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The importance of thermoperiod for proper gametogenesis and successful egg and sperm production in meagre (*Argyrosomus regius*) breeders in aquaculture

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Abstract

We examined the effect of constant water temperature throughout the year on gametogenesis, spawning success and egg/sperm/embryo quality in meagre (*Argyrosomus regius*). Two broodstocks were exposed to simulated natural photoperiod, and either attenuated seasonal water temperature (SeasT, 16.4 to 19.6ºC) or relatively constant water temperature (CoT, 19.4 ± 0.6ºC). In the spawning period (May), 4 couples per group were induced to spawn with gonadotropin releasing hormone agonist (GnRHa). Gonadal stage of development, sperm quality parameters and plasma levels of sex steroids were evaluated prior to the GnRHa treatment. Spawning success and egg/sperm quality were examined over the following 4 weeks. Constant temperature did not prevent gametogenesis, but exposure to attenuated seasonal water temperature with the inclusion of winter low temperature was beneficial to both sexes. The mean (±SD) diameter of the largest vitellogenic oocytes prior to GnRHa administration was significantly higher in the SeasT compared to the CoT group (598 ± 27 vs 520 ± 17 µm). Testosterone plasma levels in the females were significantly higher in the SeasT group, but all other hormones were similar in both sexes. SeasT females spawned more consistently with higher relative fecundity, and 24-h embryo survival of the produced eggs. A more pronounced negative effect of constant water temperature was observed in males, since CoT males exhibited a spermiation index of 0 prior to GnRHa treatment, the latter clearly having a beneficial effect over the following 4 weeks. The study demonstrated that meagre do undergo gametogenesis to a significant extent even under constant water temperatures during the year. However, a seasonal thermal regime -even an attenuated one- was necessary for the proper development of the gametes, allowing for the successful spawning induction using the established GnRHa induction protocol.

Keywords: *Argyrosomus regius*; GnRHa; spawning; temperature; gametogenesis; sperm quality.

Introduction

Meagre (*Argyrosomus regius*) belongs to the Sciaenidae family and it is a species of interest for the diversification of Mediterranean aquaculture production (Quéméner *et al.*, 2002; Stipa & Angelini, 2005). The rapid growth, excellent flesh taste and low-fat content of this fish led to its increasing production in the last decade (Poli *et al.*, 2003; Cárdenas, 2010; Chatzifotis *et al.*, 2010; Monfort, 2010; Grigorakis *et al.*, 2011; Duncan *et al.*, 2013). However, females do not reproduce readily in captivity, exhibiting dysfunctions with oocyte maturation, ovulation and spawning (Duncan *et al.*, 2012; Mylonas *et al.*, 2013a; Soares *et al.*, 2015). As a result, significant research efforts have been invested in developing appropriate methods for controlling reproduction, based on the use of gonadotropin releasing hormone agonists (GnRHa) administered in the form of multiple injections or sustained release implants, resulting in the successful production of good quality eggs (Duncan *et al.*, 2012; Mylonas *et al.*, 2013b; Mylonas *et al.*, 2013a; Fernández-Palacios *et al.*, 2014; Mylonas *et al.*, 2015; Soares *et al.*, 2015; Duncan *et al.*, 2018; Ramos-Júdez *et al.*, 2019).

The annual reproductive cycle in fishes in the temperate or higher latitudes is controlled by environmental cues, mainly photoperiod and temperature, with photoperiod being the principal environmental regulator of the process of gametogenesis, and temperature acting as a secondary cue, being more important during final maturation and spawning (Bromage *et al.*, 2001; Pankhurst & Porter, 2003; Falcon *et al.*, 2010; Migaud *et al.*, 2010; Wang *et al.*, 2010; Zohar *et al.*, 2010; Gordoa & Carreras,

2014). Changes in photoperiod modulate reproductive development through the melatonin system and the transduction of photoperiodic information to the brain-pituitary-gonad axis (Migaud *et al.*, 2010; Wang *et al.*, 2010). In the temperate zone, where progressive variations occur seasonally, temperature may work in synergy with photoperiod. These changes are reliable signals that fish can use to synchronize their biological rhythms to reproduce during the most favorable moment of the year, and exposure to abnormal temperature profiles may alter the amount and quality of the gametes (Durant *et al.*, 2007).

Although some fishes seem to have a specific thermal requirement in parallel with the photoperiod regime, others do not (Brown *et al.*, 2006). This information is of a practical importance for the aquaculture industry, which usually simulates both photic and thermal conditions to achieve seasonal reproductive development and production of eggs (Bromage *et al.*, 2001; Migaud *et al.*, 2005; Povoa *et al.*, 2011), as well as off-season spawning (Carrillo *et al.*, 1989; Bromage & Roberts, 1995; Cerdá *et al.*, 1995). Due to biosecurity requirements, aquaculture broodstocks are commonly maintained in recirculating aquaculture systems (RAS) using borehole seawater that is sterile, but has a relatively constant temperature throughout the year. As a result, significant costs for infrastructure and energy are incurred to heat and cool the water in order to provide the thermal cycling usually encountered in nature. If thermal cycling is found to be not so relevant for the gonadal development of a given species, then maintaining broodstocks under simulated photoperiods only, but under relatively constant borehole water temperatures would save in infrastructure and energy.

For example, in gilthead seabream (*Sparus aurata*) and common dentex (*Dentex dentex*), it was demonstrated that constant temperatures did not prevent full reproductive maturation or spawning of fish maintained in natural photoperiods (Pavlidis *et al.*, 2001; Karamanlidis, 2017). A similar situation was also reported in salmonids when borehole water was used (Bromage *et al.*, 2001). In an effort to reduce maintenance costs and also prolong the spawning season in meagre, previous studies have shown that females complete vitellogenesis and can spawn reliably after GnRHa administration, if exposed to a modified annual thermal cycle, with lower spring-summer-fall maxima than the natural temperature profile in the Mediterranean (Mylonas *et al.*, 2013a; Mylonas *et al.*, 2015). Similarly, males completed spermatogenesis and spermiation under these conditions (Fakriadis *et al.*, 2020). Then, spawning could be maintained for up to 17 weeks in response to weekly GnRHa injections to the females and a once-every-3-weeks GnRHa implantation to the males, under a water temperature of 19-20°C (Mylonas *et al.*, 2016).

In the present study, we examined the hypothesis that meagre, as other fishes, may also undergo vitellogenesis and spermatogenesis in captivity when exposed solely to a natural photoperiod, while exposed to the relatively constant thermal regime provided by borehole seawater. Therefore, we evaluated a) the effect of a constant thermal regime compared to an attenuated seasonal thermal regime established earlier, in the process of gametogenesis, sperm production and quality, and plasma sex steroid levels during the expected spawning period; and b) the response to GnRHa administration in terms of spawning kinetics, and egg/sperm production and quality.

Materials and Methods

Broodstock maintenance

The experiment was held in the AQUALABS facilities of the Institute of Marine Biology, Biotechnology, and Aquaculture (IMBBC) of the Hellenic Centre for Marine Research (HCMR), Crete, Greece. From May 2018 until May 2019, two mixed-sex broodstocks (N = 14-16, mean body weight ± SD of 8.7 ± 0.5 kg) were maintained in 15-m^3 rectangular tanks in recirculating aquaculture systems (RAS) under simulated natural photoperiod and supplied with borehole seawater, exposed to either a simulated "attenuated" seasonal temperature profile (SeasT group; 16.4 - 19.6º C) previously established for meagre maturation (Mylonas *et al.*, 2015; Mylonas *et al.*, 2016), or a constant temperature profile (CoT group; 19.4 ± 0.6ºC) resulting from the use of a typical borehole seawater source used in commercial hatcheries in the Mediterranean (Fig. 1). Fish were fed five days per week to apparent satiation with commercial feed (Vitalis, Skretting S.A., Norway). Monitoring of temperature was done on a daily basis, while monitoring of pH, dissolved oxygen (DO, %), NH_3-N (mg l^{-1}) and NO_2-N (mg l^{-1}) was done once a week (CoT - pH = 7.54 ± 0.06; DO = 91 ± 4%; NH_3-N = 0.32 ± 0.23 mg l^{-1}; NO_2-N = 0.039 ± 0.038 mg l^{-1} and SeasT- pH = 7.57 ± 0.06; DO = 92 ± 3%; NH_3-N = 0.32 ± 0.22 mg l^{-1}; NO_2-N = 0.031 ± 0.026 mg l^{-1}).

The experimental protocol was approved by the National Veterinary Service (PN 255356 - ΑΔΑ: 6ΛΙ17ΛΚ-ΠΛΩ). 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).

Broodstock reproductive evaluation and spawning induction

On the 6th of May 2019 both broodstocks were evaluated for reproductive stage. Fish were first tranquilized in their tanks after a 2-day starvation period with a dose of 0.01 ml l^{-1} of clove oil, and then were transferred one-by-one for complete sedation to an anesthetic bath of a dose of 0.03 ml l^{-1} clove oil (Mylonas *et al.*, 2005). Ovarian biopsies were collected using an endometrial catheter (Pipelle de Cornier, Laboratorie CCD, France) after applying gentle aspiration. A part of the biopsy was examined immediately as a wet mount under a compound

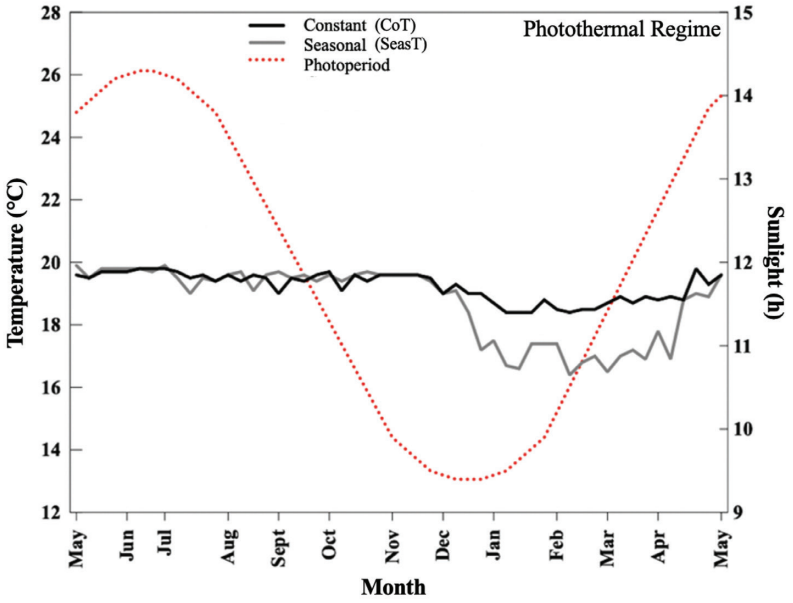

Fig. 1: Photoperiod and water temperature profiles of the Constant (CoT) regime using borehole water and the attenuated Seasonal (SeasT) regime applied to meagre (*Argyrosomus regius*) broodstocks from May 2018 to May 2019.

light microscope (x40 magnification) to evaluate the reproductive stage of the fish and to estimate the mean diameter of the most advanced vitellogenic oocytes (n = 10, at x100 magnification), and microphotographs were taken. The other part of the biopsy was stored for further histological processing (see below). Sperm production was evaluated after applying gentle abdominal pressure (stripping), using a subjective index (Spermiation Index, SI) developed earlier and used in meagre (Fakriadis *et al.*, 2020) as follows: S0 = no milt released, S1 = only a drop of milt released after multiple stripping attempts, S2 = milt released after the first stripping attempt and, S3 = copious amount of sperm released with very little pressure. Milt samples (50–100 µl) were collected for sperm quality evaluation (when the spermiation index was ≥S2) after the genital pore was rinsed with clean water and blot dried, taking care to avoid contamination of samples with feces or urine. Milt was collected using a positive displacement pipette. The collected milt sample was stored in a 500-µl centrifuge tube, placed on ice and then transferred to a 4ºC refrigerator until evaluation, immediately after the completion of the sampling.

Four females per group were selected at the expected spawning period (day 0) based on their ovarian maturity stage evaluation (mean oocyte diameter of the most advanced vitellogenic oocytes >550 µm), and were injected with desGly^{10}, DAla^{6}, Pro^{9}-GnRH-NEthylamide (GnRHa, H-4070, Bachem, Switzerland) at an effective dose of 14.3 ± 0.2 µg GnRHa kg^{-1} BW for the CoT females and 14.1 ± 0.2 µg GnRHa kg^{-1} BW for the SeasT females (Mylonas *et al.*, 2016). After treatment, females were placed individually in eight separate 5-m^3 rectangular flow-through tanks supplied with aerated borehole seawater at 19.7 ± 0.4ºC under simulated natural photoperiodic conditions. The four selected males from each thermal group (SeasT or CoT) were treated with a GnRHa implant at an effective dose of 48.0 ± 2.7 µg GnRHa kg^{-1} BW (Mylonas *et al.*, 2013b) constructed with [Ethylene-Vinyl Acetate]-copolymer and were also placed in the 5-m^3 tanks with the females from their respective temperature profile group. This way four couples per temperature group were formed and induced to spawn. The spawning tanks were fitted with passive egg collectors supplied with water from the surface outflow. All GnRHa-treated fish were sampled weekly for the following four weeks. Females were injected with the same dose of a GnRHa injection at each of the following three weeks (days 7, 14, 21). Males were treated with the same GnRHa implant of the same dose once again at the beginning of the third week (day 14). At each sampling, biopsies and sperm were collected when it was possible, mean oocyte diameters of the largest vitellogenic oocytes were calculated and the spermiation index was determined. On day 28 the experiment was completed and all fish were transferred back to their original tanks.

Sperm quality evaluation

Sperm quality was assessed evaluating the following parameters: (a) sperm density (number of spermatozoa ml^{-1} of milt), (b) survival of spermatozoa stored at 4ºC (spermatozoa survival, days), (c) duration of forward motility of ≥ 5% of the spermatozoa in the field of view (mo-

tility duration, min). Estimation of density was performed in duplicates using a Neubauer haemocytometer under a compound light microscope (200x magnification, Nikon, Eclipse 50i, Japan), after diluting sperm 2121-fold with saline. Spermatozoa survival was estimated as follows: after collection, milt was stored at 4°C and examined every other day for spermatozoa motility until forward motility was less than 5%. Motility duration evaluation was conducted in duplicates after mixing 1 µl of milt and a drop of seawater (50 µl) on a microscope slide (400x magnification) and examining spermatozoa motility until forward motility was less than 5%.

Sperm quality was also assessed using computer assisted sperm analysis (CASA, ISAS, Spain). Milt samples were activated with seawater containing 2% bovine serum albumin (1:334) to obtain 200-300 cells in the field and placed in a counting chamber with a fixed depth (Sperm-track 10). Evaluation with CASA was done immediately after milt collection, using a compound light microscope (Proiser UB 200i) under x200 magnification, on which a digital camera was mounted recording at 100 frames per second. Milt samples were evaluated in triplicates every 15 sec after activation until less than 5% of motile cells were present in the field of view. 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 the head area. Spermatozoa were considered immotile, when showing a VCL < 10 µm sec^{-1}, whereas they were classified as rapid when VCL was higher than 100 µm sec^{-1}.

Egg collection and embryo/larval survival evaluation

Egg collectors were checked every day for the presence of eggs. After spawning, all eggs in the egg collector were taken with a dip-net and placed into a 10-l bucket. After whisking the bucket content, a sub-sample of 10-ml was used to estimate the fecundity (x 1000 eggs $spawn^{-1}$) and fertilization success (%) using a stereoscope with an egg-counter plate. To evaluate embryonic development, the method of Panini *et al.* (2001) was used by placing individual fertilized eggs in a 96-well microtiter (mct) plate (one egg per well). Daily monitoring of the mct-plates was carried out using a stereoscope, taking records of embryo viability 24 h after egg collection, hatching success, and larval survival 7 days after egg collection (yolk sac absorption). Embryo viability at 24 h after egg collection was estimated as the ratio of viable eggs (having live embryos) to the number of live eggs initially placed in the mct plate. Hatching success was calculated as the ratio of the hatched larvae to the number of viable embryos 24 h after egg collection. The survival 7 days after egg collection was estimated as the ratio of live larvae to the number of hatched larvae. Determination of survival of a specific developmental stage was done having as a denominator the live individuals from the previous stage to avoid masking effects and to have a more independent evaluation (Mylonas *et al.*, 1992; Mylonas *et al.*, 2004).

Plasma sex steroid analysis

Using slightly modified enzyme-linked immunosorbent assays (ELISAs) (Cuisset *et al.*, 1994; Nash *et al.*, 2000; Rodríguez *et al.*, 2000), the plasma concentrations of testosterone (T), Estradiol (E2), 11-Ketotestosterone (11-KT) and 17,20β-P (17,20β-dihydroxy-4-pregnen-3-one) were quantified. Briefly, before running the samples in the ELISAs, plasma extraction was performed twice, by adding diethyl ether (3 ml) to plasma (300 µl) and vortexing vigorously the solution for 3 minutes (Vibramax 110, Heidolph, Germany). Once separated, the organic phase was transferred to new tubes in which it was dried under a nitrogen stream (React-vap III, Pierce, USA). Eventually, samples were reconstituted in 600 µl of assay buffer.

Histological analysis

Ovarian biopsies, obtained during the evaluation of the reproductive stage, were dehydrated in ethanol of gradually increasing concentration (70-96%), and were embedded in methacrylate resin (Technovit 7100®, Heraeus Kulzer, Germany). Using a microtome (Leica RM 2245, Germany), 3 µm sections were cut and stained with Methylene Blue (Sigma, Germany)/Azure II (Sigma, Germany)/Basic Fuchsin (Polysciences, USA) according to the procedure of Bennett *et al.* (1976). A compound optical microscope (Nikon, Eclipse 50i, Japan), with a digital camera (Jenoptik progress C12 plus, Germany) mounted on top, was used to examine and photograph the sections.

Statistical analysis

Differences in mean oocyte diameters between the two thermal profile groups (SeasT vs CoT) were tested using either a t-test for the initial sampling of all fish, or using a two-way analysis of variance (ANOVA) for the GnRHa-treated selected females during the four weeks of the experiment. Differences in mean plasma sex steroid concentrations, relative fecundity, fertilization success and egg/larval survival between the two groups were tested using a t-test. Differences in spermiation index were tested using the nonparametric Mann-Whitney's U test at the initial sampling of all fish, or the nonparametric Friedman's test for the GnRHa-treated males during the four weeks spawning experiment, followed by Dunn's post-hoc test. Statistical significance was set at $P \leq 0.05$. Normality was tested using Shapiro-Wilk test. Statistical analysis was performed using GraphPad Prism version 8.4.3 for Mac (GraphPad Software, La Jolla California USA, www.graphpad.com). Results are presented as mean ± standard error of the mean (SEM) unless mentioned otherwise.

Results

Reproductive stage after different thermal regimes

The constant thermal regime did not prevent gametogenesis, but it affected negatively oocyte development, since the mean diameter of the largest vitellogenic oocytes of females from the CoT group was significantly lower (520 ± 17 μm) compared to the females from the SeasT group (598 ± 27 μm) (t-test, P = 0.02) (Fig. 2A). Further to a larger diameter, the ovaries of SeasT females were at a more advanced stage of development than those of CoT females, since CoT ovaries had a higher occurrence of oocytes in earlier developmental stages, such as primary (PO), cortical alveoli (CA) and early vitellogenic (eVg) oocytes (Fig. 3). In males, the influence of constant temperature on spermatogenesis was more pronounced, since no milt samples could be collected (Spermiation Index = S0) after stripping males from the CoT group (Fig. 2B). On the contrary, all males from the SeasT group where spermiating, though not all at the same extent, having a significantly higher mean spermiation index compared to the CoT group (Mann-Whitney's U test, P = 0.002).

The plasma levels of T were also significantly higher in SeasT females (0.25 ± 0.01 ng ml^{-1}) compared to CoT females (0.17 ± 0.02 ng ml^{-1}) (t-test, P = 0.02), but no differences were observed in plasma levels of E_2 or 17,20β-P between the females from the two groups (Fig. 4A). No significant differences between the males from the two thermal groups were observed in the mean plasma levels of T, 11-KT or 17,20β-P (Fig. 4B).

Spawning induction after GnRHa injections

In the SeasT females, spawning commenced 2 days after the first GnRHa injection and a second spawn was obtained also the following day, with one female (No 4) spawning with a one-day delay compared to the rest (Fig. 5). On the contrary, not all females from the CoT group spawned after the first GnRHa injection, and spawning was very erratic. Fecundity was also many folds higher in the SeasT females at the first spawning induction.

In subsequent weekly ovarian evaluations, no significant differences in mean diameters of the largest vitellogenic oocytes were observed between the SeasT and CoT females, but a trend of lower values was noted in the CoT females (Fig. 6A). Spawning after the subsequent GnRHa injections was not as consistent in the SeasT females as it was after the first GnRHa injection, while in the CoT females it seemed to occur in more synchrony than before (Fig. 5), though individual fecundity was still markedly less than in the SeasT females. Overall relative fecundity of the SeasT females was significantly (t-test, P = 0.02) and 3-fold higher compared to the CoT group (Fig. 7A), while overall fertilization success was not significantly different (t-test, P = 0.31) between the two thermal groups (data not shown). Embryo survival 24-h after egg collection from SeasT females (77 ± %) was significantly higher (t-test, P = 0.003) compared to the CoT group (35 ± 21%), while no differences between the two thermal regimes were observed for hatching success and larval survival (Fig. 7B).

Sperm production and quality after GnRHa implantation

After the first GnRHa implantation, the males of the CoT group that had a Spermiation Index of S0 started progressively releasing sperm upon stripping, with 50% of males on day 7, 25% on day 14 and 75% on day 21 having a spermiation index of ≥ S2 (Fig. 6B). Still, males from the SeasT group were overall in higher spermiation

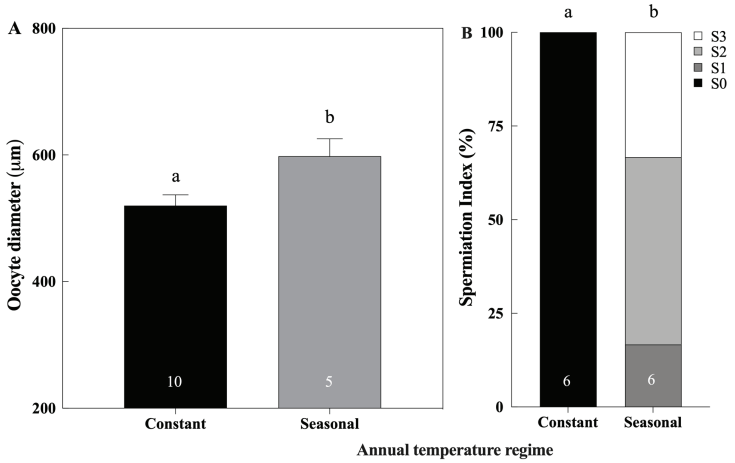

Fig. 2: Mean (± SEM) diameter of the largest vitellogenic oocytes from ovarian biopsies (A) and percentage (%) of males at different spermiation index stages (B) of meagre (*Argyrosomus regius*) breeders exposed to a Constant (CoT) or attenuated Seasonal (SeasT) thermal regime during the year and sampled at the start of the expected spawning period. Lowercase letters above the means indicate significant differences (A. t-test, P = 0.02; B. Mann-Whitney U test, P = 0.002). Numbers inside the bars indicate the N value of the means.

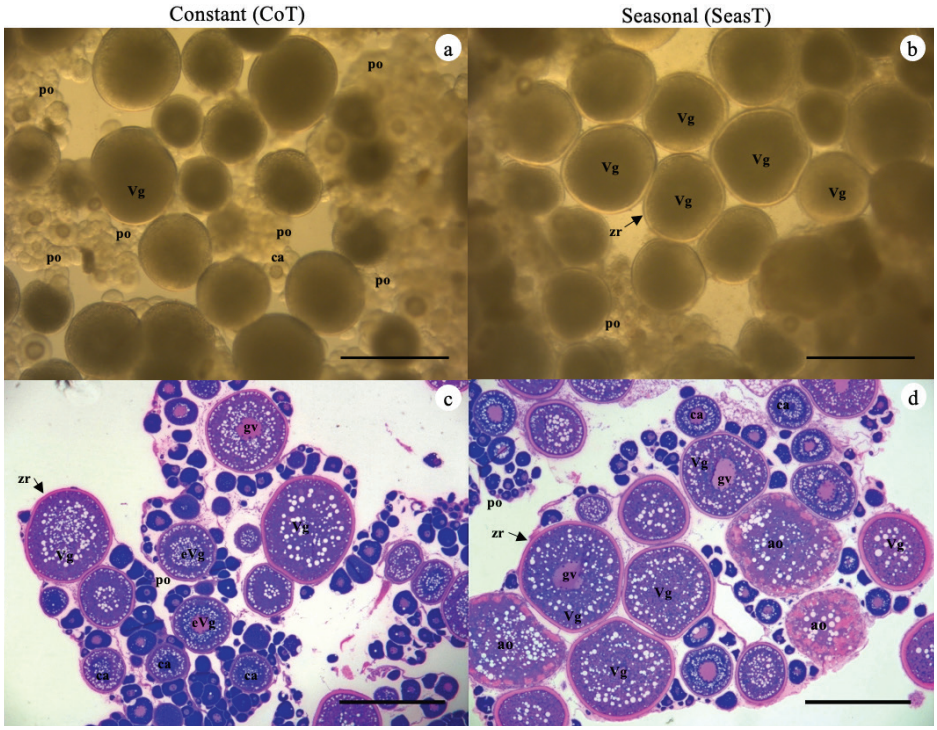

Fig. 3: Photomicrographs of wet mounts (a and b) and histological sections (c and d) of meagre (*Argyrosomus regius*) ovarian biopsies before the first GnRHa injection, from females exposed to a Constant (CoT) thermal regime (a and c) or attenuated Seasonal (SeasT) thermal regime (b and d). po – primary oocytes, Vg – Vitellogenic oocyte, ao – apoptotic oocytes, ca – cortical alveoli, gv – germinal vesicle, zr – zona radiata. Bars represent 500 µm.

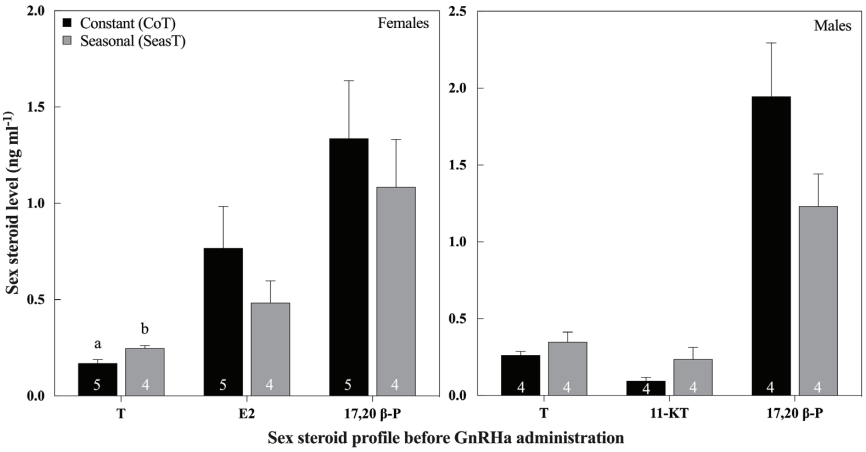

Fig. 4: Mean (± SEM) plasma testosterone (T), 11-ketotestosterone (11-KT), 17β-estradiol (E_2) and 17,20β-dihydroxy-4-pregnen-3-one (17,20β-P) at the onset of the spawning season, in meagre (*Argyrosomus regius*) breeders exposed to a Constant (CoT) or attenuated Seasonal (SeasT) thermal regime. When present, lowercase letters above the means indicate significant differences between thermal regimes (t-test, P = 0.02). Numbers inside the bars indicate the N value of the means.

index compared to their CoT counterparts during all the sampling days (Friedman's test, P = 0.002). Since day 0, all SeasT males released milt, and the percentage of males with an index equal to S3 steadily increased from 25% on day 7 to 75% on day 21. Statistical analysis on sperm quality parameters during the experiment was performed only for SeasT males (one-way ANOVA, P ≤ 0.05), due to the shortage of milt samples from CoT

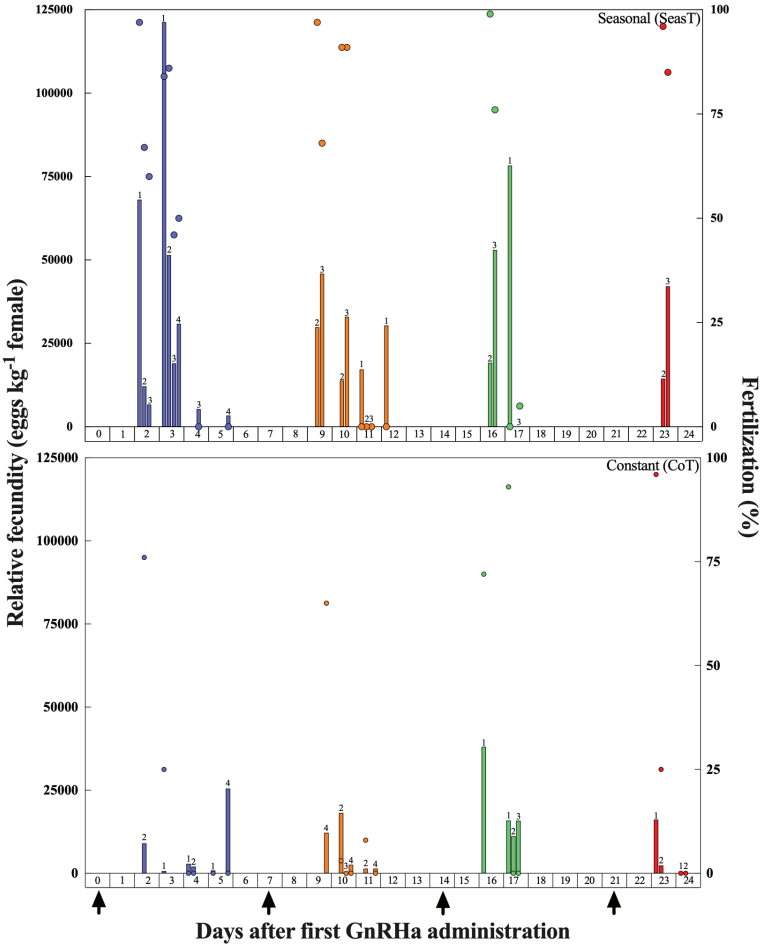

Fig. 5: Daily relative fecundity (bars, eggs kg^{-1} female) and fertilization success (circles, %) of individual meagre females (*Argyrosomus regius*) induced to spawn with multiple GnRHa injections (n = 4, arrows) after exposure to either a Constant (CoT) or attenuated Seasonal (SeasT) thermal regime (n = 4, per thermal regime). Different colors correspond to spawns after consecutive GnRHa injections. Numbers above the bars indicate the ID of the individual female, in order to show which female produced each spawn.

males (Table 1), therefore no statistical comparison could be made between the two thermal regimes. For the SeasT sperm samples, no significant differences were observed in any of the evaluated parameters during the experiment. Sperm density ranged between 10 - 20 x 10^9 szoa ml^{-1}, progressive motility between 43 - 69%, motility duration between 2.2 - 5.0 min, and survival under cold storage between 2.0 - 4.5 days (Table 1). The percentage of progressive and rapid cells evaluated by CASA was higher than 40% for the whole experiment. The VCL ranged between 145-210 µm sec^{-1}, VSL between 109-146 µm sec^{-1}, VAP between 115-170 µm sec^{-1} and STR between 86 - 95% (Table 1).

Discussion

As meagre is one of the emerging species for the aquaculture development in the Mediterranean, the existing knowledge on environmental factors influencing its reproductive success needs to be improved. In order to reduce the energy cost of maintaining broodstocks in aquaculture facilities, previous studies exposing meagre to seasonal photoperiod, but relatively constant temperatures typical of borehole seawater (18-20°C) during the spring, summer and fall did not have any negative effect on gametogenesis in males or females in earlier studies (Mylonas *et al.*, 2015). Based on these results, a very successful spawning induction protocol was proposed (Mylonas *et al.*, 2016), which involved exposing meagre broodstock to low temperatures in the winter time, followed by a gradual increase to reach those typical of

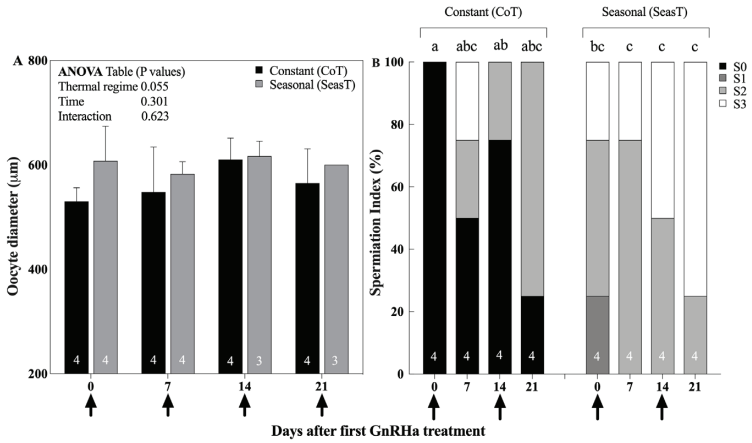

Fig. 6: Mean (± SEM) diameter of the largest vitellogenic oocytes from ovarian biopsies (A) and percentage (%) males at different spermiation index stages (B) of meagre (*Argyrosomus regius*) breeders exposed to a Constant (CoT, n = 4)) or attenuated Seasonal (SeasT, n = 4) thermal regime, and selected for spawning induction. Arrows on the x-axis indicate the time of GnRHa administration (injection in females, implantation in males). Numbers inside the bars indicate the N value of the means. No significant differences were observed during the spawning induction experiment in oocyte diameters, but significant differences in spermiation index among thermal regime/sample time combinations were observed (Friedman's test, Dunn's post hoc, P≤0.05), indicated by different lowercase letters above the bars.

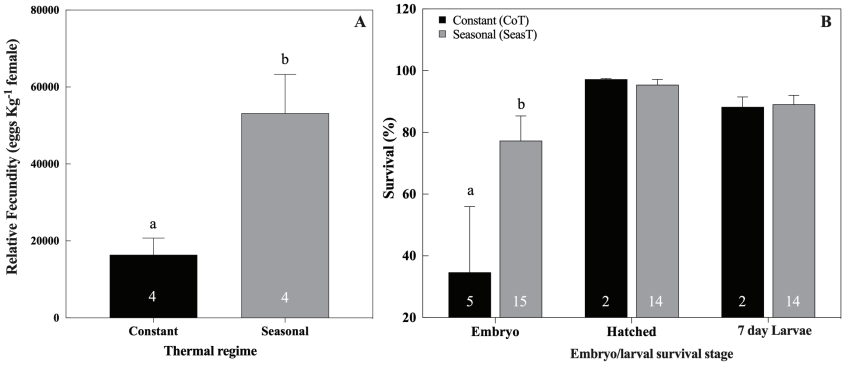

Fig. 7: A. Mean (± SEM) total relative fecundity (eggs kg^{-1} female) of meagre (*Argyrosomus regius*) exposed to Constant (CoT) or attenuated Seasonal (SeasT) thermal regimes (t-test, P = 0.02). B. Mean (± SEM) percentage of embryo viability 24-h after egg collection (t-test, P = 0.003), hatching success and larval survival 7 days after egg collection. When present, lowercase letters above the means indicate significant differences between thermal regimes. Numbers inside the bars indicate the N value of the means.

spring (18-20°C) and then inducing spawning, with up to 17 weekly injections of GnRHa in females and GnRHa implantation every 3 weeks in males. This protocol resulted in the production of eggs of high fecundity and quality, as well as the necessary amount and quality of milt to ensure high fertilization success (Mylonas *et al.*, 2015; Mylonas *et al.*, 2016; Fakriadis *et al.*, 2020). Extending these previous reports, the present study demonstrated that exposure throughout the year to relatively constant water temperatures typical of borehole water in the Mediterranean, did not prevent gametogenesis in either males or females. However, the results underlined the necessity of at least a winter thermal profile for the proper progression and completion of the gametogenic process. Therefore, maintaining broodstocks on seasonal photoperiod but constant borehole water temperature throughout the year cannot be utilized as a cost-effective method for aquaculture production for meagre.

The ability, however, of meagre -as well as other Mediterranean species (Pavlidis *et al.*, 2001; Papadaki *et al.*, 2018)- to undergo gametogenesis to a great extent responding only to photoperiod cues provides interesting information on the relative importance of temperature in controlling reproductive development in fishes in the temperate zone. At the onset of the spawning period, meagre females from the CoT group were in full vitellogenesis,

Table 1. Mean (± SEM) values of sperm quality parameters of meagre (*Argyrosomus regius*) exposed to a Constant (CoT) or attenuated Seasonal (SeasT) thermal regime and sampled at the start of the spawning period and the onset of the GnRHa spawning induction experiment (day 0) and at weekly intervals thereafter. Fish were implanted with GnRHa at days 0 and 14, and were allowed to spawn with females induced with weekly injections of GnRHa. Sperm could be collected only from males with Spermiation Index of >S2 (see Materials and Methods).

		Sperm and Computer Assisted Sperm Analysis (CASA) Parameters									
Day	**n**	**Density [1] (10^9 szoa ml^{-1})**	**Motility [1] (%)**	**Duration [1] (min)**	**Survival [1] (days)**	**Progres-sive [2] (%)**	**Rapid [2] (%)**	**VCL [2] (%)**	**VSL [2] (µm s^{-1})**	**VAP [2] (µm s^{-1})**	**STR [2] (%)**
Constant (CoT) Thermal Regime											
0	0	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a
7	2	19.6 ± 3.9	62 ± 6.6	2.8 ± 0.5	2.5 ± 1.5	53 ± 1.0	58 ± 6.1	171 ± 24.7	132 ± 9.8	142 ± 16.6	94 ± 4.0
14	1	14.2 ± 10.0	94	5	4	69.8	92.2	210.3	145.6	170.5	85.8
21	2	10.2 ± 1.0	52 ± 41.8	3.4 ± 2.1	3.0 ± 1.0	43 ± 35.7	47 ± 44.5	145 ± 46.0	110 ± 46.2	117 ± 52.4	95 ± 3.2
Seasonal (SeasT) Thermal Regime [3]											
0	3	19.0 ± 5.3	63 ± 4.8	2.2 ± 0.5	2.0 ± 0.0	47 ± 4.0	5 ± 6.5	162 ± 17.7	111 ± 16.8	124 ± 18.8	90 ± 0.3
7	4	13.3 ± 3.8	78 ± 8.7	5.8 ± 1.2	2.8 ± 0.8	66 ± 5.7	74 ± 9.4	180 ± 16.2	144 ± 12.6	154 ± 15.5	93 ± 1.6
14	4	13.7 ± 2.8	75 ± 6.5	4.0 ± 1.3	3.0 ± 0.6	62 ± 7.0	65 ± 8.0	153 ± 8.0	105 ± 11.5	115 ± 10.7	91 ± 2.2
21	4	15.4 ± 5.1	82 ± 6.8	4.3 ± 1.3	4.5 ± 0.5	66 ± 5.3	74 ± 10.4	186 ± 16.8	143 ± 17.5	157 ± 18.0	91 ± 2.0

[1] The sperm parameters analyzed were density (Density, number of spermatozoa ml^{-1} of milt), forward motility (Motility, %), duration of forward motility (Duration, min), and survival of spermatozoa stored at 4° C (Survival, days).
[2] The CASA parameters analyzed were the percentage of spermatozoa with progressive movement (Progressive, %) and rapid movement (Rapid, %), curvilinear velocity (VCL), straight-line velocity (VSL), average path velocity (VAP) (µm sec^{-1}), and straightness of spermatozoa movement (STR, %).
[3] Statistical analysis was performed only for data from the SeasT group, since very few males from the CoT group produced collectable milt (Spermiation Index ≤S1). No significant differences were observed among sampling times (one-way ANOVA, $P \leq 0.05$).

but their ovaries had high occurrence of early developmental stage oocytes and the mean diameter of the largest vitellogenic oocytes had not reached the required minimum (550 μm) for the females to be successfully induced to spawn (Duncan *et al.*, 2012; Mylonas *et al.*, 2013a; Mylonas *et al.*, 2016; Duncan *et al.*, 2018). Similarly, males from the CoT group did not produce releasable milt at this time, but they did spawn and fertilized eggs 2 days after induction with GnRHa implants, suggesting that spermiation was also completed, but not to the same extent as males exposed to the SeasT thermal regime. So, it appeared that the lack of winter temperature delayed or prevented the full progression of gametogenesis in meagre. Negative effects of inappropriate thermal conditions on gametogenesis and spawning success have been observed also in other teleosts, such as the common wolffish (*Anarhichas lupus*) (Tveiten & Johnsen, 1999), the Eurasian perch (*Perca fluviatilis*) (Migaud *et al.*, 2002), the yellow perch (*Perca flavescens*) (Shewmon *et al.*, 2007), the pikeperch (*Sander lucioperca*) (Zakęś, 2007) the European sea bass (*Dicentrarchus labrax*) (Carrillo *et al.*, 1995) and the striped bass (*Morone saxatilis*) (Clark *et al.*, 2005), which need to be exposed to a seasonal thermal regime simulating the winter season, in order to fully achieve vitellogenesis. Apparently, as shown in this and earlier studies of meagre (Mylonas *et al.*, 2013b; Mylonas *et al.*, 2013a; Mylonas *et al.*, 2015; Mylonas *et al.*, 2016), removing the summer from the thermal regime does not affect gametogenesis at all, but removing the winter has pronounced negative effects.

The fact that gametogenesis in meagre under a constant thermal regime was completed to a great extent in the present study, was also reflected in the lack of dramatic differences in plasma sex steroid hormones from the SeasT regime at the expected time of spawning, when GnRHa induction was implemented. The plasma levels measured were generally low in both sexes and thermal regimes, as observed previously in captivity (Mylonas *et al.*, 2013a). Group-synchronous species, such as meagre, possess simultaneously oocytes at different developmental stages during the spawning season, and as a result plasma sex steroid levels do not exhibit dramatic differences among them and over time, as they are all required to support the process of vitellogenesis at the same time that oocyte maturation and ovulation take place. The slightly reduced T levels observed in the CoT females may be explained by a possible disruption of the sex steroid pathway caused by the warmer water during the gametogenesis period, as shown in studies with Atlantic salmon (*Salmo salar*) (Pankhurst *et al.*, 2011), pikeperch (Hermelink *et al.*, 2011) and Atlantic halibut (*Hippoglossus hipoglossus*) (van Nes & Andersen, 2006). Although it has been reported that both expression and activity of the gonadal aromatase (Cyp19a1a) gene may be inhibited by high-temperature exposure (Anderson *et al.*, 2012; Elisio *et al.*, 2012), in the present study plasma E_2 did not differ between females of the two thermal regimes, again in support of the lack of an absolute requirement of a seasonal thermal regime in the reproductive cycle of meagre.

Similarly, in males the negative influence -but not prevention- of the constant thermal regime on spermatogenesis was not reflected by the similar levels of the measured sex steroid hormones. In other fishes, when a warmer than optimal temperature was applied for a prolonged period, a reduction or inhibition of spermatogenesis or spermiation was observed (Vikingstad *et al.*, 2016; Fenkes *et al.*, 2017; Hani *et al.*, 2019). The hormonal levels of males from both thermal groups were found to be similar to those reported in other studies of meagre in captivity (Mylonas *et al.*, 2013a; Fakriadis *et al.*, 2020). This was surprising, since the failure to express milt from the CoT males prior to the GnRHa therapy was expected to be associated with lower plasma levels of 11-KT and/or 17,20β-P, as it has been amply demonstrated that these hormones are directly responsible for spermiation and the testicular hydration associated with the increase in releasable milt during the spawning season (Schulz *et al.*, 2010).

Perhaps expectedly, the negative consequences of exposure to the constant thermal regime during the winter season were not limited to the maturation stage of both sexes at the onset of the spawning period. In fact, the negative effects extended also during the spawning season in late spring (May-June) -when both treatments were exposed to the same temperature- compromising the final reproductive output. Previous studies on meagre demonstrated that using weekly GnRHa injections can improve vitellogenesis, favor the maturation of new batches of vitellogenic oocytes, and allow multiple spawning with eggs of high quality (Fernández-Palacios *et al.*, 2014; Mylonas *et al.*, 2016). Although after the first GnRHa injection the mean diameter of the largest vitellogenic oocytes increased in the CoT females and did not differ significantly from the SeasT females, there was a clear tendency for smaller oocytes throughout the spawning induction experiment. Similar results were found in the spiny chromis (*Acanthochromis polyacanthus*), where higher temperature decreased the ability of the fish to undergo proper vitellogenesis, and in the Atlantic salmon, where higher temperature led to a large imbalance in the size of the oocytes (Donelson *et al.*, 2010; Vikingstad *et al.*, 2016). Spawning kinetics and egg production of the SeasT females were more consistent to earlier studies (Mylonas *et al.*, 2015; Mylonas *et al.*, 2016; Duncan *et al.*, 2018), although they differed from the expected two spawns per female on Days 2 and 3 after each GnRHa injection. On the contrary, CoT females spawned erratically, producing significantly and markedly less eggs, albeit of similar fertilization success to the SeasT females who experienced the low temperatures during the winter season. Additionally, although the mean fertilization success of fertilized spawns was not statistically different between the two thermal groups, in more than 50% of the spawns obtained from the CoT females, the eggs were not fertilized at all. Exposing fish during gametogenesis to a higher than optimal temperature had comparable effects on the relative fecundity of Atlantic salmon and river lamprey (*Lampetra fluvialis*) exposed to 22 and 10°C, respectively (Pankhurst *et al.*, 2011; Cejko *et al.*, 2016). Therefore, although the repeated GnRHa injections were

successful in inducing further vitellogenesis to some extent in some CoT females, the initial reproductive condition of the females due to their exposure to a constant thermal regime over the whole year had a dramatic negative influence on their reproductive performance.

Embryo development and survival to hatching was significantly and drastically reduced in the eggs obtained from the constant thermal regime, while embryos from the SeasT group had a high survival, comparable to a previous study (Mylonas *et al.*, 2016). Embryo mortality was also decreased significantly in common wolfish eggs obtained from females exposed to higher than optimal water temperature (Tveiten & Johnsen, 1999). Reduced survival at this stage could be related with altered intake of phospholipids and free fatty acids during vitellogenesis, as was observed in females of Arctic charr (*Salvelinus alpinus*) and brown trout (*Salmo trutta*) exposed to higher temperatures (Jobling *et al.*, 1995; Lahnsteiner & Leitner, 2013). Therefore, it is obvious that the overall egg production and quality from meagre females exposed to constant temperature was inadequate for profitable production in a commercial hatchery.

Regarding the males, the administration of two GnRHa implants improved steadily the spermiation index of individuals from the SeasT group, and milt could be collected and analyzed from almost all males at all sampling times. A similar, but much less pronounced effect was observed in GnRHa treated males from the CoT group. This demonstrates that the fish had the capacity to respond to the GnRHa stimulation -and the expected increase in plasma Luteinizing Hormone (LH) and sex steroid production (Mylonas *et al.*, 2017)- but their initial stage of reproductive development was such that spermiation and releasable sperm production was reduced. A significantly lower sperm production was probably one of the reasons that many spawns from CoT females were not fertilized at all, as mentioned above.

Unfortunately, it was not possible to make any sperm quality comparisons between the two thermal groups due to the limited availability of releasable milt from CoT males. The milt obtained from the SeasT groups showed comparable or higher values with other studies with meagre under similar environmental conditions. For example, the percentage of motile spermatozoa from SeasT males was between 62 and 81%, which is similar to values between 80% and 90% (Mylonas *et al.*, 2013a), 73% (Santos *et al.*, 2018) or between 53 and 74% (Schiavone *et al.*, 2012). The VCL in the present study was higher than the of 140.90 ± 7.75 μm/s reported when testing sperm extenders for cryopreservation (Santos *et al.*, 2018) or *in vitro* fertilization (Ramos-Júdez *et al.*, 2019). In the latter study VAP values ~90 μm sec^{-1} were considered satisfactory for high fertilization success, and in the present study even higher values were recorded. As has been reported for other species, such as the gilthead seabream (*Sparus aurata*) (Beirão *et al.*, 2011), the percentage of motile cells and VCL are of critical importance for fertilization success. The sperm characteristics during the study did not change, suggesting that the GnRHa enhanced milt production without affecting sperm quality, either positively or negatively, which is commonly the effect of GnRHa implants in a number of teleosts (Mylonas *et al.*, 2017).

In conclusion, although exposure throughout the year to relatively constant water temperatures did not prevent gametogenesis in either males or females, the present study demonstrated that a winter thermal profile is necessary for the proper progression and completion of the gametogenic process in meagre, in order to achieve the high reproductive performance required for successful industrial egg production. The results also point to the potential negative effects of global warming in the future reproduction of meagre in the wild.

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