Paired spawning with male rotation of meagre *Argyrosomus regius* using GnRHa injections, as a method for producing multiple families for breeding selection programs

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Highlights

- The most in-depth analysis to-date of paired spawning with male rotation for producing multiple families for fish genetic selection breeding programs.
- An advantage of paired spawning with male rotation was the production of large fecundity spawns of many different meagre (*Argyrosomus regius*) families.
- A disadvantage of paired spawning with male rotation was that many meagre females only contributed to three families.
- Meagre males produced more families than females and this appeared to be related to behavioural differences between sexes.

Abstract

Weekly gonadotropin-releasing hormone agonist (GnRHa) injections were used to induce spawning in paired male and female meagre (*Argyrosomus regius*) with a weekly rotation of the males, in order to produce a large number of families, as a method to facilitate selective breeding programs. Two different broodstocks were used (HCMR and IRTA), with females of mean weights of 11.7 ± 2.6 kg and 20.0 ± 1.8 kg, and males of 10.2 ± 1.2 kg and 15.1 ± 1.0 kg, respectively. A single GnRHa injection of 15 μg kg^{-1} was administered to each selected female, and 7.5 or 15 μg kg^{-1} to each male to induce spawning. In the subsequent weeks, maturity was checked and fish were induced as above, but males (n=18) were rotated to form a different pair with the selected females (n=21). Experiments finished when all paired combinations had been completed or a fish lost maturity status and could not be induced further. A total of 56 families were produced with a mean number of eggs from each family of 87,666 ± 11,244 eggs kg^{-1}. There was a decline in the fecundity, number of spawns and percentage of pairs that spawned successfully after consecutive weekly GnRHa injections. Relative fecundity declined significantly from 134,495 ± 25,557 eggs kg^{-1} female body weight after the first injection, to 44,252 ± 17,638 eggs kg^{-1} after the fourth injection. However, there were no differences amongst weeks in egg fertilization success, hatching success or larval survival to 5 days post hatch. The decrease in fecundity and spawning success was attributed to a loss of maturity observed in the females, which may be related to differences in mate selection strategies between male and female meagre. The study demonstrated that paired spawning with male rotation was a successful method that can be used for breeding programs to produce a limit of three families per female or as a scaling up step to produce large numbers of offspring from a limited number of selected pairs.

Keywords: *Argyrosomus regius*, meagre, reproduction, induced spawning, GnRHa, egg quality.

1. Introduction

The aquaculture production of meagre (*Argyrosomus regius*, Sciaenidae) has increased rapidly in the last decade from 859 t in 2004 to 11,770 t in 2014 (FAO 2005-2017). This increase has been in part due to the development of effective spawning induction methods (Duncan et al., 2012; Duncan et al., 2013a; Fernández-Palacios et al., 2014; Mylonas et al., 2013a; 2015; 2016), since meagre rarely undergo spontaneous oocyte maturation, ovulation and spawning in captivity (Duncan et al., 2013a; Gil et al., 2013; Mylonas et al., 2013b; Soares et al., 2015). Both liquid injections and controlled-release delivery systems that release gonadotropin-releasing hormone agonist (GnRHa) for a prolonged period of time have been shown to be effective in inducing maturation and multiple spawns in females (Duncan et al., 2012; Duncan et al., 2013a; Fernández-Palacios et al., 2014; Mylonas et al., 2013a; 2015; 2016). The differences in spawning kinetics and production characteristics showed that multiple GnRHa injections resulted in more consistent spawning results and better control of egg production than GnRHa implants, and this method offered significant advantages to control reproduction for commercial aquaculture production (Mylonas et al., 2015; 2016).

However, these methods need to be modified to give the reproductive control required for breeding programs. Commercial meagre producers have identified that a major bottleneck to the expansion of the industry is that broodstocks have been acquired from a limited number of sources (personal communications). A recent study on a wide number of broodstocks in the framework of the EU project DIVERSIFY (www.diversifyfish.eu) confirmed that the broodstocks being used in aquaculture have originated from only three different wild populations (Estévez et al., 2015). Although adequate genetic variation exists in these broodstocks, care is required with breeding programs to ensure variation is not lost, resulting in negative impacts on desired traits. The control of reproduction is an

82 essential part to a genetic breeding program (Duncan et al., 2013b) and is required both to ensure that selected broodstocks with desired traits can be bred together, as well as later to
84 scale up the production of large numbers of fertilized eggs and juveniles from broodstocks with the selected traits.

86 Tank spawning in pairs is one way to create families in breeding selection programs, since artificial "strip" spawning is a complicated operation. However, some marine species such
88 as gilthead seabream (*Sparus aurata*) and European seabass (*Dicentrarchus labrax*) do not spawn in pairs. For example, gilthead seabream spawning success was low when held in
90 pairs (Gorshkov et al., 1997; personal observation) or groups of 15 females with a single male (Gorshkov et al., 1997). However, this does not appear to be a problem in the
92 reproductive function of meagre. Duncan et al. (2012) used parentage analysis of larvae to demonstrate that some entire spawns collected from a small broodstock were actually the
94 offspring of a single pair. Mylonas et al. (2015; 2016) confirmed that paired spawning was possible when isolated single pairs were successfully induced to spawn up to 17 times on
96 a weekly basis. However, it remains to be determined if many families can be produced by changing the pairing of the male and female to produce a different family with each induced
98 spawning.

The objective of the present work was to examine the possibility of using the multiple
100 GnRHa injection method for inducing spawning in paired breeders, with a weekly rotation of the males, in order to produce a large number of families for a selective breeding
102 program. Specifically, the study examined if females would continue spawning in response to consecutive, weekly GnRHa injections, if their male partner was changed every week.

104

2. Materials and Methods

106 Experiments to induce spawning with male rotation were made in the facilities of the Institute of Marine Biology, Biotechnology and Aquaculture (previously Institute of
108 Aquaculture) of the Hellenic Centre for Marine Research (HCMR), Iraklion, Crete, Greece during 2015 and IRTA, Sant Carles de la Rapita, Spain during 2014 and 2015.

110

2.1. Broodstock maintenance

112 All work and maintenance of broodstocks was in agreement with European regulations on animal welfare (Federation of Laboratory Animal Science Associations, FELASA,
114 http://www.felasa.eu/).

Broodstocks at the HCMR facilities came from eggs produced in the hatchery in 2004,
116 2006 and 2007. Fish were fed 5 days per week to apparent satiation with industrial feed (Skretting S.A., Spain and IRIDA, S.A., Greece). During the year and outside the period
118 of the experiments, fish were maintained in a large communal tank (10 m^3) exposed to a simulated natural photo-thermal regime. Measurements of temperature and water quality
120 (dissolved oxygen, NH_3-N and NO_2-N) were conducted once per week throughout the year. For spawning induction, single pairs of fish (one male and one female) were transferred to
122 5,000-l Recirculation Aquaculture Systems (ACE, the Netherlands) supplied with seawater from a well, under simulated natural photoperiod, but controlled temperature of 19.3 ±
124 0.1°C during the induced spawning experiment. The maintenance of constant temperature was chosen based on previous experiments with meagre, showing that maintaining the
126 temperature at spring levels (19-20°C) resulted in fish maintaining vitellogenesis, and spermatogenesis and sperm production (Mylonas et al., 2016).

128 Broodstocks in IRTA were a stock of mixed wild and cultured origin, which were brought to the installation in 2008. The fish were fed Monday, Wednesday and Friday with a

broodstock diet (Vitalis Cal, Skretting S.A., Spain) and either sardines or squid to apparent satiation. During the year outside the period of the spawning induction experiments, fish were held in communal tanks, either a D-ended raceway (50 m^3) or circular tank (6 m x 3 m, 60 m^3), under natural day light and simulated natural temperature (16-25 ºC). For spawning induction, single pairs of fish (one male and one female) were transferred to 10,000-l tanks under natural photoperiod and controlled temperature of 18.7 ± 0.1 °C. All tanks used were in recirculation systems (IRTAMAR®) that controlled and registered temperature, oxygen and flow (+400% water exchange of tanks and 10-20% daily water renewal).

2.2. Broodstock selection

To select the breeders for the spawning experiments, fish were starved 2 days prior to handling. For handling, the fish were sedated using two approaches: in HCMR, fish were tranquilized initially in their tank with the use of clove oil (0.01 ml l^{-1}) and then transferred to an anesthetic bath for complete sedation with a higher concentration of clove oil (0.03 ml l^{-1}) (Mylonas et al., 2005), while in IRTA, fish were caught fully awake from the holding tank and sedated completely in a 400-l anesthetic bath of MS222 (70 mg l^{-1}) (Duncan et al., 2012). Ovarian biopsies for the evaluation of oocyte development were obtained by inserting a plastic cannula (Pipelle de Cornier, Laboratoire CCD, France or Izasa Hopsital, Barcelona, Spain) and applying gentle aspiration. A wet mount of the biopsy was first examined under a compound microscope (40 and 100X) to evaluate the stage of oogenesis and measure the mean diameter of the largest, most advanced batch of vitellogenic oocytes (n=10-20). Females were considered suitable for spawning induction if they contained oocytes in full vitellogenesis with a diameter of >550 µm and very little atresia/apoptosis present (Duncan et al., 2012; Mylonas et al., 2013a). Male fish were considered suitable

for spawning induction, if they were in full spermiation, releasing substantial amounts of sperm upon application of gentle abdominal pressure (Mylonas et al., 2016).

2.3. Spawning induction experiments

The induction experiments with male rotation began on a Monday in week 1, when mature males and females were selected from the stock. After selection, a GnRHa injection was applied to each fish and the pair was formed by placing a male and a female together in a spawning tank, where the pair was left to spawn for a week. On the next Monday and after each subsequent week the maturity status of each breeder was determined, a GnRHa injection was applied and the females were returned to the same tank, whilst males were paired with a different female in a different tank to form a different pair. An injection of 15 μg kg^{-1} GnRHa was administered to induce female spawning (both years) and male spawning in 2015, while in 2014 injections of 7.5 μg kg^{-1} GnRHa were administered to induce male spawning. This procedure was continued until all paired combinations had been completed or fish were found not to be suitable for induction. Females were unsuitable if their ovarian biopsies demonstrated an absence of vitellogenic oocytes >550 μm in diameter and/or the occurrence of extensive apoptosis. Males were considered unsuitable if they were not releasing sperm upon application of abdominal pressure.

In HCMR, four females and four males were used. The females had mean ± SD body weight 11.7 ± 2.6 kg, and the males 10.2 ± 1.2 kg. The inductions were made in the period between 4 and 25 May 2015. After this period of 4 weeks, all paired combinations had been made and the experiment ended.

In IRTA, 9 females and 7 males (respective mean body weights of 22.2 ± 1.4 kg and 14.9 ± 1.0 kg) were used in 2014 (7 April to 2 June) and 8 females and 7 males (respective mean

body weights of 17.8 ± 2.1 kg, and 15.3 ± 1.0 kg) were used in 2015 (4 May to 29 June). Some of the same fish were used both years. The spawning induction and male rotation was continued until a fish was found to be unsuitable for induction. In this case, a different suitable fish was selected from the stock tank and the series was continued. When both a new male and female were selected, a new series of induced spawnings with male rotation was initiated.

2.4. Evaluation of egg/larval quality

A passive egg collector was placed in the outflow of each spawning tank, in order to collect the spawned eggs. Eggs were collected every morning (~12 h after spawning) into a 10-l bucket and their number (fecundity) was estimated by counting the total number of eggs in a 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 50+ eggs in this 5 or 10 ml sample for the presence of a viable embryo (usually at the blastula stage) using a stereoscope.

To monitor embryo and larval survival, eggs from each spawn were placed individually in 96-well microtiter plates (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 transferred to the wells of the microtiter plates (one egg per well). The microtiter plates were then covered with a plastic lid, placed in a controlled-temperature incubator and maintained for 5 days at 19 ± 0.5ºC (HCMR) or 18

± 0.5ºC (IRTA). Using a stereoscope, embryonic and early larval development was evaluated once a day for 5 days. The number of (a) live embryos was recorded 1 day after egg collection (or ~36 h after spawning, day 1), (b) hatched larvae was recorded 2 and 3 days after egg collection (>60 h after spawning) and (c) viable larvae was recorded 4 and 5 days after egg collection (~ yolk sack absorption). For reference, hatching of meagre eggs takes place in 44-56 h at 18-20°C.

Embryo survival was calculated as the number of eggs having live embryos 1 day after egg collection / number of fertilized eggs initially loaded in the microtiter plates. Hatching success was calculated as the number of hatched larvae / the number of live 1-d embryos, and 5-d larval survival was calculated as the number of live larvae 5 days after egg collection / the number of hatched larvae. Estimating percentage survival (%) by using in the denominator the number of individuals that survived to 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., 2004).

2.7. Statistical analysis

The relative fecundity from each weekly GnRHa injection amongst the different pairs was not normally distributed and had a highly positive skew in the distribution caused by a few highly fecund fish. The data set was normalized with a square root of the square root (double square root) transformation. Differences in mean relative fecundity among GnRHa injections (weeks) for females and males were examined using one-way ANOVA at a minimum $P \leq 0.05$, followed by Duncan's Multiple Range test at $P \leq 0.05$, when appropriate. The egg performance parameters (fertilization success, hatching and 5-d larval

survival) were not normally distributed. The egg performance parameters were highly negatively skewed by a few poor batches of eggs and transformations did not normalize the data. Differences amongst egg performance parameters per GnRHa injection were examined using Kruskal-Wallis one-way ANOVA on ranks at a minimum $P \leq 0.05$, followed by DUNNS multiple comparison test at $P \leq 0.05$, when appropriate. The distributions of the number of spawns from each pair per weekly GnRHa injection were compared with the Chi squared test. Only spawns after the 1st to 4th GnRHa injection, and pairs that spawned 1, 2 and 3 times were included in the Chi squared analysis. The number of spawns after the 5th, 6th or 7th GnRHa injection or pairs that spawned 4 times were too low to be included in a Chi squared analysis. All analyses were performed with SigmaPlot (version 12, Systat Software, Inc., San Jose California USA, www.systatsoftware.com). Results are presented as mean ± SEM, unless otherwise mentioned.

3. Results

A total of 56 families were produced from different pairs formed by rotating the 18 selected males with the 21 selected females. However, during the study a number of pairs did not successfully spawn to form a family. Two pairs failed to spawn after the 1st GnRHa injection, but subsequently all four breeders spawned after the 2nd injection, when they were paired with different individuals. As the experiments progressed, and particularly after the 3rd and 4th GnRHa injection, a large number of pairs did not spawn and females were found not to be suitable for induction (absence of vitellogenic oocytes >550 μm, see Fig. 1). These unsuitable females were removed from the experiments and this caused some uneven pairing where for example a female being induced for the 1st time was paired with a male being induced for the 4th time. As a consequence of this uneven pairing, there were a maximum of five consecutive weekly GnRHa treatments in the females, whereas there

were a maximum of seven consecutive weekly GnRHa in the males. The data on spawning performance was examined both in relation to female and male participation.

3.1 Female participation in spawning

From the 21 selected females, a total of 56 families were produced with a mean number of eggs from each family of 87,666 ± 11,244 eggs kg^{-1}. There was a significant decline ($P = 0.016$, power was 0.64, with alpha = 0.05) in the relative fecundity of the spawning pairs with each consecutive GnRHa injection administered to the females, from 134,495 ± 25,557 eggs kg^{-1} after the 1st injection to 44,252±17,638 eggs kg^{-1} after the 4th injection (Fig. 2a). The decline in relative fecundity was in part related to a decline in the number of spawns per pair obtained after each weekly GnRHa injection. Whereas after the first 2 injections usually 2-3 daily spawns were obtained, later weekly GnRHa injections usually produced only a single spawn. The frequency of the number of spawns per female changed significantly ($P < 0.001$) with each GnRHa injection (Fig. 2b). After the 1st injection, most pairs spawned 3 times and the number of spawns per pair declined until the 3rd and 4th injections, when most fish over the two injections spawned once. The decline in relative fecundity and number of spawns appeared to be related also to a loss of maturity (or spawning induction suitability) status. A total of 17 females (from 21) lost advanced stages of maturity after a few weekly GnRHa injections during the experiments and either no spawning was obtained in response to the last GnRHa injection, or the females did not have large vitellogenic oocytes >550 μm in diameter and no further induced spawning could be attempted. The increasing number of females losing advanced maturity stage with increasing number of weekly GnRHa injections was evident also in the decline in the number of spawning pairs from 90% of planned pairs spawning successfully after the 1st injection to 29% after the 4th injection (Fig. 2c). Only three females still maintained their

maturity stage after four weekly GnRHa injections, though only one female was administered a 5th GnRHa injection (Fig. 2c), since the remaining two females had completed all combinations of pairs planned in the HCMR experiment (4 females x 4 males).

3.2 Male participation in spawning

The influence of males on the spawning and egg production parameters in relation to the number of weekly GnRHa injections received exhibited a less pronounced declining trend (Fig. 3). There was no significant decline in relative fecundity associated to the males, varying from 142,690 ± 30,198 eggs kg^{-1} after the 1st injection to 53,051 ± 15,905 eggs kg^{-1} after the 5th injection (Fig. 3a). It should be mentioned that due to the high variability and lower "n" per injection the power of the test was low (power was 0.243 with alpha = 0.05) making the detection of a difference difficult. The frequency in the number of spawns per male changed significantly (P = 0.01) with each weekly GnRHa injection (Fig. 3b), but the significance of the change was lower than observed in association with the females. After the 1st injection most pairs (9 from 15) spawned 3 times and this changed significantly to predominantly 1 or 2 spawns after the subsequent weekly injections. After the 4th injection four fish spawned once and three fish spawned twice (Fig. 3b). The male-related decline in the number of spawning pairs was also less pronounced compared to females, declining from 88% after the 1st injection to 39% after the 4th weekly injection and 6% after the 7th weekly injection (Fig. 3c). The decline in spawning parameters observed in association with the males appeared to be related to the decline in maturity observed in the females. As indicated above, most females had lost advanced stages of maturity by the 4th weekly induction and just three out of 21 females had advanced stages of vitellogenesis when examined after the 4th GnRHa injection (week). In comparison, almost all males (17 out of

18) were in full spermiation, releasing substantial amounts of sperm upon application of gentle abdominal pressure throughout the experiment, until all possible combinations had been completed. The combination of the males maintaining an advanced maturity status and there being less males than females resulted in individual males being used to make more paired combinations than females. Therefore, when inductions could not continue with a female and a new female was selected, the same male that had already completed inductions with other females was used, since new males were not available to pair with the new females. In this way, some females being induced for the first time were paired with males being induced for the 3rd, 4th or 5th week and consequently the spawning parameters from these inductions reflected more the characteristics of a 1st injection of a female. For example, calculating the mean from three pairs when a male being induced for the 3rd or 4th week was paired with a female being induced for the first time gave a mean of 98,005 ± 45,618 eggs kg^{-1} and 2.0 ± 0.4 spawns per induction, which was similar to other females injected for the first time and paired with a male that was injected for the first time. Another observation that supports the view that the decline in spawning performance depended principally on the females was that on four different occasions a male with flowing sperm was paired with a suitable female and after a GnRHa injection no spawn was obtained. Upon examination after the week with no spawning, the males continued to spermiate well, whilst the females became unsuitable (*i.e.* did not have vitellogenic oocytes >550 μm). Once these four males were paired with a different suitable female, the new pairs spawned successfully, confirming that the cause of the previous spawning failure was principally due to female failure.

3.3 Egg quality

There were no significant differences in mean egg quality parameters in relation to the different weekly GnRHa injections either in association to females (Fig. 4) or males (Fig. 5). Considering all spawns collected, the mean fertilization success was 88 ± 2.0%, hatching was 66 ± 3.8% and larval survival over 5 days after hatching was 71 ± 3.1%.

4. Discussion

The study demonstrated that paired spawning with male rotation is a suitable method to mate selected males and females to produce a large number of families with high number of good quality eggs for each family. From 21 females and 18 males, a total of 56 families were produced with a mean number of 87,666 ± 11,244 eggs kg^{-1} female body weight per family. Therefore, paired tank spawning with male rotation of meagre is possible for the production of multiple families from parents with known phenotypes and can be used in a breeding program to both produce a number of desired families or to scale up production of a large number of fertilized eggs and juveniles with desired phenotypes. The fact that paired spawning is possible in meagre confirms previous indications from communal spawning that paired spawning may be a natural phenomenon, even when many males and females are maintained together. For example, Duncan et al. (2012) demonstrated, using microsatellite paternity assignment, that the eggs obtained from some daily spawning events from groups of six breeders were from a single pair. Also, Mylonas et al. (2015; 2016) set up pairs of breeders and induced the same pairs to spawn each week for up to a total of 17 weeks. This is different from some marine species being produced in the Mediterranean, which do not spawn when held in isolated pairs, such as gilthead seabream (Gorshkov et al., 1997) and European seabass (unpublished data).

However, in the present study the relative fecundity and spawning success of the different pairs decreased with increasing number of weekly GnRHa injections, contrary to what has been observed in previous studies without male rotation (Mylonas et al., 2015; 2016). The spawning response of the females to the 4th weekly injection was poor (29% of pairs spawned successfully, with a relative fecundity of 44,252±17,638 eggs kg^{-1}) indicating that the method was not reliable beyond three weekly injections and male changes (62% of pairs spawned successfully, with a relative fecundity of 50,301 ± 35,993 eggs kg^{-1}). The decrease in the spawning success of the pairs was attributed to the loss of maturity observed in the females and the absence of more post-vitellogenic oocytes >550 μm in diameter, which is considered as the criterion for successful spawning induction of meagre (Duncan et al., 2012; Mylonas et al., 2013a). Just three females (from 21) exhibited vitellogenic oocytes after the 4th injection, whilst all but one male (from 18) maintained good spermiation throughout the experiment. Compared to females, male fish spawned successfully over more weekly GnRHa injections (6-7 injections) and if needed they could succeed in more weekly spawning inductions. In addition, on four occasions a male was successfully paired and spawned with a new female, after having failed to spawn with a female that had lost maturity after some initial successful weekly spawning inductions. This loss of maturity of the female when males retained the possibility to spawn indicated that the maturity status of the female was the primarily determinant of spawning success and fecundity.

The decrease in fecundity and failed spawning or decline in maturity status represents different spawning kinetics in meagre compared to other studies (Mylonas et al., 2013a; 2015; 2016; Fernández-Palacios et al., 2014), where repeated induced spawning did not result in a reduction in spawning success or fecundity, either in small groups of meagre (Mylonas et al., 2013a; Fernández-Palacios et al., 2014) or isolated pairs (Mylonas et al.,

374 2015; 2016). In these studies, fish were returned each week to the same spawning tank, in the same pairs or groups, after the fish were checked for maturity status and injected with
376 GnRHa. The only difference between the studies of Mylonas et al. (2015; 2016) and the present one was that males were rotated (*i.e.* a different male was paired with each female
378 for each weekly spawning induction) and this appears to have had a negative effect on female maturity status and fecundity. Mylonas et al. (2016) induced isolated pairs each
380 week up to 17 weeks without any decline in maturity status or fecundity. Taken together, the results from all these studies suggest that the use of a new male at each weekly
382 spawning induction may have caused a stress that disrupted oogenesis and the production of more post-vitellogenic oocytes, resulting first in the decline in fecundity, followed by a
384 loss of suitability (*i.e.* existence of oocytes >550 μm) and failure to spawn. Alternatively, the use of an inappropriate male activated a mechanism in the females that inhibited the
386 maturation, ovulation and spawning of the existing post-vitellogenic oocytes, as well as the further progression of vitellogenesis.

388 The loss in female maturity status, reduction in fecundity and the differences in the pattern of spawning success and maturity status between male and female meagre may be related
390 to the spawning behaviour of meagre and different reproductive strategies between males and females. It is very common in the animal kingdom for females with large, energy rich
392 gametes to breed with a few dominant males that are perceived to have higher biological fitness. In contrast, males producing huge numbers of small, low energy gametes aim to
394 mate with as many different females as possible, either through dominance or through "sneaking" type tactics where males join in spawning with dominant fish (Andersson,
396 1994). In fish, mate selection is possible with paired (1 female with 1 male) or group (a few fish, often 1 female with 2-3 males) spawning, which are common reproductive
398 behaviours (Domeier and Colin, 1997). The gilthead seabream is an example of a

Mediterranean marine species that presents paired spawning (Ibarra-Zatarain and Duncan, 2015), which results in females mating with dominant males (Brown et al., 2005; Chavanne et al., 2012; García-Fernández, et al., 2017). Ibarra-Zatarain and Duncan (2015) showed that 72% of spawns in a gilthead sea bream experiment were produced from single pairs, while the remaining spawns were produced from groups of one female with two or three males providing the spawning and courtship behaviour to favor mate selection. Studies on paternity of gilthead sea bream offspring demonstrated that males showed higher variance in contributions to families than females, *i.e.* females spawned with just a few males whilst some dominant males spawn with many females (Brown et al., 2005; Chavanne et al., 2012; García-Fernández, et al., 2017). The present study appeared to indicate similar strategies in meagre, where females did not complete spawning with many males, thus losing maturity after being paired with three males, whilst males were more flexible and could maintain reproductive maturity status and spawn with more females.

Once a large number of families have been obtained, an important consideration for genetic selection is to compare traits between and within families during grow-out. Ideally, a minimum of 25 --and preferably more-- families with the same age and numbers (adjusted due to differing survival rates) are compared in the same rearing conditions (Tave, 1995; Chavanne et al., 2012; Duncan et al., 2013b). Different approaches to obtaining families provide different advantages and constraints to enable these comparisons. Paired spawning with male rotation has a constraint to the number of families that can be produced on a single day. However, spontaneous tank spawning of either large (Brown et al., 2005; Chavanne et al., 2012) or small (García-Fernández, et al., 2017) broodstocks also have constraints in that families are unknown, mixed and in different proportions (numbers of individuals), and this must be determined with progeny testing. These methods to produce families can be used in combination with mathematical modelling and specially designed

424 breeding programs that adjust for different ages or numbers. However, the ideal method for implementing breeding selection programs is the use of *in vitro* fertilization (Duncan
426 et al., 2013b), but this method has not been developed for meagre and is more difficult to implement in a farm situation.

428 In conclusion, paired spawning with male rotation provided a method to cross meagre breeders with desired phenotypes to produce particular families with high fecundities for
430 the commercial production of many juveniles. However, contrary to the males, a high proportion of females could only be spawned with three different males due to the loss of
432 advanced female maturity status, which appeared to be related to differences in mate selection strategies between male and female meagre. Therefore, this method can be used
434 with a limit of three families per female or as a scaling up step to produce a large number of offspring from a limited number of pairs that have the phenotypes that are important for
436 commercial production.

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Figure legends

Figure 1. Photographs of wet mounts of ovarian biopsies taken from meagre (*Argyrosomus regius*) (40x magnification). Photographs 1a and 1b show oocytes >550 µm from females that were considered suitable and were induced to spawn. Photographs 1c and 1d show oocytes from females that were unsuitable for GnRHa induced spawning.

Figure 2. Mean (± 1 SEM) relative fecundity (a), frequency distribution of the number of spawns from each pair (b) and percentage of successfully spawning pairs (c) of meagre (*Argyrosomus regius*) females (n = 21) for each consecutive GnRHa injection (n = 5) administered each week. At every GnRHa injection, the males were moved to a different tank, being paired with a different female so that no pair of fish was repeated. The numbers within the bars indicate the "n" value (number of pairs that spawned) of each mean. The P value on Fig. 2a, indicates the significance of a one-way ANOVA statistics applied to the first four injections. The P value on Fig. 2b, indicates the significance of a Chi squared test to compare the four frequency distributions. The different letters indicate significant differences.

Figure 3. Mean (± 1 SEM) relative fecundity (a), frequency distribution of the number of spawns from each pair (b) and percentage of successfully spawning pairs (c) of meagre (*Argyrosomus regius*) males (n = 18) for each consecutive GnRHa injection (n = 7) administered each week. At every GnRHa injection, the males were moved to a different tank, being paired with a different female so that no pair of fish was repeated. The numbers within the bars indicate the "n" value (number of pairs that spawned) of each mean. The P value on Fig. 3a, indicates the significance of a one-way ANOVA statistics applied to the first five injections. The P value on Fig. 3b, indicates the significance of a Chi squared test to compare the four frequency distributions. The different letters indicate significant differences.

Figure 4. Mean (± 1 SEM) fertilization success (top), hatching (middle) and survival of larvae five days post hatch (bottom) for the spawns obtained after each consecutive GnRHa injection (week) administered to meagre (*Argyrosomus regius*) females. The numbers within the bars indicate the "n" value (number of spawns) of each mean. The P values in each graph indicate the significance of a one-way ANOVA statistics.

Figure 5. Mean (± 1 SEM) fertilization success (top), hatching (middle) and survival of larvae five days post hatch (bottom) for the spawns obtained after each consecutive GnRHa injection (week) administered to meagre (*Argyrosomus regius*) males. The numbers within the bars indicate the "n" value (number of spawns) of each mean. The P values in each graph indicate the significance of a one-way ANOVA statistics applied to the first six injections.

Figure 1.

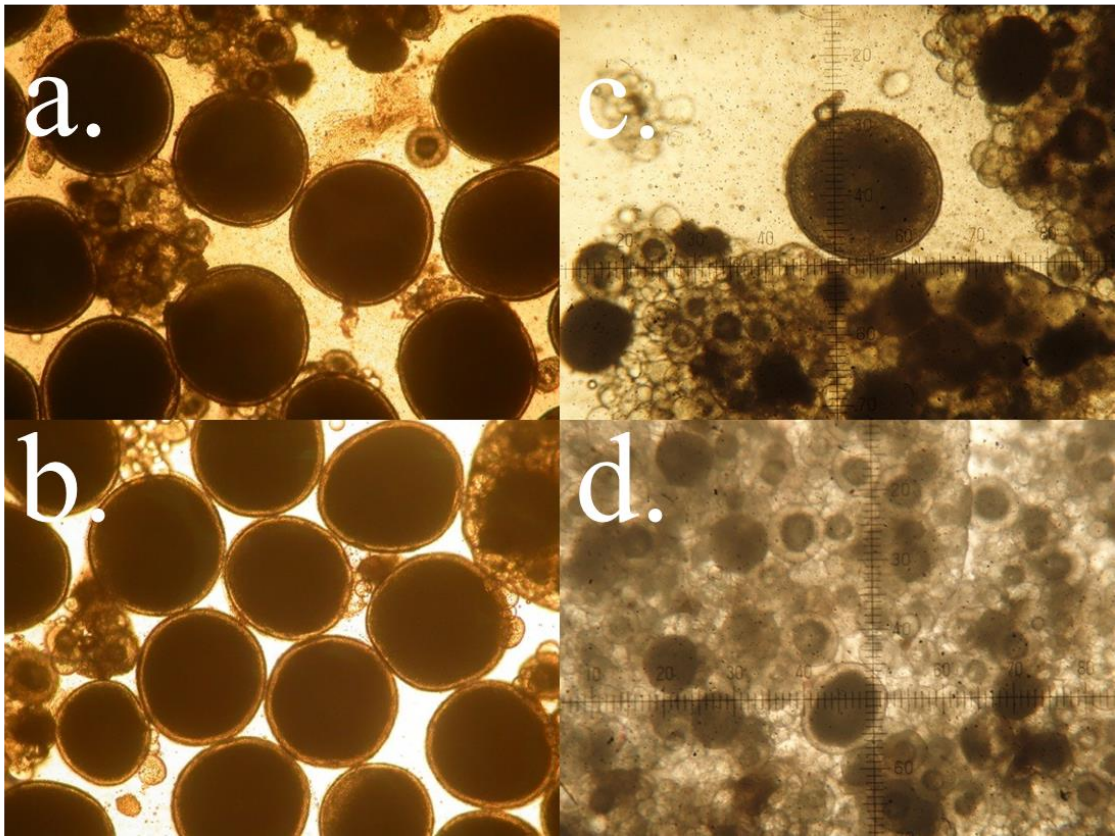
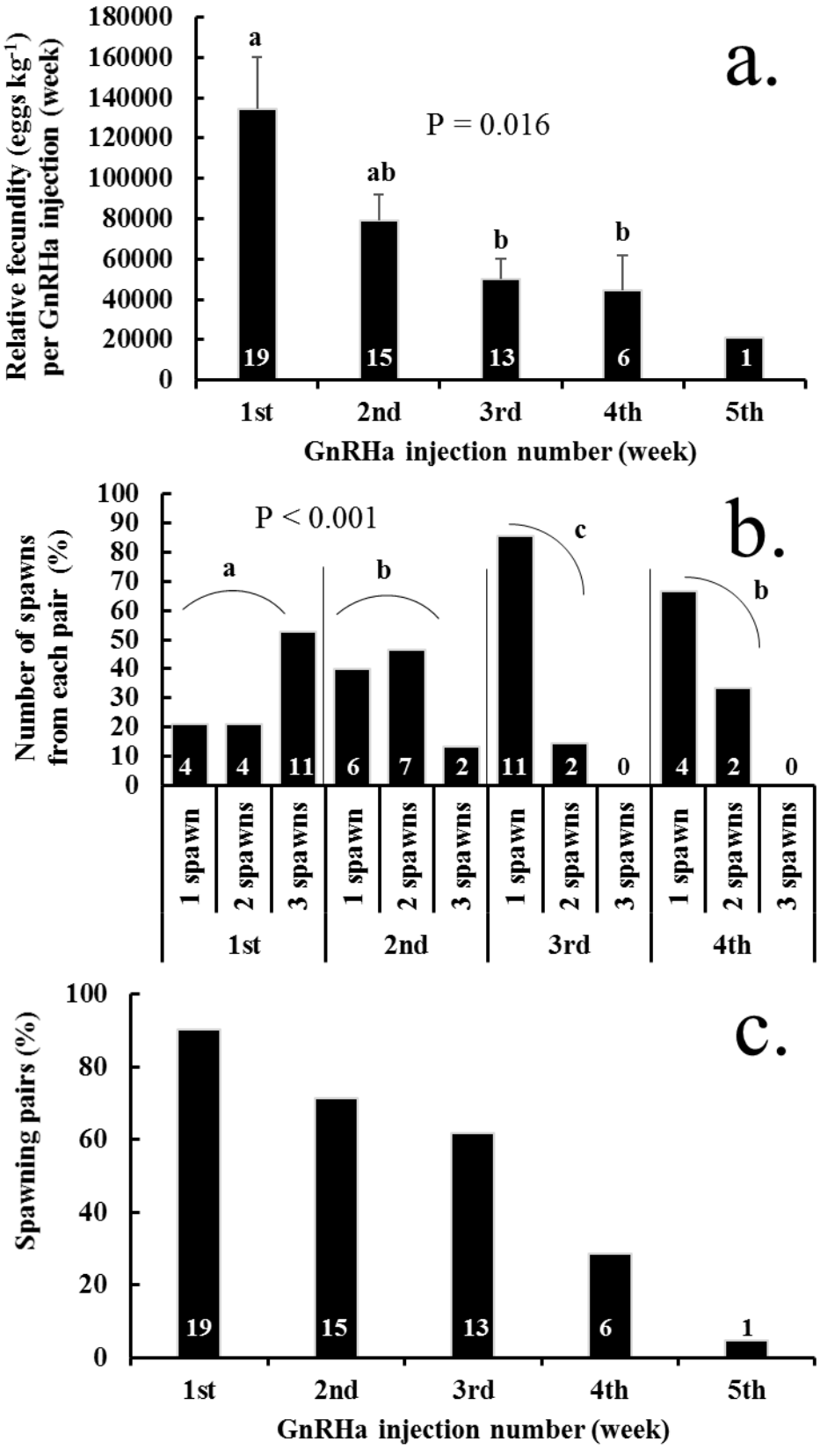
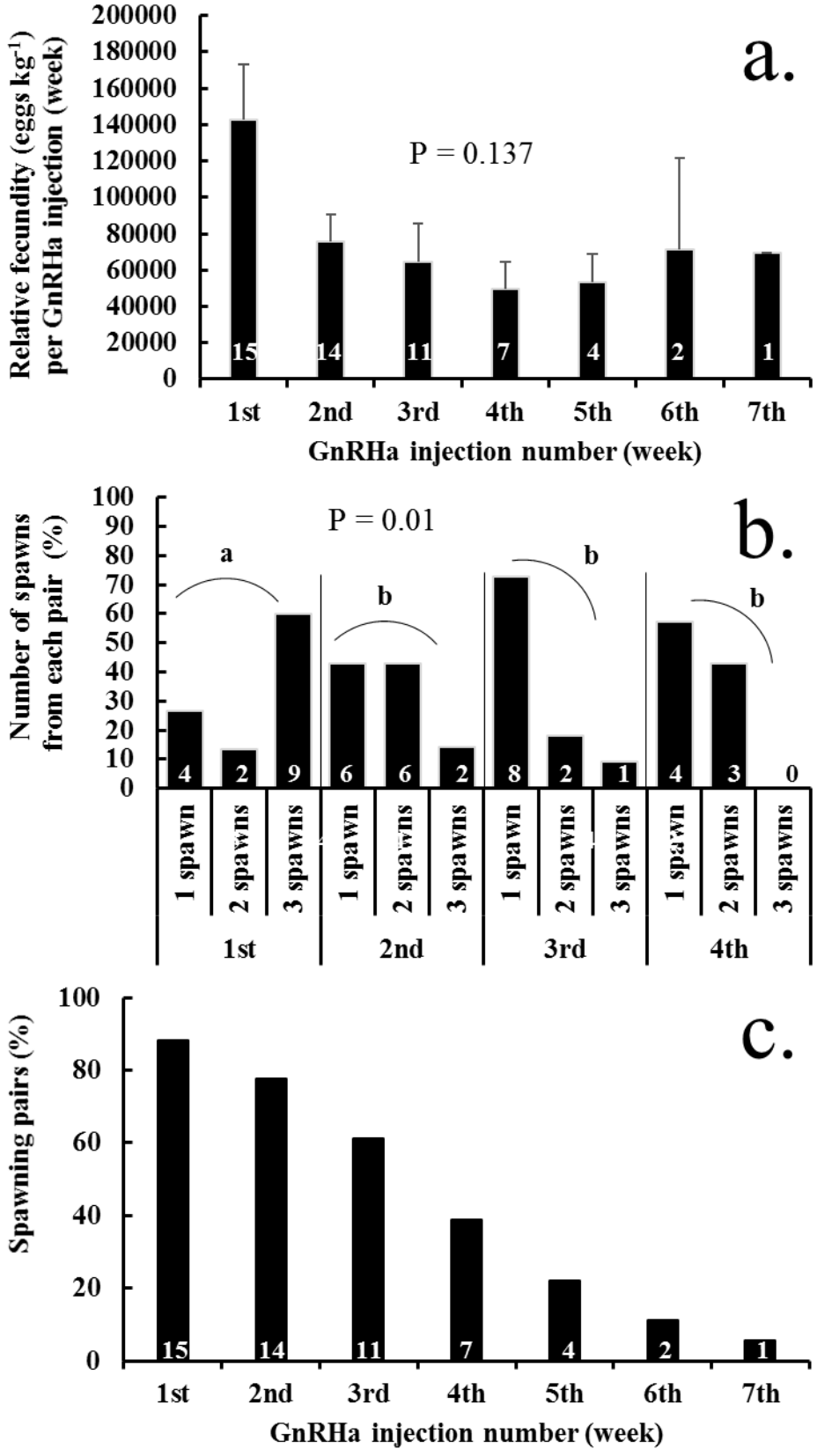


Figure 2.


a.
Relative fecundity (eggs kg-1) per GnRHa injection (week)
180000
160000
140000
120000
100000
80000
60000
40000
20000
0
a
ab
b
b
P = 0.016
19
15
13
6
1
1st
2nd
3rd
4th
5th
GnRHa injection number (week)
b.
Number of spawns from each pair (%)
100
90
80
70
60
50
40
30
20
10
0
P < 0.001
a
b
c
b
4
4
11
6
7
2
11
2
0
4
2
0
1 spawn
2 spawns
3 spawns
1st
2nd
3rd
4th
c.
Spawning pairs (%)
100
80
60
40
20
0
19
15
13
6
1
1st
2nd
3rd
4th
5th
GnRHa injection number (week)

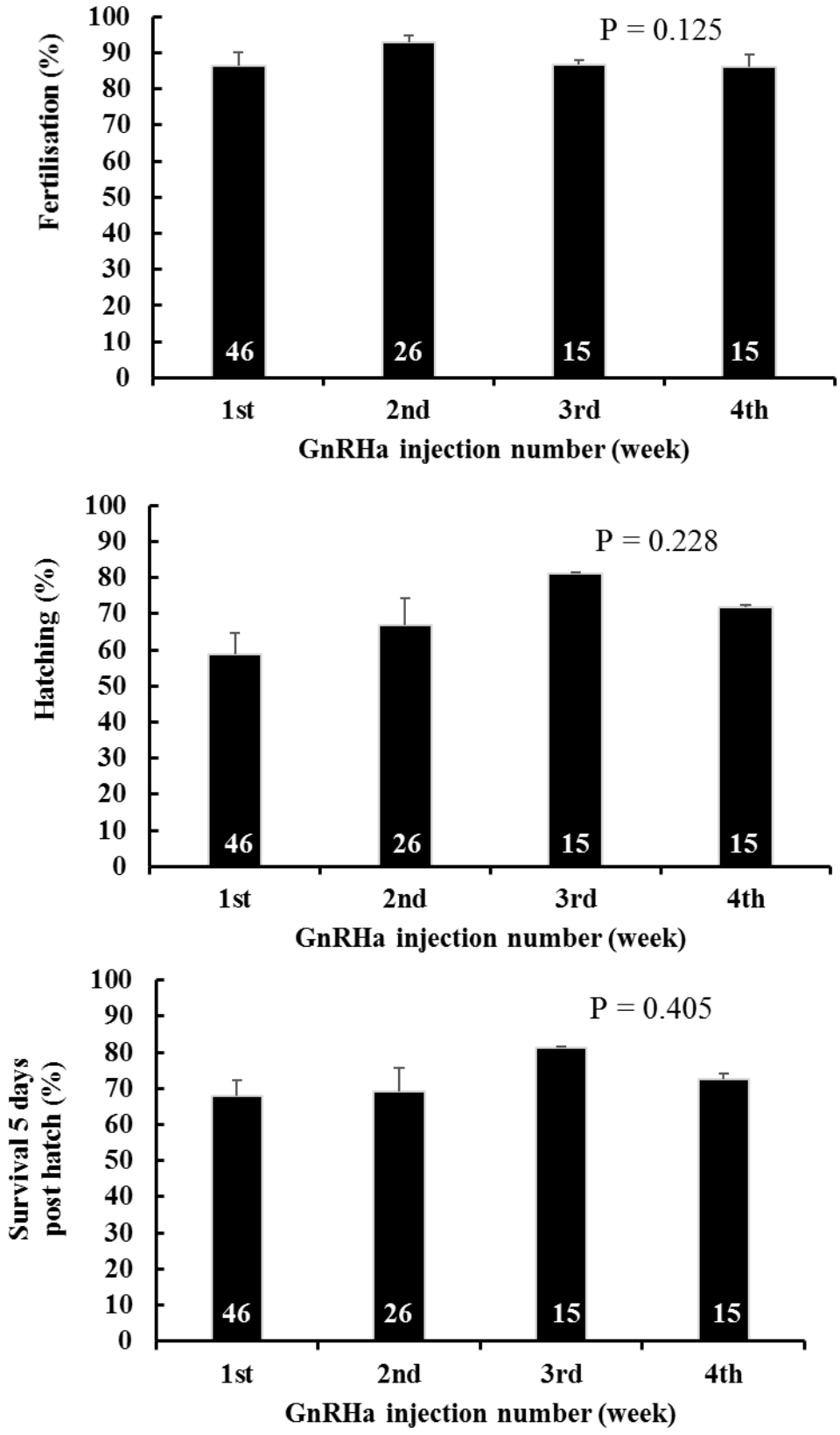
592 Figure 3.

594



596

Figure 4.


Fertilisation (%)
P = 0.125
46
26
15
15
1st
2nd
3rd
4th
GnRHa injection number (week)
Hatching (%)
P = 0.228
46
26
15
15
1st
2nd
3rd
4th
GnRHa injection number (week)
Survival 5 days post hatch (%)
P = 0.405
46
26
15
15
1st
2nd
3rd
4th
GnRHa injection number (week)

602 Figure 5.

604