, et al., 2011). Interestingly, in the 15% of the sampled captive females at late May - late June, similar GSI values were recorded with the wild fish, however extensive follicular atresia was present. Almost the same reproductive dysfunction was found in a similar study with captive and wild female jack mackerel, a carangid species as well, which was attributed to captivity induced stress, when some of the captive fish concluded vitellogenesis and others not, showing high percentages of follicular atresia (Imanaga, et al., 2014), as was observed in the present study, while common aquaculture practices and their induced stress interrupted the spawning cycle in tilapia, which had been mainly related with the suppression of LH and E_2 secretion (Chabbi & Ganesh, 2012). Taking this into account, it can be assumed that, as was supposed for jack mackerel, based on the plasticity of each species, there are individuals who respond best to captive conditions, a useful information in the process of domestication of newly cultured species (Imanaga, et al., 2014). This was emphasized for the gilthead seabream as well, a fully domesticated species today, but not 50 years ago (Zohar & Mylonas, 2001).

In the present study the levels of both GtHs gene expression and plasma GtHs did not change either in captive and wild breeders, showing that they may not reflect the situation in the ovaries or testes. Differences were observed in the pituitary GtH content, although this cannot reflect the release in the circulation also. The pituitary GtH mRNA levels were considered also as a poor indicator of the reproductive stage of the fish, compared to plasma levels of GtHs, in the Senegalese sole (Chauvigne, et al., 2016) the gilthead seabream (Meiri, et al., 2004) and European seabass (Mazón, et al., 2015), contrary to what have been observed in salmonids (Swanson, et al., 2003). In the present study, however, this observation may be related with the limited number of samplings at a restricted time period. Additionally, GtH gene expression levels, plasma levels of LH, E_2 and 17,20β-P, but not T, were shown to change significantly (even 2-fold) within a day in the gilthead seabream (Gothilf, et al., 1997) a species which follows daily cycles of maturation, ovulation and spawning. Also, *fshβ* and *lhβ* expression levels followed daily rhythms in both female and male zebrafish (Paredes, et al., 2019). In a congener of greater amberjack, the Japanese yellowtail, *fshβ* was strongly expressed in early vitellogenesis and spermatogenesis, while *lhβ* was expressed significantly at late vitellogenesis and spermatogenesis, and during spermiation (Rahman, et al., 2003). In tilapia females, a mouth brooding group synchronous species with a spawning frequency of almost 12 days, when spawned eggs were removed from the mouth of the fish, both plasma FSH and LH demonstrated two peaks, the first one on day 2 and the second, and highest, on day 12 (Aizen, et al., 2007). High levels of LH have been reported in many species during OM (Levavi-Sivan, et al., 2010; Swanson, et al., 2003), however the high levels of FSH at the same period may reflect the entrance of the next generation of oocytes entering oogenesis (Aizen, et al., 2007). The greater amberjack has been shown to spawn almost every 5 days in the Southeastern U.S. Atlantic coast (Harris, et al., 2007), similar to what has been observed both in the Mediterranean and the Canary Islands (unpublished data). Normally, fluctuations of the GtHs (gene expression and plasma levels) would have been observed if it was possible to follow a captive population of fish at the same steps of development of a batch of oocytes, however

this is still not possible with the current state of domestication of greater amberjack compared to gilthead seabream or tilapia. Interestingly, plasma FSH was shown to increase significantly in the greater amberjack at the end of the spawning season (August) and was related with the start of new gametogenic cycle (Nyuji, et al., 2016).

Plasma sex steroids were shown early to peak at the end of May-early June in the Mediterranean, with E_2 levels being higher when females were at a maturing phase, mature or partially ovulated (Mandich, et al., 2004), as observed in fish at late vitellogenesis in Japan (Nyuji, et al., 2016). Plasma T levels peaked at the maturing phase and decreased significantly by 50% at the mature and partially ovulated stages (Mandich, et al., 2004). In Senegalese sole females, a group synchronous spawner also, plasma FSH and LH have been shown to peak in the middle of the spawning season, after the peak of the E_2 and T (Chauvigne, et al., 2016). Considering the above information and the general pattern for GtH and sex steroid kinetics proposed by Habibi et.al. (2007), it was expected to be able to record clear peaks of the different hormones However, both the short half-life of these hormones in circulation (Gothilf, et al., 1997) and the small number of samples that it was possible to be collected both in the wild and captive conditions may have affected the ability to detect statistically significant changes. The latter authors reported that in the absence of GtHs, females enter into follicular atresia, and this may be enhanced by the gonadal paracrine secretion of GnRH. Interestingly, the same follicular atresia at the late vitellogenesis stage that was present in the tank-reared females (see later, Chapter 4) was possibly enhanced by the GnRHa treatment, since unfertilized eggs were spawned and follicular atresia was found to be increased 3 weeks after the GnRHa administration. This may be explained by the lack of the appropriate pituitary LH content in these females, evidenced by their ovarian biopsies which were similar to the present study, and possibly their reproductive regression was at an irreversible phase, so any GnRHa administration at this point could not stimulate a proper oocyte maturation, ovulation and spawning.

Looking at wild males now, they were found to be in corresponding reproductive stages with the females, as expected. Pituitary LH content, plasma T, 17,20β-P and GSI of wild males increased by a 3 to 4-fold. A slight increasing trend of pituitary FSH content, *lhβ* expression levels and plasma 11-KT existed between the developing and spawning capable reproductive stages, while an opposite trend was observed in plasma LH. No change between the two reproductive stages was observed in *fshβ* expression levels and plasma FSH. On the other hand, captive males fell into three categories of reproductive development according to their testicular histological analysis, showing significant differences from their wild counterparts even from the developing phase in four out of ten measured parameters. During the spawning capable phase, the situation was even worst, since in almost all measured parameters reduced levels between wild and captive males were recorded, being statistically significant or showing a strong downward trend. Taking all the above into account, the reproductive dysfunctions in captive males seem to be more pronounced than in captive females and possibly not related to the handling stress - handling had not been done up to the developing stage- since from

the developing stage the 40% of the measured parameters was significantly lower compared to their wild counterparts and increased to 60% in the spawning capable stage. In the same captive fish, seminiferous lobules of a smaller diameter, a precocious and progressive decrease of spermatogonial mitosis and a high level of germ cell apoptosis, concomitant with a many-fold higher E_2 plasma levels was reported (Zupa, et al., 2017a). In humans, apoptosis in male germ cells is suppressed by T, which is considered a survival factor (Erkkila, et al., 1997) and the same is observed in mammals in general (Schulz, et al., 2001). In fish, it was suggested that E_2 regulates spermatogonial renewal, while T and 11-KT (the main androgen in teleosts) are involved in later stages of spermatogenesis (Miura & Miura, 2003). However, the role of estrogens in male fish fertility has been suggested of being neutral (Tang, et al., 2017), although the role of the E_2 in the male apoptosis process has not been established yet. Interestingly, higher sperm volumes and frequency of spermatozoa in cysts were found in mutant zebrafish, lacking *cyp19a1a* or both *cyp19a1a* and *cyp19a1b* compared to the wilds or *cyp19a1b* mutant (Tang, et al., 2017). In human males, estrogens were shown to have negative and positive feedback roles (Guercio, et al., 2020). In fish, it is generally considered that high gonadal steroid plasma levels reduce the *fshβ* expression levels (Yaron, et al., 2003), and this is opposite to what has been observed in the present study. In coho salmon, FSH plasma levels and *fshβ* expression levels decreased after E_2 administration (Dickey & Swanson, 1998). In the yellowtail kingfish, females had increased FSH plasma levels concomitantly with significantly higher E_2 levels (Nocillado, et al., 2019) and this might be the cause of *fshβ* expression increase that was observed in the present study in the captive males at the developing stage, when the peak of E_2 was recorded. The same trend of *fshβ* expression levels and E_2 towards ovarian development was observed also in the greater amberjack females (Nyuji, et al., 2016), but relevant studies in males still lack.

In general, it is assumed that in male fish FSH regulates the early stages of spermatogenesis and LH peaks at the onset of spawning season (Schulz, et al., 2010). However, fewer studies have been carried out so far compared to females. In the Japanese yellowtail, the plasma 11-KT, *fshβ* and *lhβ* expression levels peaked at the late spermatogenesis, when their testes were at the same developmental stages with the developing phase of the present study, and remained at the same high levels after entering the spawning season, when the GSI values peaked (Higuchi, et al., 2016). In the greater amberjack in a similar study, T plasma levels peaked in mid-May and remained at the same levels until the end of June, while 11-KT levels increased in mid-May, but peaked in mid-June (Mandich, et al., 2004). In the chub mackerel, a multiple spawner also, it was considered also that FSH may play a role in early and late phases of spermatogenesis, while LH is involved during late spermatogenesis and spermiation (Nyuji, et al., 2012). In male salmonids, both *fshβ* expression levels and FSH plasma increase during early stages of maturation, while *lhβ* expression levels and LH plasma levels are very low at the early stages and increase sharply in the spawning season (Ciani, et al., 2020).

In summary, captive female greater amberjack appeared to be particularly sensitive to repeated handling, as they failed to successfully complete vitellogenesis. Although, almost no significant differences were observed in their wild counterparts in the measured endocrine parametres during the developing phase -

prior to any handling, during the spawning period and later, most females had already aborted reproductive development. Males, seemed to face reproductive dysfunctions even without handling, since significant differences were already present when compared to wild breeders at the first sampling. The greater amberjack is considered to be a “poorly spermiating” fish species, a category in which fish complete spermatogenesis in captivity, but produce small volumes of milt (Mylonas, et al., 2017b). This phenomenon seems problematic in multiple spawning fish species or with a rather long female spawning period (Mylonas, et al., 2017b), such as the greater amberjack. Furthermore, the quality of the produced sperm might be also lower, compared to other cultured species (Zupa, et al., 2017a).

Chapter 4 - Control of reproduction of greater amberjack reared in aquaculture facilities

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4.1 Introduction

Marine fish are one of the best food sources, since they contain several essential amino acids and have a unique lipid composition due to the presence of long-chain, poly-unsaturated fatty acids (Tacon & Metian, 2013). At the same time, fish are the most efficient converters of feed into high quality flesh, and their carbon footprint is lower compared to other animal production systems (Béné, et al., 2015). Seafood consumption has been increasing steadily in the last decades, with the increased supply made available to satisfy this demand coming from aquaculture and not the wild fisheries, which have plateaued since the 1980s as the majority of fish stocks have been fished beyond biological sustainability (FAO, 2018). Food security and high-quality nutrition must be ensured, and aquaculture has provided the increasing seafood demand of the last 30 years, caused by a growing and wealthier population, as well as health concerns and the advantages offered by marine fish (FAO, 2018). Further growth of the aquaculture industry is imperative, and could result both from higher production of the already established species, but also from diversification with new species that have important biological or market advantages.

Members of the genus *Seriola* have been notable species for the aquaculture diversification worldwide (Kagawa, 1989; O'Neill, et al., 2015; Sicuro & Luzzana, 2016; Symonds, et al., 2014), while the greater amberjack is one of the most prominent species given its cosmopolitan distribution (Mylonas, et al., 2017a). The greater amberjack has a rapid growth rate (Crespo, et al., 1994; Jover, et al., 1999; Mazzola, et al., 2000), which is higher than Atlantic salmon (Asche & Bjorndal, 2011). Its large body size makes it suitable for development of a variety of value-added seafood products and its late reproductive maturation (Micale, et al., 1999; Zupa, et al., 2017b) allows marketing the fish before growth is affected negatively. Furthermore, greater amberjack is found throughout the temperate zone, which is an advantage for species diversification of the aquaculture industry, compared to other potential candidate species that have a narrower geographical distribution. However, until now the production of greater amberjack has been faced with major bottlenecks, such as inconsistent and unreliable reproduction and production of juveniles for grow out. The key to incorporating a species into the aquaculture industry is the cost-effective control of its production processes (Asche & Bjorndal, 2011; Asche, et al., 2009), therefore, research in understanding the reproductive biology of greater amberjack and developing methods to control reproduction in captivity is essential.

Controlling reproduction of greater amberjack in captivity lags behind its congeners the Japanese yellowtail and yellowtail kingfish, even though some sporadic and unpredictable spawning has been observed

in greater amberjack in Japan (Kawabe, et al., 1998; Kawabe, et al., 1996) and in the Canary Islands (Jerez, et al., 2006; Sarih, et al., 2018). However, spontaneous spawning had not been reported so far in the Mediterranean region (Grau, et al., 1996). Unpredictable and variable reproductive performance in newly cultured fish species could be due to improper broodstock nutrition (Izquierdo, et al., 2001), while failure of spawning can result from improper environmental conditions or by captivity-induced stress (Mylonas, et al., 2010). As a result, pituitary LH release at the end of vitellogenesis does not take place, and thus no oocyte maturation, ovulation or spawning occurs (Mylonas, et al., 1997a; Mylonas, et al., 1997d). In greater amberjack, reproductive dysfunctions in captivity have been observed both in breeders maintained in tanks (Micale, et al., 1999; Mylonas, et al., 2004b) and in sea cages (Kozul, et al., 2001; Zupa, et al., 2017b)(Chapter 3). To overcome the observed reproductive dysfunctions and to induce spawning, various hormonal treatments have been examined (Fernández-Palacios, et al., 2015a; Kozul, et al., 2001; Mylonas, et al., 2004b; Sarih, et al., 2018), while recently the use of controlled-release delivery systems loaded with GnRHa has proven to be very effective (Chapter 5).

The objective of the present study was to develop broodstock management and spawning induction methods using controlled-release delivery systems loaded with GnRHa for greater amberjack maintained in tanks and sea cages, in typical Mediterranean aquaculture facilities. Due to the large size of greater amberjack breeders, the two methods have specific advantages. The advantages of sea cage rearing include optimal environmental conditions and welfare, and low cost of fish maintenance, while the advantages of tank rearing include biosecurity, ease of management and handling operations, and control of egg collection. In the course of this study, an alternative "hybrid" method was tested, combining rearing the breeders throughout the year in sea cages, and then putting them in tanks for spawning after hormonal induction. This method was shown to be very effective and resulted in the production of large numbers of eggs of adequate quality for commercial larval rearing of the species.

4.2 Materials and methods

4.2.1 Broodstock maintenance

Wild greater amberjacks were collected from the Ionian or Aegean Sea, Greece as juveniles (~300 g in 2010 and earlier) and were maintained at different locations. These included two research facilities: a) AQUALABS facilities of the Institute of Marine Biology, Biotechnology and Aquaculture (IMBBC) of the Hellenic Centre for Marine Research (HCMR), Heraklion, Crete, Greece, (Registration No EL91-BIObr-03 and EL91-BIOexp-04) and b) SOUDA facilities, the pilot sea cage farm of the IMBBC at Souda Bay, Chania, Crete (GR94 FISH0001). Fish were also maintained at registered commercial aquaculture facilities: ARGO, GMF and FORKYS.

The experimental protocol was approved by the National Veterinary Service (PN 255356). All procedures were conducted in accordance to the "Guidelines for the treatment of animals in behavioral research and teaching" (Anonymous, 1998), the Ethical justification for the use and treatment of fishes in research: an

update (Metcalfe & Craig, 2011) and the "Directive 2010/63/EU of the European parliament and the council of 22 September 2010 on the protection of animals used for scientific purposes" (EU, 2010).

The fish were reared either in sea cages (SOUDA, ARGO and GMF) or in land-based tanks (AQUALABS and FORKYS) for 3 consecutive years (**Table 4.1**). One hundred seventeen fish tagged with P.I.T. tags (Avid, UK) were utilized in 2014 (68 fish in sea cages and 49 in tanks) of 6.3-23.8 kg BW; 116 fish were used in 2015 (68 fish in sea cages and 48 in tanks) 8.6-23.8 kg BW; and 111 fish were used in 2016 (66 fish in sea cages and 45 fish in tanks) of 9.8-23.6 kg BW. All fish were older than 4 years old at the start of the study in 2014, and were considered reproductively mature based on their age and size (Marino, et al., 1995). The greater amberjack breeders were fed with live fish, raw frozen fish, squid, moist extruded or dry extruded feed (Skretting Vitalis CAL, 22 mm), or a combination of the above (**Table 4.1**). Over the course of the 3-year study an effort was made to switch all broodstocks feeding on live/raw fish to the same commercial broodstock diet (dry extruded feed). Live/raw fish may not be an appropriate diet for broodstock, mainly for biosecurity reasons, but also for nutritional ones (*e.g.* variable raw fish quality, rancidity, vitamin content, etc.). However, the fish did not accept dry feed easily and they were gradually moved from raw fish, to moist extruded feed and then dry feed. This was not possible for some commercial facilities (**Table 4.1**), therefore these broodstocks continued being fed raw/live fish. Feed was given 3 to 5 times a week to apparent satiation or to 1-2% of their estimated body weight in case of dry pellets.

Table 4.1 Description of the various greater amberjack broodstocks maintained in sea cages or tanks during the three years of the study.

2014

Stock	Rearing Method	Volume (m^3)	Number of individuals (males/females)	Size at sampling (range in kg)	Feeding
ARGO	sea cages	512	12/16	10.0-16.0	live, raw fish
GMF	sea cages	1018	14/14	6.0-16.0	live fish
SOUDA	sea cages	1018	8/4	7.4-14.8	moist pellets
AQUAL	tanks	40	12/15	6.5-23.8	raw fish, squid
FORKYS	tanks	25	13/9	7.7-10.3	raw fish, squid

2015

Stock	Rearing Method	Volume (m^3)	Number of individuals (males/females)	Size at sampling (range in kg)	Feeding
ARGO	sea cages	1018	12/15	10.7-19.5	moist pellets, raw fish
GMF	sea cages	1018	14/14	9.0-18.0	live fish
SOUDA	sea cages	1018	8/4	9.9-18.4	moist pellets
AQUAL	tanks	40	12/15	8.6-23.8	moist pellets, raw fish
FORKYS	tanks	25	13/8	9.4-15.9	raw fish, squid

2016

Stock	Rearing	Volume	Number of	Size at sampling	Feeding

	Method	(m^3)	individuals (males/females)	(range in kg)	
ARGO	sea cages	1018	14/15	10.7-23.6	dry pellets
GMF	sea cages	1018	14/14	11.8-21.5	live fish
AQUAL	tanks	40	12/14	9.8-18.5	dry pellets
FORKYS	tanks	25	11/8	12.6-20.3	raw fish, squid

At the SOUDA, ARGO and GMF facilities, greater amberjacks were maintained in the sea, either in rectangular (8x8x8 m) or round cages (40 m perimeter, 8 m depth). Fish were maintained in tanks of 40 m^3 (AQUALABS) and 25 m^3 (FORKYS) in volume with rearing density beginning in 2014 at 3.3 and 7.8 kg m^{-3}, respectively, and increasing in 2016 to 5.2 and 11.7 kg m^{-3}, respectively, as the fish grew in size. Fish were maintained under simulated natural (AQUALABS) or natural (FORKYS) photoperiod regime. The tanks were supplied with seawater from a well with an ambient temperature profile (FORKYS) or from a deeper well with constant water temperature (18-19°C), with the natural temperature profile being simulated using a recirculation system (AQUALABS) (**Fig. 4.1**). Monitoring of pH, NH_3-N and NO_2-N in the recirculation system was conducted once a week (pH: 6.79-7.86, NH_3-N: 0.01-1.63 mg l^{-1}, NO_2-N: 0.000-0.150 mg l^{-1}), while temperature and dissolved oxygen (D.O.: 70-99%) in all facilities were measured from 1 to 7 times a week.

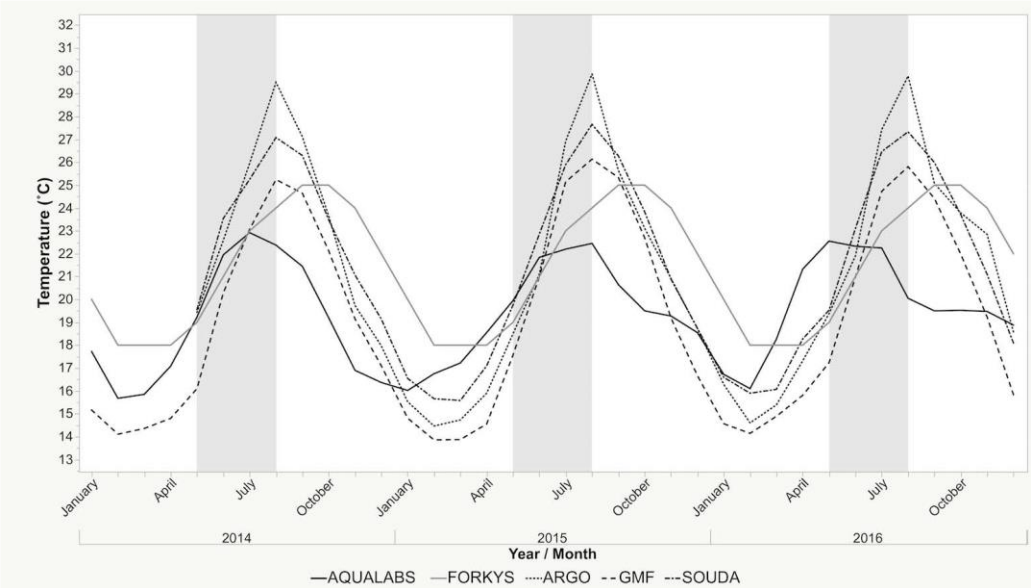

Figure 4.1 Water temperature profile in the various sites where greater amberjack broodstocks were maintained in sea cages (ARGO, GMF and SOUDA) or tanks (AQUALABS and FORKYS) for the period of 2014-2016. The shaded areas indicate the estimated breeding period in the wild in the Mediterranean Sea.

4.2.2 Evaluation of reproductive stage and selection of breeders

Evaluation of reproductive stage and selection of breeders for spawning induction experiments was done after a 2-day starvation period. Fish were initially tranquilized in their tank or in an anesthesia sack (20-40 m^3 in volume) within their sea cage, with the use of either clove oil (0.01ml l^{-1}) or 2-phenoxyethanol (0.15 ml l^{-1}). Then, the fish were transferred to a separate tank for complete sedation with a higher concentration of clove oil (0.03ml l^{-1}) or 2-phenoxyethanol (0.4 ml l^{-1}) (Mylonas, et al., 2005). Ovarian biopsies for the evaluation of oocyte development were obtained by inserting a plastic catheter (Pipelle de Cornier, Laboratorie CCD, France) and applying gentle aspiration. A wet mount of the biopsy was examined under a compound microscope (40 and 100x) to evaluate the stage of oogenesis and measure the diameter of the most advanced vitellogenic oocytes (n=10). A portion of the biopsies was fixed in a solution of 4% formaldehyde-1% glutaraldehyde for further histological processing. Females were considered eligible for spawning induction if they contained fully vitellogenic oocytes with a diameter >600 μm (Chapter 5). Sperm samples were obtained by inserting a cannula at the base of the genital pore and applying gentle aspiration, as described above for the females. Care was taken to avoid contamination of sperm with urine or feces, and prior to sampling the genital pore was rinsed and blot dried to avoid introducing water into the collected sperm. Milt was kept on ice until evaluation of various quality parameters (see later), which were done within 2-3 hours from collection. Males were considered eligible for spawning induction if milt could be obtained with the cannula.

4.2.3 Spawning induction experiments

The breeders that were in the appropriate reproductive stage for spawning induction were administered implants of GnRHa. The GnRHa implants were prepared by loading the agonist desGly^{10}, DAla^{6}, Pro^{9}-GnRH-NEthylamide (Bachem, Switzerland) into a matrix of ethyl vinyl acetate copolymer (EVAc) (Dow Corning) as reported previously (Mylonas, et al., 2007). The implants were loaded with 250, 500, 750, 900 or 1000 μg of GnRHa, and combinations of implants were administered for an effective dose of 64±17 μg kg^{-1} GnRHa for females and 48±12 μg kg^{-1} GnRHa for males. Variations in the GnRHa doses were caused by the fact that implants are loaded with fixed amounts of GnRHa. Even though implants loaded with different amounts of GnRHa were used, it was still not possible to adjust the dose exactly to the different body weights of the fish. The selected intended doses were those used successfully in previous experiments using GnRHa implants (50 μg GnRHa kg^{-1})(Mylonas, et al., 2004b).

Fish that were maintained in tanks during the year, after GnRHa administration they were returned to the same tank for spawning (tank spawning – AQUALABS, FORKYS)(**Fig. 4.2**). For breeders that were maintained in sea cages during the year, two approaches were examined. In the first approach, the breeders were moved back to a round sea cage for spawning (cage spawning – ARGO, GMF, SOUDA), which was equipped with a passive egg-retaining device to prevent the spawned eggs from being carried out of the cage by water currents (see later for description). In the second approach, immediately after GnRHa administration

the breeders were transferred to land-based tanks for spawning (cage-to-tank spawning – ARGO, GMF)(**Fig. 4.2**). For transferring to land a service boat was employed, transferring the already anesthetized breeders one-by-one in a 1000-l tank containing seawater with a low dose of anesthetic (0.01ml l^{-1} clove oil or 0.15 ml l^{-1} 2-phenoxyethanol). The spawning tanks were 21-24 m^3 in volume and were stocked with 6-8 breeders (males and females) at 3.1-5.6 kg m^{-3} density, and were supplied with surface seawater.

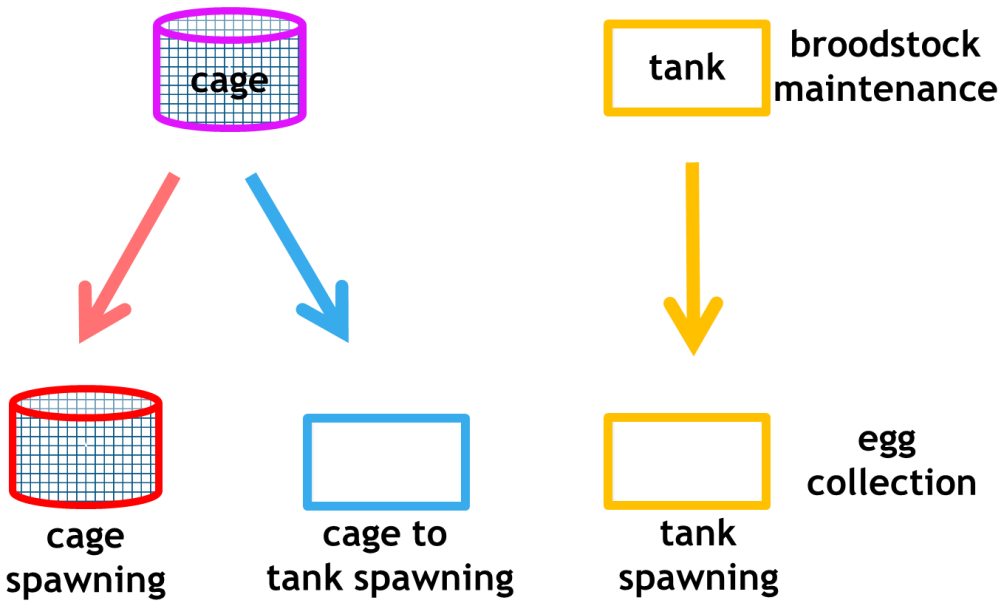

Figure 4.2 Schematic representation of the experimental design of the broodstock maintenance and spawning induction method. Greater amberjack broodstocks were maintained in sea cages or land based tanks during the year. At the expected spawning period and after reproductive evaluation and GnRHa implant administration the fish from tanks were transferred to tanks for spawning (tank spawning). The fish from cages were transferred for spawning either in cages (cage spawning) or to land based tanks (cage to tank spawning).

After the cessation of spawning (1-3 weeks), all fish were re-evaluated for reproductive stage and where returned to their original rearing facilities (tanks or sea cages). In one case in 2016 at ARGO, when the females were found to contain large numbers of vitellogenic oocytes and were eligible for further spawning, the fish were induced again with a second GnRHa administration, 14 days after the first one. Due to the significantly lower numbers of eggs obtained from cage spawning experiments and in order to examine if eggs were escaping from the passive egg-collecting device, after the 2nd GnRHa administration in 2015 at GMF, the breeders were placed in the anesthetic sack of 36 m^3 in volume (almost completely enclosed) for spawning. To ensure adequate oxygen concentration, dissolved oxygen was monitored continually and the sack was provided with gaseous oxygen. In 2016, no spawning induction was attempted in SOUDA.

4.2.4 Evaluation of sperm quality

Sampled sperm was stored in 1.5 ml micro-centrifuge tubes, which were then placed on ice and transferred to a 4°C refrigerator until evaluation. Sperm quality parameters that were evaluated included (a) spermatozoa density (number of spermatozoa ml^{-1} of sperm), (b) initial percentage of spermatozoa showing forward motility immediately after activation (spermatozoa motility, %), (c) duration of forward spermatozoa motility of ≥5% of the spermatozoa in the field of view (motility duration, min) and (d) survival of spermatozoa under cold storage at 4°C (spermatozoa survival, days) according to published and validated methods (Fauvel, et al., 1999; Mylonas, et al., 2016b; Papadaki, et al., 2008), as described below. Spermatozoa density was estimated after a 2121-2626-fold dilution with 0.9% saline using a Neubauer haemocytometer under 200X magnification (in duplicate) under a compound light microscope. Spermatozoa motility and motility duration were evaluated on a microscope slide (400X magnification) after mixing 1 μl of sperm with a drop of about 50 μl of saltwater (in duplicate). Activated sperm samples were observed under the compound light microscope for the first time 10 sec after activation. Spermatozoa motility (%) was determined subjectively using increments of 10%. Sperm was stored at 4°C for the days after collection, and was examined every other day for spermatozoa motility, until no forward motility was observed, in order to estimate spermatozoa survival time (days).

4.2.5 Egg collection and evaluation of egg quality

The egg collection device for the sea cages consisted of a two-section plastic curtain deployed around the inside perimeter of the cage. This is a passive trapping device, which restricts the movements of floating eggs within the cage, as has been used previously for bluefin tuna in Japan and the Mediterranean Sea (De Metrio, et al., 2010; Mylonas, et al., 2007; Sawada, et al., 2005). The “lower” section started at about 30 cm above the water line and goes down to about 3 m in depth, and was secured on the net of the cage throughout its perimeter through portholes in the tarpaulin every 30 cm. The “upper” section was hanging from the rails of the cage using ropes every 30 cm along the perimeter of the cage, and drapes down the cage over the lower section, overlapping with the top 1.5 m below the water surface. The objective of this two-piece design was to allow wind pressure to be relieved by allowing the upper section on the windward side to lift above the water, while the leeward side is pushed tightly against the net and the lower section, thus preventing the floating eggs from “jumping” over the cage and being lost. In 2014 (ARGO, GMF and SOUDA), the depth of the lower section was set at 3 m, while the depth of the cage net was reduced to 8 m. After realizing that egg loss was taking place, in 2015 (ARGO, GMF and SOUDA) the depth of the egg collector was set at 5 m under the surface, while the depth of the cage net was reduced further to 6 m. Finally, in 2016 (GMF), since the egg collection in cages was minimal the previous years, the depth of the egg collector was again set at 5 m, but the cage net depth was reduced further to 5.3 m, and at the same time the bottom of the cage was covered with an extra fine mesh to reduce the possible currents (though allowing some water exchange) and prevent the eggs from passing through.

The cages were examined for the presence of eggs every day beginning at dawn, and egg collection continued approximately every hour until no more eggs were collected. For the tank and cage-to-tank spawning experiments, the passive overflow egg collectors were examined three times a day, so eggs were collected within a few hours after spawning. Eggs were collected into a 10-l bucket and their number (fecundity) was estimated by counting the total number of eggs in a random sub-sample of 5 or 10 ml (depending on the total number of eggs), after vigorous agitation. Fertilization success was evaluated at the same time by examining each of the eggs in this 5 or 10 ml for the presence of an embryo (usually at the 16-128 stage) using a stereoscope. After the egg evaluation, the eggs were transferred to the hatchery for larval rearing.

4.2.6 Histological analysis

Before embedding in methacrylate resin (Technovit 7100®, Heraeus Kulzer, Germany) ovarian biopsies were dehydrated in gradually increasing ethanol solutions (70-96%). Serial sections of 3 μm were obtained with a microtome (Leica RM 2245, Germany). Sections were stained with Methylene Blue (Sigma, Germany)/Azure II (Sigma, Germany)/Basic Fuchsin (Polysciences, USA) according to Bennett et al. (Bennett, et al., 1976). Sections were examined under a light compound microscope (Nikon, Eclipse 50i) and photographed with a digital camera (Jenoptik progress C12 plus).

4.2.7 Statistical analysis

Differences in oocyte diameter were tested using 2-way ANOVA followed by Tukey's HSD post hoc test. For the "female spawning eligibility" data, when the variance distribution was not normal even after Arcsin transformation, a t-test was used to compare the two rearing conditions ($P<0.01$). Differences in mean egg production characteristics and sperm quality parameters were tested using one-way ANOVA followed by Tukey's HSD post hoc test. Percentage data were Arcsine transformed prior to statistical analyses to normalize variances. A level of $P<0.05$ was set as minimum statistical significance for the ANOVA tests. Statistical analyses were performed with JMP 12 (SAS Institute Inc., Cary, NC, USA). Results are presented as mean ± standard error (SEM), unless mentioned otherwise.

4.3 Results

Looking at the data from the fish reared in tanks throughout the year (AQUALABS and FORKYS), in 2014 most of the females contained mostly POs, some CA stage oocytes and oocytes at early vitellogenesis (**Fig. 4.3A**). Forty-four percent of the females had Vg oocytes in their ovaries with a mean diameter of 481±54 μm **(Fig. 4.4**) concomitantly with increased occurrence of follicular atresia (**Fig. 4.3C)**. At the same time, two vitellogenic females had also a small number of ovulated eggs in their ovaries, indicating that they matured and ovulated spontaneously some eggs in the previous days (**Fig. 4.3D**). A similar situation was repeated in 2015 and 2016; females in both facilities (AQUALABS and FORKYS) contained mostly POs and eVg oocytes, and only a few fish were in full vitellogenesis (**Fig. 4.3B**), but still with the mean oocyte diameter being even lower than in 2014 (**Fig. 4.4**). The GnRHa-treated fish responded only in the case of the

AQUALABS broodstock in 2014, producing 3 spawns of almost completely unfertilized eggs (**Fig. 4.5**). Only one of the females was eligible for spawning induction in 2015 (5.6%) and one of the females in 2016 (6.3%) (**Fig. 4.4**). Follicular atresia was again present in the ovaries of these females, and the 5 spawns obtained in 2015 at the AQUALABS were of 0% fertilization success (**Fig. 4.5**).

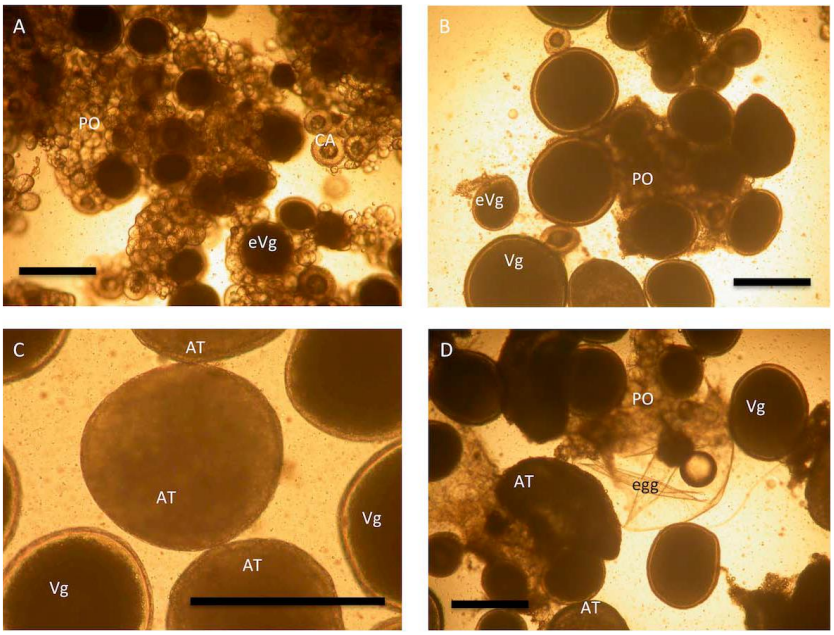

Figure 4.3 Microphotographs of representative ovarian biopsies (wet mounts) from greater amberjack broodstocks reared in tanks throughout the year (AQUALABS and FORKYS). A) a female with mainly primary oocytes (PO), some cortical alveoli stage (CA) and only a small percentage of oocytes in early vitellogenesis (eVg), B) a fully vitellogenic female, C) a vitellogenic female with high presence of follicular atresia (AT), D) a vitellogenic female with some ovulated eggs. Vg=vitellogenic oocyte. Bars=500 μm.

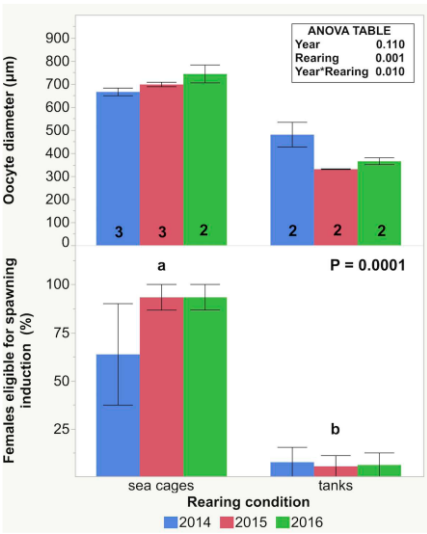

Figure 4.4 Mean diameter (± SEM) of the largest vitellogenic oocytes from biopsies obtained at the expected spawning period from female greater amberjack of different stocks reared in sea cages (n=3 or 2) or tanks (n=2) and percentage of females eligible for spawning induction (vitellogenic oocytes >600 μm in diameter).

The numbers inside the bars in the upper graph indicate the n value of the means (number of stocks examined) (2-way ANOVA, P<0.05). The "female eligible for spawning" data were analyzed with a t-test (P<0.01), because the variance distribution was not normal even after Arcsin transformation.

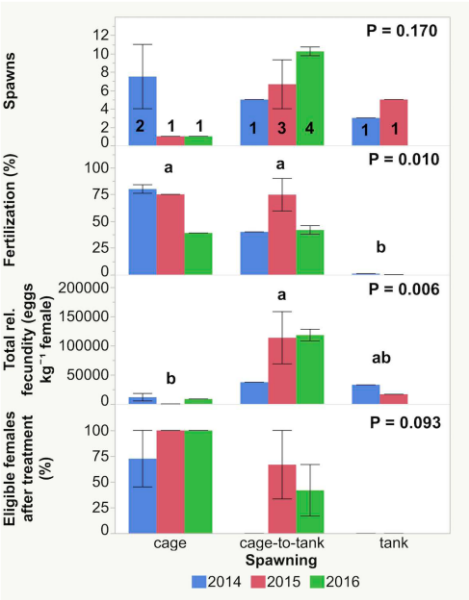

Figure 4.5 Mean (± SEM) number of spawns per annual reproductive season, fertilization success (%), total relative fecundity (eggs kg^{-1} female) and percentage of females eligible for a second hormonal treatment, for greater amberjack of different stocks reared under different conditions and induced to spawn with GnRHa implants. "Cage" – fish were maintained throughout the year and were allowed to spawn in a sea cage. "Cage-to-tank" – fish were maintained in a sea cage throughout the year, but were placed in a tank to spawn after hormonal induction. "Tank" - fish were maintained throughout the year and were allowed to spawn in a tank. Lowercase letters indicate statistically significant differences between the different spawning conditions. The numbers inside the bars in the upper graph indicate the n value of the means (number of spawning groups examined) (1-way ANOVA, Tukey's HSD, P<0.05).

Looking at the data from the greater amberjack reared in cages throughout the year, during the 3 years of the study the vast majority of females had ovaries with fully Vg oocytes (**Figs 4.6A and 4.7A**) at the start of the spawning season (June). The annual mean oocyte diameter was always >650 μm with an increasing trend over the years, and the percentage of females eligible for spawning induction was 64±46% in 2014, 93±11% in 2015 and 93±9% in 2016 (**Fig. 4.4**). A small percentage of the females had even initiated oocyte maturation (**Figs 4.6B and 4.7B**) or had pre-ovulated or ovulated eggs from a very recent spawning (**Fig. 4.6C**). The biopsied oocytes were significantly larger compared to the oocytes from fish reared in tanks throughout the year (P=0.001) **(Fig. 4.4)**.

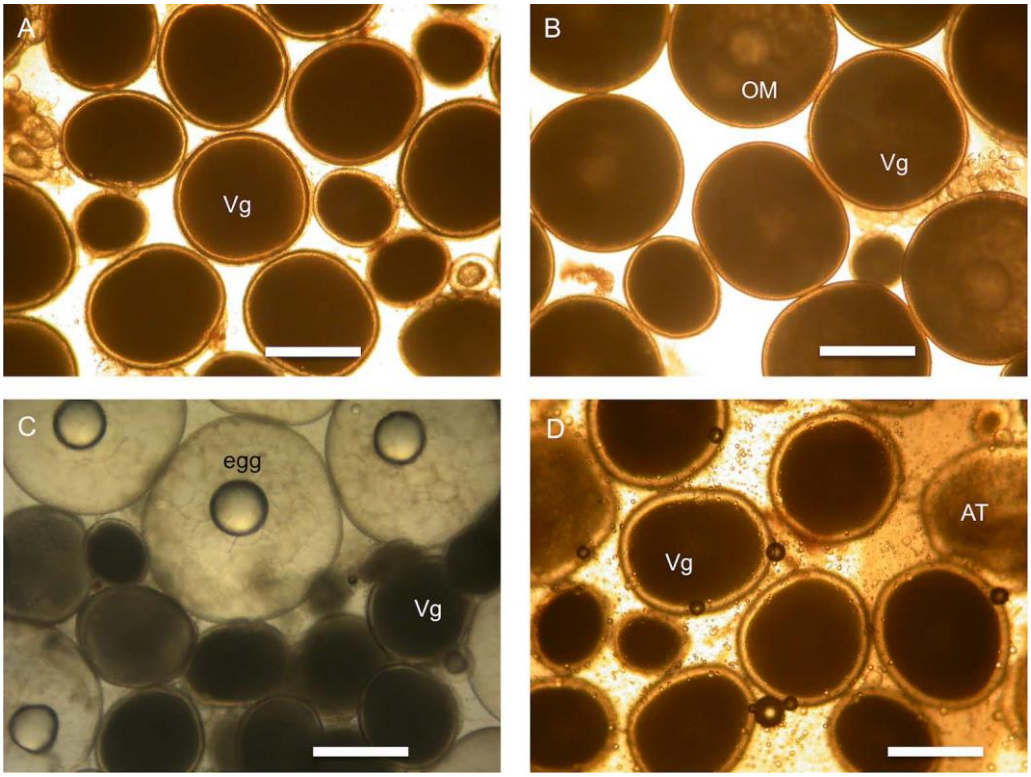

Figure 4.6 Microphotographs of representative ovarian biopsies (wet mounts) prior to hormonal induction of spawning from greater amberjack reared in sea cages throughout the year (ARGO, GMF and SOUDA). A) a female with fully vitellogenic (Vg) oocytes, B) a female in oocyte maturation (OM), C) a female with ovulated eggs and D) a female with Vg oocytes. AT=apoptotic/atretic follicle. Bars=500 μm.

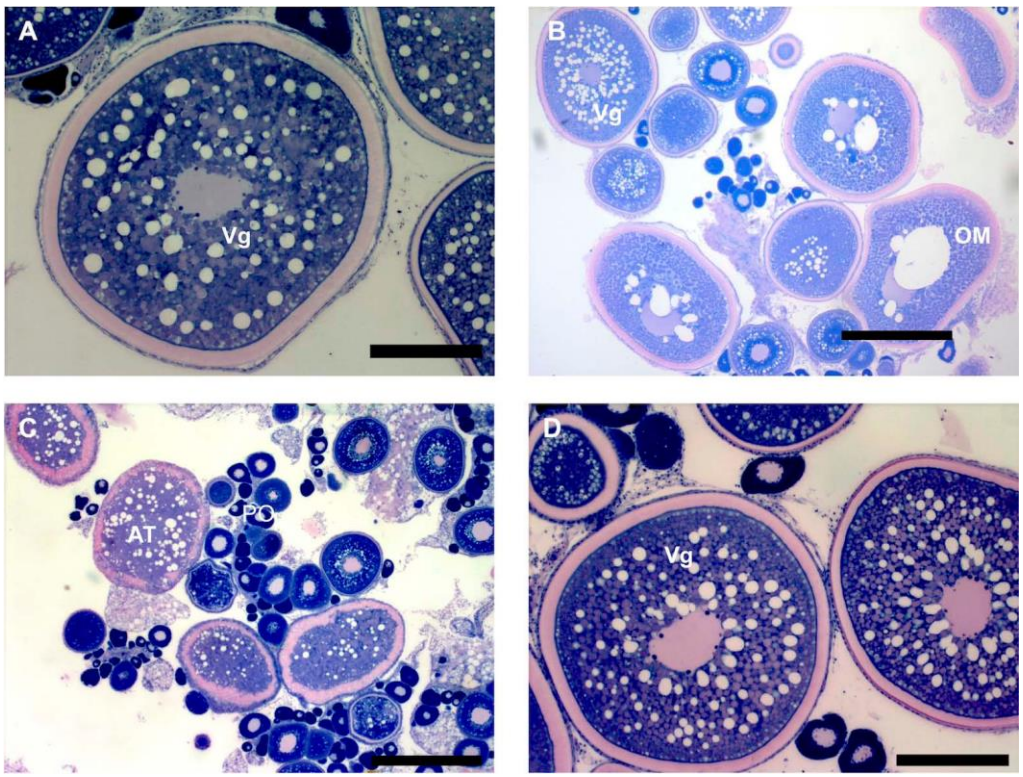

Figure 4.7 Microphotographs of representative ovarian biopsies (histological sections) from greater amberjack reared in sea cages throughout the year (ARGO, GMF and SOUDA). A) a female with oocytes in vitellogenesis (Vg), B) a female with oocytes in maturation (OM), C) a female with oocytes in follicular atresia (AT) and D) a female with Vg oocytes. PO=primary oocyte. Bars=500μm.

Males maintained in tanks or sea cages were in spermiating condition (82-100% depending on facility). Except from spermatozoa survival under cold storage (P = 0.02), there were no significant differences in the examined sperm quality parameters of males maintained in tanks versus sea cages, throughout the study (ANOVA, P<0.05). Over the 3 years of the study, males reared in tanks had mean spermatozoa motility of 80±7%, motility duration of 3.7±0.4 min, spermatozoa density of 31±2 10^9 szoa ml^{-1} and spermatozoa survival of 7±1 days **(Fig. 4.8)**. Concerning the sperm quality parameters of males reared in sea cages, mean spermatozoa motility over the three years was 81±3%, motility duration was 3.6±0.5min, spermatozoa density was 37±6 10^9 szoa ml^{-1} and spermatozoa survival was 9±1 days **(Fig. 4.8)**.

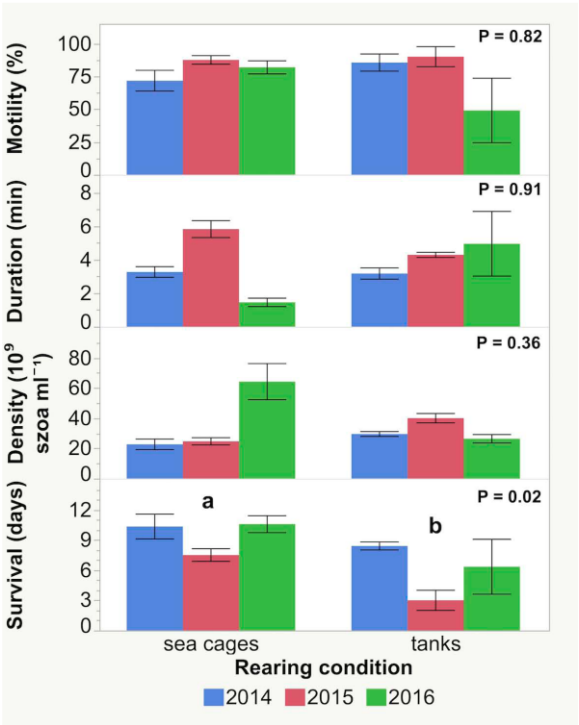

Figure 4.8 Mean (± SEM) spermatozoa motility (%), motility duration (min), density (x10^9 ml^{-1}) and survival under cold storage at 4°C (days) of sperm collected from greater amberjack males reared in tanks (n=1) or in sea cages (n=1). Statistically significant differences between males reared under different conditions (tanks vs sea cages) over the three years of the study were observed only for spermatozoa survival under cold storage (1-way ANOVA, P=0.02).

Although a dedicated analysis on the effect of broodstock feeding regime on reproductive performance was not carried out in the present study, they were not observed any startling differences between the rearing facilities that would lead us to believe that broodstock nutrition was a primary parameter influencing the results of this study. Looking at the gonadal development stage at the time of the experiments, such as ovarian biopsies and oocyte diameters (**Fig. 4.4**) and spermiation and sperm parameters (**Fig. 4.8**), the major effect -when it existed- was relevant to rearing conditions (*i.e.* sea cage vs tank) and not feeding regime.

Regarding the spawning kinetics and egg production data, in 2014 the females in the ARGO facility had a high percentage of atretic follicles (**Fig. 4.7C**), and as a consequence the percentage of the eligible females for spawning induction was only 13%. No eggs were collected after the GnRHa treatment. On the contrary, in the GMF and SOUDA facilities fish responded successfully to the GnRHa treatment and both cage spawning and cage-to-tank spawning resulted in the production of fertilized eggs (**Fig. 4.9 and 4.10**). In GMF, the eggs collected daily from the cage-to-tank spawning were 6-7 times higher compared to the eggs collected from the cage spawning (**Fig. 4.9**), but almost the same with the SOUDA facility (**Fig. 4.10**). When the GnRHa-treated fish were examined on Day 8 after treatment in SOUDA, almost all females had ovulated eggs in their ovaries, confirming that the fish did undergo oocyte maturation and spawned. At the same time, many of the fish used for spawning induction still had large numbers of fully Vg oocytes (**Fig. 4.6D and 4.7D**) or oocytes in maturation **(Fig. 4.7B**), making them eligible for another spawning induction. A 2nd GnRHa treatment was applied to the eligible fish in the SOUDA facility, producing one more spawn after 2 days (**Fig. 4.10).**

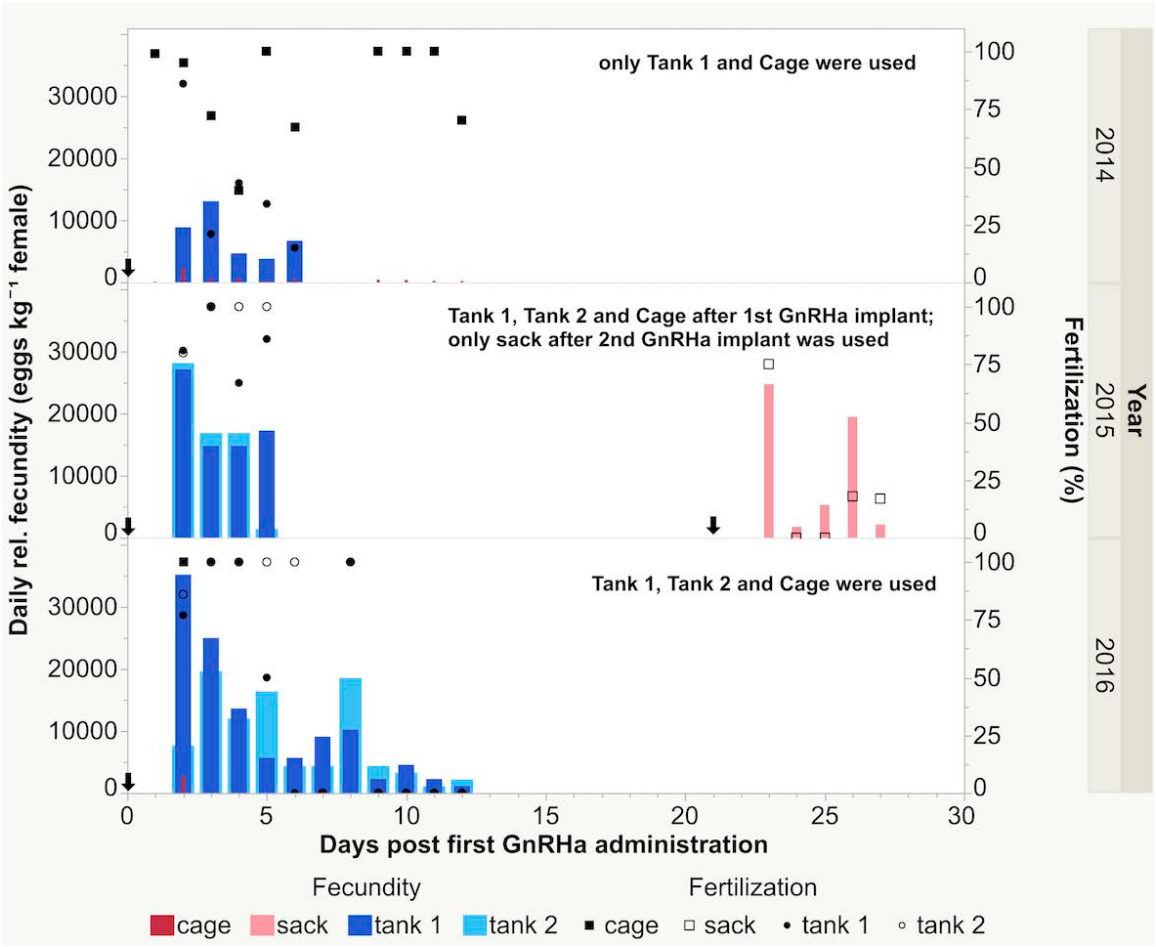

Figure 4.9 Daily relative fecundity (bars, eggs kg-1 female) and fertilization success (circles and squares, %) of greater amberjack reared in sea cages (GMF) and transferred to tank(s) for spawning after GnRHa administration (cage-to-tank, blue bars) or allowed to spawn in the sea cage (red bars). Day 0 was 26 June in 2014, 10 June in 2015 and 16 June in 2016. After the second GnRHa treatment in 2015, instead of allowing the fish to spawn in their sea cage, they were put in a large anaesthetizing tarpaulin (sack, light-red bars) within the sea cage, in order to ensure no spawned eggs were lost. Arrows on the x-axes indicate the time of GnRHa administration. The number of breeders (females/males) in 2014 was: Tank 1 = 3/2, Cage 11/12; and in 2015 it was: Tank 1 = 3/3, Tank 2 = 3/3, Cage = 8/7, Sack = 4/4; 2016: Tank 1: 3/4, Tank 2: 3/3, Cage = 8/5.

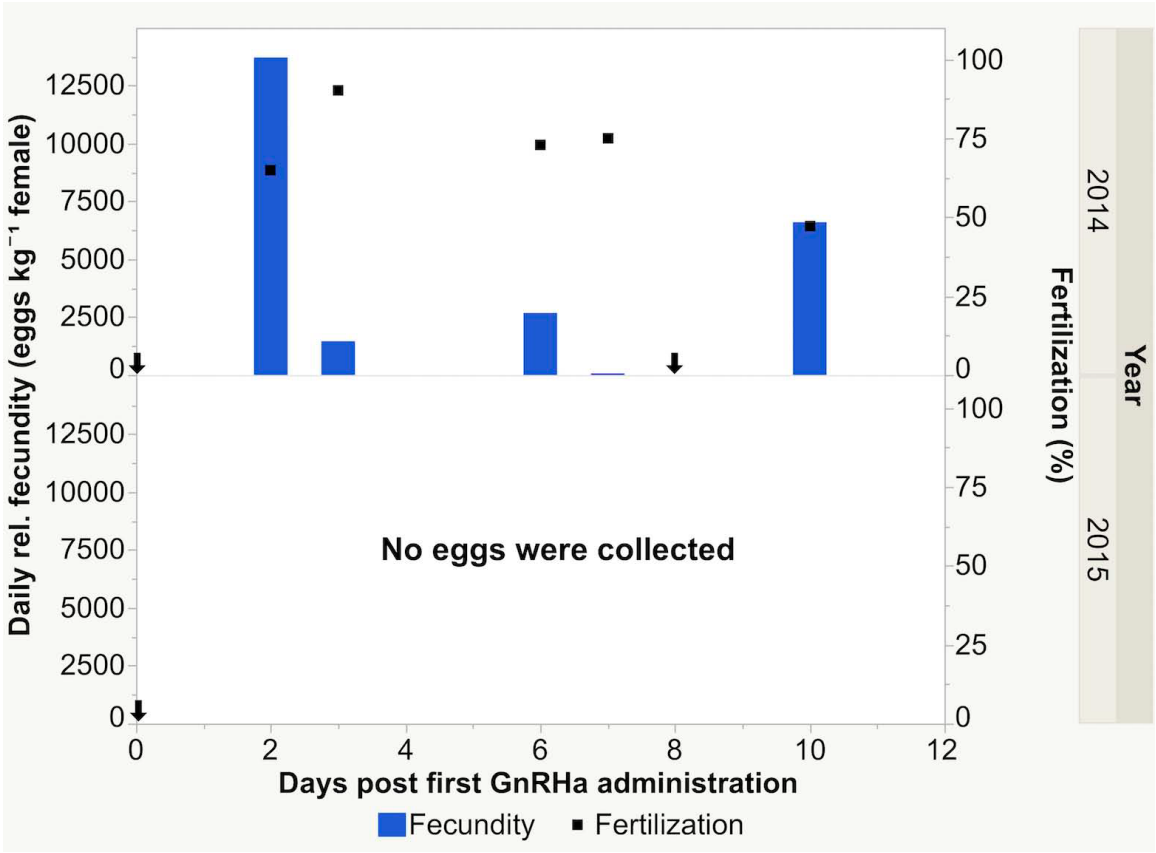

Figure 4.10 Daily relative fecundity (bars, eggs kg^{-1} female) and fertilization success (squares, %) of greater amberjack reared in sea cages (SOUDA) and allowed to spawn in the sea cage after GnRHa administration. Day 0 was 23 June both in 2014 and 2015. No GnRHa spawning induction was attempted in 2016. Arrows on the x-axes indicate the time of GnRHa administration. The number of breeders (females/males) was 4/8 in both years.

In 2015, the number of fish that had ovaries with fully Vg oocytes or oocytes in early maturation was higher than in 2014, so in the ARGO and GMF facility cage spawning and cage-to-tank spawning were tested, and cage spawning was also tested in SOUDA. Almost 30,000 eggs kg^{-1} female (GMF) (**Fig. 4.9**) or even more (ARGO) (**Fig. 4.11**) were collected from the cage-to-tank spawning the first days after the GnRHa treatment, but almost no eggs were collected from cage spawning in the three facilities (ARGO, GMF and SOUDA). At the re-evaluation of the ARGO and GMF broodstocks 3 weeks after the 1st GnRHa treatment, the majority of females still contained fully Vg oocytes (**Fig. 4.6D and 4.7D**) or oocytes in maturation, with a small percentage of females being "spent" with only POs and oocytes in follicular atresia (**Fig. 4.7C**), together with a few ovulated eggs. Therefore, a second GnRHa implant was administered to the vitellogenic females in GMF. However, since no egg collection from the sea cage spawning experiments was achieved -compared to the cage-to-tank spawning of fish from the same stock- and because the females were confirmed to have spawned after biopsying, there was a need to confirm that the failure to collect eggs was due to loss through the sea cage

net. Therefore, after the 2nd GnRHa treatment in GMF the fish were placed in the anesthesia sack, were no loss of eggs was possible. This resulted in greatly increased numbers of eggs collected the next days (**Fig. 4.9**), with 24,779 eggs kg^{-1} female collected compared to the 2,361 eggs kg^{-1} collected the previous year in the cage spawning trial. The fecundity and fertilization success obtained in the anesthesia sack was similar to the one obtained in the cage-to-tank spawning experiments of the same year (**Fig. 4.9**).

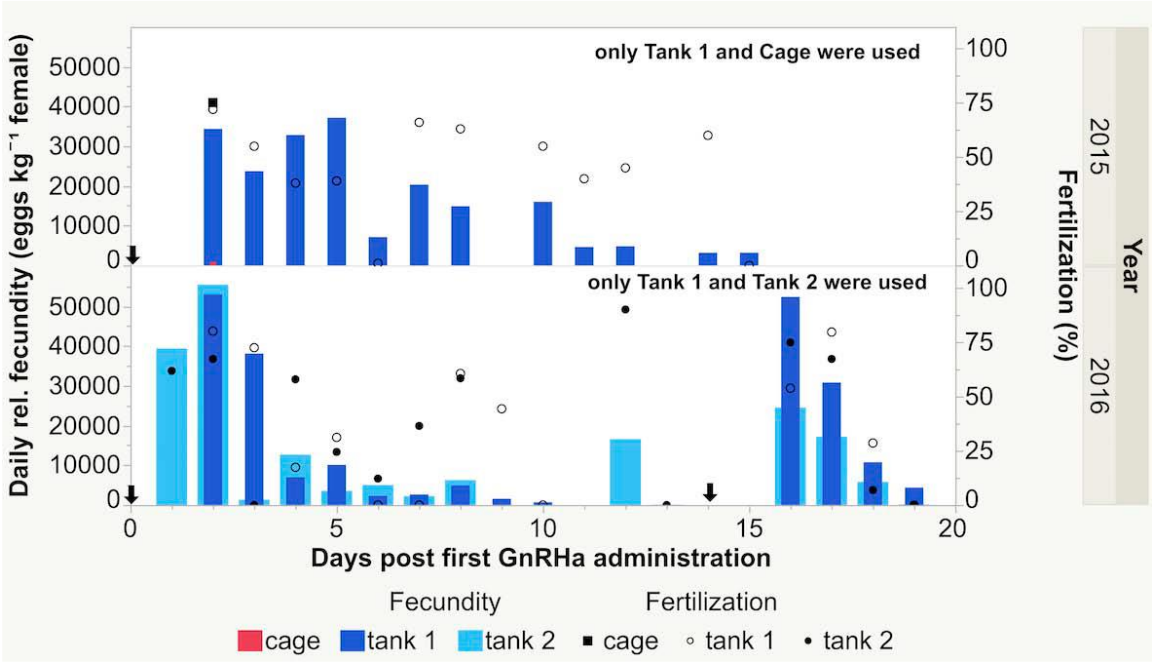

Figure 4.11 Daily relative fecundity (bars, eggs kg-1 female) and fertilization success (circles and squares, %) of greater amberjack broodstocks reared in sea cages (ARGO) and transferred to tank(s) for spawning after GnRHa administration (cage-to-tank, blue bars) or allowed to spawn in the sea cage (red bars). Day 0 was 9 June in 2015 and 7 June in 2016. Arrows on the x-axes indicate the time of GnRHa administration. The number of breeders (females/males) in 2015 was: Tank 1 = 4/3, Cage = 11/9; and in 2016 it was: Tank 1 = 3/3, Tank 2 = 4/4.

In 2016, the reproductive stage of the females reared in sea cages was the same as in 2015. Cage spawning was again tested at GMF and cage-to-tank spawning at GMF and ARGO. No spawning induction was attempted at SOUDA. Once again, cage spawning resulted in only 2,732 eggs kg^{-1} female being collected (**Fig. 4.9**). On the contrary, relative fecundity from cage-to-tank spawning was >35,000 eggs kg^{-1} female the first days after the GnRHa treatment at GMF (**Fig. 4.9**) and >55,000 eggs kg^{-1} female at ARGO (**Fig. 4.11**). At the re-evaluation of the ARGO broodstock 2 weeks after the 1st GnRHa treatment, the ovaries of most females contained fully Vg oocytes or oocytes in maturation –as in 2015- therefore the fish were given a 2nd GnRHa implant and were allowed to spawn for another week producing significant numbers of fertilized eggs (**Fig. 4.11**).

When examining the egg production data over the three years, overall fertilization success was higher in cage spawning and cage-to-tank spawning compared to tank spawning (P=0.01), but no significant difference was found for the number of spawns (P=0.17) **(Fig. 4.5)**. Total relative fecundity of cage-to-tank spawning was many-fold higher than cage spawning, but not statistically different from tank spawning (**Fig. 4.5**). The overall mean percentage of eligible females for a 2nd GnRHa spawning induction (2-3 weeks after the 1st GnRHa induction) for the cage spawning was 86±14%, for the cage-to-tank spawning was 46±18% and for tank spawning 0%.

4.4 Discussion

Given the problems encountered with egg production of this species in captivity and its large body size, here it was examined the reproductive function of greater amberjack reared both in land-based tanks as well as sea cages. Limited spontaneous spawning was documented here based on the presence of ovulated eggs in the ovarian biopsies of females reared both in sea cages and tanks, contrary to what has been reported so far for Mediterranean broodstocks kept in sea cages (Kozul, et al., 2001) or in tanks supplied with surface seawater (Grau, et al., 1996; Micale, et al., 1999; Pastor, et al., 2000). Spontaneous spawning has been reported both in the Canary Islands, Spain (Jerez, et al., 2006; Sarih, et al., 2018) and Japan (Kawabe, et al., 1998; Kawabe, et al., 1996). The broodstocks in the Canary Islands were maintained either in raceways of 500 m^3 supplied with seawater (Jerez, et al., 2006) or in circular tanks of 40 m^3 (Sarih, et al., 2018) also supplied with surface seawater, while in Japan they were maintained in sea cages and transferred to 70-m^3 tanks during the spawning season (Kawabe, et al., 1998; Kawabe, et al., 1996). However, the aquaculture industry cannot rely on such scarce spontaneous spawning (Jerez, et al., 2006; Sarih, et al., 2018) and spawning induction methods using exogenous hormones have been tested for the development of industrial protocols for the production of greater amberjack eggs throughout the world (Hamasaki, et al., 2009; Sarih, et al., 2018; Sawada, et al., 2006).

Based on the stage of gametogenesis of the females at the onset of the natural spawning season, the fish reared in tanks throughout the year in the present study exhibited a significant reproductive dysfunction compared to females reared in sea cages. This was reflected in the significantly lower maximum vitellogenic oocyte diameter achieved and the lower percentage of females being eligible for spawning induction with exogenous hormones. The fish maintained in the two different rearing conditions were provided with a similar -though not exactly the same- feeding regime and were exposed to similar environmental conditions as far as photoperiod and temperature. Also, the majority of individuals were from the exact same stock of wild juveniles captured initially in the Ionian Sea. However, fish in the sea cages were exposed to many fold larger enclosure volumes and lower stocking densities, and much less anthropogenic influences (*e.g.* noise and visual disturbances). In addition, the water quality of the supplied seawater in the tanks was not identical to the water where the sea cages were placed. The lack or unreliable oogenesis, oocyte maturation, ovulation and spawning in various fishes has been attributed to either improper or suboptimal environmental conditions, or captivity-induced stress (Mylonas, et al., 2010). Therefore, it is reasonable to assume that some of the reasons greater

amberjack reared in tanks during the year did not perform reproductively as well as fish reared in sea cages included (a) small rearing volume, (b) exposure to anthropogenic disturbances and (c) suboptimal water quality.

In terms of rearing volume and density, in the present study rearing density was higher than other studies, since the tank volumes used (25 or 40 m^3) were smaller than the 50 m^3 used for F1 broodstock where the fish completed vitellogenesis and were induced to spawn with GnRHa implants (Chapter 7). The tanks used were also many fold smaller than the 500-m^3 raceway where the same authors reported spontaneous spawning of wild-caught fish (Jerez, et al., 2006). On the other hand, they were of the same volume of a different study using a lower density, in which wild-caught fish completed vitellogenesis and were induced to spawn with GnRHa implants (Sarih, et al., 2018) or many fold larger than the 10-m^3 tank and at almost the same rearing density in which wild-caught fish were induced to spawn with GnRHa injections multiple times (Fernández-Palacios, et al., 2015a). So, it appears that tank volume or stocking density alone could probably not be the main reason females in the present study did not undergo vitellogenesis properly. Regarding the exposure to anthropogenic disturbances, there is no doubt that there are much more when fish are reared in tanks compared to sea cages. In the latter, fish are exposed visually to human presence only during feeding and the occasional net change, while noise exposure is again limited to some hours during the day when service boats are operated around the sea cage facility. For example, in a simulation experiment comparing the effect of a sea cage acoustic environment versus a tank acoustic environment, gilthead seabream grew better and had lower stress indicators in the sea cage aquaculture environment (Filiciotto, et al., 2013). In another study, negative effects of anthropogenic noise on spawning performance of Atlantic cod has been reported (Sierra-Flores, et al., 2015). Based on the available data it seems logical to expect that a sea cage provides a better environment for broodstock rearing and reproductive function.

One major difference between the environmental conditions of the tank-reared broodstocks versus the ones reared in sea cages was the chemical properties of the seawater. The water supplying the rearing tanks in both facilities was from a well (well seawater) and not directly from the sea (surface seawater). This is a common practice in the Mediterranean aquaculture for biosecurity reasons, since seawater from relatively shallow wells comes free of organisms (viruses, bacteria or eukaryotic parasites). However, well seawater may be significantly different in some chemical properties from surface seawater -mainly in dissolved CO_2 and pH- but the effect of these differences has not been yet evaluated properly in regards to fish reproduction. For example, in the AQUALABS tank facility the well seawater dissolved CO_2 was earlier determined (Katharios, et al., 2011) to be 4.3 times higher than the natural seawater (pCO_2 = 1,911 versus 460 μAtm) with a pH between 7.4-7.8 compared to the 8.2-8.3 of natural seawater. In the present study the pH ranged between 6.79 and 7.86 in the AQUALABS facility, with >80% of the registered values being between 7.15-7.76 and mean (± SD) annual values of 7.50 ± 0.24. The effects of low pH and high dissolved CO_2 in fish reproduction have not been studied extensively, but studies have shown that sensitivity varies among species, as well as

developmental stages (Ishimatsu, 2005). In a study with the marine fish two-spotted goby, which is considered a CO_2-tolerant species compared to species that inhabit the open sea, artificially elevating seawater pCO_2 to 1,400 μAtm and pH 7.6, from pCO_2 = 370 μAtm and pH 8.1 increased embryonic abnormalities and egg loss, although it did not affect spawning or clutch size (Forsgren, et al., 2013). These results suggest that marine fish may also be sensitive to increased CO_2, contrary to what has been hypothesized earlier (Melzner, et al., 2009). Therefore, it is possible that the high pCO_2 and low pH of well seawater (Katharios, et al., 2011) could be partly responsible for the abnormal gametogenesis of the greater amberjack reared in tanks, since this species and its congeners in nature inhabit the open sea where the seawater quality is stable, so the plasticity of these species to water quality modifications could be limited. Based on the wide use of well seawater -of usually much higher pCO_2 and lower pH- in marine aquaculture hatcheries, the subject is worth further examination. Interestingly, when the congeners yellowtail kingfish, longfin yellowtail and Japanese yellowtail were reared in tanks supplied with surface seawater they did complete vitellogenesis (Fernández-Palacios, et al., 2015b; Mushiake, et al., 1998; Roo, et al., 2014) and even spawned spontaneously (Moran, et al., 2007; Quiñones-Arreola, et al., 2015). Similarly, greater amberjack broodstocks reared in tanks that were supplied with surface seawater completed vitellogenesis and at times also spawned spontaneously (Jerez, et al., 2006; Sarih, et al., 2018).

On the other hand, the tank-rearing environment did not seem to have a negative effect on male greater amberjack compared to sea cages, since sperm quality parameters were not significantly different between the two rearing conditions (except for sperm survival under cold storage). The absence of or extremely low fertilization success obtained when the few eligible females reared in tanks during the year were induced to spawn was most probably related to the high occurrence of follicular atresia in these vitellogenic females, suggesting that they also produced eggs of bad quality. However, it has already been shown elsewhere that male greater amberjack reared in captivity had lower GSI, sex steroid levels (Zupa, et al., 2017b) and seminiferous lobule diameter, while they had higher number of apoptotic germ cells and an altered pattern of germ cell proliferation compared to male breeders sampled in the wild (Zupa, et al., 2017a). These results suggested that captivity affected spermatogenesis as well, causing reduced sperm production but not failure to reproduce, spawn and fertilize eggs. In the present study, neither the males reared in tanks or sea cages were releasing sperm upon application of gentle abdominal pressure (which is a common method in the majority of bony fishes), similar to most studies with captive males reared in tanks (Mylonas, et al., 2004b) or sea cages (Mylonas, et al., 2004b; Zupa, et al., 2017a), but contrary to what has been reported in one study (Kozul, et al., 2001). The present data show that even rearing in sea cages throughout the year and avoiding handling during the reproductive season (Zupa, et al., 2017a; Zupa, et al., 2017b) was not enough to prevent dysfunctions in spermatogenesis, resulting in lower sperm production. The same absence of sperm release upon application of abdominal pressure was observed also in captive-reared Atlantic bluefin tuna where intratesticular sperm was collected for the evaluation of sperm quality parameters as well (Suquet, et al., 2010).

Also, lower GSI values were observed compared to males sampled in the wild (Medina, et al., 2007), showing a similar dysfunction to the greater amberjack both in terms of GSI and produced sperm. This lower sperm production could be responsible for the relatively lower fertilization success obtained sometimes -compared to other marine aquaculture species- in the present and other studies of greater amberjack reproduction in captivity (Mylonas, et al., 2004b; Sarih, et al., 2018).

In terms of the characteristics of greater amberjack sperm, the spermatozoa density was within the same range (Zupa, et al., 2017a) or lower (Mylonas, et al., 2004b) than previous reported values for this species. Spermatozoa density of greater amberjack is at the upper range or higher than other marine species where sperm release upon abdominal pressure was possible, such as cod, hake (Cosson, et al., 2008), gilthead seabream, European eel (Kowalski & Cejko, 2019), wreckfish (Papadaki, et al., 2018b) and meagre (Mylonas, et al., 2016b), but it was within the same range with intratesticular sperm collected from Atlantic bluefin tuna (Suquet, et al., 2010). Spermatozoa motility duration was within the reported range for this species in other studies (Mylonas, et al., 2004b; Zupa, et al., 2017a). In relation to other species, spermatozoa motility duration of greater amberjack was higher than European seabass (Fauvel & Suquet, 1999), similar to meagre (Mylonas, et al., 2016b) and lower than wreckfish (Papadaki, et al., 2018b) and Atlantic bluefin tuna (Suquet, et al., 2010). Spermatozoa survival under cold storage was similar to another study with greater amberjack (Chapter 7) and to wreckfish (Papadaki, et al., 2018b), but higher than Atlantic bluefin tuna (Suquet, et al., 2010) and meagre (Mylonas, et al., 2016b). Spermatozoa motility was higher in the present study compared to other studies in the Canary Islands (Chapter 7) and the Mediterranean (Zupa, et al., 2017a), but in general it was lower than other cultured marine species such as the European seabass (Peñaranda, et al., 2008), meagre (Mylonas, et al., 2016b), wreckfish (Papadaki, et al., 2018b) and sharpsnout seabream (Papadaki, et al., 2008). These lower values could be related to the fact that sperm could not be collected with abdominal pressure, further suggesting that the spermatogenesis/spermiation process in greater amberjack was dysfunctional. Similarly, low spermatozoa motility of intratesticular sperm was reported in Atlantic bluefin tuna when abdominal pressure could not succeed in acquisition of sperm (Suquet, et al., 2010). Lack of testicular hydration and reduced spermiation was also correlated to low spermatozoa motility values in a recent study (Zupa, et al., 2017a), which fits with the relatively high spermatozoa density values reported here and also the relatively low fertilization success that was observed at some times in the present study. Therefore, the process of spermatogenesis and spermiation of greater amberjack in captivity is an area that needs to be further optimized for successful aquaculture development, and is currently under investigation.

Contrary to the situation when greater amberjack was reared in tanks, almost all females in the three different broodstocks reared in sea cages for three consecutive years concluded vitellogenesis successfully. No differences in maturity condition at the start of the experiment were observed among fish reared in the same way during the year (*i.e.* sea cage or tank), and although the experimental design was not appropriate for such evaluations, looking at the reproductive performance of all the broodstocks used in the study, there were no

marked differences that could be attributed to minor variations in husbandry implemented in the different facilities (*e.g.* water temperature profile, enclosure size and stocking density or feeding regime). The major differences were among rearing conditions during the year (*i.e.* sea cage or tank). In sea cages, in all facilities and every year some of the females matured and spawned spontaneously in addition to completing vitellogenesis and being eligible for spawning induction. The only exception was the ARGO broodstock in 2014, which had the lowest percentage of eligible females for spawning induction (13%) resulting in the reduction of the overall mean percentage of this group for the year. The biopsies taken from these females contained high percentage of follicular atresia, showing signs that the fish reached already the end of the spawning season (Marino, et al., 1995). This was due to the fact that this year the sampling was done in late June, when water temperatures had increased greatly (24-25°C), reaching a temperature where greater amberjack stop spawning (Jerez, et al., 2006; Sarih, et al., 2018). For this species, in Japan the optimum temperature for spawning was considered to be 21.5-23.5°C (Kawabe, et al., 1996) or 22.0-23.4°C (Kawabe, et al., 1998). In the GMF sea cage facility the water temperature at this time in 2014 was 22°C and 85% of the females were eligible for induction of spawning, while in the SOUDA facility the fish were at 23.3°C and 100% of the females were eligible for spawning induction. The following years the spawning induction was done earlier in June, when the seawater temperatures were within the range of the above reported values.

Eligible females from all examined broodstocks responded to the administered GnRHa treatment and spawned in all three different spawning conditions used -*i.e.* "cage", "cage-to-tank" or "tank"- but with significant differences among them in terms of effective fecundity (collected eggs), fertilization success and duration of spawning induction eligibility. The sustained-release GnRHa implants used in the present study promoted effectively oocyte maturation, ovulation and spawning, as already reported elsewhere for the same species (Mylonas, et al., 2004b; Sarih, et al., 2018) and a large number of other fishes (Mylonas & Zohar, 2001; Mylonas, et al., 2010). Moreover, the GnRHa implants promoted also vitellogenesis in many of the females, making them eligible for an additional spawning induction treatment, as was observed at the reproductive re-evaluation after the GnRHa treatments. The female greater amberjack is an asynchronous fish and spawns multiple times during the reproductive season. At the onset of the spawning season, vitellogenic oocytes of a wide range of sizes (and hence development stage) are present and while one batch may be undergoing oocyte maturation and another completing vitellogenesis, still less advanced batches (*i.e.* 400-600 μm in diameter) continue vitellogenesis for some days, until they reach the stage to enter maturation and be ovulated in subsequent batches. It appears that the sustained elevation of GnRHa release from the GnRHa implants used -which lasts for at least 2 weeks (Mylonas, et al., 2007)- induced not only oocyte maturation, but also promoted the late stages of vitellogenesis in greater amberjack as suggested recently. It is obvious that when the females were handled at the onset of the spawning season and were treated with GnRHa implants did not suffer the reproductive impairment due to handling stress reported elsewhere (Zupa, et al., 2017b), and their reproductive function continued properly. Interestingly, there was an obvious trend towards higher

numbers of females being eligible for a second GnRHa spawning induction when the fish were placed in sea cages for spawning compared to the ones transferred to tanks (cage-to-tank spawning). This longer maintenance of spawning eligibility of females maintained in cages is probably related to the better environmental conditions offered by the sea cage, as was already discussed above regarding welfare and reproductive function in general, and the process of oogenesis specifically.

Regarding spawning performance, there were significant differences in effective fecundity between cage spawning and cage-to-tank spawning -and to a lesser extent there were also fewer spawning events identified in sea cages- while fertilization success was similar in the two spawning methods. The differences were attributed to problems with egg collection in the sea cages, since significantly fewer eggs were collected, even though females exhibited clear signs of past spawns when re-evaluated a few weeks after the GnRHa induction. The loss of spawned eggs from the lower part of the sea cages was confirmed experimentally in one of the facilities (GMF in 2015), by placing the breeders in a fully closed sack after the 2nd GnRHa administration, which resulted in large numbers of eggs being collected, but with lower fertilization success. The fertilization success was possibly overestimated in the cage spawning condition due to egg loss, since non-fertilized or dead eggs are expected to have a lower buoyancy than fertilized ones, thus having less chances of being on the surface and being collected. For comparison, when the breeders were placed in the cage with the peripheral egg collection device after the 1st GnRHa administration, no egg collection was achieved. Curtain-type egg collection devices similar to the ones used in the present study have been used in the past for Atlantic and Pacific bluefin tuna (De Metrio, et al., 2010; Masuma, 2006; Mylonas, et al., 2007; Sawada, et al., 2005). Egg collection problems were reported due to currents or waves and the development of land-based breeding facilities have been proposed as a solution (Mylonas, et al., 2007) and are currently operating both in Japan and Spain. In the present study, three different sea cage sites were examined, all located very close inshore in areas protected from wind and wave action, though the existence of significant water currents could not be avoided. The failure to implement efficient egg collection for greater amberjack in sea cages -compared to bluefin tunas- was probably related to the lower buoyancy of greater amberjack eggs and the time of spawning in relation to when egg collection was attempted. Marine pelagic fish eggs are known to have increased specific gravity during the first stages after fertilization due to water loss (Jung, et al., 2014; Sundby & Kristiansen, 2015). This probably happens due to incomplete osmoregulation capability, which develops fully at a later stage of embryonic development. As a result, the eggs first become heavier soon after spawning, and then become lighter after mid-gastrulation (Jung, et al., 2014; Sundby & Kristiansen, 2015). Coupled with the relatively lower buoyancy exhibited by greater amberjack in general, the latter could be a possible explanation why it was not possible to collect eggs efficiently using the peripheral curtain-type egg collection devices, but they were collected successfully when the fully closed sack was used. The successful egg collection in one of the sea cage facilities (SOUDA in 2014), where similar numbers of eggs were collected from the sea cage compared to cage-to-tank spawning was probably because the fish spawned early in the

morning -based on the embryonic development stage at the time of collection (Tachihara, et al., 1993), exactly before the egg collection time, so the eggs were not heavy enough at that time to sink. In the other sea cage facilities, it was hypothesized that the fish spawned earlier during the night; hence the eggs became heavier and were swept by the currents when they sank lower in the water column of the cage. The observation from the cage-to-tank spawning experiments that greater amberjack may spawn between midnight to early morning supports this hypothesis.

In terms of spawning performance, the cage-to-tank method implemented here was similar or better in terms of total and daily relative fecundity than other spawning induction experiments with greater amberjack, considering the duration of the experiment, the number of spawning inductions and the differences in the duration of the spawning season of each location. In terms of fertilization success, our results were similar or worse than other studies. For example, assuming an equal sex ratio of 1:1 in the Japanese stocks (since the fish were not sexed), wild broodstock spawned spontaneously 269,918 eggs kg^{-1} female (15,877 eggs kg^{-1} female day^{-1} in 17 spawns between May 10 and July 3) of mean floating of 66% in 1990 and 281,286 eggs kg^{-1} female (17,580 eggs kg^{-1} female day^{-1} in 16 spawns between April 25 and June 4) of mean floating of 76% in 1991 (Kawabe, et al., 1996). In the Canary Islands, hatchery produced greater amberjack after three GnRHa treatments produced 118,120 eggs kg^{-1} female (2,270 eggs kg^{-1} female day^{-1} in 54 spawns over a period of 75 days) of <50% fertilization success (Chapter 7). Also in the Canary Islands, wild-caught breeders after five GnRHa treatments produced 327,425 eggs kg^{-1} female (or 25,200 eggs kg^{-1} female day^{-1} in 38 spawns between June 3 and October 14) of 32% fertilization success (Sarih, et al., 2018). In the same study, spontaneously spawning females produced 1,304,791 eggs kg^{-1} female (56,700 eggs kg^{-1} female day^{-1} in 23 spawns between June 1 to October 18) of 84% fertilization success. In the Mediterranean, a wild caught female after two GnRHa treatments produced 55,000 eggs kg^{-1} female (13,750 eggs kg^{-1} female day^{-1} in 4 spawns) of 23% fertilization success (Mylonas, et al., 2004b). Another broodstock in the same area after two GnRHa treatments produced 204,804 eggs kg^{-1} female (14,629 eggs kg^{-1} female day^{-1} in 14 spawns between June 7 to June 28) of 40% fertilization success. Females in the present study during three consecutive years after a single GnRHa treatment spawned on average 106,079 eggs kg^{-1} female (12,780 eggs kg^{-1} female day^{-1} in 8 spawns over a period of maximum 2 weeks) of 54% fertilization success. The higher fertilization success that was recorded in the above-mentioned studies with spontaneous spawning could be related to a higher male to female sex ratio. Unfortunately, the sex ratio was not reported in most of these studies (Jerez, et al., 2006; Kawabe, et al., 1996) except in the Canary Islands where a 5:2 male:female sex ratio was used in the spontaneous spawning tank (Sarih, et al., 2018). Supportive to this hypothesis is a study where a 2:1 male:female sex ratio after GnRHa injection treatment resulted in a fertilization success of 96% (Fernández-Palacios, et al., 2015a).

The cage-to-tank spawning was proven to be the most efficient combination of rearing and spawning conditions in the present study, and could be a possible solution for delivering good quality eggs to the

aquaculture industry in the short term, at least for as long as wild-caught breeders are employed. Apart from overcoming the failure of this species to undergo oocyte maturation, ovulation and spawning in captivity, the method used achieves also spawning synchronization of the broodstock and possibly greater parentage contribution to the mass spawning events, as was shown for the yellowtail kingfish (Setiawan, et al., 2016) and longfin yellowtail (Fernández-Palacios, et al., 2015b), avoiding low levels of polymorphism in the produced progeny due to a limited effective number of breeders (Nugroho, et al., 2000) when relying on scarce spontaneous spawns, of usually one or two females in the broodstock. The same cage-to-tank method was employed successfully in Japan for the greater amberjack (Kawabe, et al., 1998; Kawabe, et al., 1996), Japanese amberjack (Agius, et al., 2001; Vassallo-Agius, et al., 2002) and red sea bream (Watanabe & Kiron, 1995), but was unsuccessful for earlier attempts with captive-reared greater amberjack in the Mediterranean (Lazzari, et al., 2000) or breeders transferred directly from the fishery to land-based tanks (Pastor, et al., 2000). For the long-term development of a sustainable greater amberjack aquaculture, however, and when hatchery-produced breeders enter breeding selection programs, there is a need to control reproduction in land-based tanks with high biosecurity, where temperature and photoperiod could be manipulated in different broodstocks, so that the spawning season of greater amberjack can be extended in order to produce eggs for more than just a few months a year, as it is done routinely for many commercial aquaculture fishes.

Females reared in tanks supplied with well seawater throughout the year exhibited a significant reproductive dysfunction with limited gametogenesis, lower oocyte diameter at the peak of the reproductive season, low female eligibility for spawning induction and almost 0% fertilization success when the few eligible fish were induced to spawn using exogenous hormonal therapies. On the contrary, females reared in sea cages underwent vitellogenesis completely, and almost all females were eligible for spawning induction. No differences in sperm production and the main spermatozoa characteristics were observed between males reared in sea cages or tanks, but in general sperm production was lower compared to fish captured in the wild. For egg production, the most effective method was the rearing of breeders in sea cages throughout the year and then transferring them to land-based tanks for spawning after treatment with GnRHa implants. This method could be a possible solution for delivering good quality eggs to the aquaculture industry in the short term, at least for as long as wild-caught breeders are employed, since hatchery produced breeders may not exhibit the same reproductive dysfunction as wild-caught ones, as a result of domestication.

Chapter 5 - Spawning kinetics and egg/larval quality of greater amberjack in response to multiple GnRHa injections or implants

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5.1 Introduction

The greater amberjack is one of the most promising species for the diversification of aquaculture production, due to its cosmopolitan distribution (Paxton, et al., 1989) and acceptability, high growth rates and large size (Crespo, et al., 1994; Grau, et al., 1996; Jover, et al., 1999; Lazzari, 1991; Lazzari, et al., 2000; Mazzola, et al., 2000), and late maturation (Micale, et al., 1999; Zupa, et al., 2017b), which allows for the marketing of the fish before growth is affected by reproductive maturation. As with many other newly cultured species, reproduction control has been one of the main bottlenecks for the commercialization of greater amberjack (Mylonas, et al., 2016a). Although some natural -though unpredictable- spawning has been reported in the Canary Islands, Spain (Jerez, et al., 2006) and Japan (Kawabe, et al., 1996), this has not been the case in the Mediterranean Sea (Grau, et al., 1996). Failure of spawning in captivity can result from improper environmental conditions or by captivity-induced stress (Mylonas, et al., 2010). However, the main reproductive dysfunction observed in captive fishes is the lack or unreliable oocyte maturation, ovulation and spawning (Mylonas & Zohar, 2001) caused by inadequate pituitary LH synthesis and/or release at the end of vitellogenesis (Zohar & Mylonas, 2001). This reproductive dysfunction has been reported already in cultured greater amberjack (Kozul, et al., 2001; Micale, et al., 1999; Mylonas, et al., 2004b) and has so far barred its large-scale production in Europe. Recently, a comparative study of reproductive development in wild and captive-reared greater amberjack has shown the need for minimum handling of greater amberjack during the reproductive season, as this apparently induced significant long-term reductions in plasma T, E_2 and 17,20β-P (Zupa, et al., 2017b) in the females. As a result, the GSI was significantly reduced at the peak of the reproductive season, and extensive follicular atresia was present in the ovaries. Similarly, significant reductions in plasma T, 11-KT and 17,20β-P were observed in the males at the peak of spermatogenesis, again resulting in significant reductions in GSI (Zupa, et al., 2017b), concomitant with elevations in plasma E_2, reduction in spermatogonial mitosis and high level of apoptosis at the beginning of the reproductive season (Zupa, et al., 2017a). Therefore, more research is needed to understand the endocrine reproductive function of this species in captivity, and to overcome the observed reproductive impairments using the available endocrine therapies employed in a number of other species (Mylonas, et al., 2010; Mylonas, et al., 2017b).

Different hormonal therapies have been used in the past in order to induce oocyte maturation, ovulation and spawning, via the administration of LH (GtH or pituitary extracts), hCG or GnRHa (Mañanos, et al., 2009; Mylonas, et al., 2010; Zohar & Mylonas, 2001). Of these therapies, GnRHa is more widely used due to its advantages in relation to lower species-specificity and targeting a higher level in the brain-pituitary-gonad

axis, stimulating the release of the endogenous LH (Mylonas, et al., 2010). In addition, GnRHa may be administered in the form of a bolus (liquid injection) or in a sustained-release delivery system (Mylonas & Zohar, 2001), each method having important advantages in different species. For example in meagre, multiple injections of GnRHa were considered more advantageous compared to GnRHa implants due to better egg production control and repeatability of response (Mylonas, et al., 2015). In greater amberjack, spawning induction experiments have been done with both GnRHa implants (Mylonas, et al., 2004b) and multiple GnRHa injections (Fernández-Palacios, et al., 2015a), but a proper study comparing the two methods in greater amberjack in the Mediterranean Sea has not been carried out so far.

The aim of the present study was to examine these two endocrine methods for the induction of oocyte maturation/ovulation and spawning in greater amberjack. These methods were based on the induction of endogenous LH release from the pituitary, through the use of GnRHa either in the form of implants (sustained release) or injections (acute release). The efficacy of the two methods was evaluated in terms of oocyte development, spawning kinetics, egg production and quality, with the objective of delivering a sound and efficient protocol to the aquaculture industry. In addition, the study provides some insight into the endocrine control of the process of gametogenesis and oocyte maturation in the species, and the role of hypothalamic GnRH in acute versus sustained administrations.

5.2 Materials and methods

5.2.1 Broodstock maintenance

The amberjack broodstock was kept at the sea cage facilities of ARGO. The stock consisted of wild fish captured in 2011 at the juvenile stage with a purse seine from the area of Astakos, Greece. The fish were then moved to ARGO in May 2014 at a body size of 5-7 kg. The broodstock was maintained in a 1000-m^3 sea cage, 300 m off the coast where the on-land facilities of the company were located. At the time of the 2016 reproductive season (late spring-summer), the stock consisted of 14 females (mean ± SD body weight 18.8 ± 2.1 kg) and 14 males (mean ± SD body weight 15.1 ± 3.0 kg). Feed (Vitalis CAL, 22 mm, Skretting, Norway) was given to apparent satiation 6 days a week and fish were starved one day prior to handling.

For the spawning induction trial, fish were selected (see below in “Evaluation of reproductive stage and broodstock selection”) and induced to spawn, just prior to being transferred to the on-land facilities, to four 23-m^3 round tanks. Each GnRHa treatment was conducted in duplicates, with 6 and 8 fish per tank, respectively, at a 1:1 sex ratio, which means that each of the 4 replicates consisted of 3-4 females. The tanks were supplied with a mixture of surface (~16% exchange h^{-1}) and well water (~6% exchange h^{-1}) of ~39 ppt salinity and exposed to ambient photo-thermal conditions, with temperature ranging from 20.1°C to 24.1°C in the course of the experiment (**Fig. 5.1**). Gaseous oxygen was also provided to the tanks for safety reasons, maintaining the saturation to 110-120%. Measurements of temperature and oxygen saturation were conducted twice a day (a.m. and p.m.). Tank overflows were fitted with 250-L passive egg collectors. Feeding in the tanks was done to apparent satiation between samplings, 5 days a week.

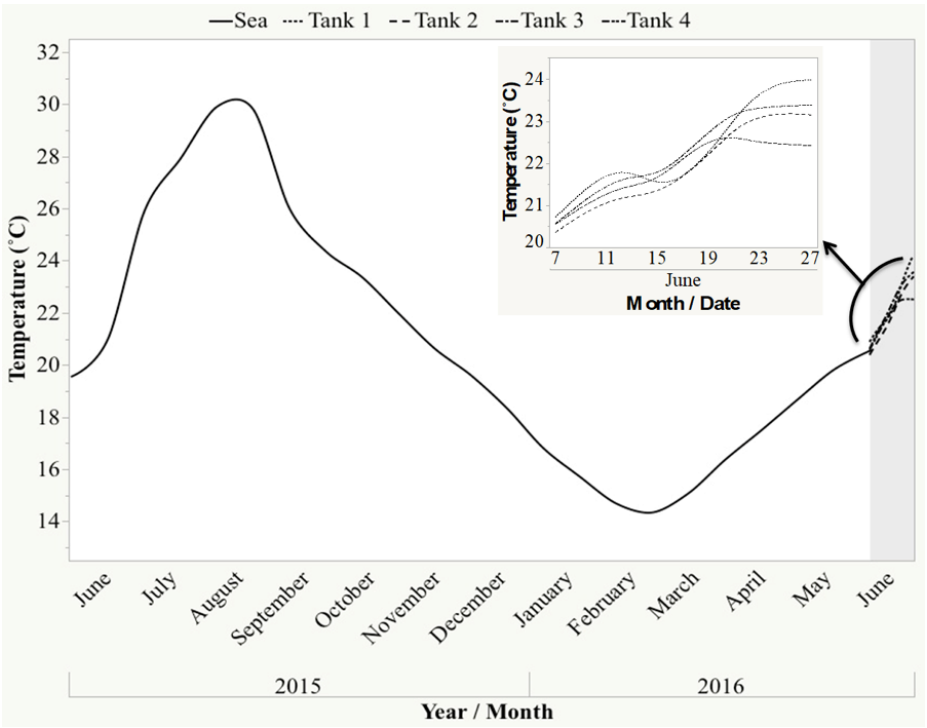

Figure 5.1 Water temperature profile for the sea cage maintaining the greater amberjack broodstock during the year (June 2015 - June 2016) or for the different tanks during the spawning induction trials (June 2016, inset). The shaded area indicates the experimental period.

For the present study, wild captive-reared greater amberjack were used. Ethical approval was not required by the relevant Greek authorities (National Veterinary Services), because the fish were maintained in a registered aquaculture facility. All procedures involving animals were conducted in accordance to the "Guidelines for the treatment of animals in behavioral research and teaching" (Anonymous, 1998), the Ethical Justification for the Use and Treatment of Fishes in Research: an Update (Metcalfe & Craig, 2011) and the "Directive 2010/63/EU of the European parliament and the council of 22 September 2010 on the protection of animals used for scientific purposes" (EU, 2010).

5.2.2 Evaluation of reproductive stage and selection of breeders

Once the fish were tranquilized inside their rearing sea cage with the use of clove oil (0.01 mL L^{-1}), they were moved for complete sedation to an anesthetic bath at a concentration of 0.03 mL L^{-1} (Mylonas, et al., 2005), on a working platform next to the sea cage. Ovarian biopsies were obtained by inserting an endometrial catheter (Pipelle de Cornier) into the ovarian cavity applying gentle aspiration. A wet mount of the biopsy was first examined under a compound microscope (40 and 100×) to evaluate the stage of oogenesis and measure the mean diameter of the largest, most advanced vitellogenic oocytes (n = 10), and pictures were taken for further evaluation. A portion of the biopsies was fixed in a solution of 4% formaldehyde-1%

glutaraldehyde for histological analysis. Females were considered eligible for spawning induction if they contained fully vitellogenic oocytes with a diameter >600 μm. Males were selected on the basis of spermiation, which was confirmed with the collection of a sample of milt using a catheter, since gentle abdominal pressure is not effective in releasing milt in greater amberjack, due to the thick muscular nature of the abdominal wall.

5.2.3 Spawning induction experiments

The spawning induction trial was conducted between 7 June and 28 June 2016. The females were treated either with GnRHa (Des-Gly^{10},D-Ala^{6}-Pro-NEth^{9}-mGnRHa, H-4070, Bachem, Switzerland) injection (20-25 μg GnRHa kg^{-1}) or with EVAc GnRHa implant (Mylonas and Zohar, 2001), loaded with 750 or 1000 μg of the same GnRHa, for an effective dose of 49-69 μg GnRHa kg^{-1} (**Fig. 5.2**). The selected doses were those used successfully in previous experiments using GnRHa implants (50 μg GnRHa kg^{-1}) (Mylonas, et al., 2004b) or GnRHa injections (20 μg GnRHa kg^{-1}) (Fernández-Palacios, et al., 2015a). In order to enhance spermiation and ensure adequate milt production, males were treated at the start of the experiment with a GnRHa implant at a dose of 45-70 μg GnRHa kg^{-1} and allocated randomly to the four tanks. Sperm quality (spermatozoa motility percentage, duration of forward motility, spermatozoa density and spermatozoa survival at 4°C) was also evaluated prior to the GnRHa administration. The dose for the first hormonal administration with GnRHa was given based on an estimated 30% growth from the last weighing time (the previous year, on 15 June 2015). As the fish did not have a uniform growth during this period, there was a variation in the effective GnRHa dose applied to each fish at the first injection. For the calculation of the GnRHa dose for the following hormonal administrations, the actual weight was used. For the GnRHa implant administrations, variations were caused also by the fact that implants are loaded with fixed amounts of GnRHa. Even though implants loaded with different amounts of GnRHa were used, it was still not possible to adjust the dose exactly to the different body weights of the fish.

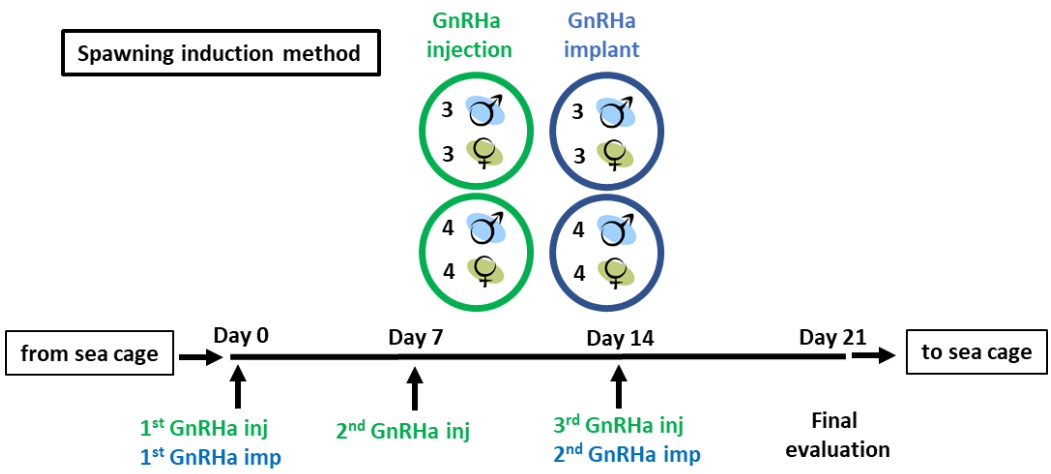

Figure 5.2 Schematic representation of the experimental design of the spawning induction method. Greater amberjack broodstocks were maintained in a sea cage during the year and were transferred to onshore tanks

after GnRHa spawning induction (arrows), and were returned to sea at the end of the experiments. Prior to GnRHa administration the fish were biopsied to evaluate stage of ovarian development and spermiation.

During the initial GnRHa administration, females were allocated to four tanks in order to have two duplicates per treatment method (3 and 4 fish per replicate). Females were treated weekly in the injected group, and biweekly in the implanted stocks (a total of 3 injections and 2 implants)(**Fig. 5.2**). The subsequent administrations and samplings were implemented with the same procedure used for the first sampling. The water level was reduced in the tanks; the fish were tranquilized and were then moved to an anesthetic bath for complete sedation prior to biopsying and GnRHa administration. Three weeks after the start of the experiment, on 28 June 2016, the final sampling was conducted and the fish were returned to the sea cage.

5.2.4 Evaluation of egg/larval quality

Egg collectors were examined three times a day (8:00 a.m., 3:00 p.m. and 8:00 p.m.). For each spawn, the date, collection time and developmental stage of the eggs (Tachihara, et al., 1993) were recorded, in order to identify different spawns and estimate an approximate spawning time. This allowed calculating the time between hormonal administration and first spawn (latency period). The eggs were collected and transferred into a 10-L bucket. Their number (fecundity) was estimated by counting the total number of eggs in a sub-sample of 10 mL, collected with a pipette after vigorous agitation. Daily relative fecundity was calculated as the number of eggs produced every day in each tank per female biomass, considering the eligible females that had vitellogenic oocytes >600 μm and were expected to spawn after each GnRHa administration. Total relative fecundity was calculated as the number of eggs produced after each hormonal administration per female biomass in each tank, as above. The fertilization percentage was evaluated at the same time by examining each egg in the subsample. After collecting the sub-sample, the eggs were transferred into a 500 L conical tank-incubator fitted with an overflow filter (250 μm mesh size), and supplied with surface seawater (~90% h^{-1} renewal) and mild aeration. The embryonated eggs were then sent to different hatcheries for larval rearing trials (Mylonas, et al., 2016a).

To monitor embryo and larval survival, embryonated eggs from each spawn were collected from the tank incubators and placed individually in 96-well mct plates (in duplicates) according to the procedure of Panini et.al (2001) with some modifications. Briefly, a sample of floating (~100% fertilized) eggs were taken from the tank incubators with a 250 μm mesh sieve, rinsed with seawater and poured in 2 L beakers filled with seawater. Using the sieve, 100-200 floating eggs were scooped from the beaker and placed in a Petri dish. Together with 200 μL of seawater, the fertilized eggs were aspirated with a micropipette one by one and transferred individually to the 96-wells of a mct plate. The plates were checked under a stereoscope and any dead eggs were replaced. Once loaded, the mct plates were covered with a plastic lid, placed in a controlled-temperature incubator and maintained for 7 days at temperatures ranging between 21.0 and 23.5°C, according

to the spawning temperature of each batch. Using a stereoscope, embryonic and early larval development was evaluated daily, recording the number of live embryos 24 hours after egg collection (or ~30 hrs after spawning), hatched larvae (examined ~55 hrs after spawning) and viable larvae on day 5 after hatching (near the time of yolk sack absorption). At 21.0-23.5°C, hatching of the amberjack eggs took place in 40-55 hrs (39.2 – 48.1 degree-days). The embryo survival was calculated as the number of eggs having live embryos 1 d after egg collection/number of fertilized eggs initially loaded in the mct plates. The hatching success was calculated as the number of hatched larvae/24-hr embryos, and the 5-d larval survival was calculated as the number of live larvae 5 d after hatching. Estimating survival percentage (%) by using as denominator the number of individuals that survived to the previous developmental stage was considered as a more accurate evaluation of survival within specific developmental stages, without the potential of a distortion effect of the previous stage.

5.2.5 Histological analysis

The samples were fixed in 4% formaldehyde: 1% glutaraldehyde (McDowell and Trump, 1976), dehydrated in a 70–95% ethanol series and embedded in glycol methacrylate resin (Technovit 7100, Heraeus Kulzer, Germany). Serial sections were obtained at a thickness of 3–5 μm on a microtome (Leica RM2245, Germany) using disposable blades. After drying, slides were stained with methylene blue/azure II/basic fuchsin (Bennett, et al., 1976), examined under a light microscope (50i Eclipse, Nikon, Japan) and photographed using a digital camera (Progres, Jenoptik AG, Germany).

5.2.6 Statistical analysis

Differences in mean oocyte diameter, relative and total fecundity and egg/larval performance parameters (fertilization success, 24-hr embryo survival, hatching, and 5-d larval survival) between the two GnRHa treatments were examined using a t-test at a $P \leq 0.05$ significance level. Within GnRHa treatment, differences among data collected in the period between GnRHa administrations were examined using one-way ANOVA followed by the post-hoc test Tukey's HSD at a $P \leq 0.05$ significance level (only for total relative fecundity due to unbalanced sample sizes for the other parameters). Data was examined for normality in the distribution of variances, in order to comply with the prerequisites of the ANOVA. Percentages were arcsin transformed before statistical analysis. All analyses were performed with a statistics software (JMP 12, SAS Institute Inc., Cary, NC, USA). Results are presented as mean ± SEM, unless otherwise stated.

5.3 Results

There were differences between the females from the two GnRHa treatments in the number of females remaining eligible for induction of spawning (*i.e.* having fully vitellogenic oocytes >600 μm) at the final sampling at the end of the experiment (**Fig. 5.3A**). However, there was no significant difference in the mean oocyte diameters between the females from the two GnRHa treatments ($t_2 = -0.71$ $P = 0.145$), since the samples represented only females eligible for induction of spawning and the evaluated oocytes were only those in advanced vitellogenesis (**Fig. 5.3B**). Specifically, in the GnRHa implant treatment, the number of eligible

females decreased from the initial administration from 7 to 6 females at the final sampling, whereas in the GnRHa injection treatment the number of eligible females decreased from 7 to 3 females.

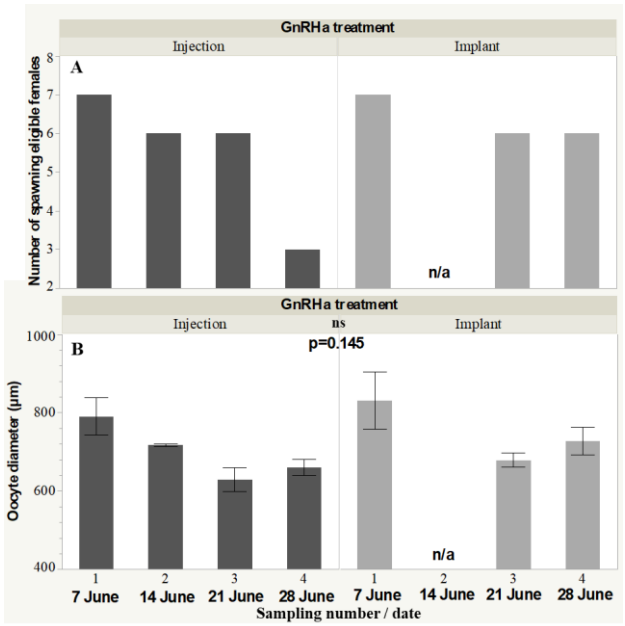

Figure 5.3 Number of spawning-eligible females (A) and mean diameter (± SEM) of the largest vitellogenic oocytes (B) from the biopsies of female greater amberjack treated with multiple administrations of a GnRHa injection or GnRHa implant. The P value of the statistical analysis (t-test) between the two GnRHa treatments is indicated on the GnRHa treatment legend (ns = no significance). n/a: not applicable.

At the time of the 1st GnRHa hormonal administration, females had mainly vitellogenic oocytes in their ovaries, while 4 out of the 14 were in oocyte maturation (**Fig. 5.4, first row**) and only a small number of atretic follicles was observed. During the 2nd sampling, only the injected fish were handled and biopsied. Vitellogenic oocytes were still present, except in one female where only ovulated eggs and atretic follicles were observed (**Fig. 5.4, second row**). Some ovulated eggs and atretic follicles were observed in almost all the biopsies taken. At the 3rd sampling, vitellogenic oocytes were visible in both treatment groups (**Fig. 5.4, third row**), except from one fish from each treatment that had only atretic follicles and ovulated eggs, while the number of primary oocytes was increased. At the 4th sampling, the implanted fish still had vitellogenic oocytes and some of them in oocyte maturation, and the proportion of atresia was minimal (**Fig. 5.4, fourth row**). On the other hand, 4 of 7 injected fish had concluded their reproductive period (*i.e.* they were spent), since only primary and early vitellogenic oocytes were present in the ovaries, together with advanced atretic follicles and unreleased, overripe eggs. The other 3 females from the injected treatment still had some vitellogenic oocytes, but with increased number of atretic follicles.

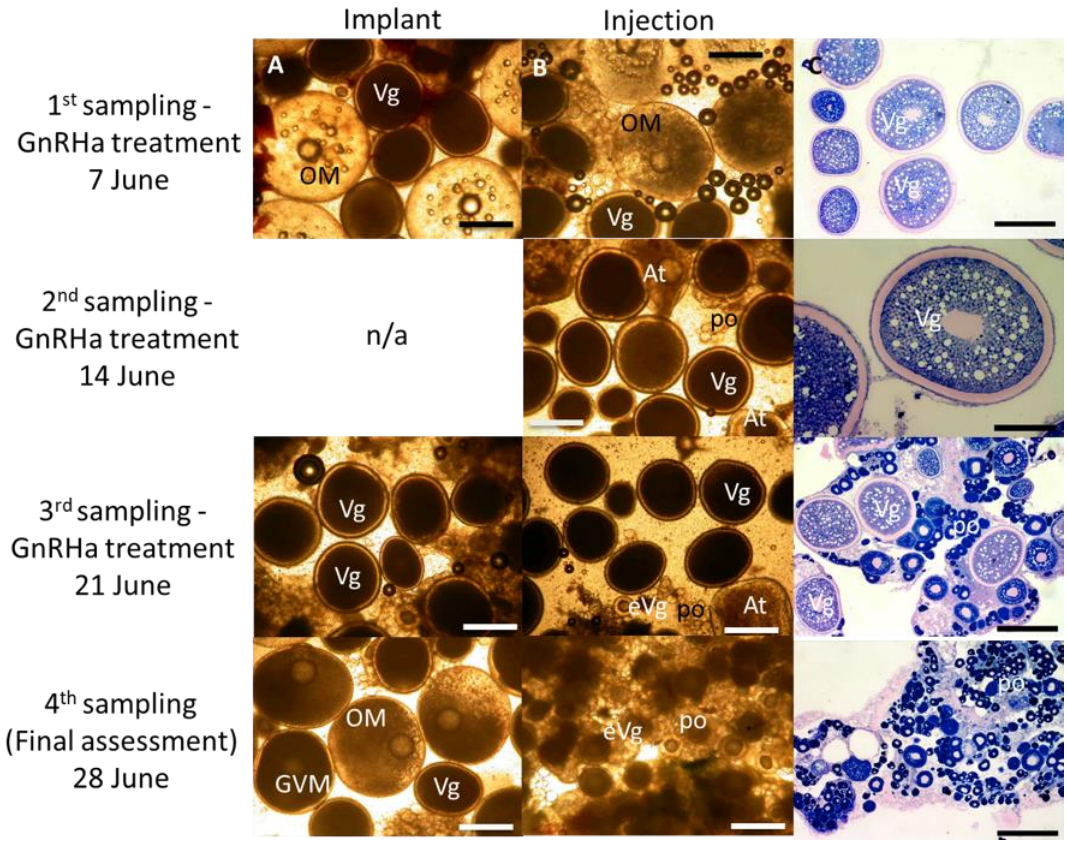

Figure 5.4 Microphotographs of representative ovarian biopsies from greater amberjack just prior to the different GnRHa administrations and the final sampling time at the end of the experiment, presented as wet mounts (left and middle columns) or after histological processing (right column). Fish were treated either with GnRHa implants or injections. po=primary oocyte, Vg=vitellogenic oocyte, eVg=early Vg, GVM=Germinal Vesicle Migration, OM= Oocyte Maturation, At=atretic follicle. Bars=500μm.

In response to both GnRHa treatments, spawning started 1 day after the 1st application, because of the existence of maturing oocytes, whereas after the following GnRHa administrations spawning commence after 2 days (**Fig. 5.5**). Implanted fish spawned for up to ten times after the 1st administration, and only four times after the 2nd administration. Injected fish spawned for seven times after the 1st administration, three to five times after the 2nd administration and one to three times after the 3rd administration.

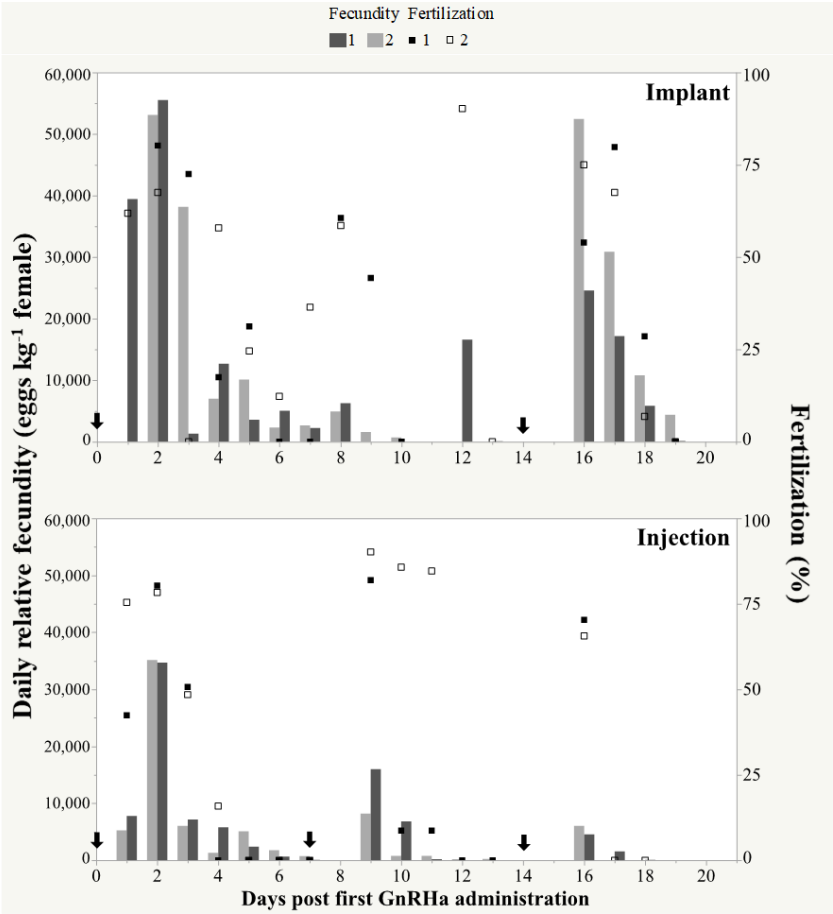

Figure 5.5 Daily relative fecundity (bars, x eggs kg^{-1} female) and fertilization success (squares, %) of GnRHa injected or implanted greater amberjack (n=2 tanks for each treatment group, numbered 1 and 2). Arrows (n=2 for GnRHa implanted and n=3 for injected fish) indicate the time of hormonal administration. The first application was done on 7 June 2016.

Mean daily relative fecundity was significantly higher ($t_{1.3}$ = -5.24, P = 0.012) in the implanted fish (15,170 ± 2,738 eggs kg^{-1} female day^{-1}) compared to the injected fish (6,119 ± 2,790 eggs kg^{-1} female day^{-1}) (**Fig. 5.6**). Total relative fecundity was also significantly higher ($t_{1.3}$ = -9.93, P = 0.003) in the implanted fish (102,402 ± 20,337 eggs kg^{-1} female) compared to the injected ones (26,517 ± 9,938 eggs kg^{-1} female) (**Fig. 5.7**). When looking separately at the total egg production in the period between different GnRHa administrations, we observed a significant decrease (ANOVA, Tuckey's HSD, $P \leq 0.05$) over time in fish from the GnRHa injected treatment, but not in the implanted treatment. The highest daily egg production was observed in implanted fish, with 4,242,000 eggs $tank^{-1}$ two days after the 1st administration, while in injected fish maximum daily egg production was 2,454,000 eggs $tank^{-1}$ at the same time.

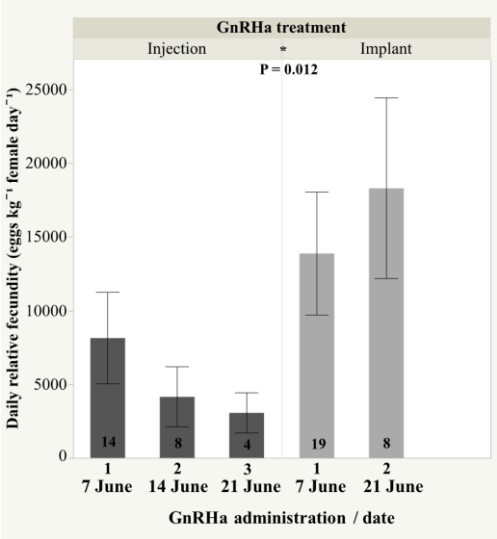

Figure 5.6 Mean (± SEM) daily relative fecundity of greater amberjack in the periods between different GnRHa injection or implant administrations. Numbers inside the bars are the spawns constituting each mean. A statistically significant difference between the two GnRHa treatments is indicated by an "*" and the P value of the t-test (n=2 replicate tanks per treatment) on the GnRHa treatment legend.

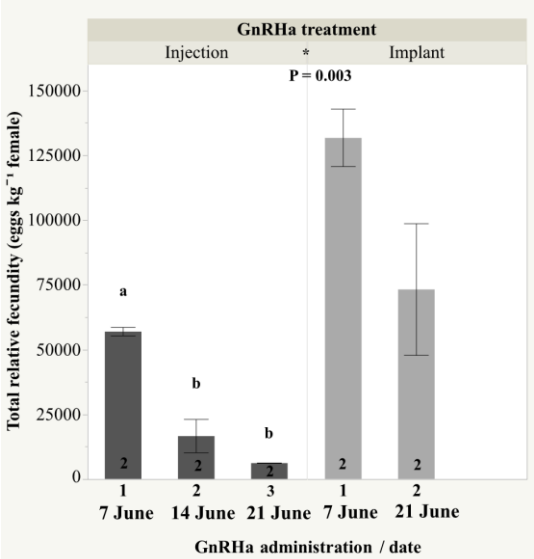

Figure 5.7 Mean (± SEM) total relative fecundity of greater amberjack in the periods between different GnRHa injection or implant administrations. Numbers inside the bars are the replicates for each mean. A statistically significant difference between the two GnRHa treatments is indicated by an "*" and the P value of the t-test (n=2 replicate tanks per treatment) on the GnRHa treatment legend. Lowercase letters above the hormonal administration means indicate significant differences within GnRHa administrations (ANOVA, Tukey's HSD, $P \leq 0.05$).

Fertilization success, 24-h embryo survival, hatching and 5-d larval survival was similar between eggs obtained with the two GnRHa treatments (**Fig. 5.8**). Overall, mean fertilization success was 36 ± 5%, 24-h embryo survival was 53 ± 7%, hatching was 70 ± 4% and 5-d larval survival was 20 ± 4%.

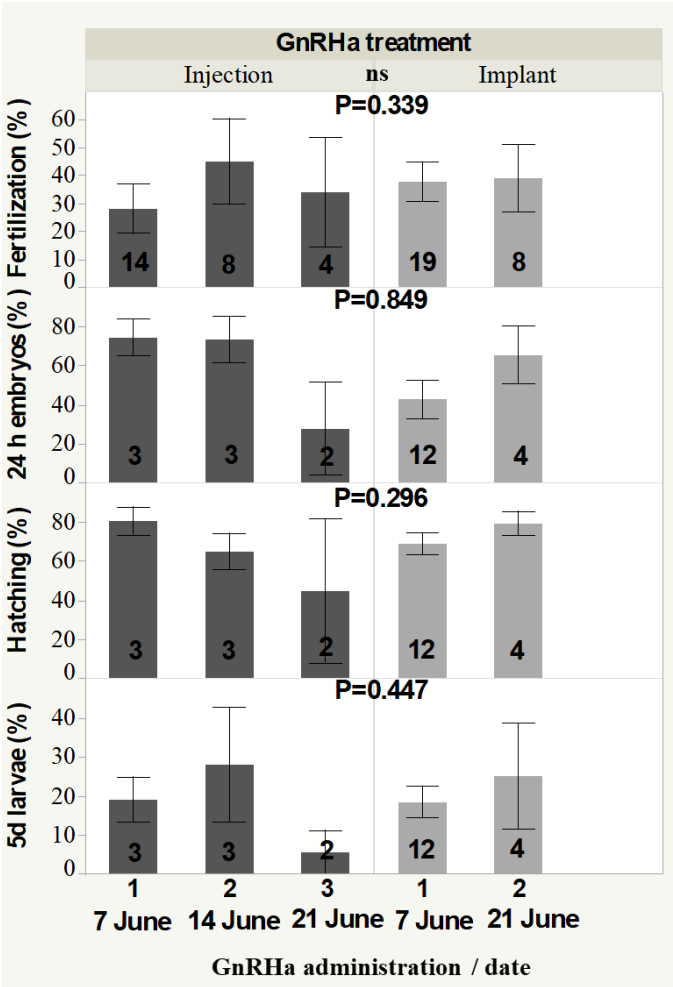

Figure 5.8 Mean (±SEM) fertilization, 24-h embryo survival, hatching and 5-d larval survival of greater amberjack eggs collected in the periods between different GnRHa injection or implant administrations. Numbers inside the bars are the number of spawns for each mean. No statistical differences were observed between different GnRHa treatment methods (t-test, n=2 replicate tanks per treatment, P value on each graph).

5.4 Discussion

Greater amberjack in the present study were induced successfully to spawn using either GnRHa implants of ~50 μg kg^{-1} fish or GnRHa injections of 20 μg kg^{-1} fish, spawning many times over the course of 3 weeks, and producing fertilized eggs of adequate quality for large scale larval rearing trials (Mylonas, et al., 2016a). These results confirmed previous studies where GnRHa implants (Mylonas, et al., 2004b) or injections (Fernández-Palacios, et al., 2015a) induced multiple spawning of viable eggs, overcoming the observed

reproductive dysfunctions of the species in captivity. The GnRHa therapy was effective in all fish treated, and resulted in many more spawns and a longer reproductive season than reported previously (Mylonas, et al., 2004b). Developing a reliable method for the production of eggs from a large number of breeders is important for the development of a sustainable industry of greater amberjack, because in Europe (and especially in the Mediterranean) spontaneous reproduction of the species is very rare (Jerez, et al., 2006; Lazzari, et al., 2000; Mylonas, et al., 2004b; Rodríguez-Barreto, et al., 2014). In addition, synchronizing spawning of a large group of breeders could mitigate the problem of disproportionate parental contribution and inbreeding, when eggs are produced from only a limited number of breeders, as it was shown for the congeners yellowtail kingfish (Setiawan, et al., 2016) and longfin yellowtail (Fernández-Palacios, et al., 2015b).

Early studies on the induction of spawning of greater amberjack utilizing chum salmon PE resulted in the production of 1.4 and 7 million eggs in two different reproductive seasons (Tachihara, et al., 1993), whereas the use of hCG injection produced mostly unfertilized eggs (Kozul, et al., 2001). The more recent use of GnRHa seems to be the most efficacious method in greater amberjack (Fernández-Palacios, et al., 2015a; Mylonas, et al., 2004b), as it was shown in other species as well (Mylonas, et al., 2010). However, the differences in response (*e.g.* spawning kinetics, egg production and quality) between the methods of administration of GnRHa have not been systematically evaluated so far. It has been shown long time ago that the *in vitro* degradation rate of even the most potent synthetic GnRHa is very rapid, with a half-life of only minutes (Zohar, et al., 1990; Zohar, et al., 1989). *In vivo*, most of the GnRHas used stimulated increases in plasma LH for only 48 hours (Zohar, et al., 1990; Zohar, et al., 1989). In other studies, injections of a bolus of GnRHa resulted in measurable levels in the plasma for a period of 2-5 days (Harmin & Crim, 1993; Mylonas, et al., 1998a), stimulating an increase in plasma LH for a similar period (Mañanos, et al., 2002; Mylonas, et al., 1998a). On the contrary, implants loaded with GnRHa resulted in measurable levels in the plasma for periods >14 days, resulting in continuously elevated plasma LH for as long as GnRHa could be measured in the plasma, without any indication of downregulation (Mañanos, et al., 2002; Mylonas, et al., 1997b; Mylonas, et al., 1998a). Due to these differences in the kinetics of plasma LH induced by a bolus versus a controlled release GnRHa administration, it was interesting both from an aquaculture production perspective, but also from a reproductive endocrinology one, to compare the effects of GnRHa injections and GnRHa implants on the reproductive function of greater amberjack, which has an asynchronous ovarian development and multiple spawning frequency during the reproductive season.

In the present study, GnRHa implanted females produced significantly more eggs of the same quality in the same number of spawns compared to the GnRHa injected females, with less handling. In addition, most of the GnRHa implanted females still contained a significant number of vitellogenic oocytes at the end of the experiment, when spawning ceased, and presumably had the potential to produce more eggs if treated with another GnRHa implant. It was shown recently that expression of the *fshβ* in the pituitary increased, and plasma FSH remained elevated throughout the reproductive season, including the spawning period, at the time that

expression of pituitary *lhβ* and plasma LH increases significantly (Nyuji, et al., 2016). Therefore, the synthesis and release of FSH from the pituitary during the peak of the reproductive period (*i.e.* during spawning) is quite relevant in this asynchronous species, presumably for the maintenance of high E_2 and vitellogenin levels in the plasma (Mandich, et al., 2004) and the support of gametogenesis and the production of multiple batches of oocytes for maturation. The results of the present study suggest that the sustained presence of GnRHa in circulation, resulting from the administration of sustained-release delivery systems was probably more physiologically sound for the continued stimulation of gametogenesis, through FSH release, compared to a multiple GnRHa injection protocol. In a similar comparison with the present study in the Canary Islands (Spain), GnRHa implants were shown to be more reliable in inducing spawning than GnRHa injections, since fish responded consistently after a GnRHa implant administration, spawning an average of 2.2±1.9 spawns per implantation (Sarih, et al., 2018). On the contrary, after GnRHa injection fish spawned only 0.8±0.5 spawns per injection, suggesting that only 80% of the injections resulted in spawning. If fish after some injections spawned more than once after the injection (this information is not provided in the article), then the success of that study was even less than 80%. Fecundity was similar in the two methods in that study, even if the number of spawns obtained after GnRHa implantation was more than 2x higher. Based on the higher fertilization success of eggs obtained from the GnRHa injected group, however, it was reported that this was a preferred method of spawning induction (Sarih, et al., 2018). Greater amberjack in the Canary Islands exhibit somewhat different reproductive characteristics with a much more prolonged reproductive period --lasting from April to October (Jerez, et al., 2006; Sarih, et al., 2018)-- presumably due to the more stable environmental conditions (photoperiod and temperature) that exist in sub-tropical regions. Perhaps the use of a bolus GnRHa administration once every ~10 days in the study of Sarih et al. (2018) was physiologically a more appropriate method under these conditions, as it was shown in meagre maintained under constant temperatures from the beginning of the spawning season and for the next 4 months (Mylonas, et al., 2016b). In the present study, two administrations of GnRHa implants spaced 14 days apart produced significantly more eggs of the same quality compared to three weekly GnRHa administrations with injections, with the GnRHa implantation approach involving less handling.

The greater amberjack is characterized as group synchronous or asynchronous regarding its ovarian development, with a multiple spawning reproductive strategy (Marino, et al., 1995). The GnRHa implant is considered as the most appropriate method of administration for this group of fishes, since it can both induce multiple cycles of oocyte maturation and ovulation, and it can support also the final stages of vitellogenesis in fish that are not quite ready to undergo oocyte maturation (Mylonas, et al., 2004b). In the multiple batch spawner Senegalese sole, two different GnRHa sustained-release delivery systems were proven to be the most effective for spawning induction when compared with a single (Guzmán, et al., 2009) or multiple injections of GnRHa (Agulleiro, et al., 2006). However, in meagre, a species which is also characterized by asynchronous oocyte development (Gil, et al., 2013; Mylonas, et al., 2013), multiple GnRHa injections were reported to be

a more appropriate method of spawning induction compared to GnRHa implants, even if the overall results of egg production and quality did not differ significantly between the two methods (Mylonas, et al., 2015). In meagre, GnRHa implants induce daily spawns for up to 3 weeks, but the majority of the eggs are produced during the first 2-3 spawns after administration, while the following spawns consist of small batchers of eggs that cannot be utilized efficiently by commercial hatcheries (Mylonas, et al., 2013), and eventually the gonad is depleted of vitellogenic oocytes and further GnRHa implantation does not produce consistent results (Mylonas, et al., 2015). On the contrary, weekly GnRHa injections induced only two high-fecundity spawns after each injection (day 2 and 3 after hormonal administration) -- presumably due to the short-lived elevation in plasma LH-- and the hormonal administration produced consistently spawns of high fecundity and egg quality for up to 17 weeks (Mylonas, et al., 2016b). So, the choice between GnRHa injection(s) or sustained-release delivery systems for the induction of spawning must be examined in each species of interest, as well as in relation to the specific environmental conditions and genetic origin of the broodstock in question. Recent genetic studies suggested that greater amberjack may be separated into an Atlantic and Mediterranean population (Šegvić-Bubić, et al., 2016), and this may explain –at least in part-- the differences in response to the GnRHa injections and implants of the fish between the present study and the one in the Canary Islands (Sarih, et al., 2018).

Group synchronous oocyte development is exhibited also by the congeners of greater amberjack such as the yellowtail kingfish (Poortenaar, et al., 2001), the Japanese yellowtail (Kagawa, 1989) and the longfin yellowtail (Fernández-Palacios, et al., 2015b), and all have been induced to spawn successfully with various hormonal therapies (Chuda, et al., 2002; Fernández-Palacios, et al., 2015a; Roo, et al., 2014; Setiawan, et al., 2016). In the Japanese yellowtail a comparison among three different hormonal therapies was examined, using a single or double hCG injection, or a single cholesterol implant with GnRHa. Even though the latter hormonal administration produced the highest fecundity, a single hCG injection was considered as the most efficient method to induce oocyte maturation and ovulation, based on the production of eggs of better quality (Chuda, et al., 2001). However, when hCG administration is used repeatedly in subsequent years, the fish may develop an immune response and the injected preparation is immune-neutralized (Zohar & Mylonas, 2001). This is an important disadvantage of the hCG over the GnRHa preparations for inducing spawning of fishes for aquaculture production. This is especially true in species such as the greater amberjack that are mainly of wild-caught origin, are difficult to acquire and they mature after >3-4 years.

Eighty-six percent of the females from the GnRHa implanted group and 43% of the injected group still had a large number of fully vitellogenic oocytes 3 weeks after the initial hormonal administration and after spawning multiple times. It was expected that these fish would produce more eggs if given another hormonal therapy. As discussed earlier, this observation demonstrates the capacity of GnRHa, primarily in a sustained-release delivery system, to not only induce maturation of the available post-vitellogenic oocytes, but also to further support vitellogenesis of the smaller oocytes, as it was shown in the dusky grouper (Marino, et al.,

2003), eventually resulting in multiple cycles of oocyte maturation, ovulation and spawning. Unfortunately, oocyte diameter data at the time of consecutive GnRHa administrations are not available from some other spawning induction studies of greater amberjack (Fernández-Palacios, et al., 2015a; Sarih, et al., 2018). In a recent study in the Canary Islands, F1 greater amberjack broodstock treated with GnRHa implants every 30-40 days maintained their maximum oocyte diameter above 650 μm for a period of 5 months (May - September) (Chapter 7). The fact that the fish in these studies spawned for a period of many months after multiple injections every 10 days in the Canary Islands underlines the capacity of GnRHa to induce vitellogenesis, in addition to oocyte maturation, probably through the induction of FSH release, in addition to LH release from the pituitary (Zohar, et al., 2010). The same capacity was evident in greater amberjack in the present study, albeit to a lesser extent, since in the Mediterranean (Zupa, et al., 2017b) and Japan (Kawabe, et al., 1998; Kawabe, et al., 1996) the rapid elevation of water temperature in the summer most likely causes a cessation of reproductive function. Perhaps this is the reason that the same species has a much longer spawning season in the Canary Islands (up to October) where the water temperature does not exceed 25°C (Jerez, et al., 2006; Sarih, et al., 2018), whereas in Japan (Kawabe, et al., 1996) and the Mediterranean Sea (Mandich, et al., 2004; Marino, et al., 1995) the spawning season ends in July. The effectiveness of GnRHa in sustained release delivery systems to promote the process of vitellogenesis has been demonstrated in only limited situations, such as in the ayu (Aida, 1983), bitterling (Shimizu, 1996), winter flounder (Harmin, et al., 1995) and the congener yellowtail kingfish (Setiawan, et al., 2016). In greater amberjack it was suggested recently that an increase in the expression of the FSH receptor in the ovarian follicles was more important for regulating vitellogenesis than elevation of FSH content in the plasma (Nyuji, et al., 2016). Again, in the present study it appears that the sustained presence of GnRHa in circulation, resulting from the administration of sustained-release delivery systems was probably more appropriate than a multiple GnRHa injection protocol in stimulating the necessary increases in FSH receptor expression.

The overall daily and total relative fecundity after the two GnRHa implantations were 2.5 and 3.8 times higher, respectively, than the three GnRHa injections. Considering only the first GnRHa implantation (9.5±0.7 spawns) and injection (7±0 spawns), the overall daily and total relative fecundity were 1.7 and 2.4 times higher, respectively. Multiple GnRHa injections in another study produced less than 45,000 eggs kg^{-1} fish per hormonal administration (Fernández-Palacios, et al., 2015a), slightly lower than the respective maximum fecundity in response to GnRHa injections in the present study. In studies using GnRHa implants in the Canary Islands (Chapter 7) and Greece (Mylonas, et al., 2004b), the respective daily relative fecundity was less than 6,000 eggs kg^{-1} and 30,000 eggs kg^{-1}, respectively, which is 9 and 2 times lower compared to the maximum observed in the present study after GnRHa implantation. Wild-caught fish spawning spontaneously in the Canary Islands produced 114,490 eggs kg^{-1} (or a total of 14,311,200 eggs) during the extended spawning period (April to October) (Jerez, et al., 2006). In the present study, the overall mean (± SD) total fecundity of the GnRHa implanted fish was 204,805 ± 20,214 eggs kg^{-1} (or a total of 13,489,000 ± 1,507,000 eggs), in a

period of only 21 days. The mean total fecundity per female was 3,889,000 ± 355,00 eggs female^{-1}, which is higher than another study in the Canary Islands using multiple GnRHa injections (2,480,000 eggs female^{-1})(Fernández-Palacios, et al., 2015a).

Fertilization success was similar in the eggs obtained using any GnRHa treatment, suggesting that in both treatments the process of oocyte maturation, ovulation and spawning was equally successful, if we assume that the performance of the males was similar in both groups. To ensure the latter, males in the two GnRHa treatment groups were treated in a similar way (GnRHa implants) and the sperm quality was found to be the same at the beginning of the experiment. Similar values of fertilization success were found in a spawning induction experiment of greater amberjack using GnRHa implants in Greece (Mylonas, et al., 2004b) or in the Canary Islands using F1 broodstock (Chapter 7). Significantly higher values, however, were reported in a multiple GnRHa injections experiment (96.0 ± 6.5%) with the same species (Fernández-Palacios, et al., 2015a). In the latter study, the sex ratio was higher in favor of the males (1:2 ♀: ♂) than the present study (which was 1:1 ♀:♂), and this could have resulted in better fertilization success. In a different study where natural (*i.e.* no hormonal administration), GnRHa injected and GnRHa implanted groups were compared, fertilization success was 84.4 ± 21.6%, 58.8 ± 26.8% and 32.5 ± 34.6%, respectively, being significantly different among the groups (Sarih, et al., 2018). Again, in the latter study the sex ratio of the most successful group (natural) was higher in favor of the males (2:5 ♀: ♂). Based also on reports from greater amberjack in Japan, it seems clear that an increased male to female ratio is preferable for this species, since more than one male appears to fertilize the eggs of one female (Tachihara, et al., 1993). In the congener yellowtail kingfish, it has been shown that in a communal tank with 14 breeders, in 50% of the recorded spawning events two males fertilized the eggs of one female, resulting in >99% fertilization success, suggesting also that a higher male to female ratio is preferable for that congener as well (Moran, et al., 2007). In the same species, a high male contribution (60% in all egg batches) in the fertilized eggs was found after parentage analysis of the eggs spawned in a communal tank with 14 females and 10 males (Setiawan, et al., 2016).

The quality of the eggs obtained in the present study --in terms of embryonic development, hatching and larval survival until yolk sack absorption—did not exhibit any significant differences between the two GnRHa treatments, but was lower than in other studies. One day after spawning, only 53 ± 7% embryos were still alive compared to 92.2 ± 9.4%, 86.4 ± 25.4% and 77.6 ± 34.0% for eggs obtained from natural, GnRHa injected or GnRHa implanted females, respectively, in a study in the Canary Islands (Sarih, et al., 2018). Hatching was also lower in the present study compared to the above-mentioned study, being 96.6 ± 6.6% for eggs from the natural, 91.1 ± 25.4% from the injected and 78.0 ± 34.9% from the implanted females. However, 5-d larval survival was higher here, compared to the 10.8±14.7% for eggs from the natural, 5.50± 7.2% from the injected and 8.0 ± 12.5% from the implanted group, respectively (Sarih, et al., 2018). Although there is room for improvement in terms of the quality of the eggs produced in response to the spawning induction therapy, the eggs produced were of adequate fecundity and quality to implement a number of larval rearing

trials, for the development of commercial production protocols (Mylonas, et al., 2016a). Improvements in egg quality are expected to result from a number of actions, such as better selection of female breeders according to their production characteristics (Symonds, et al., 2014), optimized sex ratios, tank size and perhaps above all, broodstock nutrition (Izquierdo, et al., 2001; Roo, et al., 2015; Valdebenito, et al., 2013). The latter is expected to gain more importance once a growing greater amberjack aquaculture industry is established, making it worthwhile for feed companies to invest in both specialized grow out and broodstock feeds for this species.

In conclusion, spawning induction of captive-reared greater amberjack was more effective using GnRHa implants than injections. More eggs were produced using GnRHa implants compared to injections, without altering the quality of eggs in terms of fertilization, 24-h embryo survival, hatching and 5-d larval survival. In addition to apparently promoting the proper endocrine pathways leading to multiple cycles of oocyte maturation, ovulation and spawning, the use of GnRHa implants induced also vitellogenesis. This method may be more effective in greater amberjack than multiple injections, also because of the less handling it involves (*i.e.* one handling every two weeks as opposed to one handling every week). It has recently been shown that handling greater amberjack during the reproductive season may induce significant reductions in spermatogenesis and oogenesis (Pousis, et al., 2018; Zupa, et al., 2017a; Zupa, et al., 2017b). Therefore, minimizing the handling involved when implementing spawning induction methods is important for production of good quality gametes in aquaculture.

Chapter 6 - Optimization of a GnRHa treatment for spawning commercially reared greater amberjack: dose response and extent of the reproductive season

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6.1 Introduction

In the effort to diversify and expand aquaculture production, the greater amberjack is one of the most promising species worldwide, due to its cosmopolitan distribution (Paxton, et al., 1989), rapid growth and large size (Crespo, et al., 1994; Grau, et al., 1996; Jover, et al., 1999; Lazzari, 1991; Lazzari, et al., 2000; Mazzola, et al., 2000), late maturation (Micale, et al., 1999; Zupa, et al., 2017b) and high market acceptability (Sicuro & Luzzana, 2016). As consumers show increasing interest towards value-added seafood products (Asche, et al., 2009), large-sized fish species are more appropriate for the processing and production of a number of products suitable for profitable market niches.

Reproduction control has been one of the main bottlenecks for the commercialization of greater amberjack (Mylonas, et al., 2016a), with failure of oocyte maturation, ovulation and spawning being the major problem observed (Kozul, et al., 2001; Micale, et al., 1999; Mylonas, et al., 2004b). Spontaneous spawning has been scarce and unreliable, and has been reported in the Canary islands (Jerez, et al., 2006; Sarih, et al., 2018) and Japan (Kawabe, et al., 1996), but rarely in the Mediterranean (Grau, et al., 1996). This dysfunction, which is caused by inadequate pituitary release of luteinizing hormone (LH) when vitellogenesis is completed (Mylonas, et al., 2010), has been addressed successfully in a number of recent studies using hormonal therapies in the form of multiple injections or controlled-release delivery systems of GnRHa (Fernández-Palacios, et al., 2015a; Nyuji, et al., 2019; Sarih, et al., 2018). In a recent study that evaluated the performance of both administration methods, GnRHa implants were shown to be more effective (Chapter 5). However, an optimization of the GnRHa dose through a dose–response study has not been carried out yet. Choosing the right dose can be a key to better results and increased cost-effectiveness, as demonstrated in the cyprinid Pengha, where fecundity, number of spawns, fertilization and hatching were observed to be affected by the hormonal dose (Das, et al., 2016). Similarly in the spotted rose snapper, different GnRHa doses resulted in differences in hatching rates (Ibarra-Castro & Duncan, 2007) and in meagre consecutive injections of 15 μg GnRHa kg^{-1} produced the most spawns with the highest fecundity and egg/larval quality, while lower and higher doses gave somewhat inferior results (Fernández-Palacios, et al., 2014).

Since greater amberjack do not reproduce spontaneously in the Mediterranean, the extent of their potential breeding season when reared in captivity is still not known, and must be determined for proper hatchery planning by the industry. Worldwide, greater amberjack is found throughout the temperate zone where it spawns naturally from February to April in the Gulf of Mexico (Wells & Rooker, 2004), from April

to October in the Canary Islands (Jerez, et al., 2006), from April to June in Japan (Kawabe, et al., 1998; Kawabe, et al., 1996; Nyuji, et al., 2016) and from May to July in the Mediterranean (Marino, et al., 1995) with the optimum spawning temperature supposedly ranging from 21.5°C to 23.4°C (Kawabe, et al., 1998; Kawabe, et al., 1996). An efficient spawning induction protocol was reported recently in the Mediterranean, where greater amberjack was maintained in sea cages throughout the year and were transferred to onshore tanks for spawning after administration with GnRHa implants (Chapter 4). However, a drawback of maintaining broodstocks in sea cages is the lack of temperature control, since annual variations may shift or shorten the potential reproductive period. Repeated sampling of breeders in the reproductive period to monitor reproductive status can cause significant reproductive impairment in this species (Pousis, et al., 2018; Pousis, et al., 2019; Zupa, et al., 2017a; Zupa, et al., 2017b), and handling should be limited to the spawning induction. Therefore, it is important to determine the extent of the potential breeding season during which greater amberjack maintained in sea cages can respond successfully to spawning induction with GnRHa implants.

The two objectives of the present study were to a) determine the most efficient GnRHa implant dose and b) examine the extent of the spawning induction period based on the resulting spawning kinetics, egg production and quality in greater amberjack maintained in sea cages during the year and transferred to onshore tanks for spawning. This information will contribute further to the delivery of an efficient egg production protocol to the aquaculture industry of the greater amberjack.

6.2 Materials and methods

6.2.1 Fish husbandry and evaluation of reproductive stage

Wild greater amberjack juveniles were fished in the Ionian Sea, Greece, with a commercial purse seine fishing vessel and were maintained in a sea cage of a commercial aquaculture operation at Astakos (Greece). In 2013, 3-year-old fish from this stock were transferred to two commercial operations in Greece (ARGO, GMF) and were reared in round sea cages of 40 m perimeter and 8 m in depth. Broodstocks were fed commercial extruded feed (Skretting Vitalis CAL, 22 mm). Feed was administered 3 to 5 times a week until apparent satiation. Temperature and dissolved oxygen parameters were measured 1 to 7 times a week. The seawater temperature at GMF ranged between 13.0°C and 27.4°C during the year (**Fig. 6.1**). At ARGO, the temperature at the sea cage ranged between 13.9°C - 27.0°C in 2017 (data not shown).

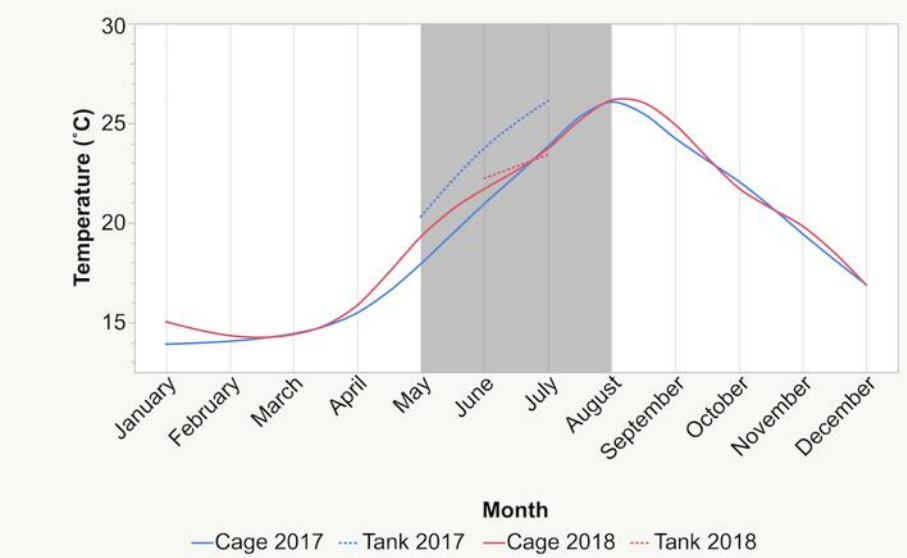

Figure 6.1 Water temperature profile in the Galaxidi Marine Farm (GMF) facilities where greater amberjack broodstocks were maintained in sea cages during the year and were transferred to onshore tanks after GnRHa spawning induction at different periods in 2017-2018. The shaded area indicates the estimated breeding period of greater amberjack in the wild in the Mediterranean Sea.

Broodstock selection for spawning induction experiments was done after a 2-day starvation period. Fish were initially tranquilized in a 10-20 m^3 anesthesia sack placed inside the cage using clove oil (0.01 ml L^{-1}), and from there the fish were transferred one by one to a 1.0 m^3 tank for complete anesthesia with a higher concentration of clove oil (0.03 ml L^{-1}) (Mylonas, et al., 2005). Ovarian biopsies for the evaluation of oocyte developmental stage were obtained by inserting a plastic catheter (Pipelle de Cornier, Laboratorie CCD, France) and applying gentle aspiration. A wet mount of the biopsy was examined under a compound microscope (40 and 100 x) to evaluate the stage of oogenesis and measure the diameter of the most advanced batch of vitellogenic oocytes (n=10). Females were considered eligible for spawning induction if they contained fully vitellogenic oocytes of >600 μm in diameter, as described previously (Chapter 5). Because of the hard musculature surrounding the abdominal cavity and the limited quantity of sperm produced by captive greater amberjack (Zupa, et al., 2017a; Zupa, et al., 2017b), milt samples were obtained by catheterization, as described above for the females. Males were considered eligible for spawning induction if milt could be obtained with the catheter.

Ethical approval for the experiment was obtained by the relevant Greek authorities (National Veterinary Services) under the license No 255356 (ΑΔΑ: 6ΛΙ17ΛΚ-ΠΛΩ). All procedures involving animals were conducted in accordance to the "Guidelines for the treatment of animals in behavioral research and teaching" (Anonymous, 1998), the Ethical justification for the use and treatment of fishes in research: an update (Metcalfe & Craig, 2011) and the "Directive 2010/63/EU of the European parliament and the council of 22 September 2010 on the protection of animals used for scientific purposes" (EU, 2010). The greater

amberjack is classified as “Least Concern” in the IUCN Red List of Threatened Species (Smith-Vaniz, et al., 2015).

6.2.2 GnRHa dose comparison

The spawning induction trial for the comparison of GnRHa doses was conducted between June 7 and July 5, 2017 at ARGO (**Fig. 6.2**). Females (n=10) of mean BW ± SD 23.0 ± 2.2 kg maintained in a sea cage were selected according to their stage of gonadal development and were allocated to four 23-m^3 round tanks onshore (2 or 3 females per tank) together with an equal number of spermiating males (1:1 sex ratio) of 18.4 ± 1.9 kg BW. Females were treated with an EVAc implant (Mylonas & Zohar, 2001; Mylonas, et al., 2007) loaded with 500–1000 μg of Des-Gly^{10}, D-Ala^{6}-Pro-NEth^{9}-mGnRHa implants (H-4070, Bachem, Switzerland), resulting in an effective dose of ~25 μg GnRHa kg^{-1} BW (group named as “LOW”, two tanks) or ~75 μg GnRHa kg^{-1} BW (group named as “HIGH”, two tanks). The effective dose for the females was 22.4 ± 2.4 μg GnRHa kg^{-1} for the LOW group and 74.4±4.5 μg GnRHa kg^{-1} for the HIGH group. In order to enhance spermiation and ensure adequate sperm production, all males regardless of female treatment were given an EVAc implant for an effective dose of 58 ± 18 μg GnRHa kg^{-1} BW. A certain degree of variability in the effective GnRHa doses occurred since the GnRHa amount is fixed in the implants, even though combinations of two implants loaded with different amounts of GnRHa were used. Both females and males were treated again two weeks later (June 21, 2017) with GnRHa implants for the same effective dose as before. Four weeks after the start of the experiment, on July 5, 2017, the final sampling was conducted and fish were returned to the sea cage.

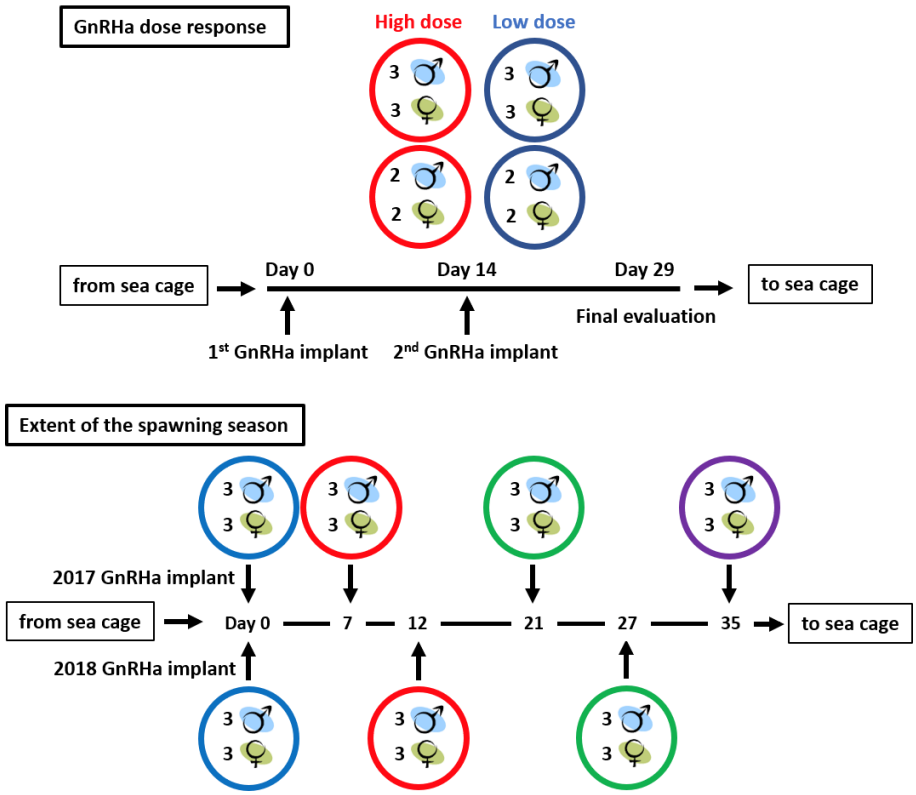

Figure 6.2 Schematic representation of the experimental design of (upper) the GnRHa implant dose response and (lower) the extent of the spawning induction period. Greater amberjack broodstocks were maintained in sea cages during the year and were transferred to onshore tanks after GnRHa spawning induction (arrows), and were returned to sea at the end of the experiments. Prior to GnRHa implantation the fish were biopsied to evaluate stage of ovarian development and spermiation.

The indoor tanks were supplied with a surface seawater (20% exchange h^{-1}) and were exposed to ambient photo-thermal conditions, with temperature ranging from 21.3°C to 23.7°C over the course of the experiment. Gaseous oxygen was also provided to the tanks for safety reasons, maintaining the saturation to 110-120%. Measurements of temperature and oxygen saturation were conducted twice a day (a.m. and p.m.). Tank overflows were fitted with 250-L passive egg collectors. Feeding in the tanks was done to apparent satiation between samplings, 5 days a week, though the fish did not accept feed readily.

6.2.3 Extent of the reproductive season and timing of GnRHa implantation

In order to estimate the extend of the reproductive season of greater amberjack in captivity when fish were maintained in sea cages, and identify potentially the best timing for GnRHa induction of maturation and spawning, two spawning induction experiments were conducted between May 30 - July 18, 2017 and June 15 – July 24, 2018 at GMF (**Fig. 6.2**). In 2017, fish were split into two sea cages from the year before. Cage A contained 6 females (mean BW ± SD of 19.4 ± 3.0 kg) and 6 males (15.7 ± 1.4 kg) and cage B contained 7 females (17.3 ± 3.1 kg) and 7 males (14.4 ± 1.3 kg). On May 30, 2017 half of the fish from cage A were evaluated for gonadal development stage (TANK IN) as described above, and three females and three males

were selected and treated with GnRHa, and were transferred to an onshore 21-m^3 round tank for spawning (1st period). A week later, on June 6, the remaining fish in cage A were evaluated as above and three females and three males were treated with GnRHa, and were transferred to a different onshore tank for spawning (2nd period). A similar scheme was followed for cage B for the 3rd and 4th periods that started on June 20 and July 4, 2017, respectively. Each group remained in the onshore facilities for 14 days (except the 1st group, which remained for 21 days) and then was transferred back to the sea cage. The gonadal development stage of the females of each spawning induction experiment was evaluated again at the end of the experiment before their transfer back to the sea cage (TANK OUT). A final (third) gonadal evaluation was done for the females used in the 1st, 2nd and 3rd spawning induction periods on July 18, 2017, when the fish remained for 14 – 28 d in the sea cage after the completion of their spawning induction experiment (CAGE).

The same series of spawning induction experiments was repeated in the 2018 reproductive season (**Fig. 6.2**) when the fish had a mean BW of 18.4 ± 2.9 kg for the females, and 15.5 ± 1.7 kg for the males. The respective dates of starting the 1st, 2nd and 3rd spawning induction periods were June 15, June 27 and July 12, 2018. This year, all fish were maintained in the same cage prior to the start of the reproductive period because it was not observed any negative effect of handling on the maturational stage of the fish in the 2017 reproductive season. For each spawning induction experiment, three females and three males were selected as described above, and were transferred to an onshore tank for spawning. As in 2017, each group remained in the onshore facilities for about 2 weeks and then was transferred back to the sea cage.

In both years, females were treated with EVAc implants (Mylonas and Zohar, 2001), loaded with 750-1000 μg of Des-Gly^{10},D-Ala^{6}-Pro-NEt^{9}-mGnRHa (H-4070, Bachem, Switzerland) for an effective dose of 58 ± 9 μg GnRHa kg^{-1} BW in 2017 and 60 ± 7 μg GnRHa kg^{-1} BW in 2018. The selected males were also treated with GnRHa implants for an effective dose of 67 ± 6 μg and 58 ± 8 μg GnRHa kg^{-1} BW in 2017 and 2018, respectively. The spawning tanks were supplied with surface seawater and exposed to ambient photo-thermal conditions, with temperature ranging from 19.8°C to 26.4°C in 2017 and 21.3°C to 25.2°C in 2018. Gaseous oxygen was also provided to the tanks for safety reasons, maintaining the saturation to 110-120 %. Measurements of temperature and oxygen saturation were conducted twice a day (a.m. and p.m.). Egg collection was done in a 250-L passive egg collector supplied with water from the tank overflow (60% of the total incoming water), as well as from a drain at the bottom of the tanks (40% of the total incoming water). Feeding of the fish while in the tank was done to apparent satiation 5 days a week, though again the fish did not accept feed readily.

6.2.4 Evaluation of egg quality parameters

Egg collectors were examined three times a day (8:00 a.m., 3:00 p.m., 8:00 p.m.). For each spawn, date, collection time and developmental stage (Tachihara, et al., 1993) were recorded in order to identify different spawns and estimate an approximate spawning time. However, for statistical analysis and presentation, the egg production and quality data (*i.e.* daily fecundity, fertilization success and embryo/larval

survival) was pooled according to spawning day. Eggs were collected and transferred into a 10-L bucket. Their number (fecundity) was estimated by counting the total number of eggs in a sub-sample of 10 mL collected with a pipette after vigorous agitation. Fertilization percentage was evaluated at the same time by examining each egg in the subsample for the presence of viable embryos. Immediately after the sub-sample was collected, eggs were placed into a 125 or 500-L conical tank-incubator fitted with an overflow filter (250 μm mesh size), and supplied with surface seawater (~90% h^{-1} renewal) and mild aeration.

To monitor embryo and larval survival, eggs from each spawn were collected from the tank incubators and placed individually in 96-well mct plates (in duplicates) according to the procedure of Panini et al. (2001) with some modifications. Briefly, a sample of floating (~100% fertilized) eggs were taken from the tank incubators with a 250 μm mesh sieve, rinsed with sterile seawater (obtained from a shallow well) and poured in 2-L beakers filled with seawater. A Petri dish was used to scoop 100-200 floating eggs from the beaker. The dish was then placed under a stereoscope and only fertilized eggs were individually aspirated in 200 μL of sterile seawater to the 96 wells of a mct plate. At the end of the process, plates were double-checked under a stereoscope and dead eggs, if any, were replaced. Once loaded, the mct plates were covered with a plastic lid, placed in a controlled-temperature incubator set at the spawning temperature of the tanks during the spawning experiment (See Section 2.1. above and **Fig. 6.2**) and maintained for 5 days. Using a stereoscope, embryonic and early larval development was evaluated daily, recording the number of live embryos 24 hours after egg collection, hatched larvae (examined ~55 hrs after spawning) and viable larvae on day 5 after hatching (near the time of yolk sack absorption). The embryo survival was calculated as the number of eggs having live embryos 1 d after egg collection/number of fertilized eggs initially loaded in the mct. The hatching success was calculated as the number of hatched larvae/24-h embryos, and the 5-d larval survival was calculated as the number of live larvae 5 days after egg collection/the number of hatched larvae. Estimating survival percentage (%) by using as denominator the number of individuals that survived to the previous developmental stage is considered as a more accurate evaluation of survival within specific developmental stages, without the potential of a distortion effect by the survival during the previous stage (Mylonas, et al., 2004a).

6.2.5 Statistical analysis

Differences in measured parameters per day of spawning were tested using 1-way or 2-way ANOVA followed by Tukey's HSD post hoc test. Data were transformed accordingly to meet the 1-way or 2-way ANOVA assumptions if not normally distributed. A level of $P \leq 0.05$ was set as minimum statistical significance for the ANOVA tests. Statistical analyses were performed with JMP 12 (SAS Institute Inc., Cary, NC, USA). Results are presented as mean ± standard error (SEM), unless mentioned otherwise.

6.3 Results

6.3.1 GnRHa dose comparison

At the start of the experiment, when the fish were selected from the sea cage (June 7, 2017) females had mostly Vg oocytes, with two fish out of 10 found at the OM stage (data not shown). A small percentage

of atretic Vg follicles was observed in almost all ovarian biopsies. At the same time, sperm could be collected from all males using a catheter. At the second sampling and GnRHa implantation two weeks after the first GnRHa therapy, three out of five females of the LOW group and four out of five females of the HIGH group still had Vg oocytes, together with atretic follicles and ovulated eggs, while the other females had only POs and atretic follicles. All males were in spermiation. At the last sampling two weeks after the second GnRHa implantation, one female per group had Vg oocytes, but with increased percentage of POs and atretic follicles. The rest of the females were devoid of Vg oocytes and had mostly POs and ovulated eggs, an indication that they had matured and released all their Vg oocytes, and reached the end of the reproductive season (referred to as "spent").

No significant differences were observed between the mean diameters of the largest Vg oocytes biopsied from females given one of the two GnRHa doses at the time of the 1st or 2nd GnRHa implantation (**Fig. 6.3**). After treatment with either GnRHa dose, spawning started one day after the 1st implantation, presumably because one female in each group had oocytes at the OM stage at the time of implantation. Females in both GnRHa doses spawned 7-9 times after the 1st implantation and 4-5 times after the 2nd implantation. The highest daily relative fecundity was observed in the LOW dose with 89,976 eggs kg^{-1} female three days after the 1st implantation (**Fig. 6.3)**. They were obtained one or two spawning events per day, and only once in one tank they were obtained three spawns in a single day. Multiple spawns occurred almost exclusively after the first GnRHa implantation, with no relation to GnRHa dose.

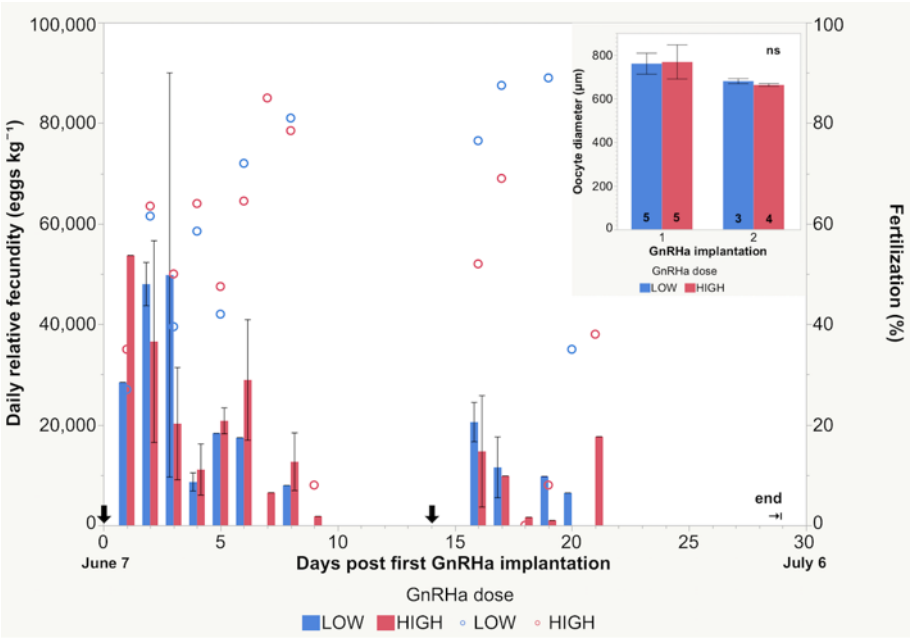

Figure 6.3 Mean (± SEM) daily relative fecundity (bars) and fertilization success (circles) of greater amberjack implanted twice (arrows) with a low (25 µg kg^{-1} body weight, LOW) or high (75 µg kg^{-1} body weight, HIGH) dose of GnRHa implants (n=2 stocks $dose^{-1}$) at ARGO. The insert shows the mean (± SEM) oocyte diameter of the largest vitellogenic oocytes at the time of the GnRHa implantations. No significant differences (ns) in oocyte diameter were observed between the LOW and HIGH doses (2-way ANOVA, $P<0.05$).

Mean daily or total relative fecundity was not significantly different between the LOW and HIGH doses (**Fig. 6.4)**. However, mean total relative fecundity in response to the two GnRHa implantations was significantly higher after the 1st implantation compared to the 2nd one. Fertilization success was similar between the two doses after the 1st GnRHa implantation, but was significantly different between the two doses after the 2nd implantation, with the LOW dose having higher fertilization success than the HIGH dose. Twenty four-hour embryo survival and 5-d larval survival were similar in the two GnRHa doses and implantations, while hatching success was significantly higher in the eggs obtained after the 2nd GnRHa implantation regardless of GnRHa dose.

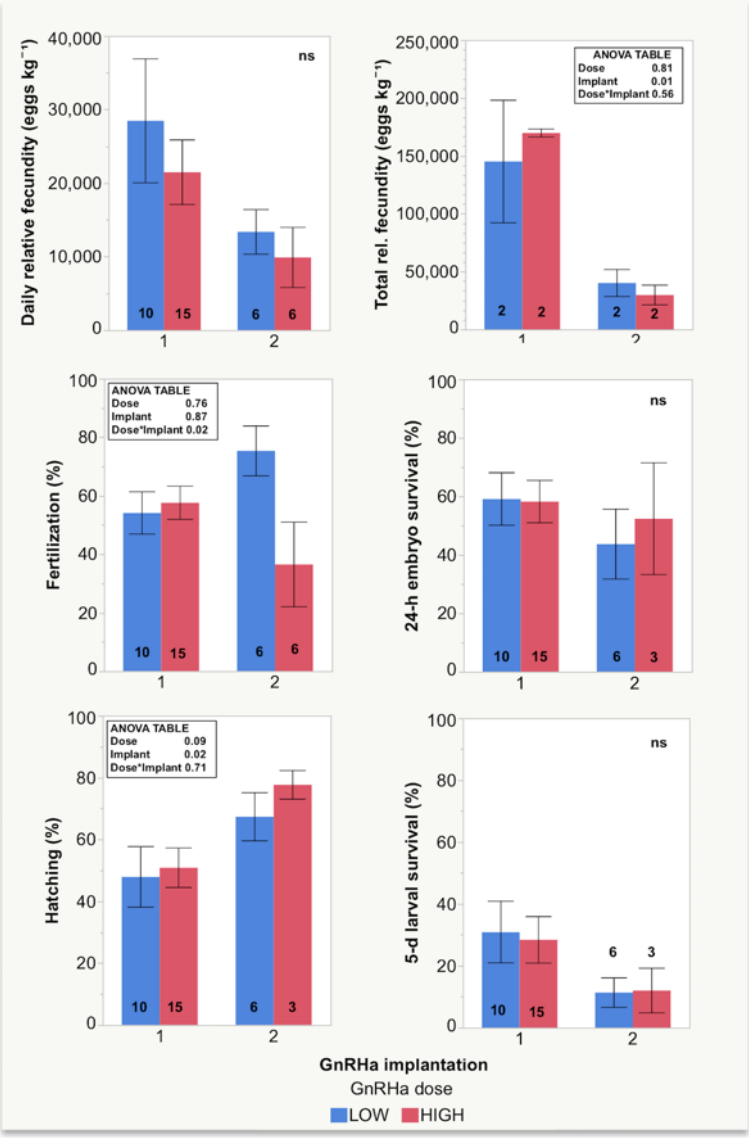

Figure 6.4 Mean (± SEM) daily and total relative fecundity, fertilization success, 24-h embryo survival, hatching and 5-d larval survival of greater amberjack after two GnRHa implantations of a LOW or HIGH dose. The numbers inside the bars indicate the n values of the means. Significant differences between means are indicated in the 2-way ANOVA tables, while absence of any statistical significance is indicated by "ns".

6.3.2 Extent of the reproductive season and timing of GnRHa implantation

At the time of the GnRHa implantations in 2017, females used at all four periods had mainly Vg oocytes in their ovaries, with few fish exhibiting also very limited follicular atresia (**Fig. 6.5**, left column**)**. At the time of the 1st, 2nd and 3rd period, one female at each time was found to be either in OM or having ovulated recently, suggesting that some maturation was taking place in the sea cage (data not shown). All males were in spermiating condition, though sperm could be collected only with a catheter. At the end of the spawning induction period when females were maintained in the onshore tanks, a certain degree of variability was observed in the ovarian biopsies taken from the different females. For example, at the end of the 1st period, two females had only POs and fully atretic follicles, while another female had fully Vg oocytes. At the end of the spawning induction of the 2nd period, two females still had fully Vg oocytes -but of relatively smaller diameter- together with ovulated eggs (**Fig. 6.5**), while another female had fully Vg oocytes and oocytes at the OM stage. At the end of the 3rd spawning induction period, one female contained mainly ovulated eggs (**Fig. 6.5**) and POs, and another one contained only POs (*i.e.* they were both spent), while a third one contained Vg oocytes of lower diameter. Finally, at the end of the 4th period all females were considered spent, having only POs, atretic follicles and ovulated eggs in their ovaries (**Fig. 6.5**).

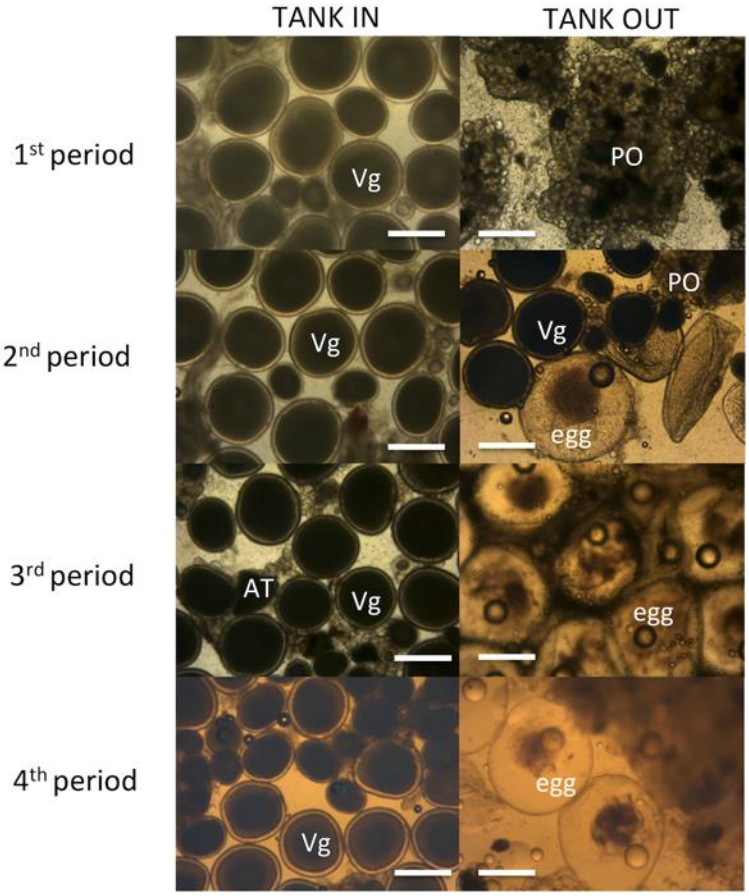

Figure 6.5 Microphotographs of representative wet mounts of ovarian biopsies from greater amberjack induced to spawn with GnRHa implants during different periods at GMF in 2017: 1st period (30/5-20/6/2017), 2nd period (6-20/6/2017), 3rd period (20/6-4/7/2017) and 4th period (4-18/7/2017). The left column shows biopsies obtained at the start of each spawning induction period when fish were moved from sea cages to onshore tanks (TANK IN), and the right column shows biopsies obtained at the end when the fish were taken back to the sea cage (TANK OUT). PO=primary oocyte, Vg=vitellogenic oocyte, AT=apoptotic/atretic follicle. Bars=500μm.

No significant differences were observed among mean oocyte diameters of the largest Vg oocyte of females examined at the beginning of the four periods (ANOVA, $P<0.05$) in 2017, when females had mainly Vg oocytes (**Fig. 6.6)**, while more spawns were obtained during the first two periods compared to 3rd and 4th (**Fig. 6.6**), though statistical confirmation could not be obtained.

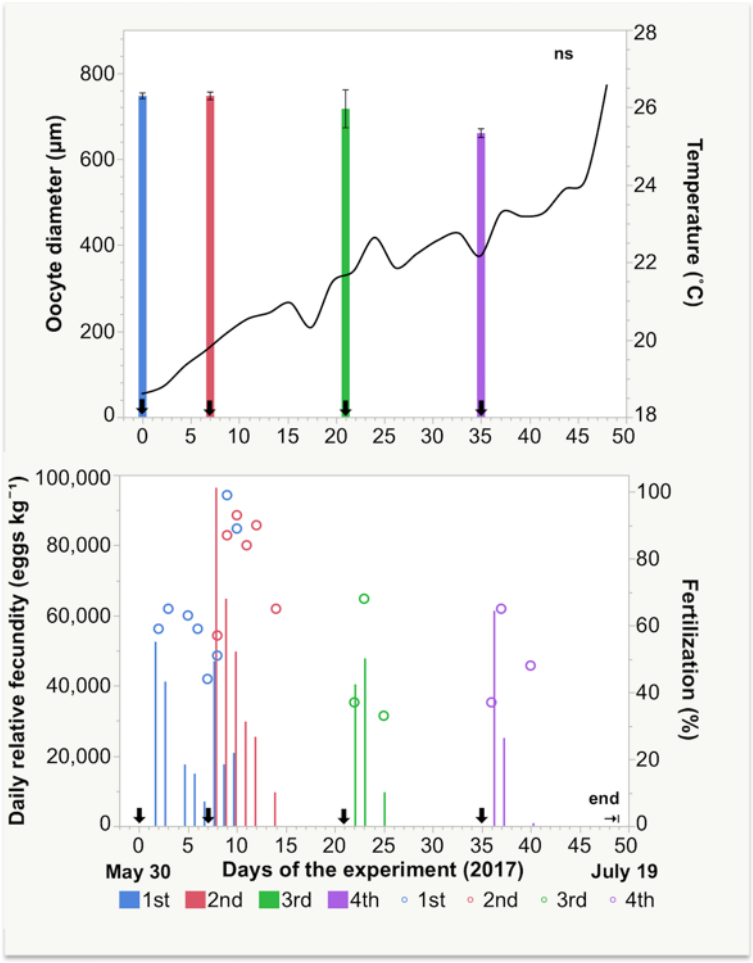

Figure 6.6 (Upper graph) Mean oocyte diameter (± SEM) of the largest vitellogenic oocytes of greater amberjack (n=3) at the time of GnRHa implantation at different periods during the reproductive season of 2017: 1st period (30/5/2017 - blue), 2nd period (6/6/2017 - red), 3rd period (20/6/2017 - green) and 4th period (4/7/2017 - purple). The seawater temperature in the sea cages during the experiment is shown by the solid black line. (Lower graph) Daily relative fecundity (bars) and fertilization success (circles) after the GnRHa implantation (arrows).

Mean daily relative fecundity in 2017 was not significantly different among the four spawning induction periods (ANOVA, P<0.05) and averaged 33,966±5,354 eggs kg^{-1} day^{-1} (**Fig. 6.7**). Similarly, mean fertilization success, 24-h embryo survival and hatching success were not significantly different among the four spawning induction periods (ANOVA, P<0.05) and averaged 65±4%, 81±5% and 56±8%, respectively (**Fig. 6.7**).

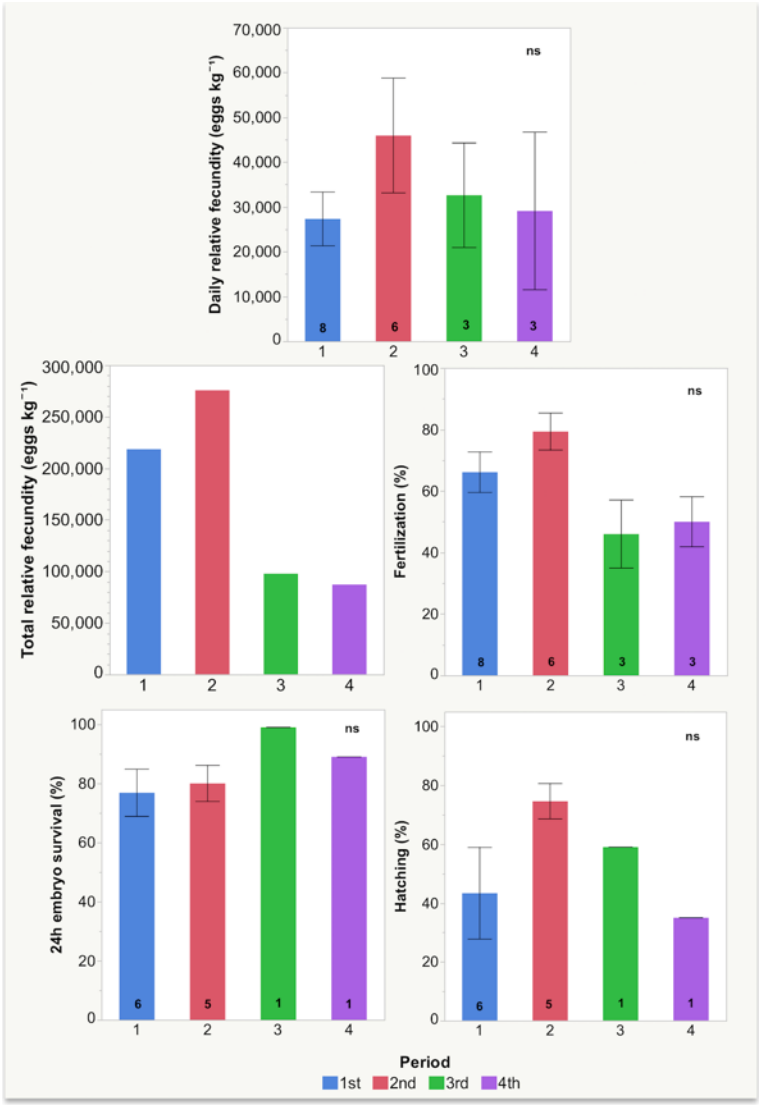

Figure 6.7 Mean (± SEM) daily and total relative fecundity, fertilization, 24-h embryo survival and hatching of eggs from greater amberjack, after GnRHa induction of spawning during four different periods during the reproductive season of 2017 (see Fig. 6). The numbers inside the bars indicate the n values of the means. There were no statistically significant (ns) differences among the four periods for any of the parameters examined (ANOVA, $P<0.05$).

In 2018, the situation was similar to the previous reproductive season, with the females examined at the start of each of the three periods having mainly Vg oocytes of 680-730 μm in diameter in their ovaries (**Fig. 6.8**), while one female at the 1st period had already ovulated and one female at the 3rd period was in OM. All males were in spermiating condition. At the end of the three spawning induction periods when females were maintained in the onshore tanks for two weeks, females had mostly POs, atretic follicles and ovulated eggs, except from one female at the end of the 1st period that was found to have also Vg oocytes in addition to

ovulated eggs. As in 2017, more spawns were produced during the first two spawning induction periods (**Fig. 6.8**).

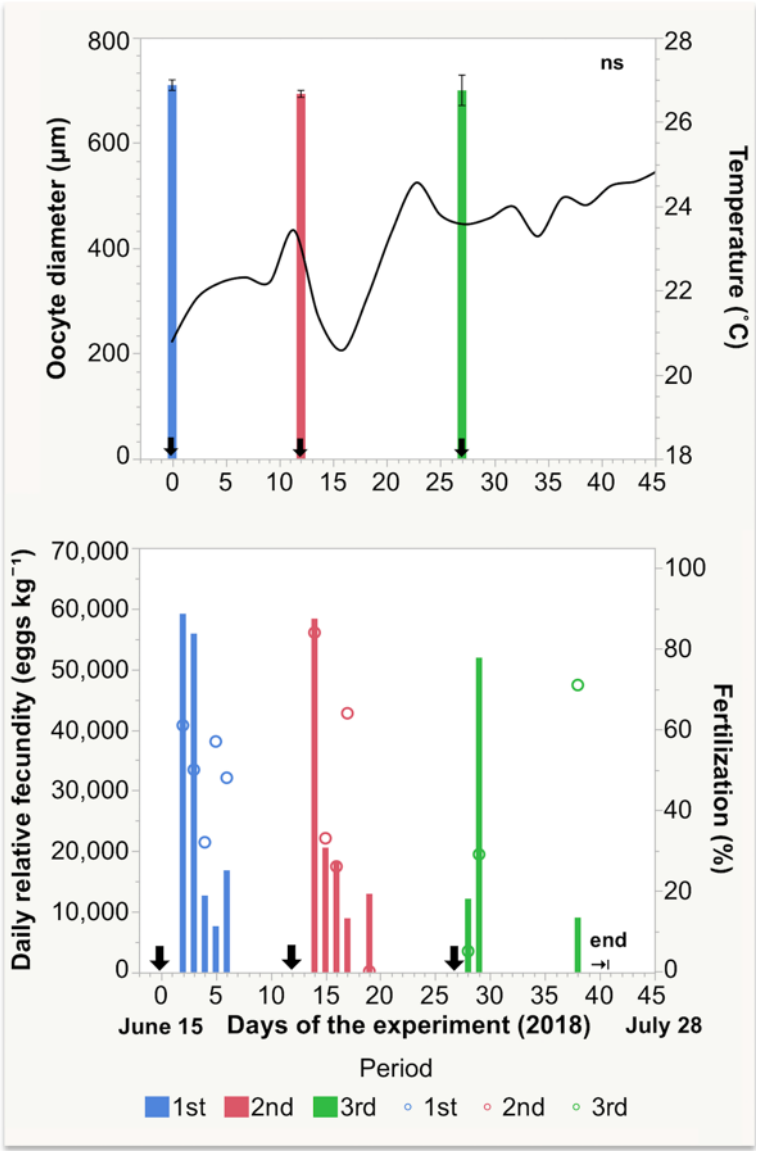

Figure 6.8 (Upper graph) Mean oocyte diameter (± SEM) of the largest vitellogenic oocytes of greater amberjack (n=3) at the time of GnRHa implantation at different periods during the reproductive season of 2018: 1st period (15/6/2018 - blue), 2nd period (27/6/2018 - red) and 3rd period (12/7/2018 - green). The seawater temperature in the sea cage during the experiment is shown by the solid black line. (Lower graph) Daily relative fecundity (bars) and fertilization success (circles) after the GnRHa implantation (arrows).

Mean daily relative fecundity in 2018 was not significantly different (ANOVA, $P<0.05$) among the three periods of GnRHa implantation and was 26,505±5,846 eggs kg^{-1} day^{-1} on average (**Fig. 6.9)**. Similarly, there were no significant differences (ANOVA, $P<0.05$) among the three spawning induction periods in mean

fertilization success, 24-h embryo survival and hatching success, which averaged 43±7%, 36±9% and 73±12%, respectively (**Fig. 6.9)**.

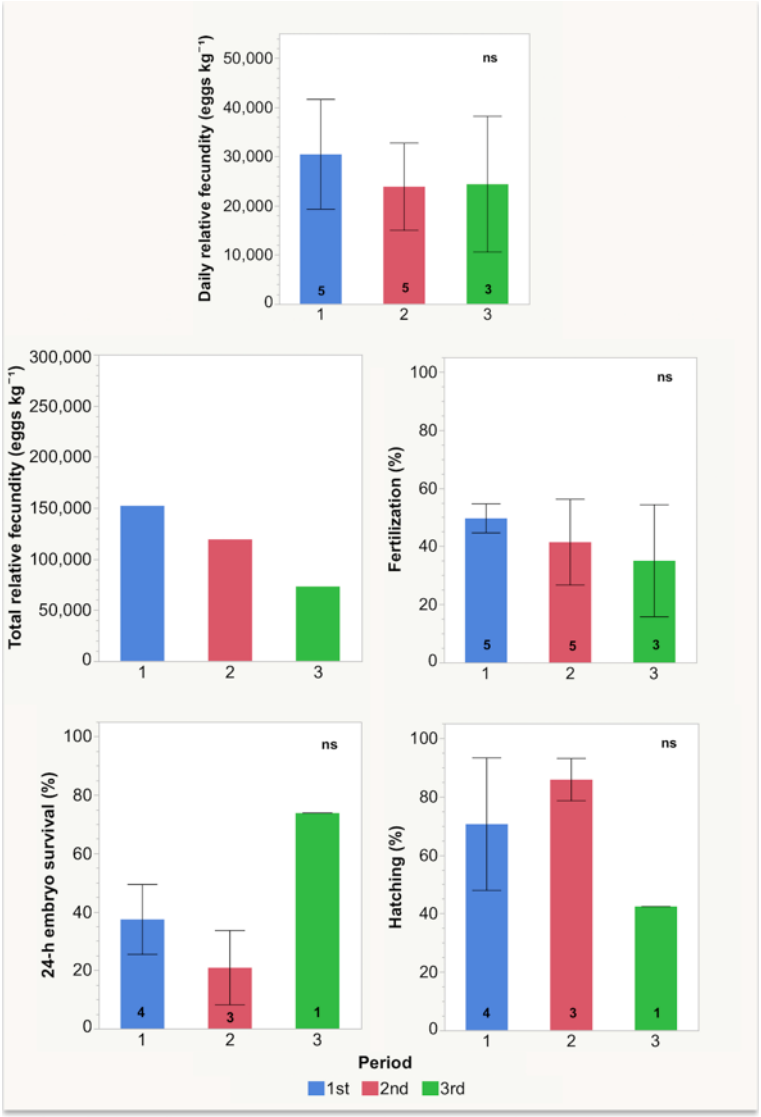

Figure 6.9 Mean (± SEM) daily and total relative fecundity, fertilization, 24-h embryo survival and hatching of eggs from greater amberjack, after GnRHa induction of spawning during three different periods during the reproductive season of 2018 (see Fig. 8). The numbers inside the bars indicate the n values of the means. There were no statistically significant differences among the three periods for any of the parameters examined (ANOVA, P<0.05).

In 2017, it was observed that regardless of the GnRHa induction period, the mean diameter of the largest Vg oocytes of the females 2-3 weeks after the spawning induction (TANK OUT) was significantly lower than what it was at the beginning of the period (TANK IN) prior to the GnRHa implantation (2-way ANOVA, P=0.01) (**Fig. 6.10**). However, when these females were examined again 2-4 weeks after they were

returned to the sea cages (CAGE), their mean oocyte diameter of the largest Vg oocytes increased significantly, and was similar to the values measured at the beginning of the spawning induction period.

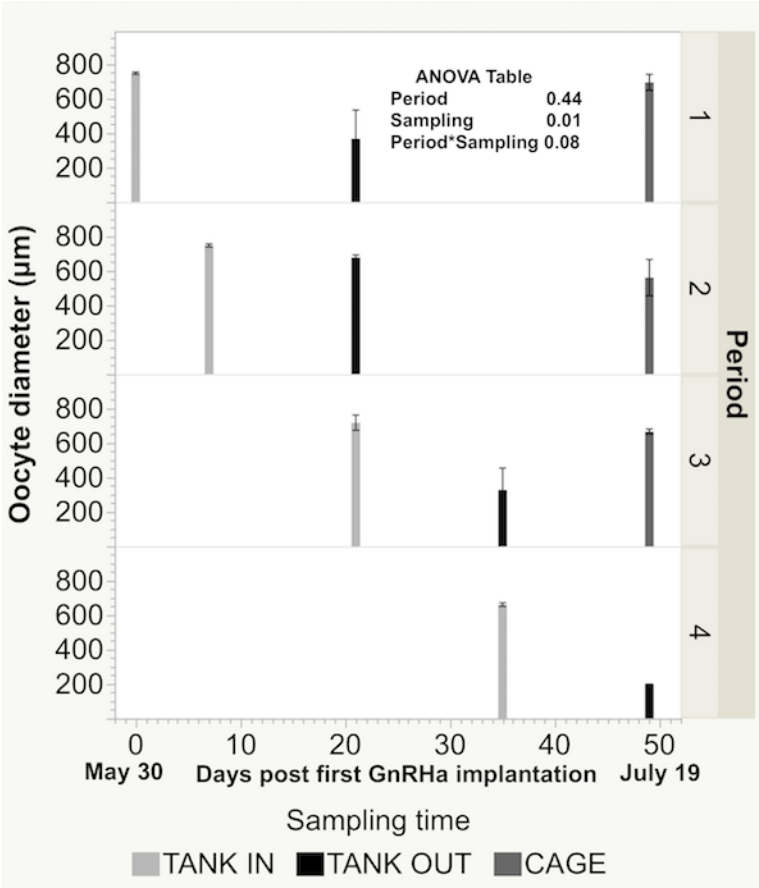

Figure 6.10 Mean oocyte diameter (± SEM) of the largest vitellogenic oocytes of greater amberjack (n=3) at the time of GnRHa implantation (TANK IN) for spawning, at the end of the spawning experiment when they were transferred back to the sea cages (TANK OUT) and 14-28 days after their return to the sea cages (CAGE) in 2017 (See Fig. 6). For the 4th spawning period, the fish were not biopsied again after they were moved to the sea cage.

6.4 Discussion

A broodstock management and spawning induction method was reported recently for greater amberjack in the Mediterranean, where the breeders were reared in sea cages throughout the year and then were transferred to onshore tanks for spawning after treatment with GnRHa implants (Chapter 4). Spawning induction with controlled-release implants was also shown to be the preferred mode of GnRHa administration compared to weekly injections, based on the less handling required and the resulting spawning kinetics and egg production (Chapter 5). This "cage-to-tank" broodstock management and spawning induction method was also used in the present study, and it combines the advantages of sea cage rearing throughout the year -*i.e.*

optimal environmental conditions and welfare, and low cost of fish maintenance- with the control of tank spawning -*i.e.* ease of handling operations and egg collection (Chapter 4). Building on the data obtained in these previous studies, here it was investigated (a) the most cost-effective GnRHa dose based on spawning performance and egg quality and (b) the extent of the potential GnRHa induction period when greater amberjacks were maintained in sea cages without environmental temperature control.

For the first objective, it was examined a lower and a higher GnRHa dose from the one used in previous experiments in the Mediterranean (Chapter 5), but also in the Canary Islands (Sarih, et al., 2019). Choosing the minimum effective GnRHa dose for any spawning induction protocol is necessary not only to reduce the cost of the therapy, but also to ensure the best egg quality. Too low GnRHa doses may be ineffective in inducing ovulation in synchrony in the broodstock or result in reduced fecundity (Chatakondi, 2017; Podhorec, et al., 2011), but too high GnRHa doses have been accused of accelerating oocyte maturation resulting in reduced egg quality (Fernández-Palacios, et al., 2014; Ibarra-Castro & Duncan, 2007; Mylonas, et al., 1992). Previous studies with GnRHa implants used a dose of 50 μg kg^{-1} (Mylonas, et al., 2004b; Sarih, et al., 2019), so in the present study it was examined 25 (LOW) and 75 μg GnRHa kg^{-1} (HIGH). Based on the achieved fecundity and the ovarian evaluation after the first GnRHa implantation, it was expected that all treated females in both the LOW and HIGH GnRHa doses spawned successfully, therefore both doses were effective in inducing oocyte maturation, ovulation and spawning. No significant differences were observed between the LOW and HIGH GnRHa doses in terms of relative fecundity and egg quality parameters. The lack of significant differences between the two doses may suggest that more than three-fold differences in GnRHa doses should have been used in order to identify both the minimum effective dose and the upper dose at which negative effects on the egg production and quality start appearing. For example, in a dose-response study for GnRHa injections in meagre (1-50 μg GnRHa kg^{-1} BW), although the optimal dose was 15 μg GnRHa kg^{-1}, similar results were obtained also with 10 and 30 μg GnRHa kg^{-1}, a three-fold difference (Fernández-Palacios, et al., 2014). Only when many-fold lower or higher doses were used, negative effects on spawning response and egg quality were observed. Similarly, using the same GnRHa implants in wild spotted rose snapper (75-300 μg GnRHa kg^{-1}), similar results were observed for the 75-225 μg GnRHa kg^{-1} doses, although the 75 μg GnRHa kg^{-1} dose was overall the optimal dose recommended (Ibarra-Castro & Duncan, 2007). Finally, in a brown trout dose-response study (0.01-10 μg GnRHa kg^{-1} BW), differences in spawning efficacy and egg quality were observed between doses differing an order of magnitude (Mylonas, et al., 1992). Unfortunately, testing a large number of different doses is not currently feasible in greater amberjack, both due to limitations in the number of available breeders in culture and large tanks to host them.

In response to two consecutive GnRHa implantations, fish receiving either the LOW or HIGH dose achieved high total relative fecundities over a period of 29 days in 11-18 spawning events. Relative batch fecundity (±SD) for these spawning events (14,797±12,666 eggs kg^{-1}) was similar to those reported by other spawning induction studies, which ranged between 13,260 and 14,629 eggs kg^{-1} $spawn^{-1}$ in the Mediterranean

(Mylonas, et al., 2004b)(Chapter 4 & 5) and between 15,878 and 17,580 eggs kg^{-1} $spawn^{-1}$ in Japan (Kawabe, et al., 1998; Kawabe, et al., 1996). On the contrary, the relative batch fecundity obtained here was lower (Sarih, et al., 2018) or seven-fold higher (Chapter 7) than those obtained after GnRHa implantation of greater amberjack in the Canary Islands. The other studies carried out in the Mediterranean and Canary Islands administered respectively 1-2 (Mylonas, et al., 2004b) (Chapter 4 & 5) and 3-5 (Sarih, et al., 2018) consecutive GnRHa implant with doses ranging between 40 and 64 μg GnRHa kg^{-1}, which is intermediate between the LOW and HIGH doses used in the present dose-response study. These results corroborate further our findings that GnRHa implant doses between 25-75 μg GnRHa kg^{-1} are equally effective at inducing spawning in greater amberjack. For commercial production, therefore, it is proposed that implants providing a 50 μg GnRHa kg^{-1} dose should be used -instead of the less expensive 25 μg GnRHa kg^{-1} one. This will result in a secure "overdose" of the fish to guard against unexpected variations in female responsiveness from year to year. In addition, it will also counteract any underestimation of expected body weight, since (a) the implants have a fixed GnRHa content and (b) it is not recommended to sample fish more than once during the reproductive season (Zupa, et al., 2017b), and the weight estimation should be based on a projection of growth since the last year's spawning induction weighing (Chapter 5). In the latter study that used this "MEDIUM" dose (50 μg kg^{-1}) to treat the same broodstock used in the present study and under exactly the same conditions in the ARGO facilities a year before (*i.e.* rearing cage, location, feeding, spawning tanks, handling, etc.), the mean daily relative fecundity was higher (40-50,000 eggs kg^{-1}) than that obtained from the LOW and HIGH doses here, especially in the 2-3 days following GnRHa implantation, which would be advantageous for synchronizing hatchery operations. Finally, the dose of 50 μg GnRHa kg^{-1} was used also in the other commercial facility utilized in the present study and resulted in higher fecundity. Similar findings have been reported in a walking catfish GnRHa dose comparison experiment using four different doses of a GnRHa and dopamine antagonist preparation (Ovatide), showing that the best results were obtained with a medium dose in terms of breeding performance and egg quality, in comparison to a lower and higher dose (Sahoo, et al., 2005).

In terms of egg quality, mean fertilization success in the LOW and HIGH doses was similar, but relatively low compared to some other cultured species induced to spawn with GnRHa implants (Mugnier, et al., 2000; Mylonas, et al., 2003; Mylonas, et al., 2015; Nyuji, et al., 2013). Similarly, no significant differences were found between the two GnRHa doses in terms of 24-h embryo survival, hatching and 5-d larval survival, with the values obtained being similar to previously reported values for GnRHa-induced greater amberjack in the Mediterranean (Chapter 5), and higher (Chapter 7) or lower (Sarih, et al., 2018) than those reported in the Canary Islands. Fertilization success -and egg quality in general- in response to spawning induction should be improved further in this species, especially considering the high variability among different spawning events, and the higher fertilization success reported from spontaneously spawning greater amberjack in the Canary Islands (Sarih, et al., 2018). Nevertheless, the obtained fertilization values were higher than other spawning induction studies (Mylonas, et al., 2004b; Sarih, et al., 2018) or within the same range of previously reported

values in the Mediterranean and Canary Islands (Chapter 4 & 7). On the other hand, higher fertilization success has been reported in the Canary Islands after multiple GnRHa injections (Fernández-Palacios, et al., 2015a). The exact underlying causes for these differences are currently unresolved, but some speculation may be made. For example, the higher fertilization success obtained in the latter reports (Fernández-Palacios, et al., 2015a; Sarih, et al., 2018) was associated with the use of fluent males (*i.e.* releasing sperm after applying abdominal pressure) at higher male:female sex ratios of 5:2 (Sarih, et al., 2018) or 2:1 (Fernández-Palacios, et al., 2015a), which we believe had a significant effect on the increased fertilization success. Supportive to this hypothesis is the fact that when a 1:1 sex ratio was used in the GnRHa induction experiment reported in one of these studies (Sarih, et al., 2018), the resulting fertilization success was lower than our study. Furthermore, a male:female sex ratio of 2:1 was also used in Japan in view of the important role of male courtship behavior (Tachihara, et al., 1993). Therefore, the already reported dysfunctional spermatogenesis and spermiation of reared male greater amberjack in the Mediterranean (Zupa, et al., 2017a; Zupa, et al., 2017b) should be examined further together with the effect of sex ratio, since the observed lower fertilization success of the collected eggs was probably related to these two parameters. Although treatment of males in the present study with GnRHa implants did improve sperm production (data not shown), either the enhancement was not enough or it is necessary to use higher male:female ratios to allow the used males to fertilize efficiently the produced eggs of a number of females spawning in synchrony in response to a GnRHa treatment.

The advantage of rearing female greater amberjack breeders in sea cages during the year -in terms of reproductive function- was corroborated also in the second commercial facility utilized in the present study to examine the duration of the spawning season. In both years, all females were at the final stage of vitellogenesis at the onset of the experiments in June, having Vg oocytes of the appropriate size to be induced with GnRHa for spawning. Based on monitoring during the expected spawning season, greater amberjack maintained in sea cages were shown to maintain their gonads in post vitellogenesis and be eligible for spawning induction using GnRHa implants for a period of at least 2 months from late May until late July. However, there was a trend towards a reduction in spawning performance -*i.e.* less spawns and lower total relative fecundity- as the season progressed and breeders were induced to spawn at a later time. This was not associated with any differences in ovarian stage of development or maximum oocyte diameter at the time of GnRHa implantation, which is probably the reason that no significant differences in egg quality parameters were observed, but was related to the time of GnRHa treatment. The decrease in egg production could be -at least in part- due to the occurrence of spontaneous spawning of the females while in the sea cage (Chapter 5), which would result in less available oocytes for spawning after GnRHa induction. In addition, the reduction in spawning performance could be in response to the increase in water temperatures that happens rapidly during this period. In the wild, greater amberjack spawn from May to July in the Mediterranean (Marino, et al., 1995) at water temperatures between 19.3 and 23.8°C (Zupa, et al., 2017a; Zupa, et al., 2017b). In the present study the ambient temperature during the experiments ranged from 18.6°C to 24.1°C. Still it is possible that better spawning performance may take

place early in the spawning season, under lower water temperatures, even though the fish may have fully Vg oocytes in their ovaries later in the season.

An important finding of the reproductive period experiment was that the females that were handled twice (in 2017) or three times (in 2018) during the process of taking some fish at a time out of the sea cage for the consecutive spawning induction trials (period 2 and 4 in 2017; period 2 and 3 in 2018) did not show any reproductive impairment. In earlier studies where captive greater amberjacks were handled in a similar way during the early gametogenesis period –*i.e.* crowded, anesthetized and some individuals also biopsied- fish exhibited severe gametogenesis impairment and increased follicular atresia during the spawning period (Zupa, et al., 2017b). Also, wild caught Atlantic bluefin tuna maintained in a sea cage exhibited severe follicular atresia as early as one day after a strong handling stress (Corriero, et al., 2011). Almost the same situation was observed in the red gurnard, with wild fish responding to capture and confinement stress with follicular atresia (Clearwater & Pankhurst, 1997). Apparently, greater amberjack may be sensitive to handling during early vitellogenesis, but not when this period is concluded, and during the spawning period females can maintain their ovaries in a functional stage after mild handling.

Another noteworthy observation from the present study was that "spent" females or with smaller remaining Vg oocytes after spawning induction in the onshore tanks, recovered to their initial reproductive stage –in terms of maximum Vg oocyte diameter- after a "resting" period of 14-28 days in sea cages. At the end of each spawning induction period, many of the females contained mostly post-ovulated eggs and POs in their ovarian biopsies, an indication that they had reached the end of the reproductive season and were considered incapable of further spawning. The continuation of the vitellogenic process in these females was probably related to the optimal rearing conditions offered by the sea cages (Chapter 4), but also to the effectiveness of the GnRHa delivery systems to promote the process of vitellogenesis (Chapter 5) in the asynchronous greater amberjack (Marino, et al., 1995) and not only to induce oocyte maturation, ovulation and spawning. The duration of the process of vitellogenesis varies among species from a single day to one year (Jalabert, 2005). In greater amberjack, it seems that fish can conclude vitellogenesis in less than two weeks, an interesting information for broodstock management of this species.

The present study demonstrated that 50 μg GnRHa kg^{-1} BW is the most appropriate dose for spawning induction of greater amberjack using EVAc implants. In addition, it was shown that it is possible to maintain greater amberjack in cages throughout the year, and then take portions of the broodstock at different times in a period of at least 2 months from the end of May to the end of July, when seawater temperature is within the range of 19-24°C, and induce them to spawn in onshore tanks.

Chapter 7 - Spawning induction of first-generation (F1) greater amberjack in the Canary Islands, Spain using GnRHa delivery systems

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7.1 Introduction

The greater amberjack is a cosmopolitan pelagic species (Paxton, et al., 1989) with high growth rate and excellent flesh quality (FAO, 2016; Jover, et al., 1999; Mazzola, et al., 2000). It has been considered a good candidate for the species diversification of aquaculture production in the Mediterranean region (Mylonas, et al., 2016a). However, commercial production is still very limited (FAO, 2016), due to bottlenecks in reproduction, larval rearing and health control during grow out. Recent research has evaluated the potential of wild-caught broodstock to mature and be induced to spawn in captivity (Pousis, et al., 2018; Zupa, et al., 2017a; Zupa, et al., 2017b). However, the development of a sustainable aquaculture industry requires the closing of the life-cycle of a potential species in captivity, and the establishment of breeding selection programs using hatchery-produced breeding stocks (Chavanne, et al., 2016; Janssen, et al., 2016).

Reproductive dysfunctions in captive broodstock occur in many cultured species (Mylonas & Zohar, 2009; Mylonas, et al., 2010; Mylonas, et al., 2017b), including the greater amberjack (Díaz, et al., 1997; Lazzari, et al., 2000; Micale, et al., 1999), preventing commercial development of seed production (Jover, et al., 1999; Mazzola, et al., 2000). In some species, reproductive dysfunctions are more severe in hatchery-produced broodstocks. This has been demonstrated in the Senegalese sole (Agulleiro, et al., 2007; Carazo, et al., 2013; Duncan, et al., 2013; Howell, et al., 2008; Morais, 2016; Rasines, et al., 2012), the greenback flounder (Pankhurst & Fitzgibbon, 2006) and the sharpsnout seabream (Papadaki, et al., 2018a). In Senegalese sole, F1 females undergo maturation, ovulation and spawning, but the obtained eggs are not fertilized, which has been related to a critical reproductive dysfunction of the F1 males (Mañanos, et al., 2009). On the other hand, in the greenback flounder a delayed release of eggs by the females after ovulation has been blamed for the failure to produce fertilized eggs (Pankhurst & Fitzgibbon, 2006). In sharpsnout seabream, females ovulate their eggs, but they do not spawn them, though it is still not known if this is associated with a dysfunction in the females or the lack of breeding behavior of the males (Papadaki, et al., 2018a). The existence of a significantly lower reproductive success of captive-produced compared to wild-caught breeding animals has been reported in a great number of animal taxa with relevance not only in aquaculture production, but also in conservation biology and laboratory experimentation (Farquharson, et al., 2018).

The most commonly observed reproductive dysfunctions in fish maintained in aquaculture facilities are the failure to undergo OM after completion of vitellogenesis in females and the low production of sperm

in males (Mañanos, et al., 2009; Mylonas & Zohar, 2001; 2007). Hormonal treatments using hCG or GnRHa have been used successfully in several species to overcome the observed reproductive dysfunctions. In greater amberjack, wild-caught fish have been shown to complete gametogenesis in captivity, and spawning could be induced using different hormonal therapies (Fernández-Palacios, et al., 2015a; García, et al., 2001; Kozul, et al., 2001; Lazzari, et al., 2000; Mylonas, et al., 2004b). In addition, spontaneous spawning has also been achieved in some individuals without any exogenous hormonal therapies (Jerez, et al., 2006). However, production of fertilized eggs from hatchery-produced broodstock has not been successful so far in Europe, although vitellogenesis and OM has been achieved (Rodríguez-Barreto, et al., 2014). This is contrary to what has been reported for the greater amberjack in Japan (Kawabe, et al., 1998) and the congener yellowtail kingfish, in which spontaneous spawning with fertilized eggs have been obtained successfully from F1 broodstock (Setiawan, et al., 2016).

The objectives of the present study were to examine the reproductive development of hatchery-produced F1 generation greater amberjack, to evaluate the potential of controlled-release GnRHa delivery systems (implants) to induce OM, spermiation and spawning of fertilized eggs, and to monitor spawning kinetics and gamete quality. The method described in this study shows great potential for the development of the aquaculture industry for greater amberjack, by enabling the use of hatchery-produced broodstocks for further breeding selection.

7.2 Materials and methods

7.2.1 Broodstock maintenance

Rearing was undertaken in the facilities of the IEO, Tenerife, Spain. The broodstock consisted of 14 hatchery-produced F1 fish (seven males and seven females), individually identified with PIT tags, from eggs obtained from wild-caught broodstock between 2005 and 2009. Fish were maintained during the year in two outdoor 50 m^3 tanks covered with shading mesh, supplied with seawater from a well (10 renewals day^{-1}) at ambient water temperature until the beginning of the experiments on 13 May 2015 (**Table 7.1**). After the first GnRHa treatment, the selected fish (see later for selection criteria) were placed in an outdoor covered (shading mesh) raceway tank of 500 m^3 with flow-through water supply (6 renewals day^{-1}) under natural photoperiod. Fish were fed three times per week to apparent satiation with raw fish (mackerel). Measurements of temperature and water quality (Dissolved Oxygen, NH_3–N and NO_2–N) were conducted once per week throughout the year. All work and maintenance of broodstocks was in agreement with European regulations on animal welfare (Federation of Laboratory Animal Science Associations, FELASA, http://www.felasa.eu/).

7.2.2 Evaluation of reproductive stage and selection of breeders

The fish were sampled four times during the 2015 spawning season (May, June, July and September). Fish were starved for two days prior to sampling and were tranquilized initially with the use of chlorobutanol (0.1 mL L^{-1}) diluted in the tank and then transferred to an anesthetic bath for complete sedation with a higher concentration of chlorobutanol (0.3 mL L^{-1}). Fish were individually identified, and biometric parameters of

length and body weight were measured. Ovarian biopsies for the evaluation of oocyte development were obtained by inserting a plastic cannula (Pipelle de Cornier) into the ovary. A wet mount of the biopsy was examined first under a compound microscope (Nikon Eclipse, Tokyo, Japan) (40 and 100×) equipped with a Nikon Digital Sight DS-Fi1 camera (Nikon Instruments Europe BV, Amsterdam, Netherlands) to evaluate the stage of oogenesis and to measure the mean diameter of the ten largest, most advanced vitellogenic oocytes. A portion of the biopsy was also fixed in a solution of 4% formaldehyde-1% glutaraldehyde for further histological processing. Maturation of the males was examined by the release of sperm upon application of gentle abdominal pressure. If this was not possible, a sperm sample was obtained by inserting a plastic catheter at the opening of the genital pore. The collected sperm was stored on ice and then transferred to a 4°C refrigerator. Evaluation of sperm parameters was done immediately after the completion of the sampling, and spermatozoa survival under storage at 4 °C was done every other day thereafter, until cessation of movement.

All the experimentation on fish was in accordance with the Spanish (R.D. 53/2013) and European regulations (Directive 2010/63/UE) for the protection of animals used for experimentation and other scientific purposes and supervised and approved by the Ethical Committee CEIBA-IEO (Comité de Ética de la Investigación y Bienestar Animal del Instituto Español de Oceanografía).

7.2.3 Spawning induction experiments

Fish were treated with an EVAc implant (Mylonas & Zohar, 2001) loaded with Des-Gly10, D-Ala6-Pro-NEth9-mGnRHa (H-4070, Bachem, Switzerland) at the sampling times of May, June and July. There were variations in the effective GnRHa dose applied to each fish (**Table 7.1**) due to the fact that the implants were loaded with fixed amounts of GnRHa. At the time of GnRHa implantation, selected females were in advanced vitellogenesis and selected males were in spermiation, although sperm was collected with a catheter.

Table 7.1 Number and mean (±SD) oocyte diameter (μm) of biopsied greater amberjack (N), and number, mean weight (±SD) and dose of GnRHa (μg kg^{-1} body weight) of implanted greater amberjack at each treatment/sampling time. All treated fish (N) were given a GnRHa implant, and slight variations in the effective GnRHa dose were due to the fact that implants were loaded with fixed amounts of GnRHa.

Sex		Females			Males		
Sampling (Month)	Treatment	N		Dose (μg kg^{-1})	N		Dose (μg kg^{-1})
		Biopsied	Treated		Biopsied	Treated	
May	First	7	4 (29.1 ± 5.1)	53.9 ± 10.9	7	7 (14.9 ± 1.9)	67.9 ± 20.3
June	Second *	7	7 (23.3 ± 4.1)	54.4 ± 8.5	7	5 (16.9 ± 1.9)	38.5 ± 4.1
July	Third	7	6 (23.9 ± 3.6)	52.7 ± 4.6	7	6 (13.9 ± 1.9)	39.9 ± 14.5
September		6			5		

* Dose estimated using the individual weights in May.

7.2.4 Evaluation of sperm quality

Sperm quality parameters that were evaluated included (a) sperm density (number of spermatozoa mL^{-1} of sperm), (b) initial percentage of spermatozoa showing forward motility immediately after activation

(sperm motility, %), (c) duration of forward sperm motility of ≥5% of the spermatozoa in the field of view (motility duration, min) and (d) survival of sperm during storage at 4 °C (sperm survival, days). Sperm density was estimated after a 2211-fold dilution with 0.9% saline using a Neubauer hemocytometer (Hirschmann, Eberstadt, Germany) under a compound light microscope at 400× magnification (in duplicates). Sperm motility and duration were evaluated on a microscope slide at 400× magnification after mixing 1 μL of sperm with a drop of seawater (~50 μL) in duplicates. Activated sperm samples were observed under a compound light microscope for the first time 10 s after activation. Sperm motility was determined subjectively using increments of 10% and sperm was considered immotile when <5% of the spermatozoa were exhibiting forward motility. Sperm was stored at 4 °C for the following days, and was examined every other day for motility, until no forward motility was observed. The survival time (days) for each sample was considered as the day before the sample was found to have lost all its motility capacity. This last parameter was evaluated only for the last two samplings in June and July.

7.2.5 Evaluation of egg/larval quality

At the expected onset of the spawning season (May 2015), a passive egg collector was placed in the outflow of the spawning tank and checked daily, in order to collect the spawned eggs. Eggs were collected every morning into a 10 L bucket and their number (fecundity) was estimated by counting the total number of eggs in 3–5 sub-samples randomly of 5 or 10 mL (depending on the total number of eggs), after vigorous agitation. Fertilization success was evaluated at the same time by examining all the eggs in this sub-sample for the presence of a viable embryo using a stereoscope. Each spawn was incubated in a 90 L tank with gentle aeration and filtered (5 μm) water supply.

To monitor embryo and larval survival, eggs from each spawn were placed individually in 96-well mct (in duplicates) according to the procedure of Panini et al. (2001), with some modifications. Briefly, floating (almost 100% fertilized) eggs were taken in a 250 μm mesh filter and were rinsed with sterilized seawater and poured in a 2 L beaker. A Petri dish was used to scoop 100–200 eggs from the beaker. The Petri dish was then placed under a stereoscope and only fertilized eggs were taken one by one with a micropipette set to 200 μL, and they were transferred to the wells of the mct (one egg per well). The mct were then covered with a plastic lid, placed in a controlled-temperature room and maintained for five days at 21 ± 0.5 °C. Using a stereoscope, embryonic and early larval development was evaluated once a day for five days. The number of (a) live embryos was recorded one day after egg collection (or ~36 h after spawning, day 1), (b) hatched larvae was recorded two and three days after egg collection (>60 h after spawning) and (c) viable larvae was recorded four and five days after egg collection (~yolk sack absorption).

Embryo survival was calculated as the proportion of live embryos one day after egg collection in the mct. Hatching success was calculated as the proportion of hatched larvae out of the number of live one day embryos, and five days larval survival was calculated as the proportion of live larvae five days after egg collection out of the number of hatched larvae. Estimating percentage survival (%) by using in the denominator

the number of individuals that survived from the previous developmental stage was considered as a more independent evaluation of survival within specific developmental stages, without the potential of a masking effect of the previous stage (Mylonas, et al., 1992; Mylonas, et al., 2004a).

7.2.6 Histological analysis

Before embedding in methacrylate resin (Technovit 7100®, Heraeus Kulzer, Hanau, Germany) ovarian biopsies were dehydrated in gradually increasing ethanol solutions (70–96%). Serial sections of 3 μm were obtained with a microtome (Leica RM 2245, Nussloch, Germany). Sections were stained with Methylene Blue (Sigma-Aldrich, Steinheim, Germany)/Azure II (Sigma)/Basic Fuchsin (Polysciences, Warrington, Pennsylvania, USA) (Bennett, et al., 1976). Sections were examined under a light compound microscope (Nikon, Eclipse 50i, Tokyo, Japan) and photographed with a digital camera (Jenoptik progress C12 plus, Jena, Germany).

7.2.7 Statistical analysis

Differences in spawning and egg and sperm quality parameters were tested using one-way ANOVA followed by Tukey's post hoc test. The data were checked for normal distribution with the one sample Kolmogorov–Smirnoff test, as well as for homogeneity of the variances with the Levene's test, and percentage data were Arcsine transformed prior to statistical analysis to normalize variances. Pearson's correlation coefficients were used to assess the relationships between egg quality variables. Percentage data were Arcsine transformed prior to statistical analyses to normalize variances. Results are presented as mean ± standard deviation (SD) of the pre-transformed data, unless mentioned otherwise. In all statistical tests used, differences with a P value of less than 0.05 were considered statistically significant. Analyses were performed with the SPSS statistics package, International Business Machines Corporation (IBM) version 20.0 for Windows (Armonk, New York, USA) and JMP 12 (SAS Institute Inc., Cary, North Carolina, USA).

7.3 Results

Almost one third of the females were in eVg stage in May (**Fig. 7.1A**), but mostly the fish (n = 4) had fully Vg oocytes (**Fig. 7.1B**), with a mean (±SEM) diameter of the largest oocytes of 685 ± 21 μm (**Fig. 7.2**). Some AT was present, as well. Only females with oocytes >600 μm were considered eligible for spawning with GnRHa implantation. In June, four of the biopsied females again had mainly Vg oocytes, but also oocytes in maturation, with the largest oocytes having a mean diameter of 723 ± 47 μm (**Fig. 7.2**), with also some AT. At the same sampling, one female was found with ovulated eggs. In July, three of the females had Vg oocytes (**Fig. 7.1C**) in the ovary, with an increased percentage of AT. The mean diameter of the largest Vg oocytes was 697 ± 26 μm (**Fig. 7.2**). The rest of the females had mainly ovulated eggs and primary or eVg oocytes at most. At the last sampling in September, most of the females (n = 4) were in Vg stage with a mean diameter

of 650 ± 37 μm (**Fig. 7.2**). Two of them had oocytes in OM (**Fig. 7.1D**). No significant differences were found in the mean diameters of the largest Vg oocytes among the different samplings.

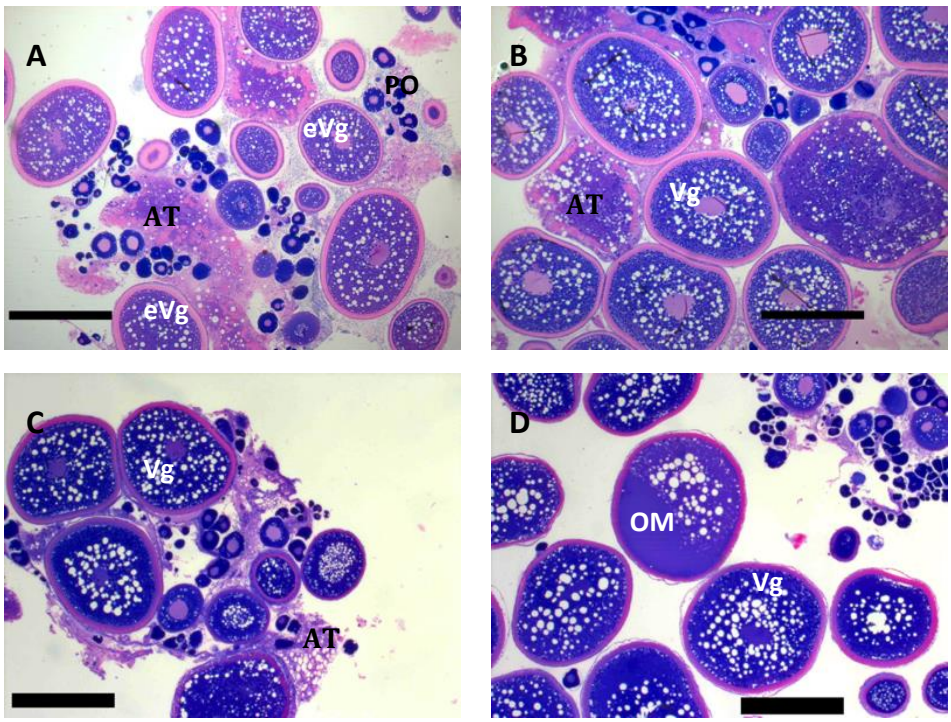

Figure 7.1 Ovarian biopsies from F1 greater amberjack maintained in land-based tanks in Tenerife, Canary Islands and processed for histology (**A**): Female in May in early Vitellogenesis (eVg) with a large number of primary oocytes (PO) as well as atresia (AT). (**B**): Female in May with oocytes in full Vg. (**C**): Female in mid-July, in full Vg. (**D**): Female in mid-September, having oocytes in oocyte maturation (OM) and Vg. Bar = 500 μm.

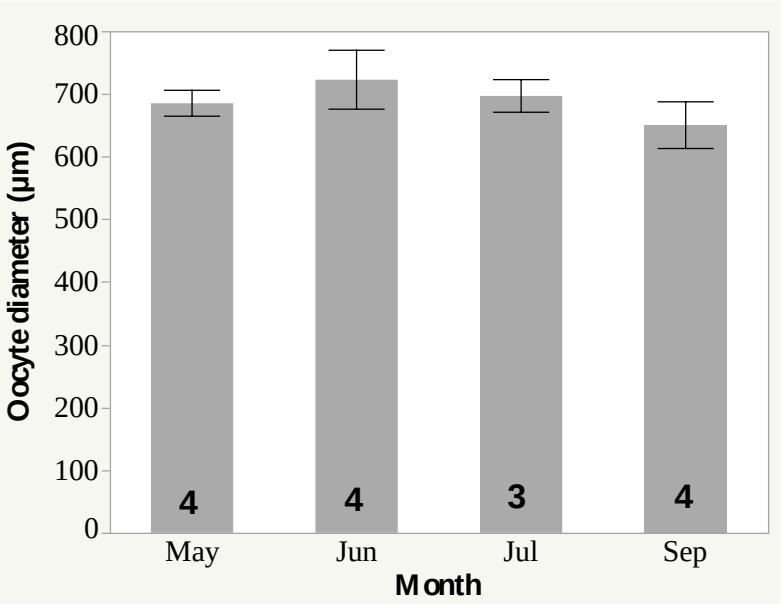

Figure 7.2 Mean (±SEM) oocyte diameter of F1 greater amberjack (*n* inside the bars) that were expected to respond to given GnRHa implant, at the time of treatment (May–Jul) and at the conclusion of the experiment (September). No statistically significant differences were observed ($P < 0.05$).

Males and females in May, June and July were implanted with a GnRHa implant at a dose of ~50 µg GnRHa kg^{-1} body weight in the successive spawning induction treatments (**Table 7.1**). In June and July, more females were given a GnRHa implant than were eligible for spawning based on their maximum Vg oocyte diameters (**Fig. 7.2**), because the measurements of the oocytes were done later in the laboratory once the fish sampling was completed.

Mean sperm motility was >50% (**Fig. 7.3**) and remained unchanged throughout the monitored period, while the duration of sperm motility was significantly higher in May (4.35 ± 1.12 min) than in June (2.44 ± 0.24 min) ($P < 0.05$). The mean sperm density was 30.8 ± 6.8 × 10^9 spermatozoa ml^{-1} in May and 78.0 ± 72.2 × 10^9 spermatozoa ml^{-1} in September, although with elevated individual variability in September (**Fig. 7.3**).

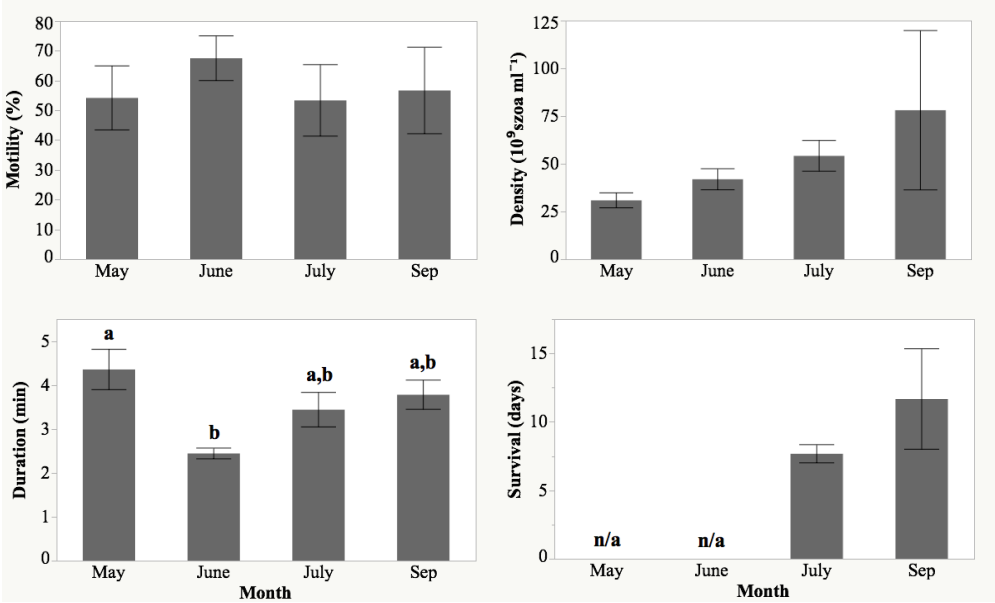

Figure 7.3 Mean (±SEM) sperm quality parameters of F1 greater amberjack at different times during the reproductive season (spermatozoa (szoa) forward motility, density, duration of motility and maximum survival during storage at 4 °C). Statistically significant differences among sampling times are indicated by different lower-case letters (a, b) ($P \leq 0.05$). n/a = not available.

The first spawn occurred between one and two days after each GnRHa treatment (**Fig. 7.4**). A total of 52 spawns were obtained during a period of 72 days (**Table 7.2**). The number of spawns and fecundity obtained after successive GnRHa implantations decreased over time and the spawning events were concentrated more and more around the application of each GnRHa treatment. In the period after the first treatment, the fish spawned 29 times. After the second treatment, a total of 15 spawns were recorded during the first 16 days, and no eggs were collected during the following days. The eggs released after the third GnRHa treatment were collected from eight spawning events that were obtained during the first nine days.

Table 7.2 Egg production (mean ± SEM where appropriate) of F1 greater amberjack induced to spawn using a GnRHa implant treatment at different times during the reproductive season (May–July). No statistically significant differences were observed among the means of relative batch fecundity obtained after the three GnRHa treatments ($p < 0.05$).

Treatment	Spawns (*n*)	Eggs spawn^{-1} kg^{-1}	Total eggs kg^{-1} (×1000 Eggs)	Total eggs (×10^6 eggs)
1	29	2087 ± 218	60.54	7.05
2	15	2828 ± 420	42.42	6.55
3	8	1895 ± 827	15.16	1.35

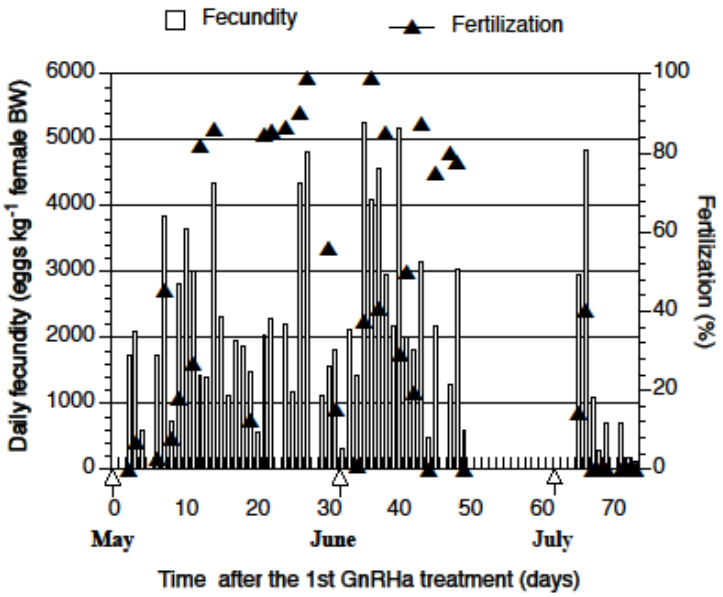

Figure 7.4 Daily fecundity (eggs kg^{-1} female body weight (BW)) and fertilization (%) of F1 greater amberjack in response to three treatments with GnRHa implants. The white arrows on the X-axis indicate the times of the GnRHa treatments.

The highest daily relative fecundity that was recorded was 5539 eggs kg^{-1} fish after the second GnRHa treatment (**Fig. 7.4**), but the total egg production was higher after the first treatment; that is, 60,540 eggs kg^{-1} fish compared to 40,180 eggs kg^{-1} fish after the second treatment (**Table 7.2**). No significant differences were found in daily relative fecundity among the three treatment periods. Almost 15 million eggs were produced from the three successive GnRHa applications.

Mean fertilization changed through the spawning period after each treatment, reaching its highest values after the first and the second GnRHa treatment, while a significant decrease was observed after the third treatment ($P< 0.05$) (**Fig. 7.5**). On the other hand, no significant differences were observed in hatching, one-day embryo survival and three-day larval survival after successive GnRHa treatment.

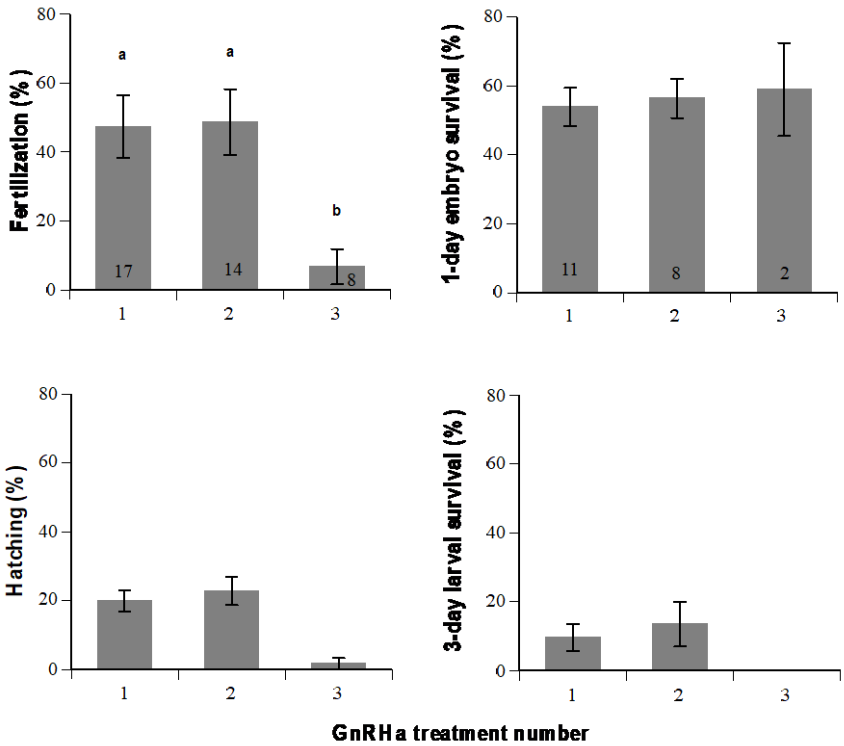

Fig. 7.5 Mean (±SEM) egg quality parameters of F1 greater amberjack after three GnRHa implantations (May–July). Numbers in bars indicate the number of samples that constitute each mean. The number of samples of each mean for hatching (%) and three-day larval survival (%) are the same as for one-day embryo survival. Statistically significant differences among GnRHa implantations are indicated by different lower-case letters (a, b) (P = 0.05).

7.4 Discussion

In the Mediterranean, the ovaries of wild-caught greater amberjack have batches of oocytes at distinct stages of development with diameters of 120–400 µm in early vitellogenesis (Marino, et al., 1995), 400–600 µm at the beginning of the spawning season in May (Lazzari, et al., 2000), and the largest diameter (650–750 µm) in June (Kozul, et al., 2001; Mylonas, et al., 2004b). Even after reaching appropriate oocyte sizes, a considerable number of females fail to undergo OM and do not ovulate, and hormonal treatments are necessary to induce OM and spawning in captivity. The efficiency of the hormonal treatments depends on the stage of ovarian development at the time of treatment, the hormone type, the dose and the method of application. Captive-reared wild greater amberjack spawned successfully after having been induced with hCG injections when females had vitellogenic oocytes of 550–600 µm in diameter (Díaz, et al., 1997; Kozul, et al., 2001), and after injection (Fernández-Palacios, et al., 2015a) or implantation (Mylonas, et al., 2004b) with GnRHa when oocytes were at 500 and 650 µm in diameter, respectively. In the present study, some of the F1 females underwent full vitellogenesis and had larger oocytes than reported before for wild-caught individuals, and were appropriate to be induced for spawning at all sampling times from May to September. This demonstrates that

under these rearing conditions and hormonal treatments, F1 greater amberjack undergo normal oogenesis and maintain their vitellogenic production for an extended period of time, as is customary for this species in the subtropical area of the Canary Islands (Fernández-Palacios, et al., 2015a; Jerez, et al., 2006). This is the first study demonstrating that F1 generation greater amberjack in Europe do undergo gametogenesis in captivity and are capable of producing fertilized eggs, albeit after exogenous hormonal therapy for the induction of OM, ovulation and spawning. The only other report of F1 greater amberjack broodstock reproducing spontaneously in captivity comes from Japan (Kawabe, et al., 1998).

Sperm could not be collected by abdominal pressure at any sampling time, even though the fish were in spermiating condition, and sperm samples were taken using a catheter introduced into the genital pore. The same situation was observed in captive male greater amberjack broodstocks that were held either in tanks or in sea cages in Greece (Mylonas, et al., 2017c; Zupa, et al., 2017b). Similar to other fast-swimming pelagic fishes, such as the Atlantic bluefin tuna, the abdominal wall of greater amberjack is thick and very muscular, and this probably makes it very difficult to apply adequate pressure to the internal organs and the testes (Zupa, et al., 2013). In addition, in captive-reared fish, it is very common for males to produce lower amounts of sperm, often of high sperm density (Mylonas & Zohar, 2001; Mylonas, et al., 1998b), making it difficult to obtain sperm with abdominal pressure. In general, treatments with GnRHa increase the milt volume by stimulating seminal plasma production, but often with a proportional decrease in sperm density (Clearwater & Crim, 1998; Vermeirssen, et al., 1998). Sustained-release GnRHa-delivery systems have been used successfully to induce an overall increase both in the expressible sperm and spermatozoa production in various cultured fishes (Mylonas & Zohar, 2001), enhancing the quality and the quantity of sperm produced (Clearwater & Crim, 1998; Goren, et al., 1995; Mylonas, et al., 1997c; Rainis, et al., 2003; Sorbera, et al., 1996), including wild-caught greater amberjack (Mylonas, et al., 2004b). In the present study, it was not observed a significant increase in sperm production in response to the GnRHa treatment over the course of the monitoring period that would translate into easier collection of sperm using abdominal pressure.

Similarly, there were, in general, no differences in the sperm quality parameters of the males during the present study, showing similar values to wild-caught GnRHa-treated greater amberjack reported earlier (Mylonas, et al., 2004b). A gradual reduction in the sperm motility duration during the reproductive season was observed in another study with wild-caught broodstock in the eastern Mediterranean Sea (Mylonas, et al., 2007). However, as water temperatures do not rise to the same levels in the summer in the Canary Islands, greater amberjack apparently remain in spermiating condition for a much longer period of time, reportedly from May to October (Fernández-Palacios, et al., 2015a; Jerez, et al., 2006). The sperm density in GnRHa-treated F1 males showed an increasing trend over the course of the spawning period, although it was always lower than the one obtained in wild-caught males treated with GnRHa implants (Mylonas, et al., 2004b); however, it was in the same range as a study using untreated males (Mylonas, et al., 2007). Different effects of GnRHa-delivery systems have been obtained in other species. For example, in GnRHa-treated Atlantic

salmon, the total expressible milt increased, while sperm density and motility did not change (Mazorra de Quero, et al., 2000), while in Atlantic halibut (Vermeirssen, et al., 2000) and common dentex (Greenwood, et al., 2001), the sperm motility was enhanced slightly and the sperm density decreased. In contrast, GnRHa implants were ineffective in enhancing sperm production in the flatfishes Southern flounder and summer flounder (Berlinsky, et al., 1997; Berlinsky, et al., 1996). Therefore, the application of GnRHa delivery systems for inducing and enhancing spermiation in greater amberjack requires more research in order to determine if a different mode, time of application or dose are required for a successful enhancement of milt production, as has been demonstrated in many other fishes (Mylonas, et al., 2017b).

Spontaneous and induced spawning of wild-caught greater amberjack has been achieved from May to October in the Canary Islands (Fernández-Palacios, et al., 2015a; Jerez, et al., 2006), but only from May to July in the Mediterranean Sea (Lazzari, 1991; Marino, et al., 1995), where summer temperatures are much higher, and from May to June in Japan, either using reared or wild-caught fish (Kawabe, et al., 1998; Kawabe, et al., 1996). In the present study, a total of 52 spawns were obtained from GnRHa-treated F1 greater amberjack, which is higher from the number reported previously (Kawabe, et al., 1996), with the number obtained after each consecutive treatment decreasing gradually. The use of GnRHa for inducing and increasing spawning frequency is well known (Mylonas, et al., 2010), and it has also been reported in wild-caught greater amberjack (Mylonas, et al., 2004b), longfin yellowtail (Roo, et al., 2014) and F1 yellowtail kingfish (Setiawan, et al., 2016). In this study, the gradual decrease in the number of spawns obtained after each implantation could be related to the vitellogenic capacity of the ovary, and potentially to a determinant type of fecundity characteristic belonging this species. In terms of a comparison between GnRHa injections and implants, recent studies in wild-caught greater amberjack have shown that a larger number of spawns can be expected in response to successive GnRHa implants compared to injections (Chapter 5), perhaps due to lower stress inflicted on the broodstock and a longer period of hormonal stimulation by the controlled-release GnRHa delivery systems.

The total number of eggs obtained from GnRHa-implanted F1 females (14.95×10^6 eggs) was less than that obtained from natural spawning of hatchery-reared broodstock in Japan (23.33×10^6 eggs) (Kawabe, et al., 1996), but similar to that of wild-caught greater amberjack reported previously (Jerez, et al., 2006), and three times higher than that obtained from wild-caught females treated with multiple GnRHa injections in the Canary Islands (Fernández-Palacios, et al., 2015a). Comparable results have been recorded in the Mediterranean Sea, where the spawning season is shorter, in wild-caught greater amberjack treated with hCG or GnRHa in the form of injections or implants (García, et al., 2001; Mylonas, et al., 2004b)(Chapter 5). Therefore, our results show that implants of GnRHa can induce a higher number of spawns and higher fecundity in the greater amberjack, than produced spontaneously or in response to simple hormonal injections. Unfortunately, no comparison of relative fecundity is possible with the other studies with hatchery-reared greater amberjack, since this information was not provided (Kawabe, et al., 1998; Kawabe, et al., 1996).

In some species, spawning induction using GnRHa has been reported to have a negative effect on egg quality (Bobe & Labbé, 2010). For example, GnRHa implantation has been associated with decreased egg buoyancy, fertilization and number of viable eggs, as well as smaller oil globule diameters (Bonnet, et al., 2007; Forniés, et al., 2000; Garber, et al., 2009), a situation that was also observed using F1 yellowtail kingfish (Setiawan, et al., 2016). The fertilization success obtained from hormonally treated wild-caught greater amberjack has been highly variable, ranging between 16–50% (Kozul, et al., 2001) and 58–99% (Tachihara, et al., 1993) for hCG-injected fish, and 22% to 96 ± 6.5% for GnRHa-implanted (Mylonas, et al., 2004b) and -injected (Fernández-Palacios, et al., 2015a) fish, respectively. In the latter study, the male:female sex ratio used was higher (2♂:1♀), and this might have improved the fertilization success (Blaxter, 1988). In the present study, the GnRHa-implanted F1 greater amberjack produced eggs with mean fertilization success of 42 ± 6.2%, a value that was slightly lower than that obtained from natural spawning of wild-caught greater amberjack maintained in the same facilities (62 ± 3.6%) at a similar sex ratio (Jerez, et al., 2006). Furthermore, the hatching success (35 ± 4.8%) was lower than that obtained from hCG-injected (52–97%) (Tachihara, et al., 1993) and GnRHa-injected (93%) (Fernández-Palacios, et al., 2015a) wild-caught greater amberjack, and also lower than that obtained in F1 broodstocks (Kawabe, et al., 1998; Kawabe, et al., 1996), but it was more than double that obtained by natural spawning in the Canary islands (Jerez, et al., 2006). Thus, the successive GnRHa implants used in this study to induce maturation and spawning of F1 greater amberjack were an adequate treatment to produce fertilized eggs, showing similar fertilization and higher hatching success to that obtained from natural spawning of a wild-caught broodstock in our facilities, but lower hatching success compared to the natural spawning of hatchery-reared greater amberjack in Japan, where the spawning season was shorter. With further optimization, this method may be a reliable and highly efficient way to obtain eggs from hatchery-produced broodstocks maintained in aquaculture facilities, enabling the development of a sustainable greater amberjack industry through breeding selection.

In some studies, lower embryo and larval survival were recorded at the first spawns after hormonal treatment (Fernández-Palacios, et al., 2015a; Kozul, et al., 2001). In contrast, in the present study, the lowest fecundity and quality of eggs were obtained after the third GnRHa treatment at the latter part of the reproductive season. Additionally, in the same treatment period, the three-day larval survival was lower than the one recorded using wild-caught greater amberjack injected with GnRHa (Fernández-Palacios, et al., 2015a) or hCG (Tachihara, et al., 1993). A reduction in embryo and larval survival may occur towards the end of the spawning period in fishes with asynchronous ovarian development (Mandich, et al., 2004; Mihelakakis, et al., 1995; Mihelakakis, et al., 2001), perhaps due to depletion of the nutritional maternal stores, as fish often reduce their feed intake during the spawning period.

The present study showed that hatchery-produced F1 greater amberjack undergo normal gametogenesis and can be induced to undergo maturation, ovulation and spawning using GnRHa delivery systems. Egg production is high and egg quality adequate for the implementation of larval rearing for

commercial purposes. The use of consecutive GnRHa-delivery systems over a long reproductive period resulted in multiple spawns of fertilized and viable eggs.

Chapter 8 - Sperm quality of greater amberjack throughout the reproductive season and in response to GnRHa treatment with controlled-release implants

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8.1 Introduction

Greater amberjack females have received much more attention than males, as it is generally the case for fish reproduction in captivity, due to the failure of females to undergo reliably spontaneous oocyte maturation, ovulation and spawning when reared (Chapter 4). A number of recent articles have focused in solving the reproductive dysfunction in females and controlling spawning, and rearing in sea cages during the year resulted in the completion of the process of gametogenesis, as well as maturation and spawning (Sarih, et al., 2018; Sarih, et al., 2019; Sarih, et al., 2020). Nevertheless, reproductive dysfunctions in captivity have been observed also in males (Zupa, et al., 2017b), and unlike what have been observed in females, sperm quantity and quality were not better in captive males reared in sea cages compared to land-based tanks (Chapter 4). Reduced percentage of motile spermatozoa, duration of motility, velocity and ATP content of milt collected from captive males, as well as reduced GSI, sex steroid levels and seminiferous lobule diameter of sea cage-reared captive males compared to fish in the wild have been reported (Zupa, et al., 2017a). These aspects are probably responsible for the relatively lower fertilization in farmed greater amberjack and should be tackled to ensure reliable egg production for this species.

To overcome this type of reproductive dysfunctions in cultured fishes, hormonal therapies have been used extensively, using GtH preparations (*i.e.* LH, hCG or carp pituitary extracts (CPE)) that act at the level of the gonad or GnRHa that act at the level of the pituitary (Mylonas, et al., 2017b). Of the two types of hormones, GnRHa has important advantages over GtH preparations, in part due to the possibility to administer GnRHa through long-term controlled-release delivery systems (Mylonas & Zohar, 2001) resulting in improved efficiency (Mylonas, et al., 2010). It is well reported in numerous species that GnRHa controlled-release delivery systems enhanced milt production (Clearwater & Crim, 1998; Mylonas, et al., 1997b; Mylonas, et al., 1997c; Sorbera, et al., 1996; Vermeirssen, et al., 2000), including species such as the Atlantic bluefin tuna (Corriero, et al., 2009), which exhibits the same problem of lack or reduced expressible milt in captivity. Implants loaded with GnRHa were also shown to improve not only milt production but also spermatogenic activity in other species of interest such as the European seabass (Rainis, et al., 2003) and meagre (Fakriadis, et al., 2020). However, the effects of these hormonal treatments on sperm quality parameters are variable among different fish species (Mylonas, et al., 2017b).

In males, fertilization capacity and reproductive success is usually linked to milt production and sperm quality (Bobe & Labbé, 2010; Cosson, et al., 2008; Fauvel, et al., 2010; Kowalski & Cejko, 2019). Sperm quality and quantity can be variable during the spawning period due to environmental factors, such as temperature and photoperiod, and nutrition (Kowalski & Cejko, 2019; Mylonas, et al., 2017b). Among sperm quality parameters, sperm motility (Rurangwa, et al., 2004) and velocity (Gage, et al., 2004) are considered the most important and, in some species, percentage of motile spermatozoa is also directly related to egg fertilization success (Gallego & Asturiano, 2018).

So far, in the greater amberjack or other congeners, sperm quality assessments, especially using CASA, are still limited (Zupa, et al., 2017a) or lacking. The first objective of the present study was to determine the extent of the spermiation period in greater amberjack maintained in sea cages during the year, characterize sperm quality parameters during the spawning season, and examine any variations over time. Secondly, it was evaluated the effect of a single or double administration of GnRHa implants on sperm quality parameters for a period of 21-29 days.

8.2 Materials and methods

8.2.1 Broodstock maintenance

Wild greater amberjack juveniles were fished (~300 g in 2010) in the Ionian Sea, Greece, with a commercial purse seine fishing vessel and were maintained in a sea cage of a commercial aquaculture operation at Astakos (Greece). In 2013-2014, 3-4-year-old fish from this stock were transferred to two commercial operations in Greece as broodstocks (ARGO, GMF) and were reared in round sea cages of 40 m perimeter and 8 m depth until the implementation of the described experiments in 2016 and 2017. Broodstocks were fed commercial extruded feed (Skretting Vitalis CAL, 22 mm) 3 to 5 times a week until apparent satiation. Temperature and dissolved oxygen parameters were measured at least once a week. The seawater temperature at GMF ranged between 13.0°C and 27.4°C during the year. At ARGO, the temperature at the sea cage ranged between 14.4°C – 30.2°C in 2016 and 13.9°C - 27.0°C in 2017 (data not shown).

Broodstock selection for spermiation enhancement experiments was done after a 2-day starvation period. Fish were initially tranquilized in a 10-20 m^3 anesthesia sack placed inside the cage using clove oil (0.01 ml L^{-1}), and from there the fish were transferred one by one to a 1 m^3 tank for complete anesthesia with a higher concentration of clove oil (0.03 ml L^{-1}) (Mylonas, et al., 2005). Because of both the hard musculature surrounding the abdominal cavity and the limited quantity of sperm produced by captive greater amberjack, and after unsuccessful attempts to collect sperm after applying pressure to fish's abdomen, milt samples were obtained by catheter inserted at the opening of the genital pore (Pipelle de Cornier, Laboratorie CCD, France). Males were selected for GnRHa administration if milt could be obtained with the catheter.

8.2.2 Determination of the extent of the spermiation period

In order to estimate the extent of the spermiation period of greater amberjack in captivity when fish were maintained in sea cages throughout the year, and identify potentially the best timing for spermiation

enhancement, one experiment was conducted in 2017 at GMF (**Fig. 8.1**). Mature breeders were split into two sea cages from the year before the experiment. Cage A contained 6 females (mean BW ± SD) of 19.4 ± 3.0 kg) and 6 males (15.7 ± 1.4 kg) and cage B contained 7 females (17.3 ± 3.1 kg) and 7 males (14.4 ± 1.3 kg). On May 30, 2017 (Day 0) half of the fish from cage A were evaluated and sperm samples were collected (**Fig. 8.1**, TANK 1 IN) as described above, and three females and three males were selected and treated with GnRHa with EVAc implants (Mylonas & Zohar, 2001; Mylonas, et al., 2007) loaded with Des-Gly^{10}, D-Ala^{6}-Pro-NEth^{9}-mGnRHa (H-4070, Bachem, Switzerland), and were transferred to an onshore 21-m^3 round tank for spawning (1st period), as reported earlier (Chapter 6). A week later, on June 6 (Day 7), the remaining fish in cage A were evaluated as above and three females and three males were treated with GnRHa, and were transferred to a different onshore tank (TANK 2 IN) for spawning (2nd period). A similar scheme was followed for cage B for the 3rd and 4th periods that started on June 20 (Day 21 – TANK 3 IN) and July 4, 2017 (Day 35 – TANK 4 IN), respectively. Each group remained in the onshore facilities for 14 days (except the 1st group, which remained for 21 days) and then was transferred back to the sea cages (**Fig. 8.1**, TANK OUT). Sperm samples were collected and evaluated also at the end of each spermiation enhancement experiment before their transfer back to the sea cage. A final (third) sperm evaluation was done for the males used in the 1st, 2nd and 3rd spermiation enhancement periods on July 18, 2017, after the fish remained for 14 – 28 days in the sea cage after the completion of the experiment (**Fig. 8.1**, CAGE). The effective GnRHa dose for the selected males was (mean ± SD) 67 ± 6 μg GnRHa kg^{-1} BW. A certain degree of variability in the effective GnRHa doses occurred since the GnRHa amount is fixed in the implants, even though combinations of two implants loaded with different amounts of GnRHa were used. For spawning, the tanks were supplied with surface seawater and exposed to ambient photo-thermal conditions. Gaseous oxygen was also provided to the tanks for safety reasons, maintaining the saturation to 110-120 %. Measurements of temperature and oxygen saturation were conducted twice a day (a.m. and p.m.). The results on the female reproductive maturation, induction of spawning and egg production were reported earlier (Chapter 6).

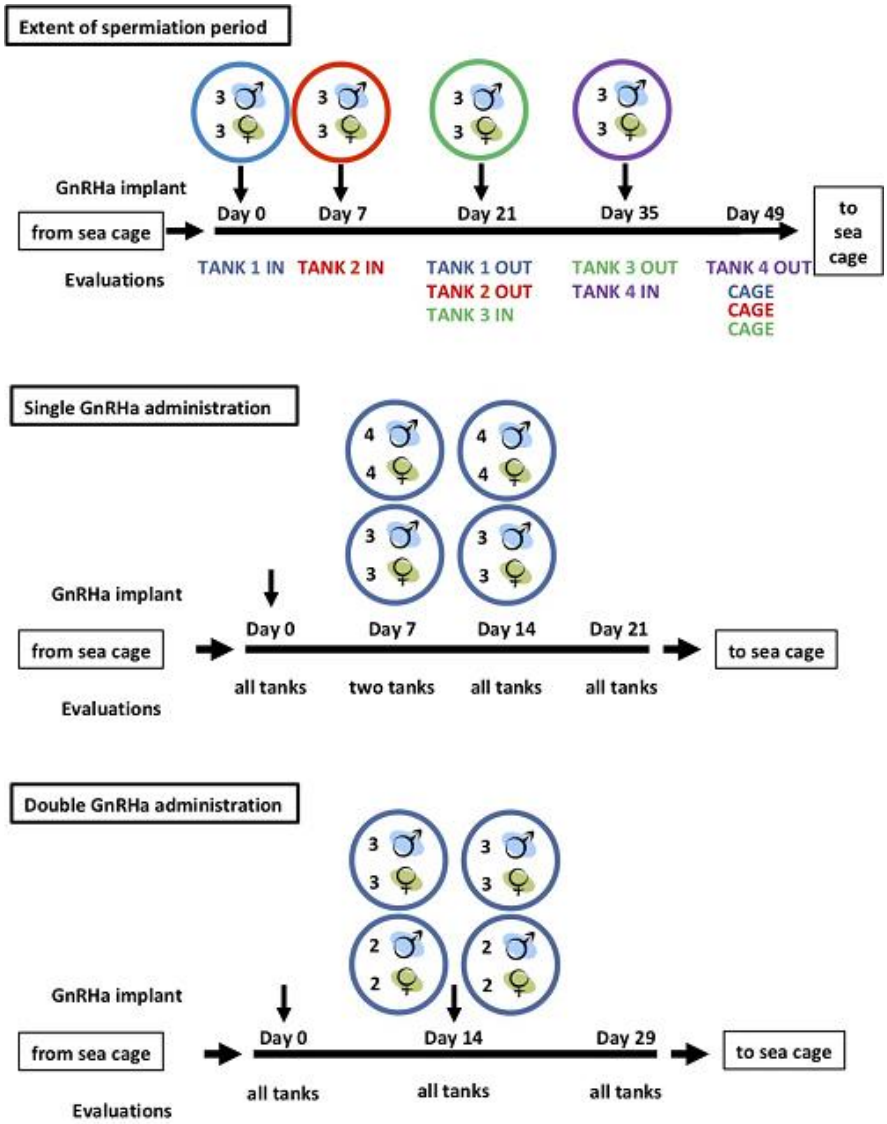

Figure 8.1 Schematic representation of the experimental design reported in Materials and Methods 2.2. Extent of the spermiation period (upper)(blue - 1st period, red – 2nd period, green – 3rd period, purple - 4th period), 2.3 Effect of a single GnRHa administration (middle) and 2.4 Effect of a double GnRHa administration (lower). Greater amberjack broodstocks were maintained in sea cages during the year and were transferred to onshore tanks after GnRHa implant treatment to enhance spermiation, and were returned to sea at the end of the experiments. Fish were sampled for sperm evaluations prior to GnRHa implantation (Day 0) and at different times thereafter, indicated below the time axis. Vertical arrows indicate the time of GnRHa administration.

8.2.3 Effect of a single GnRHa administration on sperm quantity and quality

The spermiation enhancement trial using a single GnRHa administration was conducted between 7 June and 28 June 2016 at ARGO (**Fig. 8.1**). Males (n=14) of mean ± SD BW of 15.1 ± 3.0 kg were selected and treated with a GnRHa implant as described above, resulting in a mean ± SD effective dose of 58 ±8 μg GnRHa kg^{-1} BW, just prior to being transferred to the on-land facilities, to four 23-m^3 round tanks with equal number of females. Sperm samples were collected on Day 0 for the selected fish, just prior the GnRHa

administration (n=14), on Day 7 in two of the four tanks (n=7) and on Days 14 and 21 from all the fish (**Fig. 8.1**). The tanks were supplied with a mixture of surface (~16% exchange h^{-1}) and well water (~6% exchange h^{-1}) and exposed to ambient photo-thermal conditions. Gaseous oxygen was also provided to the tanks for safety reasons, maintaining the saturation to 110-120%. Measurements of temperature and oxygen saturation were conducted twice a day (a.m. and p.m.). The results on the female reproductive maturation, induction of spawning and egg production were reported earlier (Chapter 5).

8.2.4 Effect of a double GnRHa administrations on sperm quantity and quality

The spermiation enhancement trial using a double GnRHa administration was conducted between 7 June and 5 July, 2017 at ARGO (**Fig. 8.1**). Males (n=10) of mean ± SD BW of 18.4 ± 1.9 kg were selected and treated with a GnRHa implant as described above, resulting in a mean ± SD effective dose of 58 ±18 μg GnRHa kg^{-1} BW just prior to being transferred to the on-land facilities, to four 23-m^3 round tanks with equal number of females. Sperm samples were collected from the fish on Day 0 (just prior the GnRHa administration), 14 and 29 for sperm quantity and quality evaluations. On Day 14, males were treated again with GnRHa implants for the same effective dose as before. The tanks were supplied with surface seawater (~20% exchange h^{-1}) and exposed to ambient photo-thermal conditions. Gaseous oxygen was also provided to the tanks for safety reasons, maintaining the saturation to 110-120%. Measurements of temperature and oxygen saturation were conducted twice a day (a.m. and p.m.). The results on the female reproductive maturation, induction of spawning and egg production were reported earlier (Chapter 6).

8.2.5 Evaluation of sperm quantity and quality parameters

The quantity of produced sperm was evaluated according to spermiation condition –which is a measure of the available milt in the testes- after gentle abdominal pressure was applied, determined by a subjective scale from 0 to 3, as follows: Spermiation index S0 = no milt released, S1= only a drop of milt released after multiple stripping attempts, S2= milt was released easily after the first stripping attempt and S3= copious amounts of milt released with very little pressure. The sperm quality parameters that were evaluated included: (a) sperm density (number of spermatozoa ml^{-1} (szoa ml^{-1}) of milt), (b) survival of spermatozoa under cold storage at 4°C (spermatozoa survival, days) and (c) duration of forward spermatozoa motility of ≥5% of the spermatozoa in the field of view (motility duration, min) and d) sperm motility parameters. Spermatozoa density was estimated after a 2121-fold dilution with 0.9% saline using a Neubauer haemocytometer under 200x magnification (in duplicate) under a compound light microscope. Milt was stored at 4°C, and was examined every other day for spermatozoa motility, until less than 5% of the spermatozoa were observed to be motile, in order to estimate spermatozoa survival time (days). Additionally, milt samples were assessed using CASA (ISAS, Spain). Milt samples were activated in seawater containing 2% bovine serum albumin (1:201 or 1:334) to obtain 200-300 cells in the field and spermatozoa movement was recorded on a disposable counting chamber with a fixed depth (Leja) 15 sec after activation using the digital camera The Imaging Source DMK 22BUC03 with a resolution of 744x480 pixels at 30 fps attached to a light microscope (Zeiss Primo

Star) under 200x magnification, using dark field microscopy, immediately after milt collection. Spermatozoa movement was recorded until less than 5% of the spermatozoa were observed to be motile. The analyzed parameters were VCL, VSL, VAP (μm sec^{-1}), motile cells, progressive cells (>80% STR), rapid cells and STR (%). The software settings were adjusted to: 1 to 90 μm for head area; VCL <10 μm sec^{-1} to classify a spermatozoon as immotile; and spermatozoa were considered rapid when VCL was higher than 100 μm sec^{-1}.

8.2.6 Statistical Analysis

For determining the extent of the spermiation period, differences in the measured parameters were tested using a one-way ANOVA, using as factor the period (1, 2, 3, 4) and a one-way repeated measures ANOVA using as factor the sampling time (TANK IN, TANK OUT, CAGE), followed by Tukey's HSD post hoc test. For the single and double GnRHa implantation experiments, differences in the measured parameters were tested using a one-way repeated measures ANOVA followed by Tukey's HSD post hoc test (Days 0, 7, 14 and 21 for the single GnRHa administration and Days 0, 14 and 29 for the double GnRHa administration experiment, respectively). Data were transformed (log_{10} or arcsine) accordingly to meet the ANOVA assumptions, if not normally distributed. A level of $P \leq 0.05$ was set as minimum statistical significance. Statistical analyses were performed with JMP 12 (SAS Institute Inc., Cary, NC, USA). Results are presented as mean ± standard error (SEM), unless mentioned otherwise.

8.3 Results

8.3.1 Determination of the extent of the spermiation period

Spermiation index was 0 (data not shown) at all times in all trials and in all males as milt could not be expressed with abdominal pressure and was collected with a catheter from the genital pore, both before and after the GnRHa administration. The mean percentage of motile cells, progressive cells, rapid cells, VCL, VSL, VAP and STR were similar in males sampled from sea cages at different times during the spawning season (one-way ANOVA, P>0.05) (**Fig. 8.2**). However, the duration of sperm motility, sperm density and survival under cold storage (one-way ANOVA, Tukey's HSD, P≤0.05) decreased significantly between the 1st and the 3rd or the 1st and the 4th periods, respectively, as the reproductive season progressed (**Fig. 8.2**).

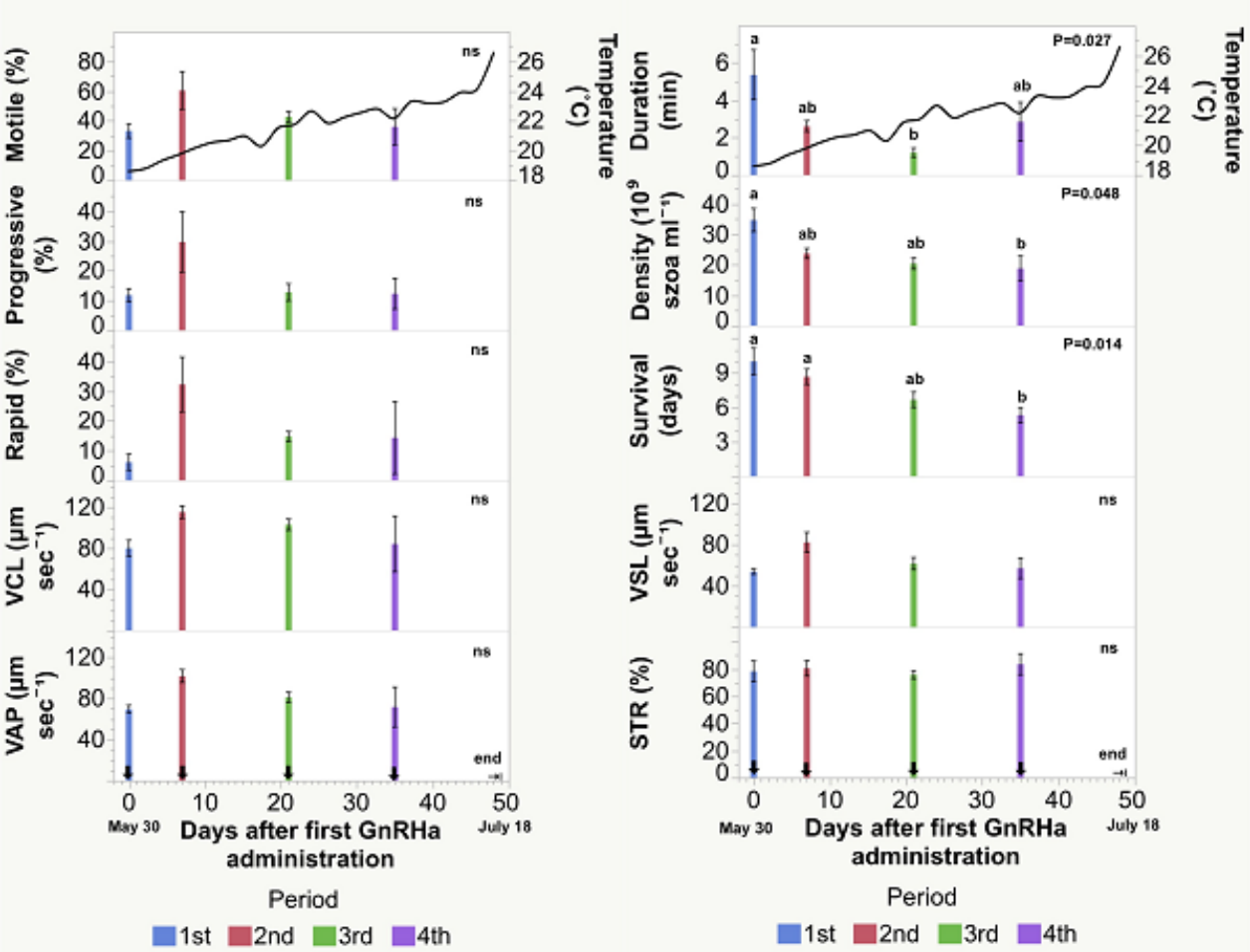

Figure 8.2 Mean (± SEM) motile cells (%), progressive cells (%), rapid cells (%), curvilinear velocity (VCL, μm sec^{-1}), average path velocity (VAP, μm sec^{-1}), spermatozoa motility duration (min), density (x10^9 szoa ml^{-1}), survival under cold storage at 4°C (days), straight line velocity (VSL, μm sec^{-1}) and straightness (STR, %) of milt collected at different times from different male greater amberjack (n=3 per period) maintained in sea cages during the reproductive season. Arrows indicate the time of GnRHa administration. Lowercase letters indicate statistically significant differences among the sampling times (one-way ANOVA, Tukey's HSD, P≤0.05). ns = not statistically significant.

The percentage of motile cells, progressive cells, rapid cells, duration of sperm motility and STR did not changed throughout the experiment from the time the fish were moved from the sea cages into the tanks for spawning (TANK IN) to the time the fish went back to the sea cages (TANK OUT) after 14-21 days and finally after an additional period of 14-28 days in the sea cage (CAGE)(one-way ANOVA, P>0.05) (**Fig. 8.3**). However, the VCL (one-way ANOVA, P=0.046, Tukey's HSD, P≤0.05), VAP (one-way ANOVA, P=0.007, Tukey's HSD, P≤0.05) and survival under cold storage (one-way ANOVA, P=0.009, Tukey's HSD, P≤0.05) were reduced from TANK IN to CAGE, while sperm density (one-way ANOVA, P=0.013, Tukey's HSD, P≤0.05) and VSL (one-way ANOVA, P=0.033, Tukey's HSD, P≤0.05) remained the same between TANK IN and CAGE with fluctuations in TANK OUT (**Fig. 8.3**).

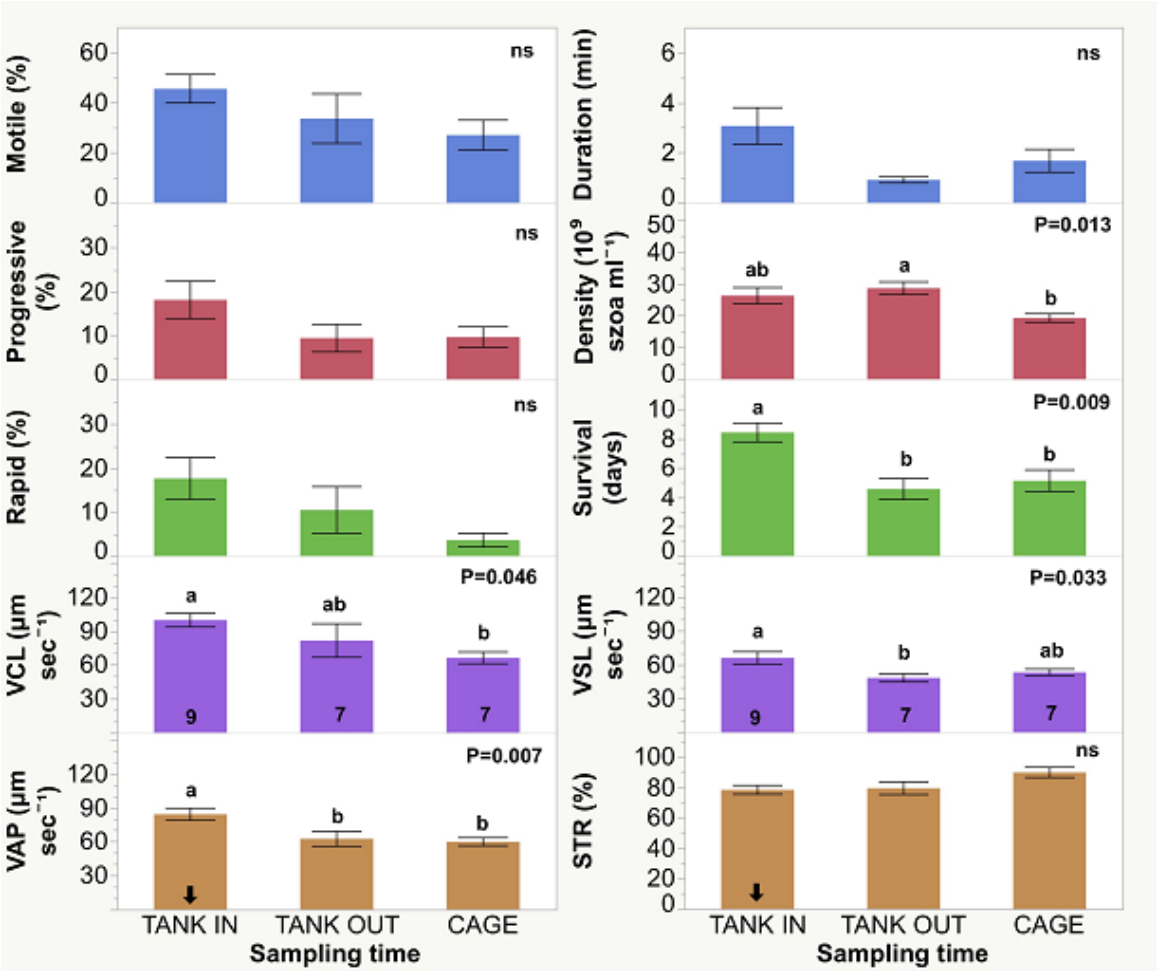

Figure 8.3 Mean (± SEM) motile cells (%), progressive cells (%), rapid cells (%), curvilinear velocity (VCL, μm sec^{-1}), average path velocity (VAP, μm sec^{-1}), spermatozoa motility duration (min), density (x10^9 szoa ml^{-1}), survival under cold storage at 4°C (days), straight line velocity (VSL, μm sec^{-1}) and straightness (STR, %) of milt collected at three different times, from male greater amberjack treated with GnRHa implants (arrows) when placed in tanks for spawning (TANK IN), moved back to sea cages after 14-21 days (TANK OUT) and after 14-28 days without any handling in the sea cages (CAGE). The numbers inside the bars indicate the number of the analyzed sperm samples in each sampling. Lowercase letters indicate statistically significant differences among the sampling times (one-way ANOVA, Tukey's HSD, P≤0.05). ns = not statistically significant.

8.3.2 Effect of a single GnRHa administration on sperm quantity and quality

Motility duration was not different among the sampling days after GnRHa treatment, while density (one-way ANOVA, P=0.002, Tukey's HSD, P≤0.05) and survival under cold storage (one-way ANOVA, P<0.001, Tukey's HSD, P≤0.05) decreased significantly at the final day of the experiment (**Fig. 8.4**). An improvement trend was observed in the quality of the milt collected on Day 7 after GnRHa administration, but overall mean motility values remained unchanged throughout the experiment (**Fig. 8.4**). The percentage of

progressive cells was significantly higher on Day 7 compared to Days 14 and 21 after GnRHa administration (one-way ANOVA, P=0.011, Tukey's HSD, P≤0.05), while the percentage of rapid cells was higher on Day 7 compared to Days 0 and 14 after GnRHa administration (one-way ANOVA, P=0.026, Tukey's HSD, P≤0.05) (**Fig. 8.4**). Similarly, at the end of the experiment there was a significant reduction in sperm velocity after GnRHa administration (**Fig. 8.4**). The VCL (one-way ANOVA, P=0.019, Tukey's HSD, P≤0.05), VSL (one-way ANOVA, P<0.001, Tukey's HSD, P≤0.05) and VAP (one-way ANOVA, P=0.002, Tukey's HSD, P≤0.05) were higher on Day 7 compared to Day 21 after GnRHa administration, while VSL was also higher on Day 0 compared to the final sampling day. No differences were observed in STR (**Fig. 8.4**).

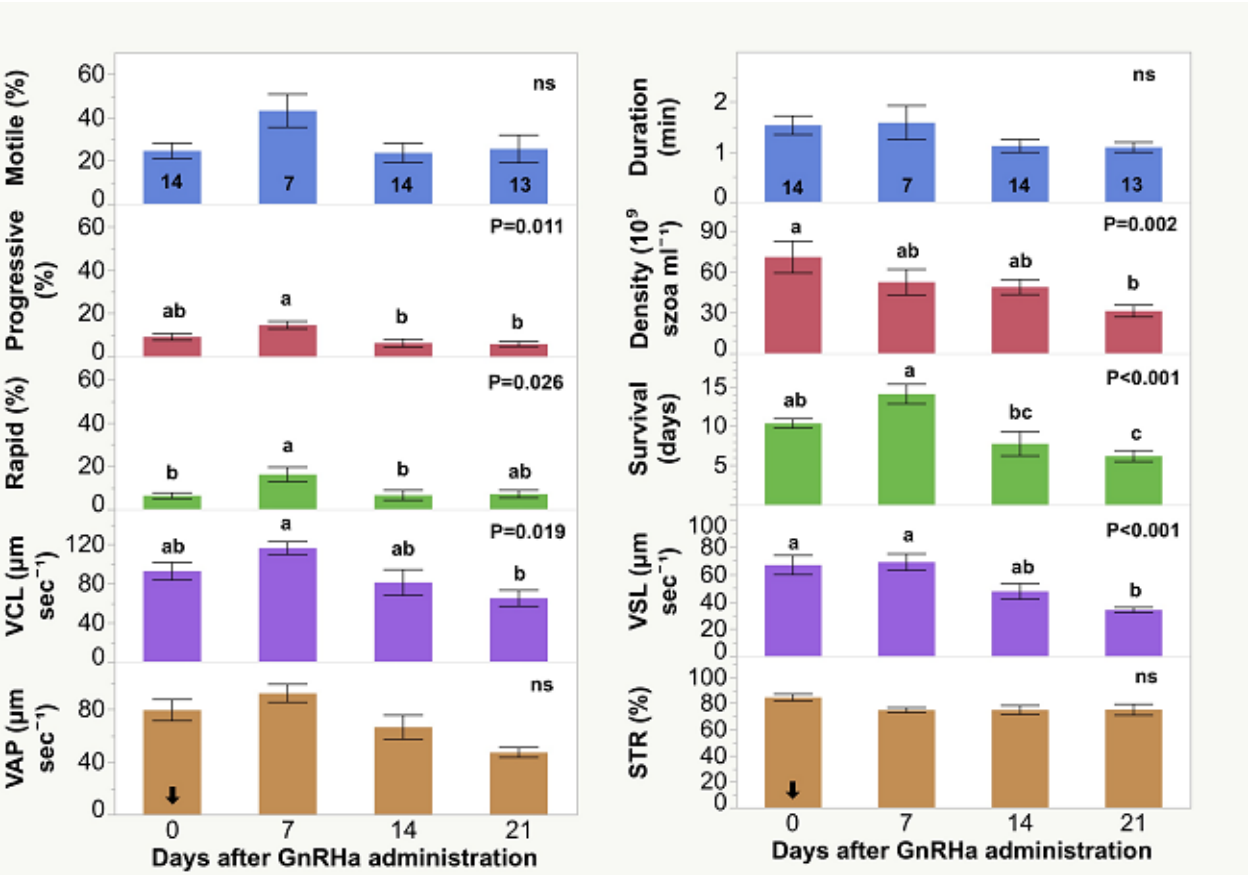

Figure 8.4 Mean (± SEM) motile cells (%), progressive cells (%), rapid cells (%), curvilinear velocity (VCL, μm sec^{-1}), average path velocity (VAP, μm sec^{-1}), spermatozoa motility duration (min), density ($x10^9$ szoa ml^{-1}), survival under cold storage at 4°C (days), straight line velocity (VSL, μm sec^{-1}) and straightness (STR, %) of milt collected from male greater amberjack treated with GnRHa implants on Day 0 (arrow). The numbers inside the bars indicate the number of the analyzed sperm samples in each sampling. Lowercase letters indicate statistically significant differences among the sampling times (one-way ANOVA, Tukey's HSD, P≤0.05). ns = not statistically significant.

8.3.3 Effect of a double GnRHa administration on sperm quantity and quality

The motility duration was not different among the sampling days in response to two GnRHa administrations. Density (one-way ANOVA, P=0.040, Tukey's HSD, P≤0.05) and survival under cold storage (one-way ANOVA, P=0.008, Tukey's HSD, P≤0.05) decreased significantly on Day 29 compared to Day 14 after the first GnRHa administration, however these parameters were not different on Day 29 compared to Day 0 (**Fig. 8.5**). The percentage of motile cells, progressive cells and rapid cells remained unchanged throughout the experiment and the same trend was observed in the VCL, VSL, VAP and STR (one-way ANOVA, P>0.05) (**Fig. 8.5**).

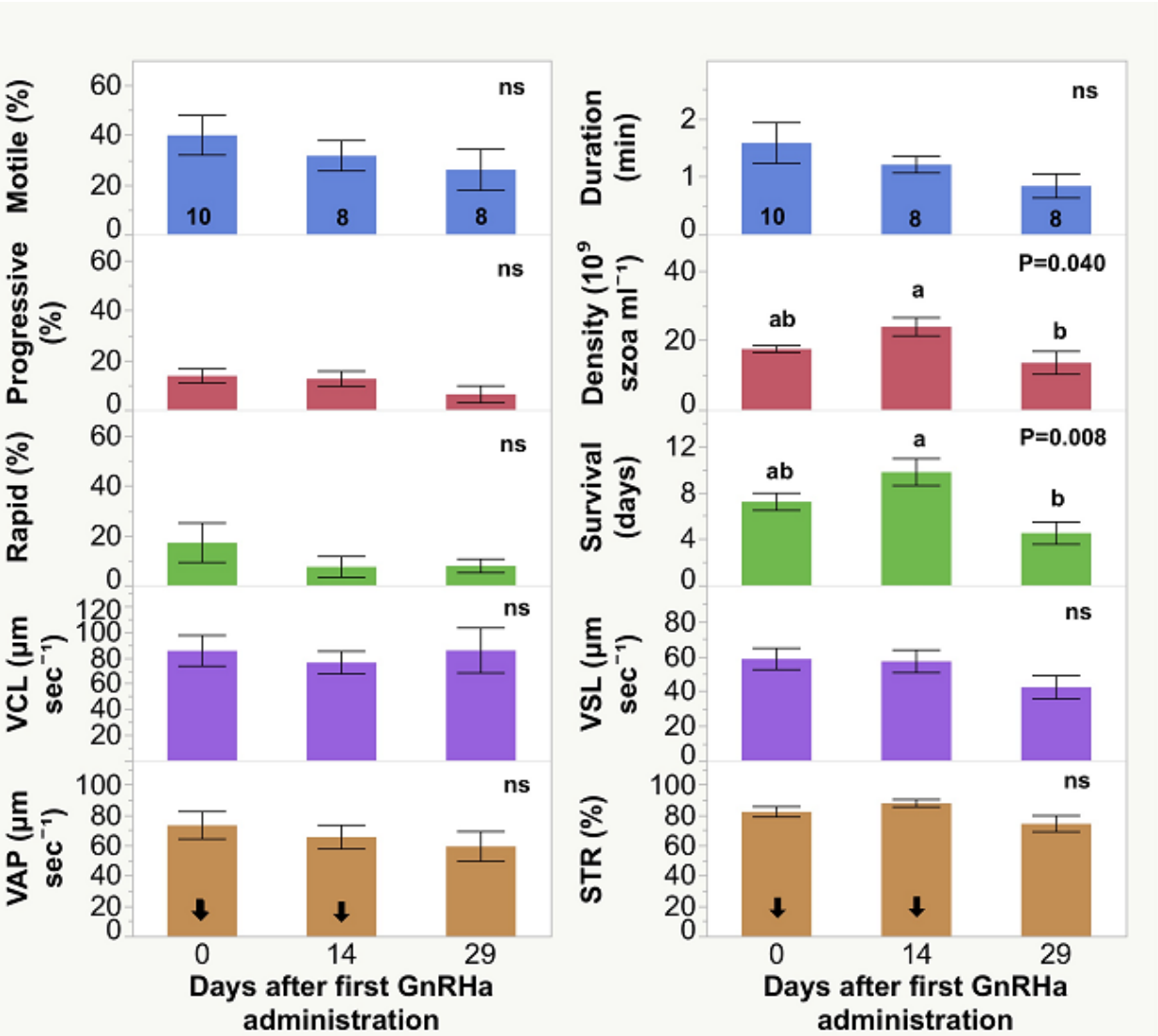

Figure 8.5 Mean (± SEM) motile cells (%), progressive cells (%), rapid cells (%), curvilinear velocity (VCL, μm sec^{-1}), average path velocity (VAP, μm sec^{-1}), spermatozoa motility duration (min), density (x10^9 szoa ml^{-1}), survival under cold storage at 4°C (days), straight line velocity (VSL, μm sec^{-1}) and straightness (STR, %) of milt collected from male greater amberjack treated with GnRHa implants on Days 0 and 14 (arrows). The numbers inside the bars indicate the number of the analyzed sperm samples in each sampling. Lowercase

letters indicate statistically significant differences among the sampling times (one-way ANOVA, Tukey's HSD, $P \leq 0.05$). ns = not statistically significant.

8.4 Discussion

In the present study, we report on (a) the extent of the spermiation period and the sperm quality of captive-reared greater amberjack reared in sea cages throughout the year and transferred to tanks for spawning, and (b) the use of GnRHa therapy to enhance sperm quality, given that the available data suggest that captivity *per se* affects significantly the spermatogenesis/spermiation process in greater amberjack (Zupa, et al., 2017a; Zupa, et al., 2017b). In terms of the characteristics of greater amberjack sperm, sperm motility duration ranged between 0.5 to 5.2 min among the experiments, and was generally lower than reported values of 1.5 to 6 min (Chapter 4) or 1 to 8 min (Zupa, et al., 2017a). Spermatozoa density ranged between 18 to 70 x 10^9 szoa ml^{-1} in the present study, similar to other studies both with wild (Zupa, et al., 2017a) and F1 broodstock (Chapter 7). Regarding sperm survival under cold storage, it ranged from 5 to 14 days, as recorded both in the Mediterranean (Chapter 4) and in the Canary Islands (Chapter 7). Concerning sperm motility parameters of greater amberjack, the only available data in the literature is for VAP and percentage of motile cells in a study conducted with wild-caught broodstock in the Mediterranean (Zupa, et al., 2017a), where similar values with the present study were recorded. Sperm quality parameters evaluations are lacking also for other species of genus *Seriola*. The mean values of VCL (80-120 μm sec^{-1}) and VSL (40-90 μm sec^{-1}) in greater amberjack here were lower compared to other marine fishes, such as the European seabass (VCL~140 μm $sec^{-1}$and VSL~100 μm sec^{-1}) (Abascal, et al., 2007) and gilthead seabream (VCL~160 μm $sec^{-1}$and VSL~110 μm sec^{-1}) (Forner-Piquer, et al., 2019a; Forner-Piquer, et al., 2019b), but similar to the Atlantic cod (VCL 70-95 μm sec^{-1}) (Tuset, et al., 2008).

Sperm production remained unchanged 7 to 14 days after the GnRHa administration, in terms of spermiation index. The males were not releasing sperm after abdominal pressure in the present study, as reported elsewhere for captive males (Mylonas, et al., 2004b; Zupa, et al., 2017a), but contrary to what was reported by Kozul et al. (Kozul, et al., 2001), showing that even rearing in sea cages throughout the year and avoiding handling during the reproductive season (Zupa, et al., 2017a; Zupa, et al., 2017b) was not enough to prevent dysfunctions in spermatogenesis, resulting in lower sperm production. The same absence of sperm release upon application of abdominal pressure was observed also in captive-reared Atlantic bluefin tuna (Suquet, et al., 2010), where milt for the evaluation of sperm quality parameters was collected after dissecting the testes. The number of spermiating males in this species was found to have increased 2-3 days after GnRHa administration, but decreased 8 days afterwards (Mylonas, et al., 2007), while increased plasma LH levels were recorded 2 days after the implantation and returned to baseline 8 days after the GnRHa implantation (Rosenfeld, et al., 2012). It is known that the increased LH plasma levels caused by GnRHa administration

lead to increased testicular steroids, which in turn regulate spermiation in fish (including milt hydration, sperm migration to the sperm duct or increase of milt volume), even if the exact mechanism of action is still unclear (Schulz, et al., 2010). Perhaps if the fish have been checked for sperm production 2-3 days after the GnRHa administration it might have observed higher sperm production, however this would have influenced spawning, which was the main target of these experiments. Also, it is conceivable that a significant increase in sperm production was not observed because the males were actively spawning in the days prior to the samplings, and based on the fertilization success of the obtained eggs (Chapters 5-6) they had expectedly released significant amounts of milt in the process. In any case, a spermiation enhancement experiment that will not include females and spawning in the tanks should be conducted in the future to study the kinetics of sperm production in more detail in the greater amberjack.

Sperm activation medium beyond the optimal water temperatures usually increases cell metabolism and leads to an increase in velocity with rapid depletion of energy resources, promoting early cessation of movement (Alavi & Cosson, 2005; Dadras, et al., 2017). In the experiment of the extent of the spermiation period, apart from the sperm density and survival under cold storage, no significant differences in the other evaluated sperm quality parameters were observed between the 1st and the 4th period, even though the water temperature increased significantly during this time. There was a significant decrease of sperm motility duration from the 1st to the 3rd period, however in the 4th period the values were not different from the 1st period. Since no differences in STR, motility (percentage of motile, progressive and rapid cells) and velocity (VCL, VSL, VAP) parameters were recorded throughout the experiment and fertilized eggs were collected in all 4 periods (Chapter 6), males could be used successfully at temperatures between 19-24ºC. However, increased number of spawns was recorded in the 1st and 2nd period while in the 3rd and 4th period a decreasing trend of fertilization success was observed (Chapter 6), which matches with the observed decreased sperm quality, at least in some parameters. Reduced sperm quality was observed also in mid-July after the stay of the fish for 14-28 days in the sea cage, which could be related either with the high temperatures of this period or the repetitive handling of the fish. Deterioration of sperm quality was observed also in early July in a similar study in the Mediterranean, when temperature was 25.5ºC (Zupa, et al., 2017a; Zupa, et al., 2017b), while stable sperm quality was reported throughout the spawning season from May to September in the Canary Islands, when temperatures were recorded between 20.4-24.5º. However, possible negative consequences of increased temperatures on sperm quality -which can decrease the extent of the reproductive season- should not be overlooked, although females were not observed to face similar problems (Chapter 6). Control of temperature in land-based tanks supplied with seawater of the proper quality (Chapter 4), which is a prerequisite for the domestication of fish in aquaculture conditions in general, may minimize the negative effect of high temperatures observed in the Mediterranean from July onwards.

Regarding the effect of the GnRHa administration on the sperm quality of greater amberjack, in the present study there was no clear enhancement in the examined sperm quality parameters, though a general

trend towards improvement was noted one week after the GnRHa implantation. After that, a general diminishing trend was observed, showing that one administration of GnRHa implant cannot sustain most of the evaluated parameters of sperm quality for more than two weeks. In a similar experiment with meagre, a single GnRHa implantation resulted in improvement of sperm production and stable sperm quality (sperm motility, motility duration, density and survival under cold storage) for 19 days (Fakriadis, et al., 2020), albeit at lower water temperatures. In the present study, during the determination of the spermiation period experiment it was also observed that the general declining trend observed 14-21 days after GnRHa administration could not be reversed even by the return of fish to sea cages and their maintenance there for 14-28 days, contrary to what was observed for the females (Chapter 6). A double GnRHa administration spaced 14 days apart, on the other hand, maintained the same sperm quality for a longer period of 29 days. Spermiation enhancement was achieved for almost 2.5 months in a similar experiment with meagre, where GnRHa administration was given every three weeks (Fakriadis, et al., 2020). The difference in treatment time between administrations in the two experiments is related to their different temperature, *i.e.* 21.3 – 23.7ºC in the present study compared to 19.5±0.1ºC in meagre. At high temperatures GnRHa is released from the implants and is metabolised faster, as it was shown with the shi drum maintained at 24ºC, where GnRHa was significantly higher for 7 days after GnRHa implantation compared to the control and then reached almost the same levels (Mylonas, et al., 2007), while at 18ºC GnRHa was maintained in the plasma for more than 14 days compared to controls in striped bass (Mylonas, et al., 1997b).

In conclusion, the sperm of greater amberjack in captivity seems to be of better quality at the beginning of the breeding season, with an observed deterioration later on in some of the studied parameters, such as sperm motility duration, density and survival under cold storage. Sperm quality was not improved when spawning fish placed in tanks were returned to their sea cages for 14-28 days, contrary to what was observed for the females, which recovered to their initial reproductive stage in terms of maximum vitellogenic oocyte diameter. Milt production can be maintained for 29 days without sperm quality deterioration, by using a double administration of GnRHa implants spaced 14 days apart.

Chapter 9 – Conclusions

The greater amberjack is one of the most promising species for aquaculture, showing advantages compared to other cultured or candidate wild species. For example, it has a higher growth rate compared to Atlantic salmon, it can be found throughout the temperate zone, thus it has acceptance from consumers worldwide. Its large body size makes it suitable for development of a variety of value-added seafood products. However, until now the production of greater amberjack has been faced with major bottlenecks, such as inconsistent and unreliable reproduction and production of juveniles for grow out.

In the present study, it was examined solving some of the reproduction-related issues and develop proper broodstock management protocols, based first on the study of gametogenesis in captive and wild breeders, in order to identify any reproductive dysfunctions and the extent to which they can be overcome with management approaches. More specifically, the present study has described the reproductive status of greater amberjack in the wild and in captivity, looking at gonadal development in association with pituitary and gonadal endocrine function. Captive females reared in sea cages were shown to be susceptible to repeated handling during vitellogenesis, since extensive follicular atresia was observed and most of the fish ceased their reproductive activity without any spawning. This reproductive failure was associated with almost no significant differences in the endocrine measured parameters during the developing phase compared to their wild counterparts. On the contrary, captive females reared in sea cages without any handling during the gametogenesis stage, underwent vitellogenesis completely, and almost all individuals were eligible for spawning induction and some also underwent spontaneous OM, ovulation and spawning. Males, on the other hand, did complete spermatogenesis and produced viable sperm, but with markedly reduced volume compared to wild fish, regardless of handling during the gametogenesis period. Also, females reared in tanks supplied with well seawater throughout the year exhibited a significant reproductive dysfunction with limited gametogenesis, lower oocyte diameter at the peak of the reproductive season, low female eligibility for spawning induction and almost 0% fertilization success when the few eligible fish were induced to spawn using GnRHa implants. In contrast, males had the same sperm quality when reared in tanks or in sea cages, but with reduced quantity in both rearing methods. So, rearing in captivity results in some reproductive dysfunctions in greater amberjack, which need to be overcome. A modern technology for inducing gametogenesis using recombinant hormones could be used in existing land based facilities. Recent studies in species such as grey mullet, European eel and Senegalese sole have shown that gametogenesis can be induced in both females and males. Administration of rGtHs may work better compared to other gametogenesis induction technologies, although the results vary considerably. The greater amberjack, where vitellogenesis is not properly completed in tanks could be considered a candidate species for the application of such methods.

Building on the above knowledge, it was demonstrated that when females were allowed to complete vitellogenesis without any handling in sea cages, they were not affected by handling during the spawning

period. No follicular atresia was observed, and females maintained their oocyte developmental stage of late vitellogenesis and were eligible for spawning induction. It is noteworthy, that captive females maintained in sea cages and transferred to land-based tanks for spawning after GnRHa implantation, seemed to be spent 2 weeks afterward, but they recovered to their initial oocyte developmental stage (in terms of the mean diameter of their largest late Vg oocytes) after been maintained for 14-28 days in sea cages. So, the sea cage environment seemed to fit better to captive females' reproductive function and further research is needed for providing the appropriate conditions in the land-based tanks to complete vitellogenesis successfully. This can be achieved by providing firstly surface seawater compared to the well water and secondly larger volume tanks, or a combination of both, since both may affect normal gametogenesis in greater amberjack.

Since spontaneous OM, ovulation and spawning was not consistent and the production of large numbers of fertilized eggs necessary for larval rearing could be not guaranteed, spawning induction methods were developed. Induction of spawning in captive-reared greater amberjack was more effective using GnRHa implants than injections. More eggs were produced using GnRHa implants compared to injections, without altering the quality of eggs in terms of fertilization, 24-h embryo survival, hatching and 5-d larval survival. In addition to apparently promoting the proper endocrine pathways leading to multiple cycles of OM, ovulation and spawning, the use of GnRHa implants induced also vitellogenesis. This method may be more effective in greater amberjack than multiple injections, also because of the less handling it involves (*i.e.* one handling every two weeks as opposed to one handling every week). It was also shown that 50 μg GnRHa kg^{-1} BW is the most appropriate dose for spawning induction of greater amberjack using implants. In addition, it was shown that it is possible to maintain greater amberjack in cages throughout the year, and then take portions of the broodstock at different times in a period of at least 2 months in the Mediterranean from the end of May to the end of July, when seawater temperature is within the range of 19-24°C, and induce them to spawn in onshore tanks. At similar temperatures in the Canary Islands, it was also shown that spawning can last from May to August in land-based tanks supplied with surface seawater, and even to September when conditions are appropriate. However, recent genetic studies suggested that greater amberjack may be separated into an Atlantic and Mediterranean population, and this may explain the differences in the extent of spawning period between the present study and the one in the Canary Islands. Considering all the above, a protocol for the Mediterranean that will include the maintenance of the breeders in sea cages, spawning induction with 50 μg GnRHa kg^{-1} BW during end of May- late July and transferring the breeders to land-based tanks can meet the needs of fish hatcheries in eggs. However, more research is needed to close the reproductive cycle in land-based tanks and conclude successfully gametogenesis.

In the same direction of the domestication of the species, are the experiments that were held in the present study with F1 fish. Breeding selection and use of hatchery-produced breeding stocks is a prerequisite for a sustainable aquaculture industry. Breeding selection aims to improve production traits, such as growth, feed conversion ratio and disease resistance, and is done through reproducing individuals or their offspring

which have the desirable traits. However, the establishment of breeding selection programs requires the closing of the life-cycle of a potential species in captivity and can be applied only when hatchery-reared fish can be successfully reproduced, and there is no further need for the acquisition of breeders from the wild. Nevertheless, reproductive dysfunctions occur in many cultured species, such as Senegalese sole, sharpsnout seabream and greenback flounder. The present study showed that hatchery-produced F1 greater amberjack undergo normal gametogenesis and can be induced to undergo OM, ovulation and spawning using GnRHa delivery systems. Egg production is high and egg quality adequate for the implementation of larval rearing for commercial purposes. The use of consecutive GnRHa-delivery systems over a long reproductive period resulted in multiple spawns of fertilized and viable eggs. Even if this was achieved and group spawning was successfully induced, both in wild-caught and F1 breeders, still there is a need for reproducing specific individuals with the desirable traits, as implemented using *in vitro* fertilization in other species of interest, such as gilthead seabream, European seabass and Atlantic salmon. However, due to the thick muscular nature of the abdominal wall in the greater amberjack the *in vitro* fertilization is almost impossible, in contrary to other cultured species such as those mentioned above. Individual pair spawning could be the solution to this problem and should be tested to enable family production with parents from a known origin and, thus specific traits would be possible to be selected.

As mentioned earlier, males also exhibit reproductive dysfunctions in captivity, which are not ameliorated by rearing in sea cages or avoiding handling during the gametogenesis period, since from the first sampling four out of the ten measured parameters (plasma FSH, LH, 17,20β-P and GSI) were reduced compared to their wild counterparts. The greater amberjack is considered to be a “poorly spermiating” fish species, a category in which fish complete spermatogenesis in captivity, but produce small volumes of milt. Even so, large numbers of fertilized eggs were collected, but fertilization success could possibly have been even better if the males produced larger amounts of sperm. Interestingly, no differences in sperm production and the main spermatozoa movement characteristics were observed between males reared in sea cages or tanks, but in general sperm production was lower compared to fish captured in the wild. The sperm of greater amberjack in captivity seems to be of better quality at the beginning of the breeding season, with an observed deterioration later on in some of the studied parameters, such as sperm motility duration, density and survival under cold storage. Sperm quality was not improved when spawning fish placed in tanks were returned to their sea cages for 14-28 days, contrary to what was observed for the females, which recovered to their initial reproductive stage in terms of maximum vitellogenic oocyte diameter. Milt production can be maintained for 29 days without sperm quality deterioration, by using a double administration of GnRHa implants spaced 14 days apart. It is conceivable that a significant increase in sperm production was not observed because the males were actively spawning in the days prior to the samplings, and based on the fertilization success of the obtained eggs they had expectedly released significant amounts of milt in the process. Although the study provided useful information on spermiation and sperm quality of greater amberjack in captivity, with relevance to the

aquaculture industry and fisheries biology, a specific spermiation enhancement experiment should be conducted in the future to effectively study sperm production kinetics and quality in the greater amberjack, without the influence of spawning. This may involve the use of other hormones, apart from GnRHa, that have been successfully used to enhance spermiation, such as hCG and recombinant GtHs, to study their effect in milt production and quality, without the males spending quantities of milt to fertilize the eggs.

In addition to those examined in the present study, further studies are needed in other aspects of reproductive biology for the successful reproduction of a species and the production of eggs of high quality that will be supplied to the larval rearing. For example, the information regarding greater amberjack nutritional needs is limited and the greater amberjack offspring nutrition is based mainly on the results obtained so far from experiments on other species of interest, such as gilthead seabream and European seabass. From the broodstock aspect, it is well known that broodstock nutrition affect the composition of the gonads and reproduction physiology, and it is the first step to a successful aquaculture production. Therefore, the production of a specialized broodstock diet based on the specific nutritional requirements of the greater amberjack and its effectiveness compared to the common broodstock diet used so far is of primary importance. An additional study topic is the breeding behavior of this species. It is well known that in nature the greater amberjack reproduces in groups that are formed during the spawning season. There is limited available data on the optimal sex ratio in captivity and, not at all, on the number of individuals involved in each spawn. Therefore, studies that will include parentage assignment of each spawn, following various sex ratio and number of breeders would provide new insights and data on how to form a broodstock to expect the best possible outcome.

References

Abascal, F.J., Cosson, J., Fauvel, C., 2007. Characterization of sperm motility in sea bass: the effect of heavy metals and physicochemical variables on sperm motility. J. Fish Biol. 70, 509-522.

Agius, R.V., Watanabe, T., Satoh, S., Kiron, V., Imaizumi, H., Yamazaki, T., Kawano, K., 2001. Supplementation of paprika as a carotenoid source in soft-dry pellets for broodstock yellowtail *Seriola quinqueradiata* (Temminck & Schlegel). Aquacult. Res. 32, 263-272.

Agulleiro, M.J., Scott, A.P., Duncan, N.J., Mylonas, C.C., Cerdá, J., 2007. Treatment of GnRHa-implanted Senegalese sole (*Solea senegalensis*) with 11-ketoandrostenedione stimulates spermatogenesis and increases sperm motility. Comp. Biochem. Physiol. A147, 885-892.

Agulleiro, M.J., Anguis, V., Cañavate, J.P., Martínez-Rodríguez, G., Mylonas, C.C., Cerdà, J., 2006. Induction of spawning of captive-reared Senegal sole (*Solea senegalensis*) using different administration methods for gonadotropin-releasing hormone agonist. Aquaculture. 257, 511-524.

Aida, K., 1983. Effect of LH-releasing hormone on gonadal development in a salmonid fish, the ayu. Bull. Jap. Soc. Sci. Fish. 49: 711-718.

Aizen, J., Kasuto, H., Levavi-Sivan, B., 2007. Development of specific enzyme-linked immunosorbent assay for determining LH and FSH levels in tilapia, using recombinant gonadotropins. Gen Comp Endocrinol. 153, 323-332.

Alavi, H.S.M., Cosson, J., 2005. Sperm motility in fishes. I. Effects of temperature and pH: a review. Cell Biol. Int. 29, 101-110.

Alavi, S.M.H., Linhart, O., Cowand, K., Rodina, M., 2007. Fish spermatology: implications for aquaculture management. in: Alavi, S.M.H., Cosson, J., Coward, K., Rafiee, G. (Eds.), Fish Spermatology. Alpha Science Intl, Oxford (UK), pp. 397-461.

Anonymous, 1998. Guidelines for the treatment of animals in behavioural research and teaching. Anim. Behav. 55, 251-257.

Asche, F., Bjorndal, T., 2011. The Economics of Salmon Aquaculture, 2nd ed. Wiley-Blackwell, Oxford, UK, Oxford, UK.

Asche, F., Roll, K.H., Trollvik, T., 2009. New aquaculture species - the whitefish market. Aquaculture Economics and Management. 13, *76-93*.

Avery, T.S., Killen, S.S., Hollinger, T.R., 2009. The relationship of embryonic development, mortality, hatching success, and larval quality to normal or abnormal early embryonic cleavage in Atlantic cod, *Gadus morhua*. Aquaculture. 289, 265-273.

Béné, C., Barange, M., Subasinghe, S., Pinstrup-Andersen, P., Merino, G., Hemre, G.I., Williams, M., 2015. Feeding 9 billion by 2050 – Putting fish back on the menu. Food Security. 7, 261-274.

Bennett, H.S., Wyrick, A.D., Lee, S.W., McNeil, J.H., 1976. Science and art in preparing tissues embedded in plastic for light microscopy, with special reference to glycol methacrylate, glass knives and simple stains. Stain Technol. 51, 71-97.

Berkovich, N., Corriero, A., Santamaria, C.A., Mylonas, C.C., Vassallo-Agius, R., De la Gándara, F., Meiri-Ashkenazi, I., Gordin, H., Bridges, C.R., Rosenfeld, H., 2013. The intra-pituitary relationship of follicle stiulating hormone and luteinizing hormone during pubertal development in Atlantic bluefin tuna (*Thunnus thynnus*). Gen. Comp. Endocrinol. 194: 10-23.

Berlinsky, D.L., William, K., Hodson, R.G., Sullivan, C.V., 1997. Hormone induced spawning of summer flounder *Paralichthys dentatus*. J. World Aquacult. Soc. 28, 79-86.

Berlinsky, D.L., King, W.V., Smith, T.I.J., Hamilton, R.D., II, Holloway, J., Jr., Sullivan, C.V., 1996. Induced ovulation of Southern flounder *Paralichthys lethostigma* using gonadotropin releasing hormone analogue implants. J. World Aquacult. Soc. 27, 143-152.

Blaxter, J.H.S., 1988. Pattern and variety in development. in: Hoar, W.S., Randall, D.J. (Eds.), Fish Physiology. Academic Press Inc., New York.

Bobe, J., Labbé, C., 2010. Egg and sperm quality in fish. Gen. Comp. Endocrinol. 165, 535-548.

Bonnet, E., Fostier, A., Bobe, J., 2007. Microarray-based analysis of fish egg quality after natural or controlled ovulation. BMC Genomics. 8, 55.

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.

Bromage, N., Bruce, M., Basavaraja, N., Rana, K., Shields, R., Young, C., Dye, J., Smith, P., Gillespie, M., Gamble, J., 1994. Egg quality determinants in finfish: the role of overripening with special reference to the timing of stripping in the Atlantic halibut *Hippoglossus hippoglossus*. J. World Aquacult. Soc. 25, 13-21.

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

Brown-Peterson, N.J., Wyanski, D.M., Saborido-Rey, F., Macewicz, B.J., Lowerre-Barbieri, S.K., 2011. A Standardized Terminology for Describing Reproductive Development in Fishes. Marine and Coastal Fisheries. 3, 52-70.

Cabrita, E., Robles, V., Herraez, P., 2009. Sperm quality assessment. in: Cabrita, E., Robles, V., Herraez, P. (Eds.), Methods in Reproductive Aquaculture: Marine and Freshwater Species. CRC Press Taylor and Francis Group, Boca Raton, pp. 93-148.

Carazo, I., Norambuena, F., Oliveira, C., Sanchez-Vazquez, F.J., Duncan, N.J., 2013. The effect of night illumination, red and infrared light, on locomotor activity, behaviour and melatonin of Senegalese sole (*Solea senegalensis*) broodstock. Physiol Behav. 118, 201-207.

Cerdá, J., Carrillo, M., Zanuy, S., Ramos, J., de la Higuera, M., 1994. Influence of nutritional composition of diet on sea bass, *Dicentrarchus labrax* L., reproductive performance and egg and larval quality. Aquaculture. 128, 345-361.

Chabbi, A., Ganesh, C.B., 2012. Stress-induced inhibition of recruitment of ovarian follicles for vitellogenic growth and interruption of spawning cycle in the fish *Oreochromis mossambicus*. Fish Physiol Biochem. 38, 1521-1532.

Chatakondi, N.G., 2017. Retracted: Effective dose of salmon GnRHa for Induction of ovulation in channel catfish. N. Am. J. Aquacult. 79, 310-316.

Chatzifotis, S., Gutiérrez, A.G., M., P., Caruzo, F., Sigelaki, I., Mylonas, C.C., 2020. Lack of negative effects of feed deprivation during the spawning period on egg quality and early larval development of gilthead seabream (*Sparus aurata*). in preparation.

Chauvigne, F., Fatsini, E., Duncan, N., Olle, J., Zanuy, S., Gomez, A., Cerda, J., 2016. Plasma levels of follicle-stimulating and luteinizing hormones during the reproductive cycle of wild and cultured Senegalese sole (*Solea senegalensis*). Comp. Biochem. Physiol. A Mol. Integr. Physiol. 191, 35-43.

Chavanne, H., Janssen, K., Hofherr, J., Contini, F., Haffray, P., Komen, H., Nielsen, E.E., Bargelloni, L., 2016. A comprehensive survey on selective breeding programs and seed market in the European aquaculture fish industry. Aquacult. Int. 24, 1287-1307.

Chuda, H., Imayoshi, T., Arakawa, T., Matsuyama, M., 2001. Hormonal treatment for induction of oocyte maturation and ovulation in cultured yellowtail, *Seriola quinqueradiata*. Scientific Bulletin of the Faculty of Agriculture, Kyushu University. 55, 169-177.

Chuda, H., Nakao, T., Arakawa, T., Matsuyama, M., 2002. Effects of hCG injection on oocyte maturation and ovulation in cultured two-year-old yellowtail, *Seriola quinqueradiata*. Suisanzoshoku. 50, 235-236.

Ciani, E., Fontaine, R., Maugars, G., Nourizadeh-Lillabadi, R., Andersson, E., Bogerd, J., von Krogh, K., Weltzien, F.A., 2020. Gnrh receptor gnrhr2bbalpha is expressed exclusively in lhb-expressing cells in Atlantic salmon male parr. Gen Comp Endocrinol. 285, 113293.

Ciereszko, A., Wojtczak, M., Dietrich, G.J., Kuźmiński, H., Dobosz, S., 2009. A lack of consistent relationship between distribution of lipid droplets and egg quality in hatchery-raised rainbow trout, *Oncorhynchus mykiss*. Aquaculture. 289, 150-153.

Clearwater, S.J., Pankhurst, N.W., 1997. The response to capture and confinement stress of plasma cortisol, plasma sex steroids and vitellogenic oocytes in the marine teleost, red gurnard. J. Fish Biol. 50, 429-441.

Clearwater, S.J., Crim, L.W., 1998. Gonadotropin releasing hormone-analogue treatment increases sperm motility, seminal plasma pH and sperm production in yellowtail flounder *Pleuronectes ferrugineus*. Fish Physiol. Biochem. 19, 349-357.

Corriero, A., Zupa, P., Bello, G., Mylonas, C.C., Santamaria, C.A., Deflorio, M., Genovese, S., Basilone, G., Buscaino, G., Buffa, G., Pousis, C., De Metrio, G., 2011. Evidence that severe acute stress and starvation induce rapid atresia of ovarian vitellogenic follicles in Atlantic bluefin tuna, *Thunnus thynnus* (L.) (Osteichthyes: Scombridae). J. Fish Dis. 34, 853-860.

Corriero, A., Medina, A., Mylonas, C.C., Bridges, C.R., Santamaria, N., Deflorio, M., Losurdo, M., Zupa, R., Gordin, H., de la Gándara, F., Belmonte Ríos, A., Pousis, C., De Metrio, G., 2009. Proliferation and apoptosis of male germ cells in captive Atlantic bluefin tuna (*Thunnus thynnus L.*) treated with gonadotroping releasing hormone agonist (GnRHa). Anim. Reprod. Sci. 116, 346-357.

Cosson, J., 2007. Methods to analyse the movements of fish spermatozoa and their flagella. in: Alavi, S.M.H., Cosson, J., Coward, K., Rafiee, G. (Eds.), Fish Spermatology. Alpha Science Intl, Oxford, UK, pp. 63-102.

Cosson, J., Groison, A.L., Suquet, M., Fauvel, C., Dreanno, C., Billard, R., 2008. Studying sperm motility in marine fish: an overview on the state of the art. J. Appl. Ichthyol. 24, 460-486.

Crespo, S., Grau, A., Padrós, F., 1994. The intensive culture of 0-group amberjack in the western Mediterranean is compromised by disease problems. Aquacult. Int. 2, 262-265.

Dadras, H., Dzyuba, B., Cosson, J., Golpour, A., Siddique, M.A.M., Linhart, O., 2017. Effect of water temperature on the physiology of fish spermatozoon function: a brief review. Aquacult. Res. 48, 729-740.

Das, P., Behera, B.K., Meena, D.K., Singh, S.K., Mandal, S.C., Das, S.S., Yadav, A.K., Bhattacharjya, B.K., 2016. Comparative efficacy of different inducing agents on breeding performance of a near threatened cyprinid *Osteobrama belangeri* in captivity. Aquaculture Reports. 4, 178-182.

De Mello, P.H., Lancerotto, S., Fakriadis, I., Tsoukali, P., Papadaki, M., Mylonas, C.C., 2020. The importance of thermoperiod for proper gametogenesis and successful egg and sperm production in meagre (*Argyrosomus regius*). Mediterr. Mar. Sci., (under review).

De Metrio, G., Bridges, C.R., Mylonas, C.C., Caggiano, M., Deflorio, M., Santamaria, N., Zupa, P., Pousis, C., Vassallo-Agius, R., Gordin, H., Corriero, A., 2010. Spawning induction and large-scale collection of fertilized eggs in captive Atlantic bluefin tuna (*Thunnus thynnus* L.) and the first larval rearing efforts. J. Appl. Ichthyol. 26, 596-599.

Díaz, M.V., García, A., Agulleiro, B., 1997. Características histológicas del ovario de la seriola Mediterránea (*Seriola dumerilii*, Risso) mantenida en cautividad, durante su ciclo reproductivo anual. in: Ruiz, J.C., Abellan Martinez, E., García García, B., Ortega Ros, A., Zamora Navarro, S. (Eds.), VI Congresso National de Acuicultura. Ministerio de Agricultura, Pesca y Alimentación, Madrid, Cartagena, Spain, pp. 389-394.

Dickey, J.T., Swanson, P., 1998. Effects of sex steroids on gonadotropin (FSH and LH) regulation in coho salmon (*Oncorhynchus kisutch*). J. Mol. Endocrinol. 21, 291-306.

Domínguez‐Petit, R., Anastasopoulou, A., Cubillo, s.L., Gerritsen, H.D., Gonçalves, P., Hidalgo, M., Kennedy, J., Korta, M., Marteinsdottir, G., Morgado, C., Muñoz, M., Quincoces, I., Saínza, M., Thorsen, A., F., V., 2017. Chapter 3: Maturity. . in: Domínguez‐Petit, R., Murua, H., Saborido‐Rey, F., Trippel, E. (Eds.), Handbook of applied fisheries reproductive biology for stock assessment and management. Digital CSIC, Vigo, Spain.

Duncan, N., Sonesson, A., Chavanne, H., 2013. Principles of finfish broodstock management in aquaculture: control of reproduction and genetic improvement. in: Allan, G., Burnell, G. (Eds.), Advances in Aquaculture Hatchery Technology. Woodhead Publishing Limited, Cambridge, UK, pp. 23-75.

Edwards, P., Kwe Ilin, C., Mac Intosh, J.D., Leong Wee, K., Litile, D., Inees-Taylor, N.L., 1988. Fish farming and aquaculture. Nature. 333, 545-546.

Erkkila, K., Henriksén, K., Hirvonen, V., Rannikko, S., Salo, J., Parvinen, M., Dunkel, L., 1997. Testosterone regulates apoptosis in adult human seminiferous tubules *in vitro*. J. Clin. Endocrinol. Metab. 82, 2314-2321.

EU, 2010. Directive 2010/63/EU of the European parliament and the council of 22 September 2010 on the protection of animals used for scientific purposes. Official Journal of the European Union. L 276, 33-79.

Fakriadis, I., Zanatta, E.M., Fleck, R., Sena Mateo, D.L., Papadaki, M., Mylonas, C.C., 2020. Endocrine regulation of long-term enhancement of spermiation in meagre (*Argyrosomus regius*) with GnRHa controlled-delivery systems. Gen Comp Endocrinol. 297, 113549.

FAO, 1988. Rural Aquaculture: Overview and Framework for Country Reviews, Bangkok, Thailand.

FAO, 2016. Cultured Aquatic Species Information Programme: *Seriola dumerili.* FAO, Rome.

FAO, 2018. The State of World Fisheries and Aquaculture 2018 - Meeting the sustainable development goals. Food and Agriculture Organization of the United Nations, Rome.

Farquharson, K.A., Hogg, C.J., Grueber, C.E., 2018. A meta-analysis of birth-origin effects on reproduction in diverse captive environments. Nat Commun. 9, 1055.

Fauvel, C., Suquet, M., 1999. La qualité des gamètes chez le bar et quelques déterminants en aquaculture. La Pisciculture Française. 134: 5-10.

Fauvel, C., Suquet, M., Cosson, J., 2010. Evaluation of fish sperm quality. J. Appl. Ichthyol. 26, 636-643.

Fauvel, C., Savoye, O., Dreanno, C., Cosson, J., Suquet, M., 1999. Characteristics of sperm of captive seabass in relation to its fertilization potential. J. Fish Biol. 54, 356-369.

Fernández-Palacios, H., Schuchardt, D., Roo, J., Hernández-Cruz, C.M., Izquierdo, M., 2015a. Multiple GnRHa injections to induce successful spawning of wild caught greater amberjack (*Seriola dumerili*) matured in captivity. Aquacult. Res. 46, 1748-1759.

Fernández-Palacios, H., Schuchardt, D., Roo, J., Hernández-Cruz, C., Izquierdo, M., 2015b. Spawn quality and GnRHa induction efficiency in longfin yellowtail (*Seriola rivoliana*) broodstock kept in captivity. Aquaculture. 435, 167-172.

Fernández-Palacios, H., Schuchardt, D., Roo, J., Izquierdo, M., Hernández-Cruz, C., Duncan, N., 2014. Dose dependent effect of a single GnRHa injection on the spawning of meagre (*Argyrosomus regius*) broodstock reared in captivity. Span J Agric Res. 12, 1038-1048.

Filiciotto, F., Giacalone, V.M., Fazio, F., Buffa, G., Piccione, G., Maccarrone, V., Di Stefano, V., Mazzola, S., Buscaino, G., 2013. Effect of acoustic environment on gilthead sea bream (*Sparus aurata*): Sea and onshore aquaculture background noise. Aquaculture. 414-415, 36-45.

Fish, F.F., 1936. Founders of fish culture European origins. N. Am. J. Aquacult. 3, 8-10.

Forner-Piquer, I., Mylonas, C.C., Fakriadis, I., Papadaki, M., Piscitelli, F., Di Marzo, V., Calduch-Giner, J., Pérez-Sánchez, J., Carnevali, O., 2019a. Effects of diisononyl phthalate (DiNP) on the endocannabinoid and reproductive systems of male gilthead sea bream (*Sparus aurata*) during the spawning season. Arch. Toxicol., 1-15.

Forner-Piquer, I., Fakriadis, I., Mylonas, C.C., Piscitelli, F., Di Marzo, V., Maradonna, F., Calduch-Giner, J., Pérez-Sánchez, J., Carnevali, O., 2019b. Effects of Dietary Bisphenol A on the reproductive function of gilthead sea bream (*Sparus aurata*) testes. Int. J. Mol. Sci. 20, 5003.

Forniés, M.A., Mañanos, E., Laureau, S., do Santos, A., Carrillo, M., Mylonas, C.C., Zohar, Y., Zanuy, S., 2000. Different delivery systems for the optimization of the induction of the spawn in sea bass (*Dicentrarchus labrax*). in: Flos, R., Creswell, L. (Eds.), Aqua 2000. European Aquaculture Society, Nice, France.

Forsgren, E., Dupont, S., Jutfelt, F., Amundsen, T., 2013. Elevated CO2 affects embryonic development and larval phototaxis in a temperate marine fish. Ecological Evolution. 3, 3637-3646.

Gage, M.J.G., Macfarlane, C.P., Yeates, S., Ward, R.G., Searle, J.B., Parker, G.A., 2004. Spermatozoal traits and sperm competition in Atlantic salmon: relative sperm velocity is the primary determinant of fertilization success. Curr. Biol. 14, 44-47.

Gallego, V., Asturiano, J.F., 2018. Sperm motility in fish: technical applications and perspectives through CASA-Mot systems. Reprod. Fertil. Dev. 30, 820-832.

Ganias, K., Lowerre-Barbieri, S., 2018. Oocyte recruitment and fecundity type in fishes: Refining terms to reflect underlying processes and drivers. Fish Fish. 19, 562-572.

Garber, A.F., Fordham, S.E., Symonds, J.E., Trippel, E.A., Berlinsky, D.L., 2009. Hormonal induction of ovulation and spermiation in Atlantic cod (*Gadus morhua*). Aquaculture. 296, 179-183.

García-Fernández, C., Sánchez, J.A., Blanco, G., 2018. Early assessment of gilthead sea bream (*Sparus aurata*) spawning dynamics by mini-broodstocks. Aquacult. Res. 49, 36-47.

García, A., Díaz, M.V., Agulleiro, B., 2001. Inducción hormonal de la puesta y desarrollo embrionario de la seriola Mediterranea (*Seriola dumerilii*, Risso). Monografías del Instituto Canario de Ciencias Marinas. 4, 561-566.

Gil, M.D.M., Grau, A., Basilone, G., Ferreri, R., Palmer, M., 2013. Reproductive strategies and fecundity of meagre *Argyrosomus regius* Asso, 1801 (Pisces: Sciaenidae): implications for restocking programs. Sci Mar. 77, 105-118.

Goetz, F.W., Garczynski, M., 1997. The ovarian regulation of ovulation in teleost fish. Fish Physiol. Biochem. 17, 33-38.

Gore, A., 2002. GnRH: The Master Molecule of Reproduction. Kluwer Academic. Kluwer Academic Publishers.

Goren, A., Gustafson, H., Doering, D., 1995. Field trials demonstrate the efficacy and commercial benefit of a GnRHa implant to control ovulation and spermiation in salmonids. in: Goetz, F.W., Thomas, P. (Eds.), Reproductive Physiology of Fish. Fish Symposium 95, Austin, Texas.

Gothilf, Y., Meiri, I., Elizur, A., Zohar, Y., 1997. Preovulatory changes in the levels of three gonadotropin-releasing hormone-encoding messenger ribonucleic acids (mRNAs), gonadotropin ß-subunit mRNAs, plasma gonadotropin, and steroids in the female gilthead seabream, *Sparus aurata*. Biol. Reprod. 57, 1145-1154.

Grau, A., Crespo, S., Riera, F., Pou, S., Sarasquete, M.C., 1996. Oogenesis in the amberjack *Seriola dumerili* Risso, 1810. A histological, histochemical and ultrastructural study of oocyte development. Sci Mar. 60, 391-406.

Greenwood, L.N., Scott, A.P., Vermeirssen, E.L.M., Mylonas, C.C., Pavlides, M., 2001. Plasma steroids in mature common dentex (*Dentex dentex*) stimulated with a gonadotropin-releasing hormone agonist. Gen. Comp. Endocrinol. 123, 1-12.

Guercio, G., Saraco, N., Costanzo, M., Marino, R., Ramirez, P., Berensztein, E., Rivarola, M.A., Belgorosky, A., 2020. Estrogens in human male gonadotropin secretion and testicular physiology from infancy to late puberty. Front. Endocrinol. (Lausanne). 11, 72.

Guy, S.V., Thomas, T., Irit, Z., Andreas, P., Dorit, S., Omri, L., Ayelet, G., Guy, B.O., 2018. Tooth oxygen isotopes reveal Late Bronze Age origin of Mediterranean fish aquaculture and trade. Sci. Rep. 8, 14086.

Guzmán, J.M., Ramos, J., Mylonas, C.C., Manaños, E., 2009. Spawning performance and plasma levels of GnRHa and sex steroids in cultured female Senegalese sole (*Solea senegalensis*) treated with different GnRHa-delivery systems. Aquaculture. 291, 200-209.

Habibi, H.R., Andreu-Vieyra, C.V., 2007. Hormonal regulation of follicular atresia in teleost fish. in: Babin, P., Cerda, J., Lubzens, E. (Eds.), The Fish Oocyte: From Basic Studies to Biotechnological Applications. Springer, The Netherlands, pp. 235-254.

Hamasaki, K., Tsuruoka, K., Teruya, K., Hashimoto, H., Hamada, K., Hotta, T., Mushiake, K., 2009. Feeding habits of hatchery-reared larvae of greater amberjack *Seriola dumerili*. Aquaculture.

Harmin, S.A., Crim, L.W., 1993. Influence of gonadotropin hormone-releasing hormone analog (GnRH-A) on plasma sex steroid profiles and milt production in male winter flounder, *Pseudopleuronectes americanus* (Walbaum). Fish Physiol. Biochem. 10, 399-407.

Harmin, S.A., Crim, L.W., Wiegand, M.D., 1995. Manipulation of the seasonal reproductive cycle in winter flounder, *Pleuronectes americanus*, using a gonadotropic hormone releasing hormone. Mar. Biol. 121: 611-619.

Harris, P.J., Wyanski, D.M., White, D.B., Mikell, P.P., Eyo, P.B., 2007. Age, growth, and reproduction of greater amberjack off the Southeastern U.S. Atlantic Coast. Trans. Am. Fish. Soc. 136: 1534-1545.

Higuchi, K., Gen, K., Izumida, D., Kazeto, Y., Hotta, T., Takashi, T., Aono, H., Soyano, K., 2016. Changes in plasma steroid levels and gene expression of pituitary gonadotropins, testicular steroidogenesis-related proteins and insulin-like growth factors during spermatogenesis of the yellowtail *Seriola quinqueradiata*. Fish. Sci. 83, 35-46.

Hollander-Cohen, L., Golan, M., Aizen, J., Shpilman, M., Levavi-Sivan, B., 2018. Characterization of carp gonadotropins: Structure, annual profile, and carp and zebrafish pituitary topographic organization. Gen Comp Endocrinol. 264, 28-38.

Howell, B.R., Conceicao, L.E.C., Prickett, R., Cañavate, P., Mananos, E., 2008. The Cultivation of Soles, Report of the 4rd Workshop held at CCMAR, University of the Algarve, Faro, Portugal, Faro, Portugal.

Ibarra-Castro, L., Duncan, N.J., 2007. GnRHa-induced spawning of wild-caught spotted rose snapper *Lutjanus guttatus*. Aquaculture. 272, 737-746.

Imanaga, Y., Nyuji, M., Amano, M., Takahashi, A., Kitano, H., Yamaguchi, A., Matsuyama, M., 2014. Characterization of gonadotropin-releasing hormone and gonadotropin in jack mackerel (*Trachurus japonicus*): Comparative gene expression analysis with respect to reproductive dysfunction in captive and wild fish. Aquaculture. 428-429, 226-235.

Ishimatsu, A., 2005. Physiological effects on fishes in a high-CO2 world. J. Geophys. Res. 110.

Izquierdo, M.S., Fernández-Palacios , H., Tacon, A.G.J., 2001. Effect of broodstock nutrition on reproductive performance of fish. Aquaculture. 197, 25-42.

Jalabert, B., 2005. Particularities of reproduction and oogenesis in teleost fish compared to mammals. Reprod. Nutr. Dev. 45, 261-279.

Janssen, K., Chavanne, H., Berentsen, P., Komen, H., 2016. Impact of selective breeding on European aquaculture. Aquaculture.

Jerez, S., Samper, M., Santamaría, F.J., Villamados, J.E., Cejas, J.R., Felipe, B.C., 2006. Natural spawning of greater amberjack (*Seriola dumerili*) kept in captivity in the Canary Islands. Aquaculture. 252, 199-207.

Jover, M., García-Gómez, A., Tomás, A., De la Gándara, F., Pérez, L., 1999. Growth of Mediterranean yellowtail (*Seriola dumerili*) fed extruded diets containing different levels of protein and lipid. Aquaculture. 179, 25-33.

Jung, K.M., Folkvord, A., Kjesbu, O.S., Sundby, S., 2014. Experimental parameterisation of principal physics in buoyancy variations of marine teleost eggs. PLoS One. 9, e104089.

Kagawa, H., 1989. Reproductive physiology and induced spawning in yellowtail (*Seriola quinqueradiata*). NOOA Technical Reports NMFS. 106, 15-18.

Kagawa, H., 2013. Oogenesis in Teleost Fish. Aqua-BioScience Monographs. 6, 99-127.

Karine, R., Patrick, P., Sylvie, D., 2020. Special features of neuroendocrine interactions between stress and reproduction in teleosts. Gen. Comp. Endocrinol.

Katharios, P., Papadaki, M., Ternengo, S., Kantham, P.K., Zeri, C., Petraki, P.E., Divanach, P., 2011. Chronic ulcerative dermatopathy in cultured marine fishes. Comparative study in sharpsnout sea bream, *Diplodus puntazzo* (Walbaum). J. Fish Dis. 34, 459-474.

Kawabe, K., Kimura, J., Ando, K., Kakiuchi, K., 1998. Natural spawning from 2-year-old reared amberjack, *Seriola dumerili* in Chichijima Ogasawara Islands, Southern Japan. Aquaculture Science. 46, 31-36.

Kawabe, K., Kato, K., Kimura, J., Okamura, Y., Ando, K., Saito, M., Yoshida, K., 1996. Rearing of broodstock fish and egg-taking from amberjack *Seriola dumerili* in Chichijima, Ogasawara Islands, Southern Japan. Aquaculture Science. 44, 151-157.

Kemp, D.A., 2004. Inclusion of places in the national heritage list, Gazette. Commonwealth of Australia.

Kopeika, J., Kopeika, E., Zhang, T., Rawson, D.M., Holt, W.V., 2003. Detrimental effects of cryopreservation of loach (*Misgurnus fossilis*) sperm on subsequent embryo development are reversed by incubating fertilised eggs in caffeine. Cryobiology. 46, 43-52.

Kowalski, R.K., Cejko, B.I., 2019. Sperm quality in fish: Determinants and affecting factors. Theriogenology. 135, 94-108.

Kozul, V., Skaramuca, B., Glamuzina, B., Glavic, N., Tutman, P., 2001. Comparative gonadogenesis and hormonal induction of spawning of cultured and wild mediterranean amberjack (*Seriola dumerili*, Risso 1810). Sci Mar. 65, 215-220.

Lazzari, A., 1991. Some notes to the aquaculture development of the new Mediterranean species: the yellowtail (*Seriola dumerilii*) case and strategy to come. in: De Pauw, N., Joyce, J. (Eds.), Aquaculture Europe 91. European Aquaculture Society, Special Publication No. 14, Dublin, Ireland, pp. 183-184.

Lazzari, A., Fusari, A., Boglione, A., Marino, G., Di Francesco, M., 2000. Recent advances in reproductional and rearing aspects of *Seriola dumerilii*. in: Basurco, B. (Ed.), Cahiers Options Méditerranéennes, vol. 47: Mediterranean Marine Aquaculture Finfish Species Diversification. C.I.H.E.A.M., Zaragoza, Spain, pp. 241-247.

Levavi-Sivan, B., Bogerd, J., Mañanós, E.L., Gómez, A., Lareyre, J.J., 2010. Perspectives on fish gonadotropins and their receptors. Gen. Comp. Endocrinol. 165, 412-437.

Lubzens, E., Young, G., Bobe, J., Cerdá, J., 2010. Oogenesis in teleosts: how fish eggs are formed. Gen. Comp. Endocrinol. 165, 36-389.

Lubzens, E., Bobe, J., Young, G., Sullivan, C.V., 2017. Maternal investment in fish oocytes and eggs: The molecular cargo and its contributions to fertility and early development. Aquaculture. 472, 107-143.

Luckenbach, J.A., Dickey, J.T., Swanson, P., 2011. Follicle-stimulating hormone regulation of ovarian transcripts for steroidogenesis-related proteins and cell survival, growth and differentiation factors in vitro during early secondary oocyte growth in coho salmon. Gen Comp Endocrinol. 171, 52-63.

Mañanos, E., Duncan, N., Mylonas, C.C., 2009. Reproduction and control of ovulation, spermiation and spawning in cultured fish. in: Cabrita, E., Robles, V., Herraez, P. (Eds.), Methods in Reproductive Aquaculture. CRC Press Taylor and Francis Group, Boca Raton, pp. 3-80.

Mañanos, E., Swanson, P., Stubblefield, J., Zohar, Y., 1997. Purification of gonadotropin II from a teleost fish, the hybrid striped bass, and development of a specific enzyme-linked immunosorbent assay. Gen. Comp. Endocrinol. 108, 209-222.

Mañanos, E., Carrillo, M., Sorbera, L.S., Mylonas, C.C., Asturiano, J.F., Bayarri, M.J., Zohar, Y., Zanuy, S., 2002. Luteinizing hormone and sexual steroid plasma levels after treatment of European sea bass with sustained-release delivery systems for gonadotropin-releasing hormone analogue. J. Fish Biol. 60, 328-339.

Mandich, A., Massari, A., Bottero, S., Pizzicori, P., Goos, H., Marino, G., 2004. Plasma sex steroid and vitellogenin profiles during gonad development in wild Mediterranean amberjack (*Seriola dumerilii*, Risso). Mar. Biol. 144, 127-138.

Marino, G., Mandich, A., Massari, A., Andaloro, F., Porrello, S., 1995. Aspects of reproductive biology of the Mediterranean amberjack (*Seriola dumerilii* Risso) during the spawning period. J. Appl. Ichthyol. 11, 9-24.

Marino, G., Panini, E., Longobardi, A., Mandich, A., Finoia, M.G., Zohar, Y., Mylonas, C.C., 2003. fakriInduction of ovulation in captive-reared dusky grouper, *Epinephelus marginatus* (Lowe, 1834) with a sustained-release GnRHa implant. Aquaculture. 219, 841-858.

Masuma, S., 2006. Maturation and spawning of bluefin tuna in captivity. in: Sakamoto, W., Miyashita, S., Nakagawa, Y. (Eds.), Ecology and Aquaculture of Bluefin Tuna. Proceedings of the Joint International Symposium on Bluefin Tuna, 2006. Fisheries Laboratory, Kinki University, Shirahama, Wakayam, Japan, pp. 15-20.

Mazón, M.J., Molés, G., Rocha, A., Crespo, B., Lan-Chow-Wing, O., Espigares, F., Muñoz, I., Felip, A., Carrillo, M., Zanuy, S., Gómez, A., 2015. Gonadotropins in European sea bass: Endocrine roles and biotechnological applications. Gen. Comp. Endocrinol. 221, 31-41.

Mazorra de Quero, C., Shields, R.J., Scott, A.P., Mylonas, C.C., Zohar, Y., Bromage, N., 2000. Effects of late season GnRHa implants on male Atlantic halibut *Hippoglossus hippoglossus* spermiation. in: Flos, R., Cresswell, L. (Eds.), Aqua 2000. European Aquaculture Society, Nice, France, pp. 455.

Mazzola, A., Favaloro, E., Sará, G., 2000. Cultivation of the Mediterranean amberjack, *Seriola dumerili* (Risso, 1810), in submerged cages in the Western Mediterranean Sea. Aquaculture. 181, 257-268.

McCann, A.M., 1979. The Harbor and Fishery Remains at Cosa, Italy. J Field Archaeol. 6, 391-411.

Medina, A., Abascal, F.J., Aragón, L., Mourente, G., Aranda, G., Galaz, T., Belmonte, A., Miguel de la Serna, J., García, S., 2007. Influence of sampling gear in assessment of reproductive parameteres for bluefin tuna in the western Mediterranean. Mar. Ecol. Prog. Ser. 337, 221-230.

Meiri, I., Knibb, W.R., Zohar, Y., Elizur, A., 2004. Temporal profile of b follicle-stimulating hormone, b luteinizing hormone, and growth hormone gene expression in the protandrous hermaphrodite, gilthead seabream, *Sparus aurata*. Gen. Comp. Endocrinol. 137, 288-299.

Melzner, F., Gutowska, M.A., Langenbuch, M., Dupont, S., Lucassen, M., Thorndyke, M.C., Bleich, M., Pörtner, H.-O., 2009. Physiological basis for high CO2 tolerance in marine ectothermic animals: pre-adaptation through lifestyle and ontogeny? Biogeosciences. 6, 2313-2331.

Metcalfe, J.D., Craig, J.F., 2011. Ethical justification for the use and treatment of fishes in research: an update. J. Fish Biol. 78, 393-394.

Micale, V., Maricchiolo, G., Genovese, L., 1999. The reproductive biology of the amberjack, *Seriola dumerilii* (Risso 1810). I. Oocyte development in captivity. Aquacult. Res. 30, 349-355.

Migaud, H., Bell, G., Cabrita, E., McAndrew, B., Davie, A., Bobe, J., Herráez, M.P., Carrillo, M., 2013. Gamete quality and broodstock management in temperate fish. Reviews in Aquaculture. 5, S194-S223.

Mihelakakis, A., Yoshimatsu, T., Kitajima, C., 1995. Change in egg size of Japanese flounder during one spawning season. Journal of the Faculty of Agriculture, Kyushu University. 40, 53-59.

Mihelakakis, A., Yoshimatsu, T., Tsolkas, C., 2001. Spawning in captivity and early life history of cultured red porgy, *Pagrus pagrus*. Aquaculture. 199, 333-352.

Miura, T., Miura, C.I., 2003. Molecular control mechanisms of fish spermatogenesis. Fish Physiol. Biochem. 28, 181-186.

Molés, G., Gómez, A., Carrillo, M., Rocha, A., Mylonas, C.C., Zanuy, S., 2011. Determination of Fsh quantity and bioactivity during sex differentiation and oogenesis in European sea bass using a newly developed immunoassay and an in vitro bioassay. Biol. Reprod. 85, 848-857.

Morais, S., 2016. The physiology of taste in fish: Potential implications for feeding stimulation and gut chemical sensing. Reviews in Fisheries Science & Aquaculture. 25, 133-149.

Moran, D., Smith, C.K., Gara, B., Poortenaar, C.W., 2007. Reproductive behaviour and early development in yellowtail kingfish (*Seriola lalandi* Valenciennes 1833). Aquaculture. 262, 95-104.

Mugnier, C., Gaignon, J.L., Lebegue, E., Fostier, A., Breton, B., 2000. Induction and synchronisation of spawning in cultivated turbot (*Scophthalmus maximus* L.) broodstock by implantation of sustained-release GnRH-a pellet. Aquaculture. 181, 241-255.

Munoz-Cueto, J.A., Zmora, N., Paullada-Salmeron, J.A., Marvel, M., Mananos, E., Zohar, Y., 2020. The gonadotropin-releasing hormones: Lessons from fish. Gen. Comp. Endocrinol. 291, 113422.

Mushiake, K., Kawano, K., Kobayashi, T., Yamasaki, T., 1998. Advanced Spawning in Yellowtail, *Seriola quinqueradiata*, by Manipulations of the Photoperiod and Water Temperature. Fish. Sci. 64, 727-731.

Mylonas, C., Robles, R., Estévez, E., Papandroulakis, N., Fontaine, P., Norberg, B., Alvarez-Blázquez, B., Koven, W., Tachen, G., 2017a. Exploring the biological and socio-economic potential of new/emerging candidate fish species for the expansion of the European aquaculture industry, European Aquaculture. European Aquaculture Society, Belgium, pp. 1-20.

Mylonas, C., Katharios, P., Grigorakis, K., Papandroulakis, N., Robles, R., Corriero, A., Pousis, C., Zupa, P., Fernandez-Palacios, H., Montero, D., Izquierdo, M., Roo, F.J., Hernandez-Cruz, C.M., Rodriguez, C., Peres, J.A., Jerez, S., Secombes, C.J., Guerrero, L., 2016a. Advances in greater amberjack (*Seriola dumerili*) research: the DIVERSIFY project, Aquaculture Europe. European Aquaculture Society, Belgium, pp. 12-19.

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

Mylonas, C.C., Zohar, Y., 2007. Promoting oocyte maturation, ovulation and spawning in farmed fish. in: Babin, P.J., Cerdá, J., Lubzens, E. (Eds.), The Fish Oocyte: From Basic Studies to Biotechnological Applications. Kluwer Academic Publishers, Dordrecht, The Netherlands, pp. 433-470.

Mylonas, C.C., Zohar, Y., 2009. Controlling reproduction in aquaculture. in: Burnell, G., Allan, G. (Eds.), New Technologies in Aquaculture: Improving Production Efficiency, Quality and Environmental Management. Woodhead Publishing Ltd, Cambridge, UK, pp. 109-142.

Mylonas, C.C., Hinshaw, J.M., Sullivan, C.V., 1992. GnRHa-induced ovulation of brown trout (*Salmo trutta*) and tts effects on egg quality. Aquaculture. 106, 379-392.

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

Mylonas, C.C., Fostier, A., Zanuy, S., 2010. Broodstock management and hormonal manipulations of fish reproduction. Gen. Comp. Endocrinol. 165, 516-534.

Mylonas, C.C., Duncan, N.J., Asturiano, J.F., 2017b. Hormonal manipulations for the enhancement of sperm production in cultured fish and evaluation of sperm quality. Aquaculture. 472, 21-44.

Mylonas, C.C., Scott, A.P., Vermeirssen, E.L., Zohar, Y., 1997b. Changes in plasma gonadotropin II and sex steroid hormones, and sperm production of striped bass after treatment with controlled-release gonadotropin-releasing hormone agonist-delivery systems. Biol. Reprod. 57, 669-675.

Mylonas, C.C., Gissis, A., Magnus, Y., Zohar, Y., 1997c. Hormonal changes in male white bass (*Morone chrysops*) and evaluation of milt quality after treatment with a sustained-release GnRHa delivery system. Aquaculture. 153, 301-313.

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

Mylonas, C.C., Papadaki, M., Pavlidis, M., Divanach, P., 2004a. Evaluation of egg production and quality in the Mediterranean red porgy (*Pagrus pagrus*) during two consecutive spawning seasons. Aquaculture. 232, 637-649.

Mylonas, C.C., Cardinaletti, G., Sigelaki, I., Polzonetti-Magni, A., 2005. Comparative efficacy of clove oil and 2-phenoxyethanol as anesthetics in the aquaculture of European sea bass (*Dicentrarchus labrax*) and gilthead sea bream (*Sparus aurata*) at different temperatures. Aquaculture. 246, 467-481.

Mylonas, C.C., Magnus, Y., Klebanov, Y., Gissis, A., Zohar, Y., 1997d. Reproductive biology and endocrine regulation of final oocyte maturation of captive white bass. J. Fish Biol. 51, 234-250.

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

Mylonas, C.C., Papandroulakis, N., Smboukis, A., Papadaki, M., Divanach, P., 2004b. Induction of spawning of cultured greater amberjack (*Seriola dumerili*) using GnRHa implants. Aquaculture. 237, 141-154.

Mylonas, C.C., Sigelaki, I., Divanach, P., Mananos, E., Carrillo, M., Afonso-Polyviou, A., 2003. Multiple spawning and egg quality of individual European sea bass (*Dicentrarchus labrax*) females after repeated injections of GnRHa. Aquaculture. 221, 605-620.

Mylonas, C.C., Mitrizakis, N., Castaldo, C.A., Cerviño, C.P., Papadaki, M., Sigelaki, I., 2013. Reproduction of hatchery-produced meagre *Argyrosomus regius* in captivity II. Hormonal induction of spawning and monitoring of spawning kinetics, egg production and egg quality. Aquaculture. 414–415, 318-327.

Mylonas, C.C., Fatira, E., Karkut, P., Sigelaki, I., Papadaki, M., Duncan, N., 2015. Reproduction of hatchery-produced meagre *Argyrosomus regius* in captivity III. Comparison between GnRHa implants and injections on spawning kinetics and egg/larval performance parameters. Aquaculture. 448, 44-53.

Mylonas, C.C., Salone, S., Biglino, T., de Mello, P.H., Fakriadis, I., Sigelaki, I., Duncan, N., 2016b. Enhancement of oogenesis/spermatogenesis in meagre *Argyrosomus regius* using a combination of temperature control and GnRHa treatments. Aquaculture. 464, 323-330.

Mylonas, C.C., Fakriadis, I., Papandroulakis, N., Raftopoulos, A., Iakovopoulos, G., Papadaki, M., Sigelaki, I., 2017c. Broodstock management and spawning induction of greater amberjack *Seriola dumerili* reared in tanks and sea cages in Greece. in: EAS (Ed.), Aquaculture Europe 2017, Dubrovnik, Croatia, pp. 791.

Mylonas, C.C., Bridges, C., Gordin, H., Rios, A.B., Garcia, A., de la Gandara, F., Fauvel, C., Suquet, M., Medina, A., Papadaki, M., Heinisch, G., De Metrio, G., Corriero, A., Vassallo-Agius, R., Guzman, J.M., Mananos, 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.

Nguyen, A.T., Chia, J.H.Z., Kazeto, Y., Lokman, P.M., 2019. Expression of gonadotropin subunit and gonadotropin receptor genes in wild female New Zealand shortfinned eel (*Anguilla australis*) during yellow and silver stages. Gen Comp Endocrinol. 272, 83-92.

Nijdam, D., Rood, T., Westhoek, H., 2012. The price of protein: Review of land use and carbon footprints from life cycle assessments of animal food products and their substitutes. Food Policy. 37, 760-770.

Nocillado, J., Palma, P., Fielder, S., Zanardini, M., Dennis, L.P., Elizur, A., 2019. Development of specific enzyme-linked immunosorbent assay for yellowtail kingfish (*Seriola lalandi*) follicle stimulating hormone using recombinant gonadotropins. Gen Comp Endocrinol. 282, 113208.

Nugroho, E., Taniguchi, N., Kato, K., Miyashita, S., 2000. Genetic Difference among Seed Populations of Greater Amberjack Used in Aquaculture Farm of Japan. SUISANZOSHOKU. 48, 665-674.

Nyuji, M., Fujisawa, K., Imanaga, Y., Matsuyama, M., 2013. GnRHa-induced spawning of wild-caught jack mackerel *Trachurus japonicus*. Fish. Sci. 79, 251-258.

Nyuji, M., Yamamoto, I., Hamada, K., Kazeto, Y., Okuzawa, K., 2019. Effect of GnRHa on plasma levels of Fsh and Lh in the female greater amberjack *Seriola dumerili*. J. Fish Biol. 95, 1350-1354.

Nyuji, M., Selvaraj, S., Kitano, H., Shiraishi, T., Yamaguchi, A., Shimizu, A., Matsuyama, M., 2012. Immunoreactivity of gonadotrophs (FSH and LH cells) and gonadotropin subunit gene expression in the male chub mackerel *Scomber japonicus* pituitary during the reproductive cycle. Zool. Sci. 29, 623-629.

Nyuji, M., Hamada, K., Kazeto, Y., Mekuchi, M., Gen, K., Soyano, K., Okuzawa, K., 2018. Photoperiodic regulation of plasma gonadotropin levels in previtellogenic greater amberjack (*Seriola dumerili*). Gen Comp Endocrinol. 269, 149-155.

Nyuji, M., Kazeto, Y., Izumida, D., Tani, K., Suzuki, H., Hamada, K., Mekuchi, M., Gen, K., Soyano, K., Okuzawa, K., 2016. Greater amberjack Fsh, Lh, and their receptors: Plasma and mRNA profiles during ovarian development. Gen. Comp. Endocrinol. 225, 224-234.

O'Neill, B., Le Roux, A., Hoffman, L.C., 2015. Comparative study of the nutritional composition of wild versus farmed yellowtail (*Seriola lalandi*). Aquaculture. 448, 169-175.

Panini, E., Mylonas, C.C., Zanuy, S., Carrillo, M., Ramos, J., Bruce, M., 2001. Incubation of embryos and larvae of marine fish using microtiter plates. Aquacult. Int. 9, 189-196.

Pankhurst, N.W., Fitzgibbon, Q.P., 2006. Characteristics of spawning behaviour in cultured greenback flounder, *Rhombosa tapirina*. Aquacultrue. 253, 279-289.

Papadaki, M., Papadopoulou, M., Siggelaki, I., Mylonas, C.C., 2008. Egg and sperm production and quality of sharpsnout sea bream (*Diplodus puntazzo*) in captivity. Aquaculture. 276, 187-197.

Papadaki, M., Mazzella, D., Santinelli, V., Fakriadis, I., Sigelaki, I., Mylonas, C.C., 2018a. Hermaphroditism and reproductive function of hatchery-produced sharpsnout seabream (*Diplodus puntazzo*) under attenuated annual thermal cycles. Aquaculture. 482, 231-240.

Papadaki, M., Peleteiro, J.B., Alvarez-Blázquez, B., Villanueva, J.L.R., Linares, F., Vilar, A., Rial, E.P., Lluch, N., Fakriadis, I., Sigelaki, I., Mylonas, C.C., 2018b. Description of the Annual Reproductive Cycle of Wreckfish *Polyprion americanus* in Captivity. Fishes. 3, 1-20.

Paredes, J.F., Cowan, M., Lopez-Olmeda, J.F., Munoz-Cueto, J.A., Sanchez-Vazquez, F.J., 2019. Daily rhythms of expression in reproductive genes along the brain-pituitary-gonad axis and liver of zebrafish. Comp. Biochem. Physiol. A Mol. Integr. Physiol. 231, 158-169.

Parker, C.G., Cheung, E., 2020. Metabolic control of teleost reproduction by leptin and its complements: Current insights from mammals. Gen. Comp. Endocrinol.

Pastor, E., Grau, A., Riera, F., Pou, S., Massuti, E., Grau, A.M., 2000. Experiences in the culture of new species in the 'Estacion de Acuicultura' of the Balearic Government (1980-1998). in: Basurco, B. (Ed.), Cahiers Options Méditerranéennes, vol. 47: Mediterranean Marine Aquaculture Finfish Species Diversification. C.I.H.E.A.M., Zaragoza, Spain, pp. 371-379.

Patiño, R., Sullivan, C.V., 2002. Ovarian follicle growth, maturation, and ovulation in teleost fish. Fish Physiol. Biochem. 26, 57-70.

Paxton, J.R., Hoese, D.F., Allen, G.R., Hanley, J.E., 1989. Pisces. Petromyzontidae to Carangidae. Australian Government Publishing Service, Canberra.

Peñaranda, D.S., Pérez, L., Fakriadis, G., Mylonas, C.C., Asturiano, J.F., 2008. Effects of extenders and cryoprotectant combinations on motility and morphometry of sea bass (*Dicentrarchus labrax*) spermatozoa. J. Appl. Ichthyol. 24, 450-455.

Podhorec, P., Socha, M., Sokolowska-Mikolajczyk, M., Drozd, B., Policar, T., Stejskal, V., Kouril, J., 2011. Effective dose of mGnRHa for induction of ovulation in tench (*Tinca tinca* L.). Aquaculture. 319, 184-187.

Poortenaar, C.W., Hooker, S.H., Sharp, N., 2001. Assessment of yellowtail kingfish (*Seriola lalandi lalandi*) reproductive physiology, as a basis for aquaculture development. Aquaculture. 201, 271-286.

Pousis, C., Mylonas, C.C., De Virgilio, C., Gadaleta, G., Santamaria, N., Passantino, L., Zupa, R., Papadaki, M., Fakriadis, I., Ferreri, R., Corriero, A., 2018. The observed oogenesis impairment in greater amberjack *Seriola dumerili* (Risso, 1810) reared in captivity is not related to an insufficient liver transcription or oocyte uptake of vitellogenin. Aquacult. Res. 49, 243-252.

Pousis, C., Rodríguez, C., De Ruvo, P., De Virgilio, C., Pérez, J.A., Mylonas, C.C., Zupa, R., Passantino, L., Santamaria, N., Valentini, L., Corriero, A., 2019. Vitellogenin receptor and fatty acid profiles of individual lipid classes of oocytes from wild and captive-reared greater amberjack (*Seriola dumerili*) during the reproductive cycle. Theriogenology. 140, 73-83.

Quiñones-Arreola, M.F., Arcos-Ortega, G.F., Gracia-López, V., Casillas-Hernández, R., Weirich, C., Morris, T., Díaz-Tenorio, M., Ibarra-Gámez, C., 2015. Reproductive broodstock performance and egg quality of wild-caught and first-generation domesticated *Seriola rivoliana* reared under same culture conditions. Lat. Am. J. Aquat. Res. 43, 953-962.

Rahman, M.A., Ohta, K., Yamaguchi, A., Chuda, H., Hirai, T., Matsuyama, M., 2003. Gonadotropins, gonadotropin receptors and their expressions during sexual maturation in yellowtail, a carangid fish. 28, 81–83,.

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.

Rasines, I., Gómez, M., Martín, I., Rodríguez, C., Mañanós, E., Chereguini, O., 2012. Artificial fertilization of Senegalese sole (*Solea senegalensis*): Hormone therapy administration methods, timing of ovulation and viability of eggs retained in the ovarian cavity. Aquaculture. 326-329, 129-135.

Rocha, M.J., Arukwe, A., Kapoor, B.G., 2008. Fish Reproduction. Science Publishers, Enfield, NH, USA.

Rodríguez-Barreto, D., Jerez, S., Cejas, J.R., Martin, M.V., Acosta, N.G., Bolaños, A., Lorenzo, A., 2014. Ovary and egg fatty acid composition of greater amberjack broodstock (*Seriola dumerili*) fed different dietary fatty acids profiles. Eur. J. Lipid Sci. Technol. 116, 584-595.

Roo, J., Fernández-Palacios, H., Schuchardt, D., Hernández-Cruz, C.M., Izquierdo, M.S., 2015. Influence of hormonal induction and broodstock feeding on longfin yellowtail *Seriola rivoliana* maturation, spawning quality and egg biochemical composition. Aquacult. Nutr. 21, 614-624.

Roo, J., Fernández-Palacios, H., Hernández-Cruz, C., Mesa-Rodriguez, A., Schuchardt, D., Izquierdo, M., 2014. First results of spawning and larval rearing of longfin yelowtail *Seriola rivoliana* as a fast-growing candidate for European marine finfish aquaculture diversification. Aquacult. Res. 45, 689-700.

Rosenfeld, H., Meiri, I., Elizur, A., 2007. Gonadotropin regulation of oocyte development. in: Babin, P.J., Cerdá, J., Lubzens, E. (Eds.), The Fish Oocyte: from Basic Studies to Biotechnological Applications. Kluwer Academic Publishers, Dordrecht, The Netherlands, pp. 171-198.

Rosenfeld, H., Mylonas, C.C., Bridges, C.R., Heinisch, G., Corriero, A., Vassallo-Agius, R., Medina, A., Belmonte Ríos, A., Garcia, A., De la Gándara, F., Fauvel, C., De Metrio, G., Meiri-Ashkenazi, I., Gordin, H., Zohar, Y., 2012. GnRHa-mediated stimulation of the reproductive endocrine axis in captive Atlantic bluefin tuna, *Thunnus thynnus*. Gen. Comp. Endocrinol. 175, 55-64.

Rurangwa, E., Kime, D.E., Ollevier, F., Nash, J.P., 2004. The measurement of sperm motility and factors affecting sperm quality in cultured fish. Aquaculture. 234, 1-28.

Sahoo, S.K., Giri, S.S., Sahu, A.K., 2005. Effect on breeding performance and egg quality of *Clarias batrachus* (Linn.) at various doses of ovatide during spawning induction. Asian Fish. Sci. 18, 77-83.

Sarih, S., Djellata, A., La Barbera, A., Fernández-Palacios Vallejo, H., Roo, J., Izquierdo, M., Fernández-Palacios, H., 2018. High-quality spontaneous spawning in greater amberjack (*Seriola dumerili*, Risso 1810) and its comparison with GnRHa implants or injections. Aquacult. Res. 49, 3442-3450.

Sarih, S., Djellata, A., Roo, J., Hernández-Cruz, C.M., Fontanillas, R., Rosenlund, G., Izquierdo, M., Fernández-Palacios, H., 2019. Effects of increased protein, histidine and taurine dietary levels on egg quality of greater amberjack (*Seriola dumerili*, Risso, 1810). Aquaculture. 499, 72-79.

Sarih, S., Djellata, A., Fernández-Palacios, H., Ginés, R., Fontanillas, R., Rosenlund, G., Izquierdo, M., Roo, J., 2020. Adequate n-3 LC-PUFA levels in broodstock diets optimize reproductive performance in

GnRH injected greater amberjack (*Seriola dumerili*) equaling to spontaneously spawning broodstock. Aquaculture. 520, 735007.

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.

Sawada, Y., Hattori, M., Iteya, M., Takagi, Y., Ura, K., Seoka, M., Kato, K., Kurata, M., Mitakake, H., Katayama, S., KUMAI, H., 2006. Induction of centrum defects in amberjack *Seriola dumerili* by exposure of embryos to hypoxia. Fish. Sci. 72, 364-372.

Schulz, R.W., Miura, T., 2002. Spermatogenesis and its endocrine regulation. Fish Physiol. Biochem. 26, 43-56.

Schulz, R.W., Vischer, R.F., Cavaco, J.E.B., Santos, E.M., Tyler, C.R., Goos, H., Bogerd, J., 2001. Gonadotropins, their receptors, and the regulation of testicular functions in fish. Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology. 129, 407-417.

Schulz, R.W., de França, L.R., Lareyre, J.-J., LeGac, F., Chiarini-Garcia, H., Nobrega, R.H., Miura, T., 2010. Spermatogenesis in fish. Gen. Comp. Endocrinol. 165, 390-411.

Šegvić-Bubić, T., Marrone, F., Grubišić, L., Izquierdo-Gomez, D., Katavić, I., Arculeo, M., Lo Brutto, S., 2016. Two seas, two lineages: How genetic diversity is structured in Atlantic and Mediterranean greater amberjack *Seriola dumerili* Risso, 1810 (Perciformes, Carangidae). Fisheries Research. 179, 271-279.

Selman, K., Wallace, R.A., Sarka, A., Qi, X., 1993. Stages of oocyte development in the zebrafish, *Brachydanio rerio*. J. Morphol. 218: 203-224, Brachydanio, zebrafish, oogenesis, FOM, histology.

Setiawan, A.N., Muncaster, S., Pether, S., King, A., Irvine, G.W., Lokman, P.M., Symonds, J.E., 2016. The effects of gonadotropin-releasing hormone analog on yellowtail kingfish *Seriola lalandi* (Valenciennes, 1833) spawning and egg quality. Aquaculture Reports. 4, 1-9.

Shimizu, A., 1996. Long-term efects of a luteinizing hormone-releasing hormone analogue and/or a dopamine antagonist, pimozide, on gonadal activity in an autumn-spawning bitterling, *Acheilognathus rhombea*, during various phases of the annual reproductive cycle. J. Exp. Zool. 276: 279-286.

Sicuro, B., Luzzana, U., 2016. The state of Seriola spp. other than yellowtail (*S. quinqueradiata*) farming in the world. Reviews in Fisheries Science and Aquaculture. 24, 314-325.

Sierra-Flores, R., Atack, T., Migaud, H., Davie, A., 2015. Stress response to anthropogenic noise in Atlantic cod *Gadus morhua* L. Aquacult. Eng. 67, 67-76.

Smith-Vaniz, W.F., Pina Amargos, F., Brown, J., Curtis, M., Williams, J.T., 2015. *Seriola dumerili*. The IUCN Red List of Threatened Species 2015.

Sorbera, L.A., Mylonas, C.C., Zanuy, S., Carrillo, M., Zohar, Y., 1996. Sustained administration of GnRHa increases milt volume without altering sperm counts in the sea bass. J. Exp. Zool. 276, 361-368.

Sullivan, C.V., Yilmaz, O., 2018. Vitellogenesis and Yolk Proteins, Fish, Encyclopedia of Reproduction, pp. 266-277.

Sundby, S., Kristiansen, T., 2015. The Principles of Buoyancy in Marine Fish Eggs and Their Vertical Distributions across the World Oceans. PLoS One. 10, e0138821.

Superio, J., I., F., Tsigenopoulos, C.S., Lancerotto, S., Rodriguez, A.V., Vervelakis, E., Mylonas, C.C., 2021. Spawning kinetics and parentage contribution of European sea bass (*Dicentrarchus labrax*) broodstocks and influence of GnRHa-induced spawning. Aquaculture Reports, (under review).

Suquet, M., Cosson, J., de la Gándara, F., Mylonas, C.C., Papadaki, M., Lallemant, S., Fauvel, C., 2010. Sperm features of captive Atlantic bluefin tuna (*Thunnus thynnus*). J. Appl. Ichthyol. 26, 775-778.

Swanson, P., Dickey, J.T., Campbell, B., 2003. Biochemistry and physiology of fish gonadotropins. Fish Physiol. Biochem. 28, 53–59.

Symonds, J.E., Walker, S.P., Pether, S., Gublin, Y., McQueen, D., King, A., Irvine, G.W., Setiawan, A.N., Forsythe, J.A., Bruce, M., 2014. Developing yellowtail kingfish (*Seriola lalandi*) and hāpuku (*Polyprion oxygeneios*) for New Zealand aquaculture. N. Z. J. Mar. Freshwat. Res. 48, 371-384.

Tachihara, K., Ebisu, R., Tukashima, Y., 1993. Spawning, eggs, larvae and juveniles of the purplish amberjack *Seriola dumerili*. Nippon Suisan Gakkaishi. 59, 1479-1488.

Tacon, A.G.J., Metian, M., 2013. Fish Matters: Importance of Aquatic Foods in Human Nutrition and Global Food Supply. Rev. Fish. Sci. 21, 22-38.

Tang, H., Chen, Y., Liu, Y., Yin, Y., Li, G., Guo, Y., Liu, X., Lin, H., 2017. New Insights Into the Role of Estrogens in Male Fertility Based on Findings in Aromatase-Deficient Zebrafish. Endocrinology. 158, 3042-3054.

Teletchea, F., Fontaine, P., 2014. Levels of domestication in fish: implications for the sustainable future of aquaculture. Fish Fish. 15: 181-195.

Trudeau, V.L., 2018. Facing the Challenges of Neuropeptide Gene Knockouts: Why Do They Not Inhibit Reproduction in Adult Teleost Fish? Front. Neurosci. 12, 302.

Trudeau, V.L., Somoza, G.M., 2020. Multimodal hypothalamo-hypophysial communication in the vertebrates. Gen. Comp. Endocrinol.

Tuset, V.M., Trippel, E.A., De Monserrat, J., 2008. Sperm morphology and its influence on swimming speed in Atlantic cod. J. Appl. Ichthyol. 24, 398-405.

Tyler, J.R., Sumpter, J.P., 1996. Oocyte growth and development in teleosts. Rev. Fish Biol. Fish. 6, 287-318.

Uribe, M.C., Grier, H.J., Mejia-Roa, V., 2014. Comparative testicular structure and spermatogenesis in bony fishes. Spermatogenesis. 4, e983400.

Valdebenito, I.I., Gallegos, P.C., Effer, B.R., 2013. Gamete quality in fish: evaluation parameters and determining factors. Zygote. 23, 177-197.

Vassallo-Agius, R., Watanabe, T., Imaizumi, H., Yamazaki, T., 2002. Spawning performance of yellowtail *Seriola quinqueradiata* fed dry pellets containing paprika and squid meal. Fish. Sci. 68, 230-232.

Vermeirssen, E.L.M., Scott, A.P., Mylonas, C.C., Zohar, Y., 1998. Gonadotrophin-releasing hormone agonist stimulates milt fluidity and plasma concentrations of 17,20ß-dihydroxylated and 5ß-reduced, 3a-hydroxylated C21 steroids in male plaice (*Pleuronectes platessa*). Gen. Comp. Endocrinol. 112, 163-177.

Vermeirssen, E.L.M., Shields, R.J., Mazorra de Quero, C., Scott, A.P., 2000. Gonadotrophin-releasing hormone agonist raises plasma concentrations of progestogens and enhances milt fluidity in male Atlantic halibut (*Hippoglossus hippoglossus*). Fish Physiol. Biochem. 22, 77-87.

Vizziano, D., Fostier, A., Loir, M., Le Gac, F., 2008. Testis development, its hormonal regulation and spermiation induction in teleost fish. in: Alavi, S.M.H., Cosson, J., Coward, K., Rafiee, G. (Eds.), Fish Spermatology. Alpha Science Intl, Oxford (UK), pp. 103-139.

Wallace, R.A., Selman, K., 1990. Ultrastructural aspects of oogenesis and oocyte growth in fish and amphibians. J Electron Microsc Tech. 16, 175-201.

Watanabe, T., Kiron, V., 1995. Red sea bream (*Pagrus major*). in: Bromage, N.R., Roberts, R.J. (Eds.), Broodstock Management and Egg and Larval Quality. Blackwell Science, Oxford, pp. 398-413.

Wells, R.J.D., Rooker, J.R., 2004. Distribution, age, and growth of young-of-the-year greater amberjack (*Seriola dumerili*) associated with pelagic Sargassum. Fish. Bull. 102, 545-554.

Weltzien, F., Andersson, E., Anderson, O., Shalchian-Tabrizi, K., Norberg, B., 2004. The brain-pituitary-gonad axis in male teleosts, with special emphasis on flatfish (*Pleuronectiformes*). Comparative Biochemistry and Physiology Part A. 127, 447-477.

Whitlock, K.E., Postlethwait, J., Ewer, J., 2019. Neuroendocrinology of reproduction: Is gonadotropin-releasing hormone (GnRH) dispensable? Front. Neuroendocrinol. 53, 100738.

Wootton, R.J., Smith, C., 2015. Reproductive Biology of Teleost Fishes. Wiley Blackwell, UK.

Yaron, Z., Gur, G., Melamed, P., Rosenfeld, H., Elizur, A., Levavi-Sivan, B., 2003. Regulation of fish gonadotropins. Int. Rev. Cytol. 225: 131-185, reproduction, GtH, GnRH.

Zadmajid, V., Myers, J.N., Sorensen, S.R., Ernest Butts, I.A., 2019. Ovarian fluid and its impacts on spermatozoa performance in fish: A review. Theriogenology. 132, 144-152.

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

Zohar, Y., Muñoz-Cueto, J.A., Elizur, A., Kah, O., 2010. Neuroendocrinology of reproduction in teleost fish. Gen. Comp. Endocrinol. 165, 438-455.

Zohar, Y., Goren, A., Fridkin, M., Elhanati, E., Koch, Y., 1990. Degradation of gonadotropin-releasing hormones in the gilthead seabream *Sparus aurata* II. Cleavage of native salmon GnRH, mammalian LHRH and their analogs in the pituitary, kidney and liver. Gen. Comp. Endocrinol. 79, 306-319.

Zohar, Y., Goren, A., Tosky, M., Pagelson, G., Leibovitz, D., Koch, Y., 1989. The bioactivity of gonadotropin-releasing hormones and its regulation in the gilthead seabream, *Sparus aurata: in vivo* and *in vitro* studies. Fish Physiol. Biochem. 7, 59-67.

Zupa, P., Fauvel, C., Mylonas, C.C., Pousis, C., Santamaría, C.A., Papadaki, M., Fakriadis, I., V., C., 2017a. Rearing in captivity affects spermatogenesis and sperm quality in greater amberjack, *Seriola dumerili* (Risso, 1810). J. Anim. Sci. 95, 4085-4100.

Zupa, P., Fauvel, C., Mylonas, C.C., Santamaria, N., Valentini, L., Pousis, C., Papadaki, M., Suquet, M., De la Gándara, F., Bello, G., De Metrio, G., Corriero, A., 2013. Comparative analysis of male germ cell proliferation and apoptosis in wild and captive Atlantic bluefin tuna (*Thunnus thynnus*). J. Appl. Ichthyol. 29, 71-81.

Zupa, R., Rodríguez, C., Mylonas, C.C., Rosenfeld, H., Fakriadis, I., Papadaki, M., Pérez, J.A., Pousis, C., Basilone, G., Corriero, A., 2017b. Comparative study of reproductive development in wild and captive-reared greater amberjack *Seriola dumerili* (Risso, 1810). PLoS One. 12, e0169645.

Appendix – Photos

Figure 1 A greater amberjack breeder in ARGO, Salamina, Greece.

Figure 2 Tranquilization of greater amberjack broodstock in a sack attached to the sea cage in ARGO, Salamina, Greece.

Figure 3 Sperm sample collection using a catheter in ARGO, Salamina, Greece.

Figure 4 Oocyte evaluation in greater amberjack in ARGO, Salamina, Greece

Figure 5 Blood collection from greater amberjack in ARGO, Salamina, Greece.

Figure 6 Administration of GnRHa implant in greater amberjack in SOUDA, Crete, Greece.

Figure 7 Transferring to land-based tanks greater amberjack breeders using a service boat in GMF, Galaxidi, Greece.

Figure 8 Computer assisted sperm analysis (CASA) in greater amberjack sperm in ARGO, Salamina, Greece.

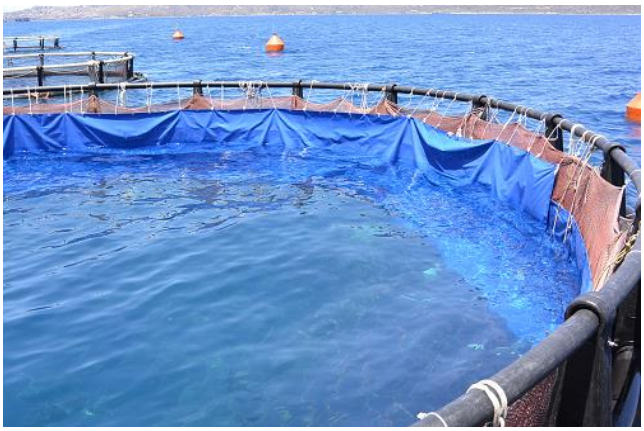

Figure 9 The egg collection trapping device attached in a sea cage in SOUDA, Crete, Greece.

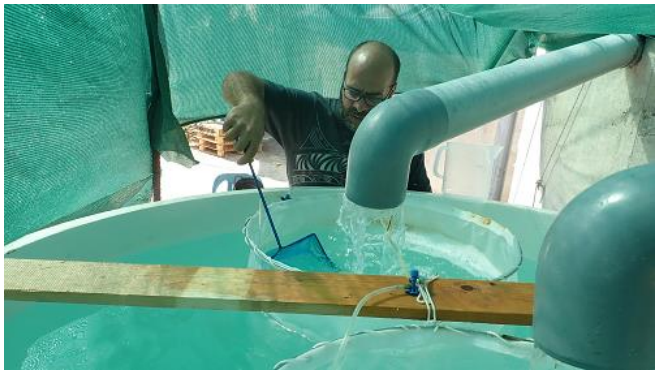

Figure 10 Egg collection in ARGO, Salamina, Greece.

Figure 11 Running the ELISAs in the National Center for Mariculture, Eilat, Israel.