Deliverable Report

Deliverable No:	D2.3	**Delivery Month:**	22
Deliverable Title	Protocol for paired spontaneous tank spawning of meagre		
WP No:	2	**WP Lead beneficiary:**	P3. IRTA
WP Title:	Title of WP from DOW		
Task No:	2.2	**Task Lead beneficiary:**	P3. IRTA
Task Title:	Development of protocols for paired crossing in spontaneous spawning		
Other beneficiaries:	P1. HCMR		
Status:	Delivered/delayed	**Expected month:**	21
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Other Scientists participating: Mylonas, C.C. (HCMR), Fakriades I. (HCMR), Sigelaki, I. (HCMR) and the students (IRTA) Edwards Milton Sullon, Zohar Ibarra, Marco Chiumento and Olinser Aviles Carrillo

Objective:

The objective of this Deliverable was to develop a protocol for the induction of spontaneous tank spawning of pairs of meagre (one male and one female), in order to allow breeding of selected individuals for the production of multiple families for implementing breeding selection programs. The deliverable presents the procedures, description of holding environment, stage of maturity required, dosage and timing of hormone application, and egg collection. In addition, the deliverable includes the results from repeated trials, including data on success rate of paired spawning, relative fecundity, latency period and egg quality parameters.

Deviations:

Originally, only work undertaken by P3. IRTA was planned in the DOW, but then additional work was included in order to obtain more data with work from another broodstock in a different facility, which would make the results more reliable. So, additional experiments were added in order to (a) examine better the potential of repeated injections to induce multiple spawnings in meagre and (b) to replicate the paired-spawning experiments of IRTA.

So, the deliverable includes additional work that was undertaken by P1. HCMR **at no extra charge to DIVERSIFY**. The additional tasks were implemented by taking advantage of the maintenance during the project of a broodstock by P1. HCMR, which was used to produce eggs and fingerlings for the Larval Husbandry, Grow out husbandry and Fish health WPs. Since spawning induction is anyway needed for the production of eggs by meagre in captivity, the inclusion of some additional spawning induction experiments for this task was considered as a good way to get the most benefit from the resources provided by the project, with some **free-of-charge** contribution from the existing personnel of P1. HCMR.

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Summary:

Five paired spawning experiments were completed to determine the potential of paired spawning inductions with male rotation to perform a dialled cross mating design as the basis of a breeding program. In one experiment the same pairs were induced repeatedly to spawn on a weekly basis. In four subsequent experiments three 4 x 4 (experiments I, IIIa and IIIb) and one 6 x 6 (experiment II) dialled crosses of pairs with weekly male rotation and induced spawning were completed. Fecundity and percentage fertilization were determined when eggs were collected and percentage hatching and 5-d (day) larval survival were determined by incubating eggs from each spawn in replicated 96-well microtiter plates. In the same-pair experiment, the 4 pairs spawned up to 17 weeks in succession with high fecundity (>400,000 eggs kg^{-1} $spawn^{-1}$) and egg quality (> 80% fertilization). The efficacy of spawning pairs with male rotation was high 76% (Experiment 1, 14 pairs spawned out of 16 (87%); experiment II, 22 pairs out of 37 (59%); experiments IIIa and IIIb, 25 pairs out of 27 (93%)) and across the three experiments a total of 61 families out of 84 (full and half-sib) were produced that had >200,000 eggs of >80% fertilization success. However, not all paired crosses with male rotation were successful and a number of females after consecutive successful spawning inductions either failed to spawn or did not present vitellogenic oocytes and could not be induced as planned in the dialled cross design. This failure to spawn or maintain maturity status after successive successful spawning inductions appeared to represent a change in spawning kinetics from the prolonged (up to 17 weeks) induced spawning period observed in the same-pair experiment and previous studies. This change in kinetics may be attributed to the stress of male rotation and consideration should be made that as the number of rotations increases, spawning pairs may fail or induced spawning may not be possible. However, together these experiments have shown that paired spawning of meagre is possible for the production of known families from parents with known phenotypes. Obtaining a large number of families with adequate fecundities that can be used on a commercial scale from crosses of selected breeders with desired phenotypes is a prerequisite for a breeding program. The present studies were a successful "proof of concept" for this approach, highlighting the both the positive potential of the approach and possible drawbacks.

Introduction:

The meagre is a sciaenid fish found in the Mediterranean and Black Sea, and along the eastern Atlantic coast (Haffray et al., 2012). This fish has attractive attributes for the market that include large size, good processing yield, low fat content, excellent taste and firm texture (Monfort, 2010). The species also has the biological characteristics required for commercial aquaculture using well-established gilthead sea bream (*Sparus aurata*) and European sea bass (*Dicentrarchus labrax*) culture technologies (Duncan et al., 2013a). These characteristics include a fast growth of ~1 kg per year (Duncan et al., 2013a), a low feed conversion ratio of 0.9-1.2 (Monfort, 2010; Duncan et al., 2013a), relatively easy larval rearing (Roo et al., 2010; Vallés and Estévez, 2013) and established induced spawning protocols for the production of viable eggs (Duncan et al., 2012, 2013a; Mylonas et al., 2013a; 2013b; 2015; Fernandez-Palacios et al., 2014). Meagre was first produced in 1997 in a commercial hatchery in France and since then it has exhibited annual production increases as high as 7 fold (FAO). In 2010, European meagre aquaculture production was 2,387 t, produced mainly in Spain, with smaller quantities produced in France, Portugal, Italy, Greece, Cyprus and Croatia (FAO).

A survey of meagre producers that was conducted in preparation of **DIVERSIFY,** identified that one of the major bottlenecks to the expansion of the industry was that the distribution of this fish only in specific areas in the Mediterranean region has resulted in the acquisition of fish for developing broodstocks from a limited number of sources (mainly a hatchery in France), resulting perhaps in a limited genetic variation of the available broodstocks. This was recently demonstrated by **DIVERSIFY** (see **Deliverable D2.2**), which confirmed that the breeders being used in aquaculture have originated from two different wild populations. Although adequate genetic variation exists in the broodstocks being held by aquaculture companies and research centers, the few populations providing breeders (two) and in some cases few families from these populations clearly highlight the needs to establish genetic breeding programs that will avoid the problems associated with inbreeding. The control of reproduction is an essential part to a genetic breeding program (Duncan et al., 2013b).

Breeding in pairs is one way to create families in breeding selection programs. However, some marine species such as gilthead seabream and European seabass do not spawn in pairs and artificial "strip" spawning is also complicated. For example, gilthead seabream spawning success was low when held in pairs (Gorshkov et al., 1997; personal observation N. Duncan) or groups of 15 females with a single male (Gorshkov et al., 1997). Various authors have suggested large groups of breeders were required for successful spawning of gilthead seabream (Gorshkov et al., 1997; Duncan et al., 2013b) and Sparidae in general (Pankhurst, 1998; Mylonas et al., 2011). The spawning in large groups of breeders complicates the establishment of breeding programs that often require that specific breeders with desired phenotypes are breed together in many paired crosses to produce the required number of families. Therefore, an essential part to establishing a breeding program for meagre is to establish the control of reproduction that enable pairs to be selected and breed together. Since reproduction in captive meagre is almost always induced by exogenous hormone treatments followed by tank spawning, the objective of this work was to examine the potential of meagre to spawn repeatedly and reliably in pairs with male rotation.

Experiments undertaken:

A total of five experiments were completed during years 2014 and 2015 (Y1 and 2 of the project). The experiments are reported in 4 sections,

1. Section 1 reports an experiment that examined the maximum number of spawnings in response to weekly GnRHa injections undertaken by P1. HCMR in 2014,
2. Section 2, reports an experiment to examine paired spawnings with male rotation on a weekly basis that were conducted by P1. HCMR in 2015,
3. Sections 3 and 4 report experiments to examine paired spawnings with male rotation on a weekly basis that were conducted by P3. IRTA in 2014 (Section 3) and 2015 (Section 4).

1. Maximum number of spawns in response to weekly GnRHa injections (P1. HCMR).

In 2014 (Y1 of the project), single pairs of fish (one male and one female) were transferred to 5,000-l tanks under simulated natural photoperiod, but controlled temperature ranging between 19 and 20°C. Females were considered eligible for spawning induction if they contained oocytes in full vitellogenesis with a diameter of >550 µm (Mylonas et al., 2013b). Male fish were considered eligible for spawning induction, if they were releasing substantial amounts of sperm upon application of gentle abdominal pressure (Mylonas et al., 2013a). Injections of GnRHa were administered once a week (every Monday) between 7 May and 28 August 2014 using four pairs of fish per treatment (n=4). Females (mean ±SD body weight 9.7 ± 1.0 kg) were treated with a GnRHa injection of 15 µg kg^{-1}. Four males (7.9 ± 1.0 kg body weight) were treated at the start of the experiment with 43–57 µg kg^{-1} using a 450-500 µg GnRHa implant for an effective dose of ~50 µg kg^{-1} GnRHa, in order to enhance spermiation. GnRHa implantation of males was repeated at subsequent samplings (at the time of the GnRHa injection of the females), if sperm production was considered low. After treatment with GnRHa, fish were placed in tanks connected to overflow egg collectors and were allowed to spawn. If a female failed to spawn in response to 2 consecutive injections, it was removed and was not considered for the remaining experiment. When a cumulative total of two females (*i.e.*, 50%) failed to spawn in response to 2 consecutive injections, the experiment was concluded, and no further injections were given.

Eggs were collected every morning into a 10-l bucket and their number (fecundity) and fertilization success was evaluated at the same time by examining each of the eggs in this 10 ml sample for the presence of a viable embryo (usually at the blastula stage) using a stereoscope. In order to monitor embryo and larval survival, eggs from each spawning were placed individually in 96-well microtiter plates (in duplicates). The microtiter plates were then placed in a controlled-temperature incubator and maintained for 5 days at 19 ± 0.5°C. Using a stereoscope, embryonic and early larval development was evaluated once a day, recording the number of live embryos 24 h after egg collection (or ~36 h after spawning), hatched larvae (examined ~60 h after spawning) and viable larvae on day 5 after egg collection (~ yolk sack absorption). At 18-20°C, hatching of meagre eggs takes place in 44-56 h. 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 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 d 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).

Mean (± SEM) oocyte diameters at the onset of the study were 590 ± 10 µm, and throughout the study ranged between 550 ± 9 µm and 620 ± 6 µm, with some small, but statistically significant variations (ANOVA, P=0.04). Large numbers of vitellogenic oocytes, could be seen in the biopsies of all four females until week 5, of three females until week 16 and of two females until week 18 --one week after the last GnRHa injection (**Fig. 1.1**). One female failed to spawn in response to GnRHa injections on weeks 6 and 7, and was thus not used again for the study. Another female failed to spawn in response to GnRHa injections on weeks 16 and 17, at which time the experiment was concluded.

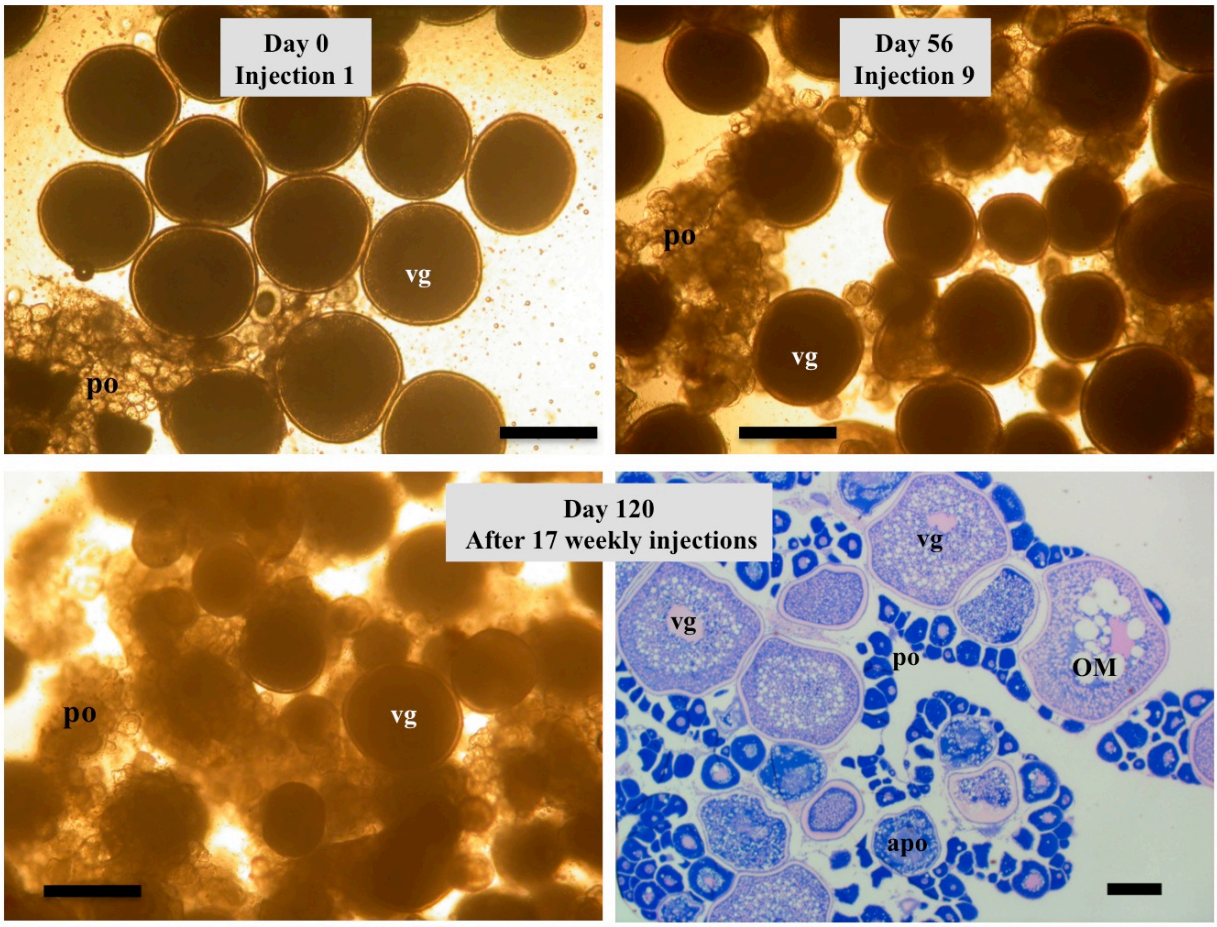

Figure 1.1. Wet mount and histological evaluations of representative ovarian biopsies obtained from meagre injected weekly with GnRHa, prior to injection 1 and 9, and a week after injection 17. apo = apoptotic, Vg = vitellogenic, OM = oocyte maturation, po = primary oocyte. The black bar in the microphotographs of the wet mounts and histological section indicate 500 and 200 µm, respectively.

The GnRHa injected females spawned for up to 17 consecutive weeks, most of the times spawning both on the 2nd and 3rd day after each weekly injection (**Fig. 1.2**). The first spawns obtained 2 d after each injection had significantly higher fecundity compared to the second spawns obtained 3 d after each injection (ANOVA, $P < 0.001$). Overall, there was no significant effect of injection number on mean fecundity (ANOVA, $P=0.83$), but there was a slight negative linear correlation ($n=32$, $R^2=0.38$, $P < 0.01$, not shown) between 2nd spawn fecundity values and GnRHa injection number. Fertilization success was high during the experiment (**Fig. 1.2**), without any significant effect of either GnRHa injection week (ANOVA, $P= 0.16$) or spawn number after each injection (ANOVA, $P=0.21$), and with the exception of the 2nd spawn of the last GnRHa injection (54%) it was always >80% and most of the times >90%.

ANOVA Table (P values)	Fecundity	Fertilization
GnRHa injection (week)	0.834	0.159
Spawn No after injection	0.001	0.215
Interaction	0.737	0.564

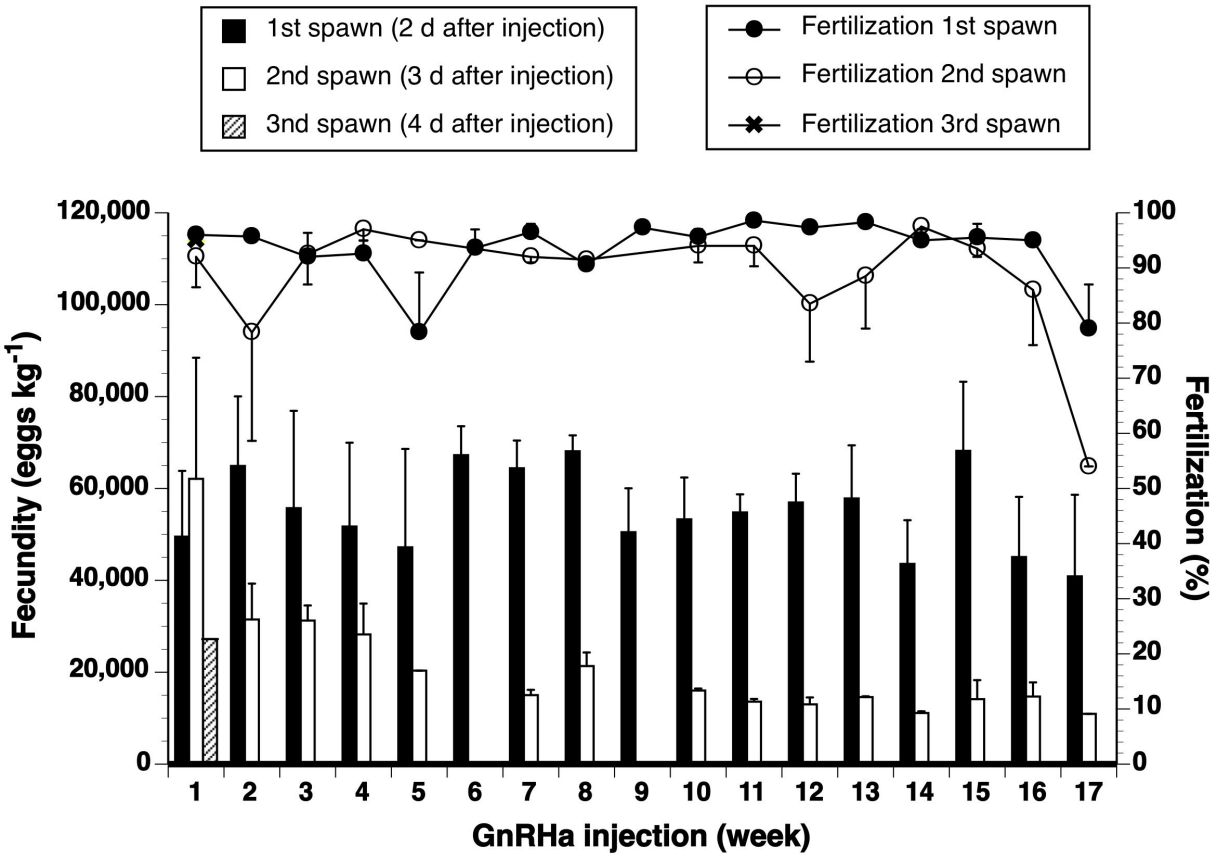

Figure 1.2 Mean (±SEM) daily batch fecundity and fertilization of meagre (n=1-4) induced to spawn with GnRHa injections (n=17, once every week) during 2014. The two-way ANOVA (GnRHa injection number vs Spawn number after each injection) indicated the existence of a significant interaction (P=0.001) in fecundity only, while the two main factors did not have any significant effect (either in fecundity or fertilization). Linear regression analysis indicated the existence of a significant negative relation between GnRHa injection number and fecundity for the 2nd spawn data (n=32, R^2=0.37, P =0.001, data not shown).

Embryonic development was very high overall and did not seem to differ significantly between eggs obtained in the first or second spawn after the GnRHa injections (data not shown), in terms of 24-h embryo survival (ANOVA, P=0.54), hatching (ANOVA, P=0.50) or 5-d larval survival (ANOVA, P=0.80). Similarly, there were no significant differences over the course of the study in response to the consecutive GnRHa injections, in terms of 24-h embryo survival (ANOVA, P=0.99), hatching (ANOVA, P=0.88) or 5-d larval survival (ANOVA, P=0.33), even after 17 weekly injections of GnRHa (**Fig. 1.3**).

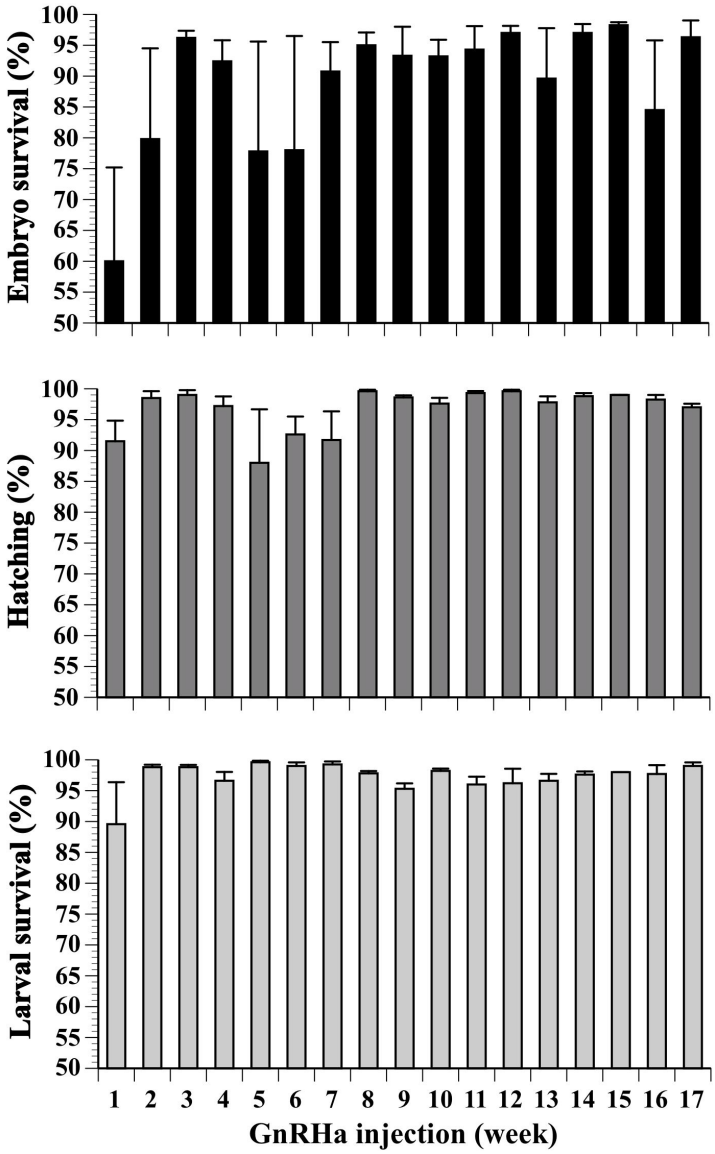

Figure 1.3 Embryonic development and larval survival eggs obtained from females (n=4) treated with consecutive weekly GnRHa injections (n=17) for a period of 125 days between May and September 2014. There were no significant differences over the course of the study in response to the consecutive GnRHa injections, either in 24-h embryo survival (ANOVA, P=0.99), hatching (ANOVA, P=0.88) or 5-d larval survival (ANOVA, P=0.33).

In a recently published work, multiple injections of GnRHa were shown to induce up to 7 weekly spawning cycles in meagre (Mylonas et al., 2015). The present experiment corroborated and extended the results, demonstrating that meagre are able to spawn multiple times and in a predictable fashion for up to 17 weeks during the reproductive season to produce large numbers of high quality eggs. It is not known if these results reflect the situation in the wild, since fish were maintained at a constant temperature of ~21°C during the trial, which was found to be optimal for spawning in earlier trials (Mylonas et al., 2013b; Mylonas et al., 2015). Meagre in the wild has an asynchronous ovarian development (Gil et al., 2013) and depending on the location its spawning season extends from April to October. However, it is not clear what is the natural spawning kinetics of meagre, what is the inter-spawn interval and how many times a fish may spawn during a single season (Gil et al., 2013; González-Quirós et al., 2011). As meagre do not usually spawn spontaneously in captivity, it is also not possible to obtain reliable information from fish in captivity

(Duncan et al., 2013). Nevertheless, the present study demonstrates that if the fish are maintained at the appropriate spawning temperature and are given a weekly GnRHa injection, they can continue to spawn for a very long period of time producing mean (±SD) relative fecundity values of 1.42±0.15 million eggs kg^{-1} body weight per year. This is probably achieved by an indeterminate recruitment of oocytes into vitellogenesis during the reproductive period, in response to gonadotropic stimulation by the multiple GnRHa injections. Further studies should be undertaken to describe the endocrine changes that take place in response to this treatment, supporting such a tremendous production of eggs.

2. Paired spawnings with male rotation on a weekly basis, Experiment I (P1. HCMR).

This experiment was run in parallel with a similar one by partner P3. IRTA in 2015 (Y2), in order to develop a method that optimizes the number of families produced by a given number of individual breeders. Four pairs of fish (one male and one female) were transferred to 5,000-l tanks under simulated natural photoperiod and constant temperature (19 and 20°C). Fish were considered eligible for spawning induction using the same criteria as in the previous experiment (section 1) (Mylonas et al., 2013b). Injections of GnRHa (15 μg kg^{-1}) were administered once a week (every Monday) between 4 and 25 May 2015 to the four females (named Juliet, Cleopatra, Cecy and Helena) of mean ±SD body weight 11.7 ± 2.6 kg, and the four males (named Romeo, Cesar, Peri and Paris) of mean ±SD body weight of 10.2 ± 1.2 kg body weight. Every week, the males were paired with a different female and after treatment with GnRHa they were placed in the separate spawning tanks and were allowed to spawn for a week. Eggs were collected and evaluated as described in the previous section 1 for fecundity and fertilization success, and then for embryo survival 24 h after egg collection, hatching success and larval survival on day 5 after egg collection.

After the first GnRHa injection, all females spawned on three consecutive days (**Fig. 2.1**), similar to what was observed in the previous year and presented above (**Fig. 1.2**). Similarly, after the second GnRHa injection all females spawned again for two consecutive days. However, from the third GnRHa injection, significant variations appeared among the four females. Some spawned only once at this time, while after the 4th injection only two fish continued spawning. These results are different than from the previous year (**Fig. 1.2**), but also from other published work from our laboratory (Mylonas et al., 2015). Examination of the ovaries of the two females that did not respond at the 4th injection ("Helena" and "Cecy") indicated that the females did not contain any more vitellogenic oocytes that could be induced to mature and spawn.

So, apparently these two females had matured and ovulated all the initially available vitellogenic oocytes in response to the first three GnRHa injections, and did not recruit any more oocytes, as was the case with the other two females ("Cleopatra" and "Juliet") and in earlier experiments (**Fig. 1.2**) and published work (Mylonas et al., 2015). The only difference between this experiment and the previous ones is the fact that males were changed and rotated among females after each injection, and one can assume that this disruption in the breeding behavior of the fish might have affected negatively some of the females. It is interesting to note, nevertheless, that the overall fecundity of the four females was not significantly different among them (**Fig. 2.2**), suggesting that the females that spawned fewer times produced more eggs per batch.

Apart from the significant differences in the response to the 4th GnRHa injection and the overall number of spawns among the four females, there were no significant differences in the quality of the eggs produced (**Fig. 2.2**). These results suggest that female meagre is able to produce good quality eggs with different males and in response to weekly change of "partners", without any significant negative impact.

Similarly, there were no significant differences in the fecundity or egg quality of the eggs obtained when females were paired with a specific male (**Fig. 2.3**), again demonstrating that all males had equal potential of successful spawning and producing eggs of good quality. It has been demonstrated from in vitro fertilization experiments in other species, that there are some male-female incompatibilities when it comes to fertilization, resulting in lower fertilization success when specific males are crossed with specific females (Saillant et al., 2001). However, in the present experiment only a small number of individuals was used, so the possibility still exists that male-female fertilization incompatibilities may exist among some individuals of this species.

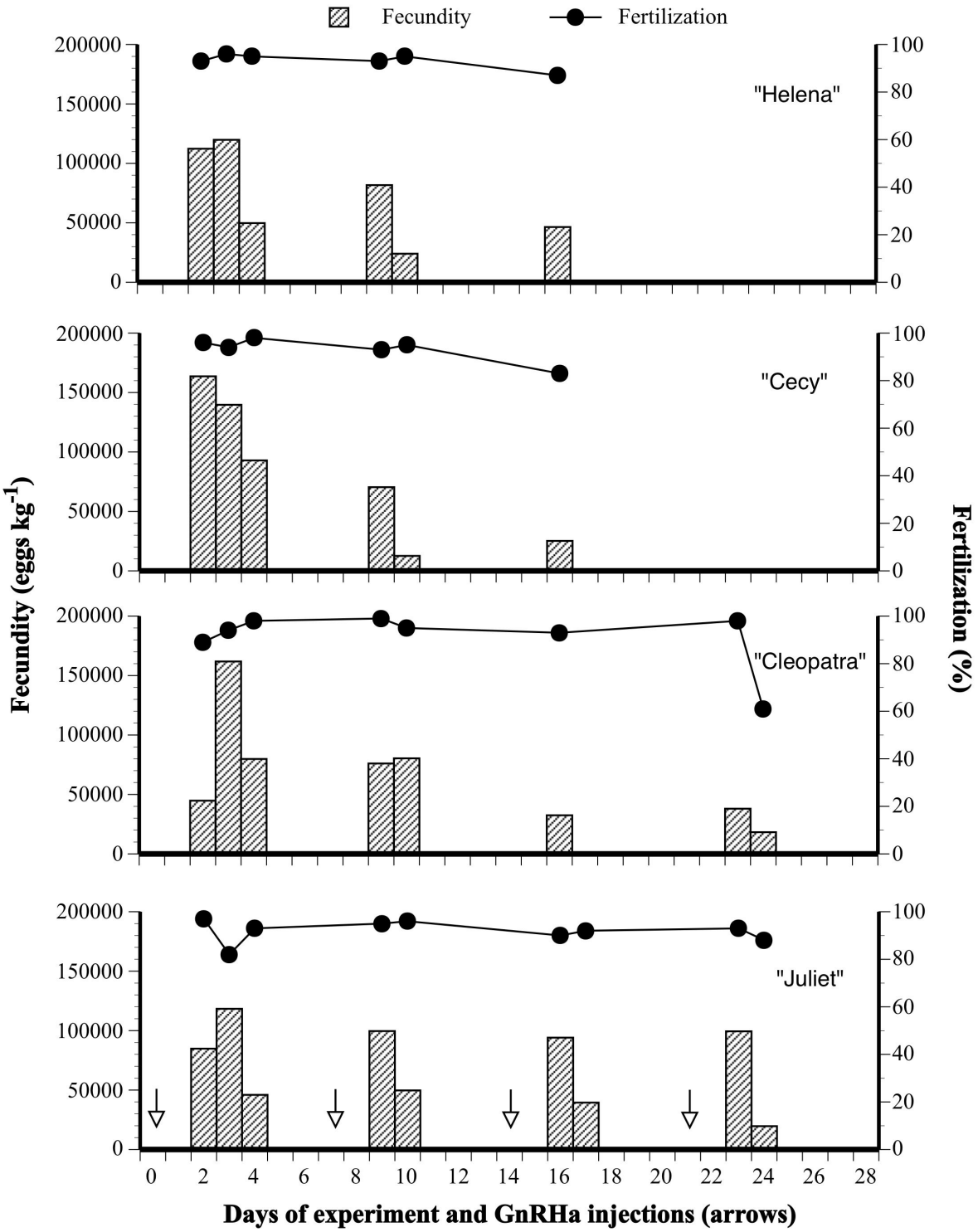

Figure 2.1 Daily batch relative fecundity and fertilization success of individual meagre females (n=4) induced to spawn with multiple GnRHa injections (n=4, once every week) during 2015 and paired with four males (named Romeo, Cesar, Peri and Paris). At every GnRHa injection, the males were moved to a different tank, being paired with a different female so at the end all males were paired with all females. The first GnRHa treatment was made on 4 May 2015.

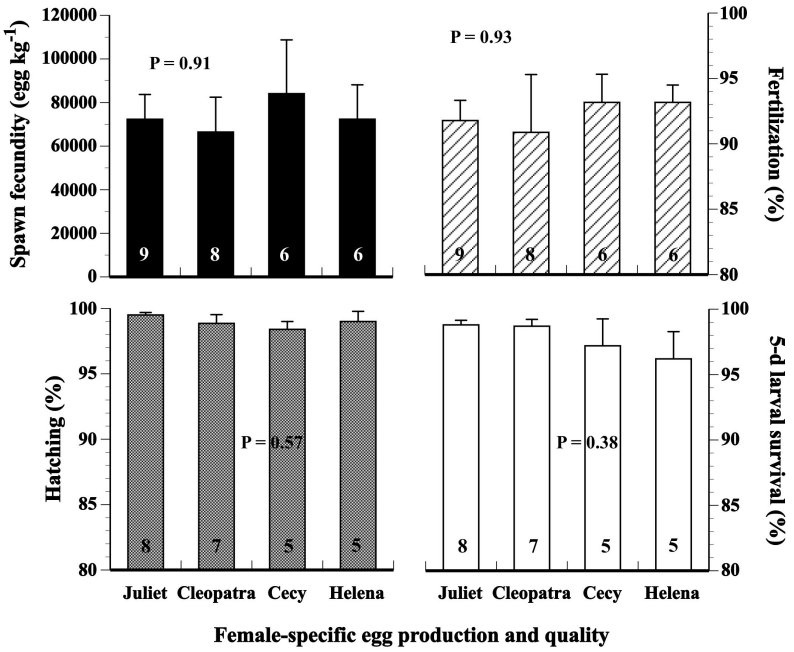

Figure 2.2 Mean (±SEM) daily batch relative fecundity, fertilization success, hatching and larval survival of individual meagre females (n=4) after each GnRHa injection (n=4, once every week) during 2015 (see **Fig. 2.1** for individual spawns). The numbers within the bars indicate the number of individual spawns making the mean. The P values in each graph indicate the significance of a one-way ANOVA statistics.

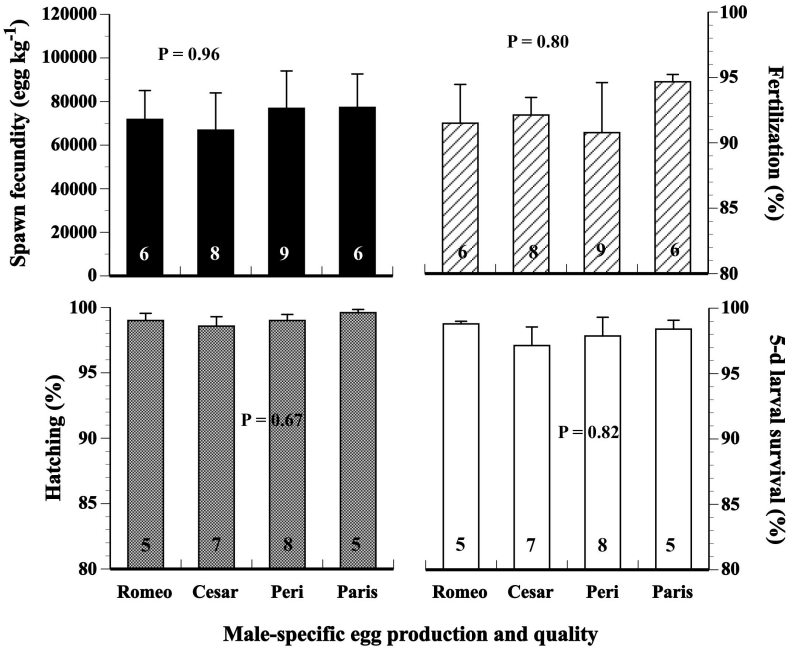

Figure 2.3 Mean (±SEM) daily batch relative fecundity, fertilization success, hatching and larval survival of eggs produced in the presence of different meagre males (n=4) after each GnRHa injection (n=4, once every week) during 2015 (see **Fig. 2.1** for individual spawns). The numbers within the bars indicate the number of individual spawns making the mean. The P values in each graph indicate the significance of a one-way ANOVA statistics.

As expected based on previous published work (Mylonas et al., 2015), but not according to the experiments done in 2014 (**Fig. 1.**2) there was a significant reduction in the fecundity obtained after consecutive GnRHa injections, while there was no difference in the fertilization success (**Fig. 2.4**) and subsequent egg development. As far as seed production by a commercial hatchery, this result is considered undesirable, since a hatchery cannot expect to maintain a stable egg production over the course of the reproductive season of meagre. Similar results have also been reported for European seabass, where egg fecundity decreased by ~50% after each subsequent spawning induction with GnRHa injections (Mylonas et al., 2003).

However, from the point of view of the objectives of the present experiment, that is the production of adequate numbers of eggs from as large number of parents as possible in order to create multiple families for breeding selection programs, we believe the results can be considered successful. A total of 14 families (half-sib) were produced, out of a possible maximum of 16 (4 males x 4 females), each family consisting of at least 200,000 eggs of >80% fertilization success. So, this method of pairing male and female meagre with a weekly rotation of the males can be used by commercial hatcheries in order to produce multiple families and build a breeding selection program that can maintain genetic variability. Obviously, in a commercial situation a much larger number of males and females should be used, but the experiment was a successful "proof of concept" for this approach.

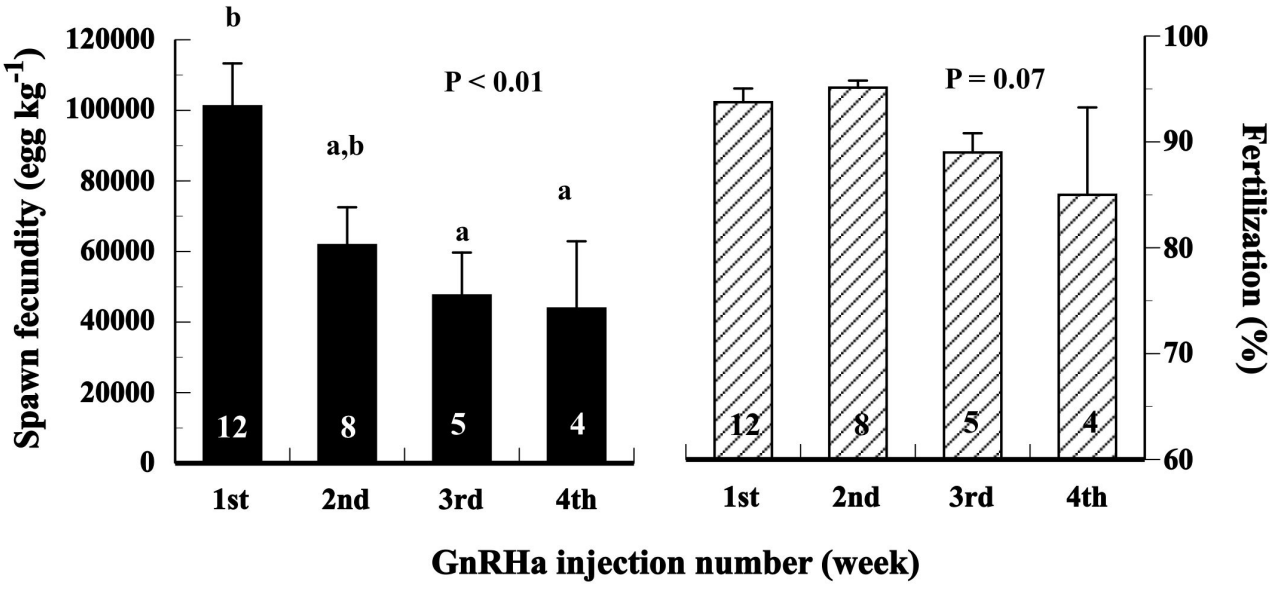

Figure 2.4 Mean (±SEM) daily batch relative fecundity and fertilization success, of meagre pairs (n=4) after each GnRHa injection (n=4, once every week) during 2015 (see **Fig. 2.1** for individual spawns). The numbers within the bars indicate the number of individual spawns making the mean. The P values in each graph indicate the significance of a one-way ANOVA statistics. Different letter superscripts indicate significant differences between means.

3. Paired spawnings with male rotation on a weekly basis, Experiment II (P3. IRTA).

A total of six pairs were selected, with females that had ovaries with vitellogenic oocytes >500 µm and males with flowing milt index of 2 or 3 (where 0 = no sperm, 1 = sperm present but not flowing, 2 = sperm flows and 3 = sperm flows easily and abundantly). Breeders were selected from a stock of wild and captivity-bred fish, and females had a mean weight of 21.24±3.69 kg and males 16.12 ±2.61 kg. To induce spawning, GnRHa (des-Gly10, [D-Ala6]-gonadotropin releasing hormone, Sigma, España), was administered to the selected breeders at doses of 15 µg kg^{-1} to females and 7.5 µg kg^{-1} to males. The pairs of breeders were placed in separate tanks 10-16 m^3 with >400% water exchange, natural photoperiod and temperature (16-21ºC maintained <21ºC) and a surface water egg collector. Every 7-10 days the breeders were sampled, maturity status was determined, breeders in advanced stages of maturity were induced as above and males were exchanged to form a different pair for each induced spawning. Breeders were induced to spawn at eight different times between 7 April (day 0) and 2 June 2014 (day 56). Breeders that did not have advanced stages of maturity were replaced with new breeders and breeders that did not spawn over 2-3 induced spawning dates were replaced. On 29 April (day 22), 3 females and 2 males were replaced with fish that had not been previously induced. On 15 May two females were rejected to leave 4 pairs. On 23 May (day 46) all females and three males were rejected and two females were introduced to form just two pairs. The introduced females were one female that had not been previously induced and one female that had been previously induced (6-W, wild). Even though female 6-W was rejected as immature on 29 April (day 22) the ovaries were found to contain large vitellogenic oocytes (637±67 µm) ready to be induced on 23 May (day 46). On the 2 June (day 56) one pair was rejected to leave just one pair. On the 9th June the last pair was rejected. Therefore, a total of 10 different females and 8 different males were induced over 8 weeks, on the first 5 weeks six pairs were induced, on week 6 four pairs, on week 7 two pairs and lastly on week 8 one pair to give a total of 37 pairs induced to spawn.

Eggs were collected every morning into a 10-l bucket and the number (fecundity) was evaluated by counting the number of eggs in 3 x 10 ml samples. When the volume of eggs exceeded 1 l (approx >1 million eggs) the 10 ml sample was diluted in 1 l and the number of eggs were counted in 3 sub-samples from the 1-l dilution. At the same time as counting the eggs, 50-100 eggs were examined for cellular development (usually at the blastula stage) using a stereoscope and eggs were recorded as either developing or not developing to determine percentage fertilization. In order to monitor embryo and larval survival, eggs from each spawn were incubated in 96-well microtiter plates (in duplicates). The 96-well plates were then placed in a controlled-temperature incubator (17-19ºC) and maintained until all larvae had died. Hatching rate (plates and incubator) and survival at 5 days post hatch were recorded.

Oocyte size varied from 506±96 to 630±63 µm and sperm index from 2 to 3 with 60±20 to 92±11 % sperm motility and motility duration from 1.15 to 3.5 min. Efficacy of spawning was 22 (59%) pairs spawned out of a total of 37 pairs that were induced (**Fig. 3.1** and **3.2**). Different individuals had clear differences in spawning response and fecundity to the GnRHa application. One female with oocyte diameters of 561±65 µm did not spawn during three inductions (data not displayed in figures). One female (6-W (wild)) produced 12 spawns from 5 different inductions with 5 different males (**Fig. 3.1**). The other 8 females responded positively to 4 inductions (female 13-C (cultured) and 7-W), 3 inductions (female 5-W), 2 inductions (females 1-W, 2-W and 16-C) and 1 induction (females 8-W and 11-C). Some of these 8 females did not respond to either the first induction (females 5-W and 13-C) and/or the last inductions (females 5-W, 7-W, 8-W, 13-C, 1-W, 2-W and 16-C). Failed spawning on the first induction was only observed on day 0 (7 April 2014) possibly indicating that this date when temperatures were 16.6 ± 0.3ºC was too early in the spawning season. The failed spawning at the end was after 4 (female 13-C and 7-W), 3 (females 5-W) or 2 (females 1-W, 2-W and 16-C) successful spawning inductions and for many of the females this coincided with temperatures rising above 20ºC. On 15 May 2014 when the temperature was > 20ºC there was no successful spawning and when the females were assessed for maturity one week after the induction all females had a low level of maturity with <500 µm oocyte diameter and were replaced. One female (11-C) spawned 4 times after the first induction (day 46) and the following week (day 56) did not present vitellogenic oocytes.

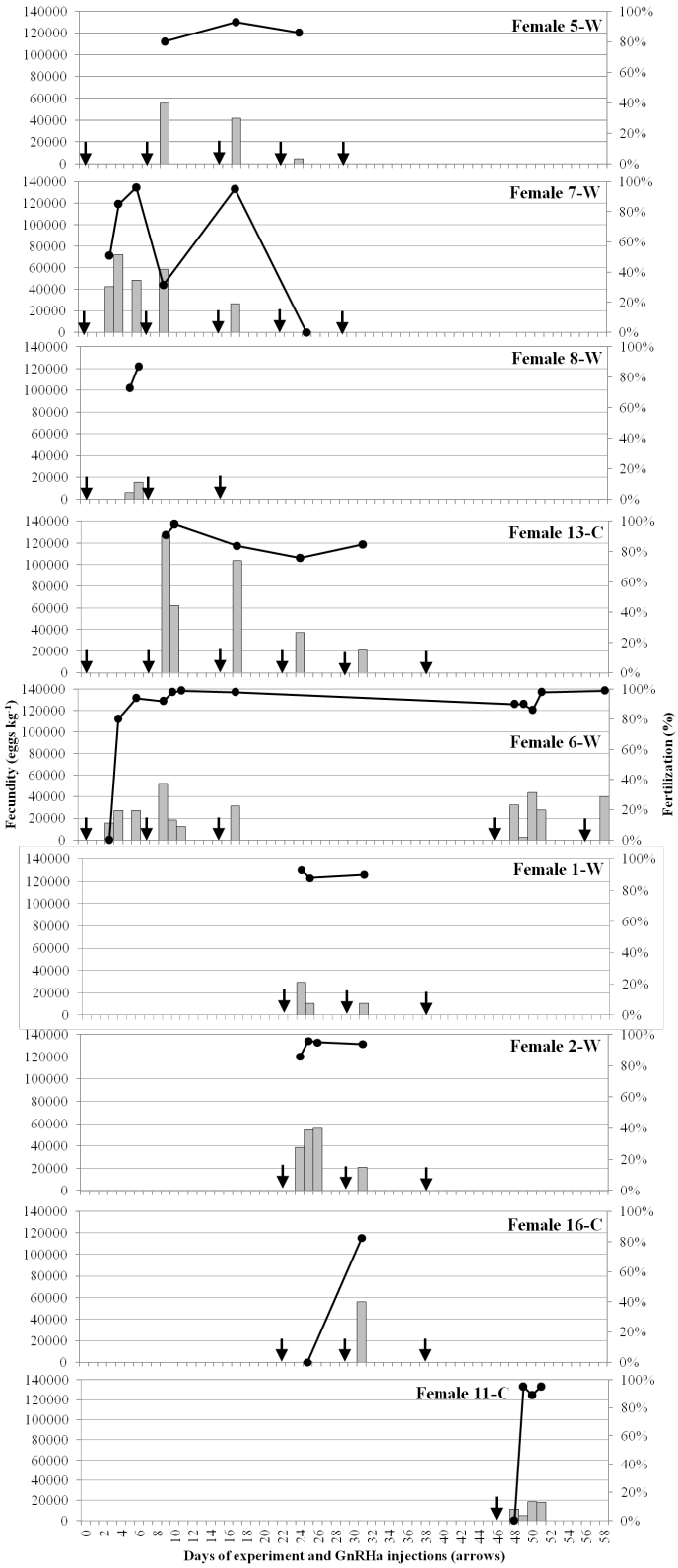

Figure 3.1 Daily batch relative fecundity (bars) and fertilization success (solid circles) of individual meagre females (n=9) induced to spawn with multiple GnRHa injections (n=1-6, once every week) during 2014 and paired with eight different males. At every GnRHa injection, the males were moved to a different tank, being paired with a different female so all pairs produced a full-sib or half-sib family. The first GnRHa treatment was on 7 April 2014.

There was also variation in egg quality between females (**Figure 3.2**) even though no statistically significant differences were observed between females. However, this should be evaluated cautiously as the statistical power was low due to some females having a low "n" of spawns and the associated high variation. Data from two females (8-W and 16-C) that spawned less than 3 times was not included. Female 8-W spawned twice, 10,806±4661 eggs kg^{-1} with fertilization of 80±7%. Female 16-C spawned twice, 28,185±28,000 eggs kg^{-1} with fertilization of 41±41%. Percentage hatching ranged from 32.2±16% (n=3) for female 5-W to 79.5±12% (n=4) for female 2-W and 5-d larval survival ranged from 31.2±15% (n=6) for female 7-W to 64.7±22% (n=4) for female 11-C.

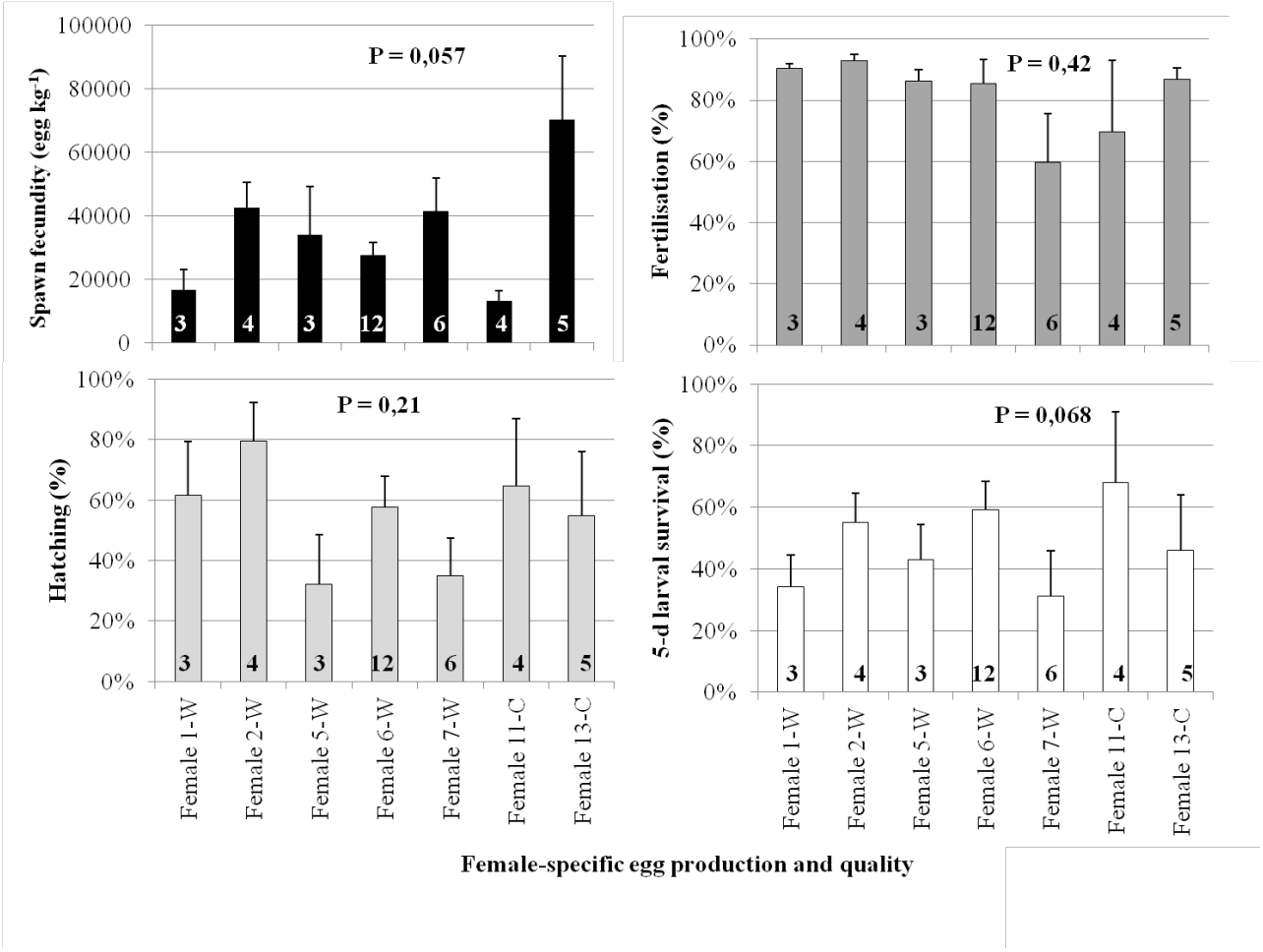

Figure 3.2 Mean (±SEM) daily batch relative fecundity, fertilization success, hatching and larval survival of individual meagre females (n=7) after each GnRHa injection (once every week, n=1-6,) during 2014 (see **Fig. 3.1** for individual spawns). The numbers within the bars indicate the number of individual spawns making the mean. The P values in each graph indicate the significance of a one-way ANOVA statistics parametric or non-parametric.

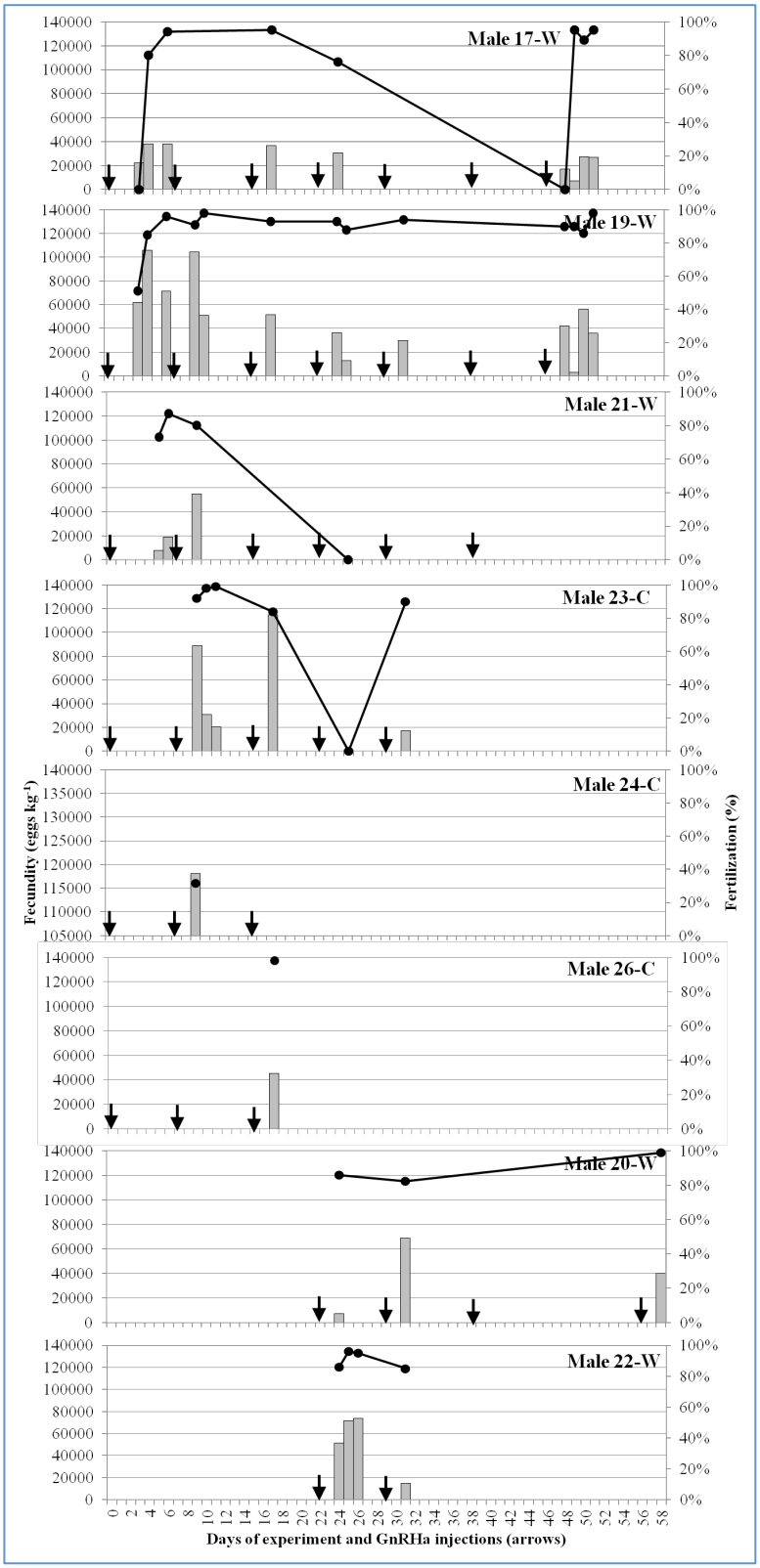

Figure 3.3 Daily batch relative fecundity (bars) and fertilization success (solid circles) of individual meagre males (n=8) induced to spawn with multiple GnRHa injections (n=1-6, once every week) during 2014 and paired with ten different females. At every GnRHa injection, the males were moved to a different tank, being paired with a different female so all pairs produced a full-sib or half-sib family. The first GnRHa treatment was on 7 April 2014.

The variation in spawning success, fecundity (**Figure 3.3**) and egg quality (**Figure 3.4**) was obviously similar for the males with the same data redistributed depending on the pair that was formed. Male 19-W, fertilized 13 spawns in six different inductions with six different females and the only date on which no spawn after an induction was day 38 when temperatures rose over 20ºC. Failures to spawn did not appear to have a pattern (first and last spawns as observed in females) and may have been related to female spawning. However, 3 males (Males 21-W, 24-C and 26-C) exhibited both poor spawning response and low fecundities suggesting that males as well as females contributed to poor spawning performance. Variation in egg quality associated to male fertilization (**Figure 3.2**) was variable as observed in the females. No significant differences were observed, however, again this should be evaluated cautiously as power was low due to some females having a low "n" of spawns and the associated high variation. Data from two males (24-C and 26-C) that spawned less than 3 times was not included. Male 24-C spawned once, 118,066 eggs kg^{-1} with fertilization of 31%. Male 26-C spawned once, 45,113 eggs kg^{-1} with fertilization of 98%. Percentage hatching ranged from 36.6±19% (n=4) for male 21-W to 79.7±13% (n=4) for male 22-W and 5-d larval survival ranged from 25.8±5% (n=3) for male 20-W to 62.1±12% (n=4) for male 22-W.

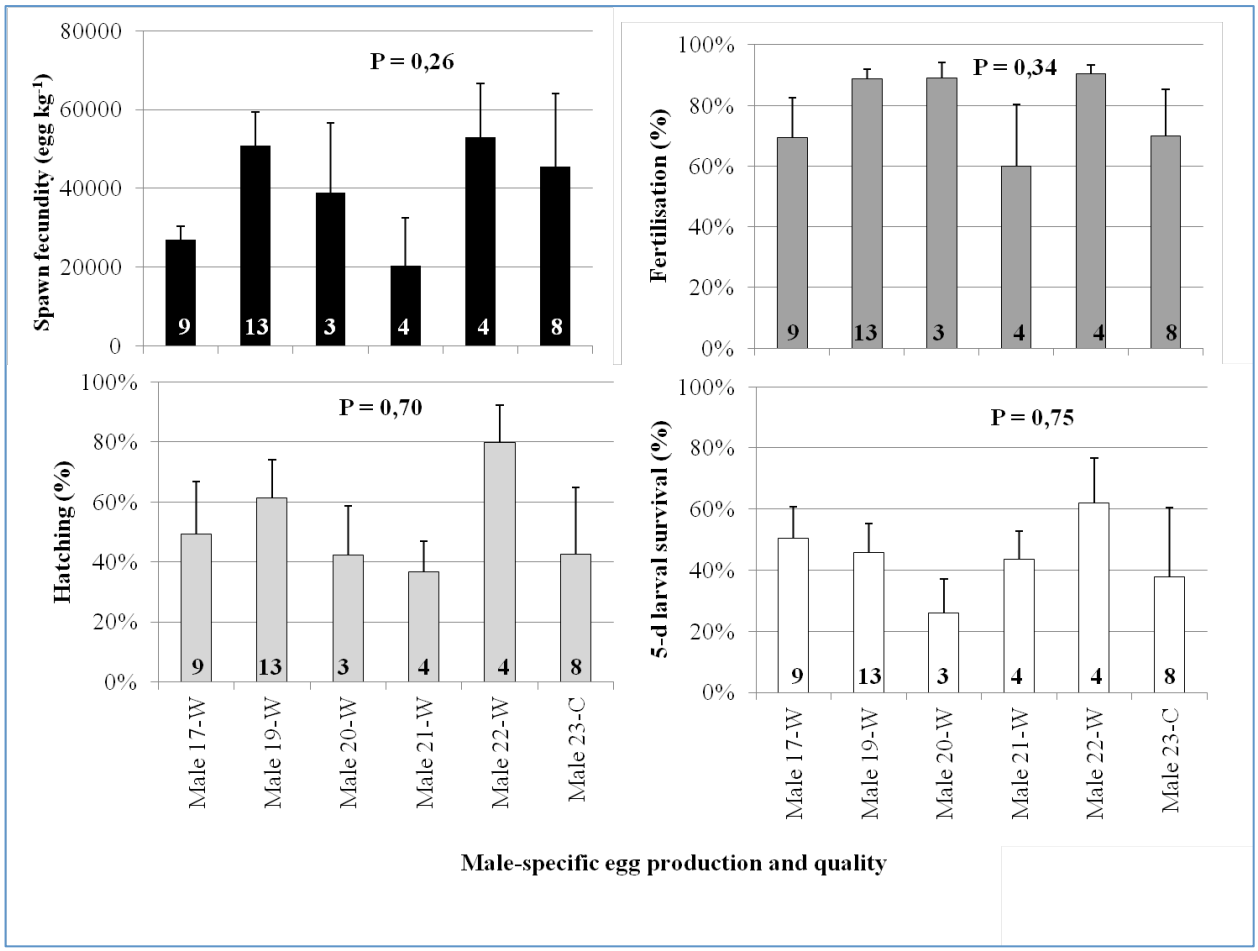

Figure 3.4 Mean (±SEM) daily batch relative fecundity, fertilization success, hatching and larval survival of individual meagre males (n=6) after each GnRHa injection (n=8, once every week) during 2014 (see **Fig. 3.3** for individual spawns). The numbers within the bars indicate the number of individual spawns making the mean. The P values in each graph indicate the significance of a one-way ANOVA statistics parametric or non-parametric.

Although there appeared to be clear failure of some individuals to spawn, there was no correlation between maturity assessment (oocyte diameter and sperm quality) and egg quality (fertilization, hatching and 5-d larval survival) and quantity (fecundity) parameters suggesting poor egg quality or failed spawning was related to other factors. In addition to this individual variation, the efficacy, fecundity and quality varied with time in relation to date of induction or temperature (**Figure 3.5**). Mean latency period was negatively correlated (R=-0.76) with mean temperature ranging from 111 ± 31 hours at 16.6 ± 0.3ºC to 48 hours at 18.2ºC. The first spawning induction (7 April 2014) and the 6th (not included in the figure) may have been affected by environmental temperatures. On the 15 May 2014 when the temperature was >20ºC there was no successful spawning and when the females were assessed for maturity one week after the induction all females had a low level of maturity with <500 µm oocyte diameter and were replaced.

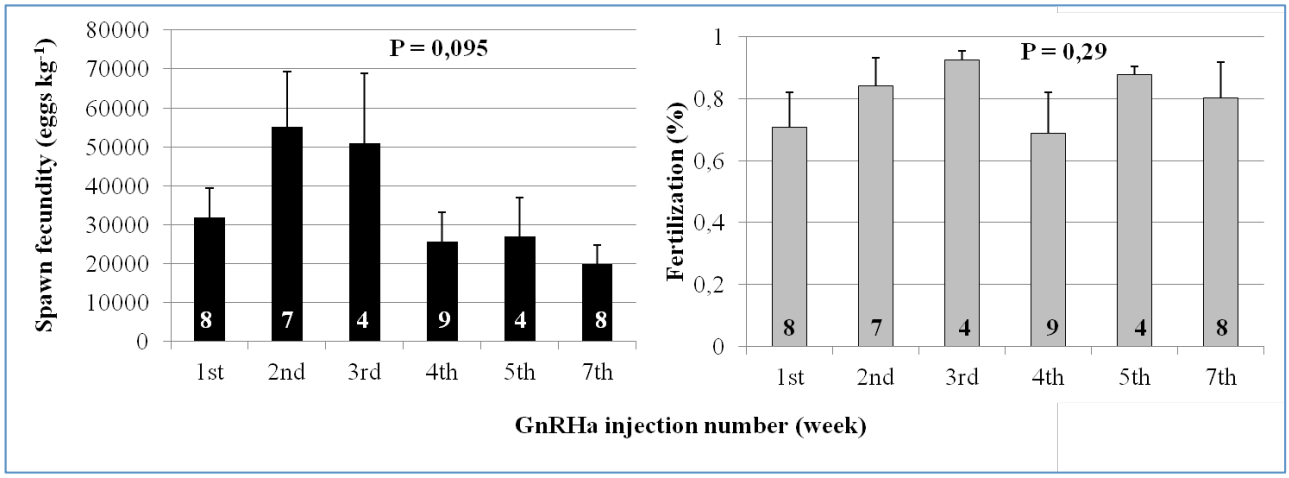

Figure 3.5 Mean (±SEM) daily batch relative fecundity and fertilization success, of meagre pairs (n=1-6) after each weekly GnRHa injection (n=8, once every week) during 2014 (see **Fig. 3.1 and 3.2** for individual spawns). The numbers within the bars indicate the number of individual spawns making the mean. The P values in each graph indicate the significance of a one-way ANOVA statistics.

Although no statistically significant differences were observed in fecundity, there was an initial increasing trend, which then dropped after the third induced spawning. However, a number of females were introduced during the course of the experiment and when the data was organized to show the fecundity from the first induction of a female (**Figure 3.6**) the differences were less pronounced with more similar fecundities over the first 3 inductions before a decrease in the 4th induction.

Despite of these failures apparently related to individual performance, a high number of pairs spawned successfully to produce a large number of full and half-sib families. The success of pairs to produce hatching eggs was 22 (59%) out of 37 pairs that were induced. A total of 10 (27%) pairs produced >500,000 eggs that hatched, 16 (43%) pairs produced >250,000 eggs that hatched and 19 (51%) pairs produced >100,000 eggs that hatched. These data indicate that meagre can be pair spawned to produce commercially viable numbers of eggs. A total of 21 families (full and half-sib) were produced that had >200,000 eggs of >80% fertilization success.

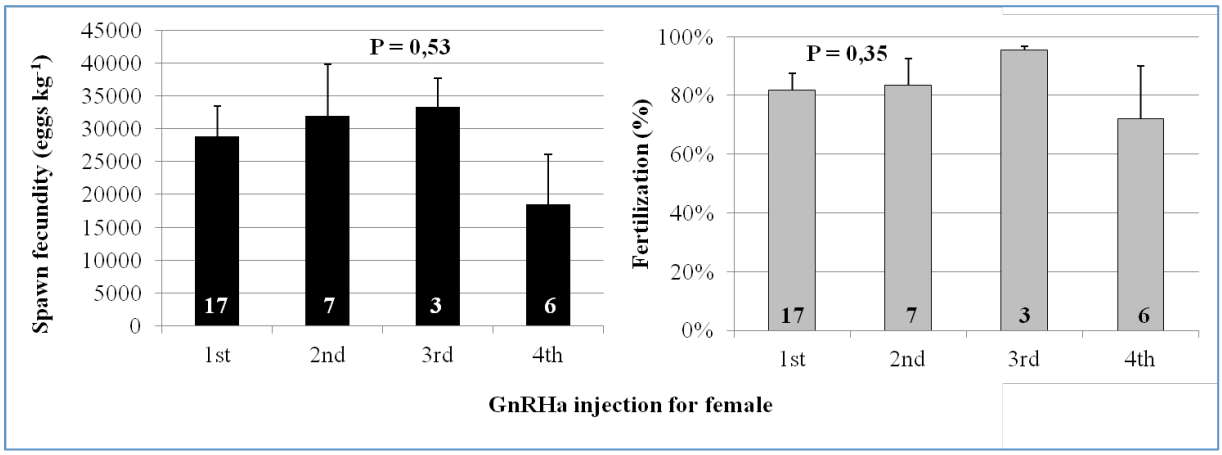

Figure 3.6 Mean (±SEM) daily batch relative fecundity and fertilization success, of meagre pairs induced per week (n=1-6) after each GnRHa injection given to a female (n=10) considering that some females were introduced later in the experiment and induced for the first time on days 22 and 46 during 2014 (see **Fig. 3.1 and 3.2** for individual spawns). The numbers within the bars indicate the number of individual spawns making the mean. The P values in each graph indicate the significance of a one-way ANOVA statistics.

4. Paired spawnings with male rotation on a weekly basis, Experiment III (P3. IRTA).

Two experiments (IIIa and IIIb) were set up with a total of four pairs selected for each experiment (IIIa and IIIb) and the experiments were conducted in a similar way to Experiment II. Females and males were selected by the same criteria (vitellogenic oocytes and flowing milt) and induced with a single injection of GnRHa at a dose of 15 µg kg^{-1} to females and males. Females had a mean weight of 18.4±5.4 kg and males 15.3±2.9 kg. The pairs of breeders were placed in separate 10 m^3 tanks as describe previously. Temperature was maintained at 19ºC. Every 6-8 days the breeders were sampled, maturity status was determined, breeders in advanced stages of maturity were induced as above and males were exchanged to form a different pair for each induced spawning. Breeders were induced to spawn on 5 dates between the 7 May 2015 (day 0) and the 20 May 2015 (day 28) in experiment IIIa and 4 dates between the 8 June 2015 (day 0) and the 29 June 2015 (day 21) in experiment IIIb. Breeders that did not have advanced stages of maturity were replaced with new breeders. In experiment IIIa, one female was replaced on each of the following dates 11 May, 18 May and 25 May 2015. Therefore, a total of 7 different females and 4 different males were induced over 5 weeks, in experiment IIIa. In experiment IIIb, one male was replaced on the 23 June 2015 and, therefore, a total of 4 different females and 5 different males were induced over 4 weeks. Eggs were collected every morning and treated as in experiment II to provide the following information, fecundity, percentage fertilization, percentage hatching and 5-d larval survival.

The efficacy of spawning across the two experiments was 25 (93%) pairs spawned out of a total of 27 pairs that were induced (**Fig. 4.1, 4.2, 4.3** and **4.4**). However, the aim was two dial crosses of paired inductions of 4 females and 4 males to form 2 x 16 full and half-sib families. This was not possible as two pairs did not spawn (female 8-W with male 23-C and female 3-W with male 22-W) and a number of females did not present vitellogenic oocytes after 3 inductions (females 1-W, 5W and 8W), 2 inductions (female 6-W) and 1 induction (females 28-C) and further inductions with these females could not be completed.

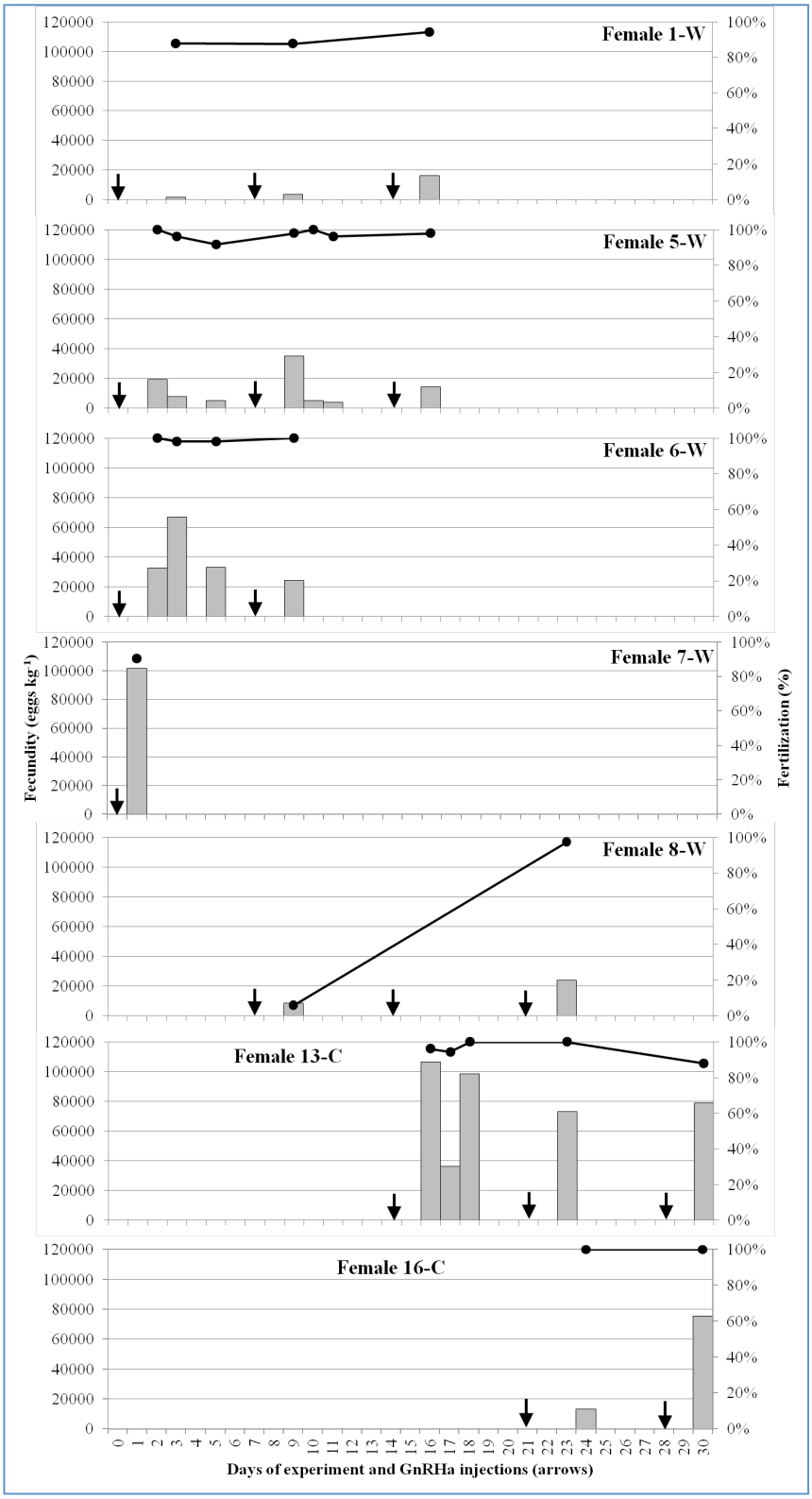

Figure 4.1 Daily batch relative fecundity and fertilization success of individual meagre females (n=7) induced to spawn with multiple GnRHa injections (n=5, once every week) in experiment IIIa during 2015 and paired with four different males. At every GnRHa injection, the males were moved to a different tank, being paired with a different female so all pairs produced a full-sib or half-sib family. The first GnRHa treatment was done on 4 May 2015.

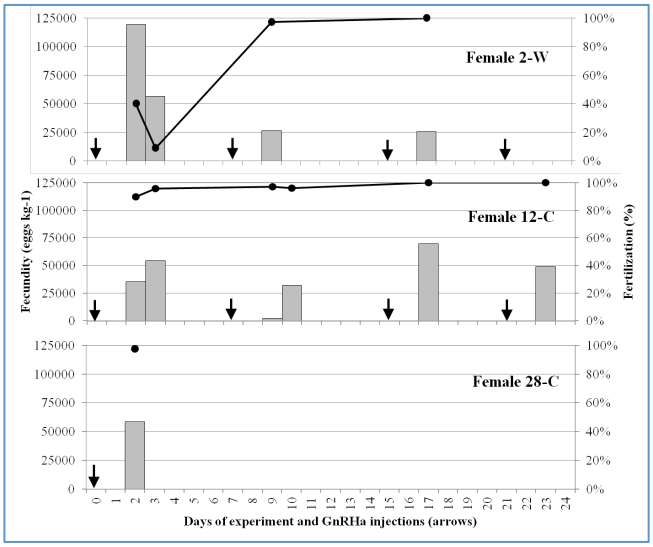

Figure 4.2 Daily batch relative fecundity and fertilization success of individual meagre females (n=3) induced to spawn with multiple GnRHa injections (n=4, once every week) in experiment IIIb during 2015 and paired with five different males. At every GnRHa injection, the males were moved to a different tank, being paired with a different female so all pairs produced a full-sib or half-sib family. The first GnRHa treatment was done on 8 June 2015.

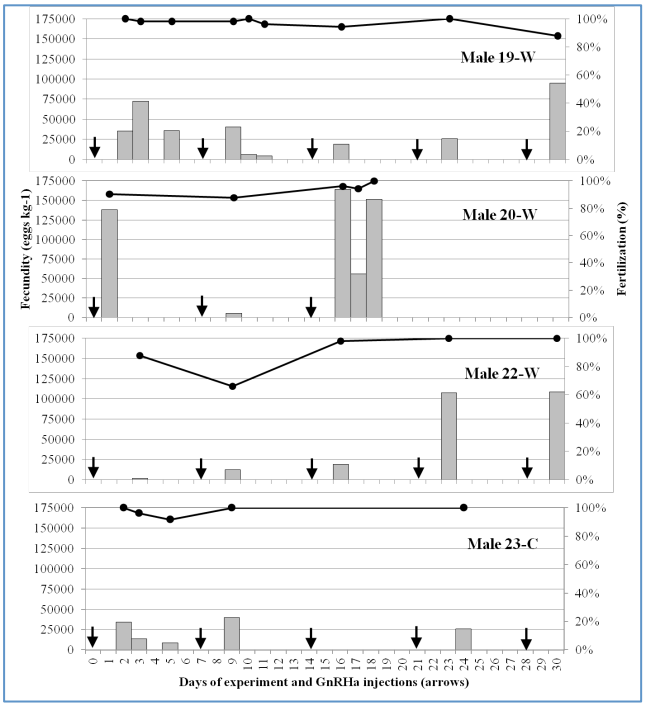

Figure 4.3 Daily batch relative fecundity and fertilization success of individual meagre males (n=4) induced to spawn with multiple GnRHa injections (n=5, once every week) in experiment IIIa during 2015 and paired with seven different females. At every GnRHa injection, the males were moved to a different tank, being paired with a different female so all pairs produced a full-sib or half-sib family. The first GnRHa treatment was done on 4 May 2015.

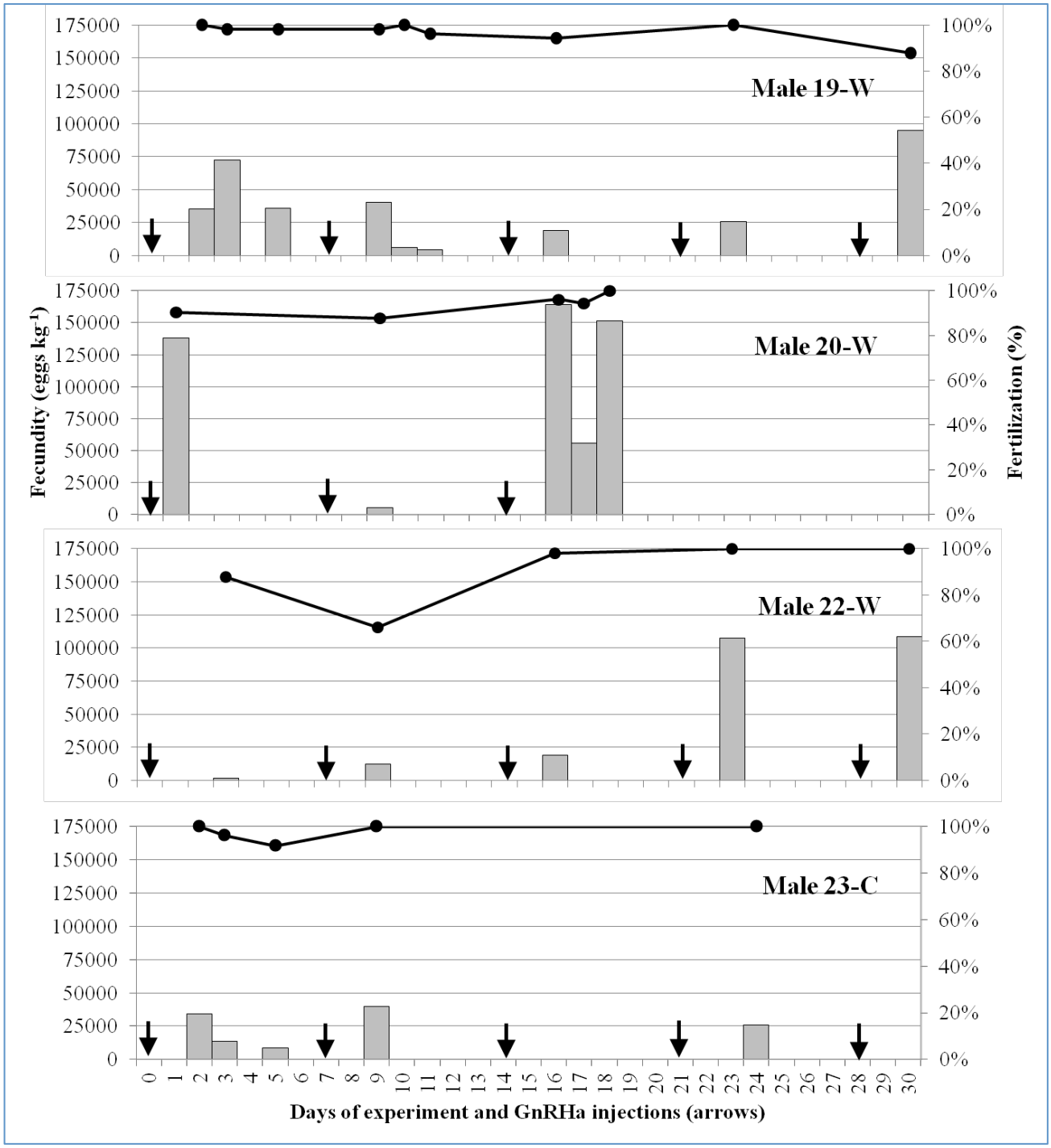

Figure 4.4 Daily batch relative fecundity and fertilization success of individual meagre males (n=5) induced to spawn with multiple GnRHa injections (n=4, once every week) in experiment IIIb during 2015 and paired with four different females. At every GnRHa injection, the males were moved to a different tank, being paired with a different female so all pairs produced a full-sib or half-sib family. The first GnRHa treatment was done on 8 June 2015.

There was variation in egg quality between females and when group by males (**Figure 4.5 and 4.6**). In experiment IIIa, the data from three females (7-W, 8-W and 16-C) that spawned less than 3 times was not included in the figures and statistical analysis. Female 7-W spawned once, 101,448 eggs kg^{-1} with fertilization of 90%. Female 8-W spawned twice, 16,359±7692 eggs kg^{-1} with fertilization of 83±17%. Female 16-C spawned twice, 44,230±31,216 eggs kg^{-1} with fertilization of 100±0%. There were significant differences in the fecundities between females in experiment IIIa and females 1-W and 5-W spawning significantly fewer eggs than female 13-C while female 6-W was intermediate (**Figure 4.5**). There were also significant differences in the percentage fertilization between females in experiment IIIa and females 5-W and 6-W had significantly higher fertilization than female 1-W, while female 13-C was intermediate (**Figure 4.5**). No significant differences were observed between hatching and 5-d larval survival for females in experiment IIIa, however, this should be evaluated cautiously as power was low due to some females having a low "n" of spawns and the associated high variation. Percentage hatching for female 1-W was low (8±7%), while other females ranged from 41±14% for female 6-W to 46±17% for female 5-W and 5-d larval survival ranged from 50±5% for female 6-W to 74±16% for female 13-C.

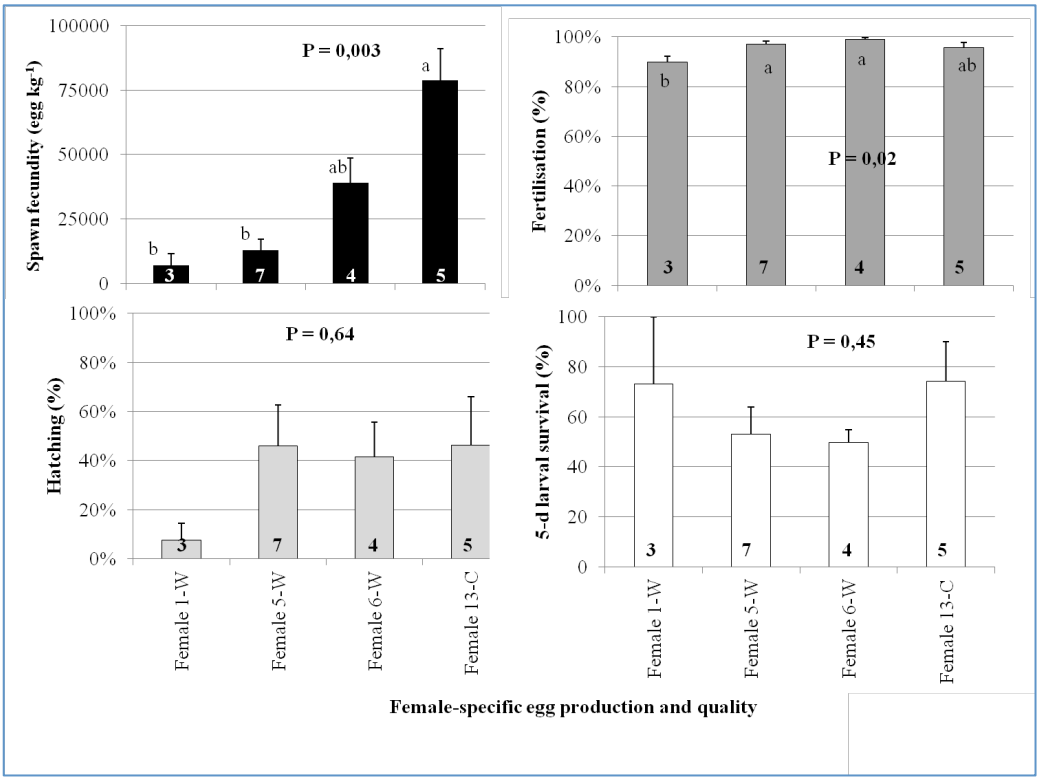

Figure 4.5 Mean (±SEM) daily batch relative fecundity, fertilization success, hatching and larval survival of individual meagre females (n=7) after each GnRHa injection (n=2-4, once every week) in experiment IIIa during 2015 (see **Fig. 4.1** for individual spawns). The numbers within the bars indicate the number of individual spawns making the mean. The P values in each graph indicate the significance of a one-way ANOVA statistics parametric or non-parametric.

In experiment IIIb, the data from two females (3-W and 28-C) that spawned less than 3 times was not included. Female 3-W did not spawn. Female 28-C spawned once, 53,333 eggs kg^{-1} with fertilization of 97%. No significant differences were observed in egg quality parameter for the two remaining females in experiment IIIa (**Figure 4.6**), however, this should be evaluated cautiously as power was low due to some females having a low "n" of spawns and the associated high variation.

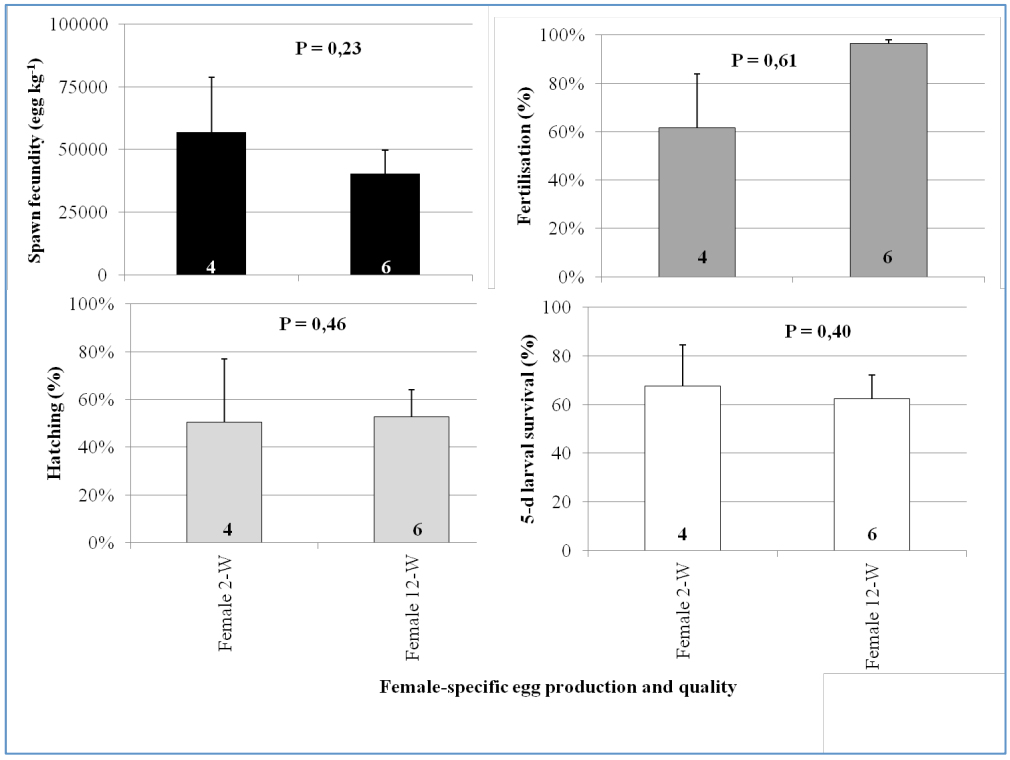

Figure 4.6 Mean (±SEM) daily batch relative fecundity, fertilization success, hatching and larval survival of individual meagre females (n=2) after each GnRHa injection (n=4, once every week) in experiment IIIb during 2015 (see **Fig. 4.2** for individual spawns). The numbers within the bars indicate the number of individual spawns making the mean. The P values in each graph indicate the significance of a one-way ANOVA statistics parametric or non-parametric.

The variation in spawning success, fecundity (**Figure 4.3 and 4.4**) and egg quality (**Figure 4.7 and 4.8**) was obviously similar for the males with the same data redistributed depending on the pair that was formed. In experiment IIIa, there were significant differences in fecundity in relation to male participation, however, a pair wise analysis did not identify differences. Spawns in which male 20-W participated had higher fecundity (102,942±30,876 eggs kg^{-1}) than when other males participated ranging from 24,436±5,885 eggs kg^{-1} (male 23-W) to 49,712±23,904 eggs kg^{-1} (male 22-W). No significant differences were observed in the egg quality in relation to male participation in experiment IIIa (**Figure 4.7**), however, again this should be evaluated cautiously as power was low due to some males having a low "n" of spawns and the associated high variation. Percentage fertilization ranged from 90±6% for male 22-W to 98±2% for male 23-W, percentage hatching ranged from 27±23% for male 22-W to 46±13% for male 19-W and 5-d larval survival ranged from 44±4% for male 23-W to 66±9% for male 19-W.

In experiment IIIb, three males (22-W, 17-W and 26-C) that spawned less than 3 times were excluded from statistical analysis. Male 22-W spawned once, 111,596 eggs kg^{-1} with fertilization of 100%. Female 17-W spawned twice, 38,325±14,251 eggs kg^{-1} with fertilization of 100±0%. Male 26-C spawned twice, 157,471±56,321 eggs kg^{-1} with fertilization of 24±16%. No significant differences were observed in the egg quality in relation to male participation (**Figure 4.7**) of the two males that spawned three times each, however, again this should be evaluated cautiously as power was low due to some males having a low "n" of spawns and the associated high variation.

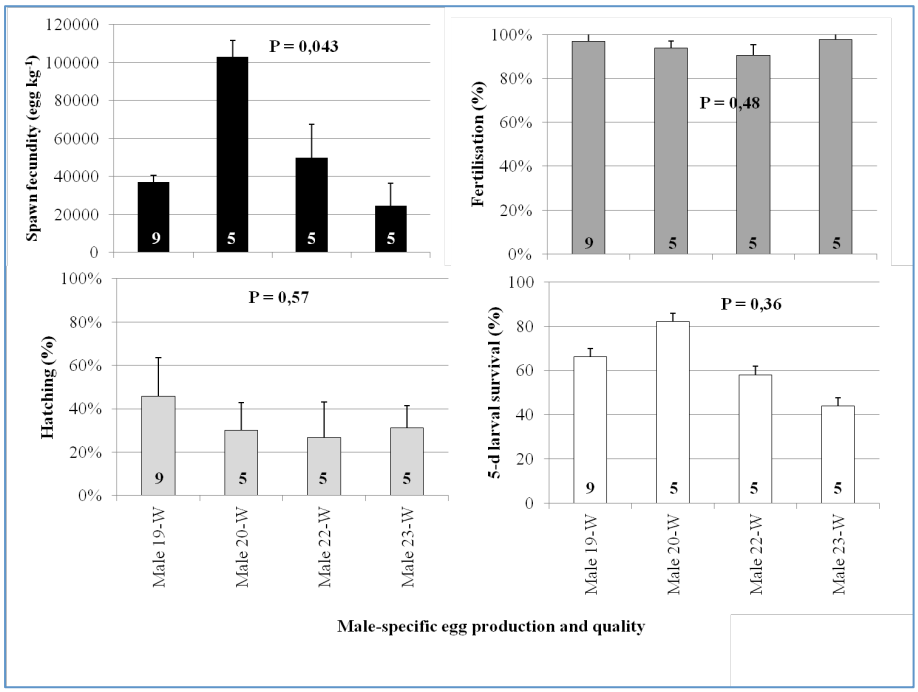

Figure 4.7 Mean (±SEM) daily batch relative fecundity, fertilization success, hatching and larval survival of individual meagre males (n=4) after each GnRHa injection (n=5, once every week) in experiment IIIa during 2015 (see **Fig. 4.3** for individual spawns). The numbers within the bars indicate the number of individual spawns making the mean. The P values in each graph indicate the significance of a one-way ANOVA statistics parametric or non-parametric.

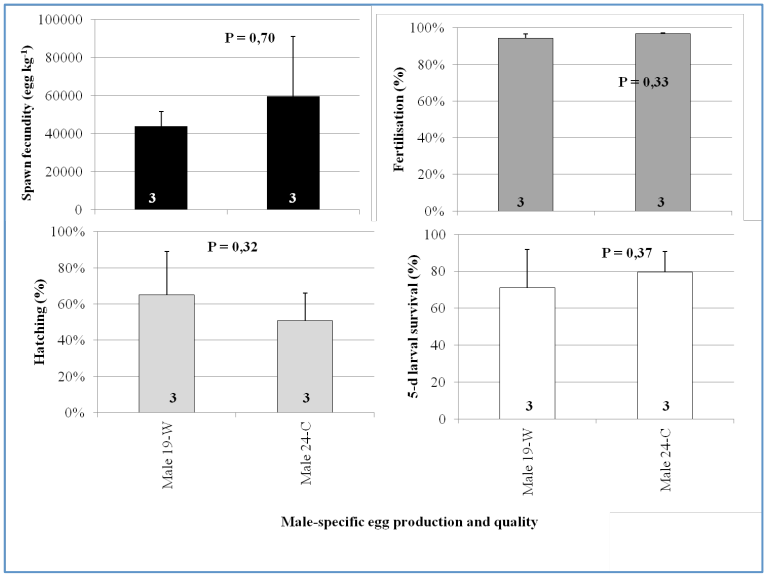

Figure 4.8 Mean (±SEM) daily batch relative fecundity, fertilization success, hatching and larval survival of individual meagre males (n=2) after each GnRHa injection (n=4, once every week) in experiment IIIb during 2015 (see **Fig. 4.4** for individual spawns). The numbers within the bars indicate the number of individual spawns making the mean. The P values in each graph indicate the significance of a one-way ANOVA statistics parametric or non-parametric.

As in experiment II (section 2) there were differences in individual contributions and some individuals appeared to perform poorly in both years such as females 1-W and 8-W and male 26-C. In relation to time, there again appeared to be some poor spawning in relation to the first induced spawning (**Figure 4.9**) in experiment IIIa. However, as in experiment II a number of females were introduced during the course of the experiment in experiment IIIa and when the data was organized to show the fecundity from the first induction of a female (**Figure 4.10**) the differences were less pronounced with more similar fecundities over the first 3 inductions. In experiment IIIb, all females were induced for the first time at the start of the experiment and a clear decrease in fecundity was observed from the first induction to the second induction (**Figure 4.11**).

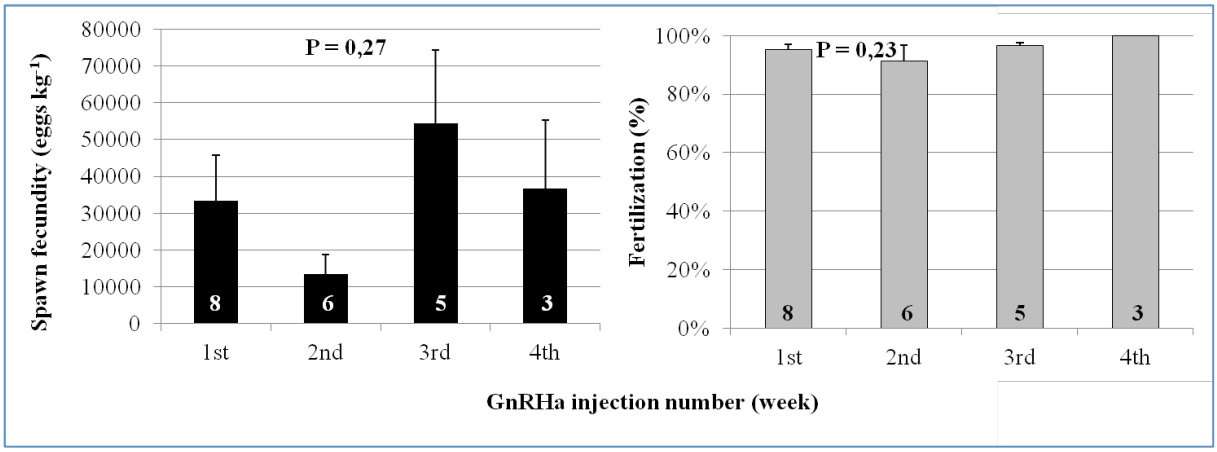

Figure 4.9 Mean (±SEM) daily batch relative fecundity and fertilization success, of meagre pairs (n=2-4) after each weekly GnRHa injection (n=5, once every week) in experiment IIIa during 2015 (see **Fig. 4.1 and 4.2** for individual spawns). The numbers within the bars indicate the number of individual spawns making the mean. The P values in each graph indicate the significance of a one-way ANOVA statistics.

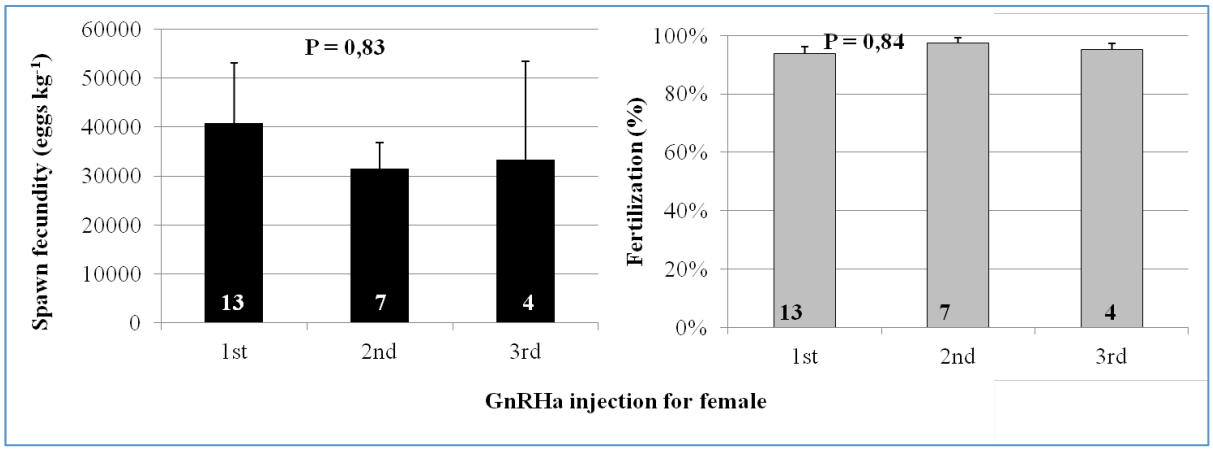

Figure 4.10 Mean (±SEM) daily batch relative fecundity and fertilization success, of meagre pairs (n=2-4) after each GnRHa injection given to a female (n=7) considering that some females were introduced later in the experiment and induced for the first time on days 7, 14 and 21 during 2015 (see **Fig. 4.1 and 4.2** for individual spawns). The numbers within the bars indicate the number of individual spawns making the mean. The P values in each graph indicate the significance of a one-way ANOVA statistics.

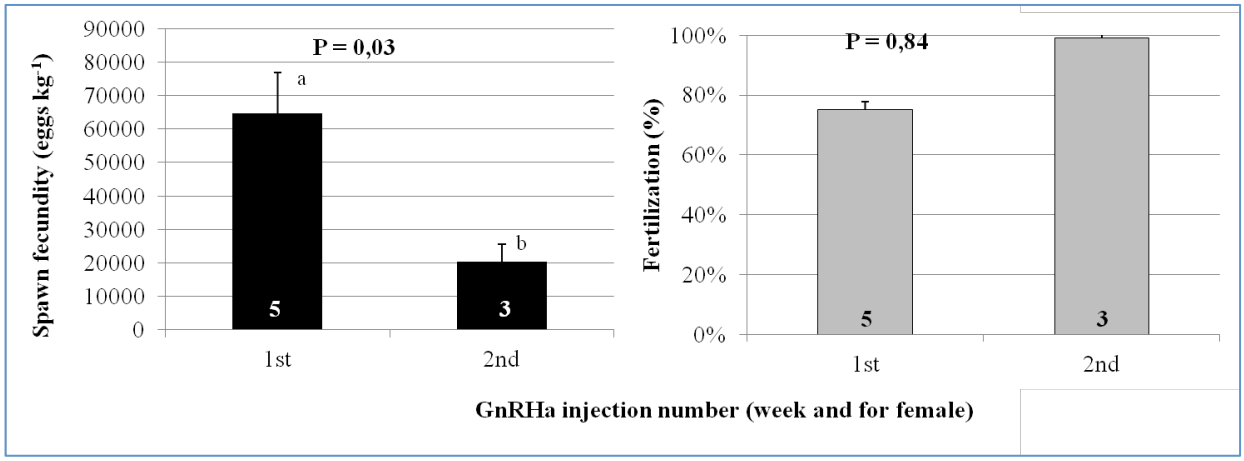

Figure 4.11 Mean (±SEM) daily batch relative fecundity and fertilization success, of meagre pairs (n=2-4) after each GnRHa (weekly) injection given to a female (n=4), all females were induced for the first time on day 0) in experiment IIIb during 2015 (see **Fig. 4.3 and 4.4** for individual spawns). The numbers within the bars indicate the number of individual spawns making the mean. The P values in each graph indicate the significance of a one-way ANOVA statistics.

These two experiments again demonstrated that paired spawning in meagre was possible. However, again some individuals or pairs did not spawn or spawned poor quality eggs indicating that although in the majority of pairs spawning was successful some pairings due to the intrinsic qualities of the individuals in the pair or the combination of the two fish in a pair produced a poor spawning response and/or poor egg quality. A total of 25 (93%) pairs were spawned out of a total of 27 pairs that were induced, which was lower than the aim of two dial crosses of paired inductions of 4 females and 4 males to form 32 (2 x 16) full and half-sib families. A total of 16 families (full and half-sib) were produced that had >200,000 eggs of >80% fertilization success.

Discussion

Together, these experiments have shown that paired tank spawning of meagre is possible for the production of multiple families from parents with known phenotypes. The production of desired families forms the basis of a genetic improvement program (Duncan et al., 2013b) and this has been a bottleneck in setting up breeding programs in some marine species such as gilthead seabream (Gorshkov et al., 1997) and European seabass. That paired spawning is possible in meagre confirms previous indications from communal spawnings, that paired spawning may be a natural phenomenon. For example, Duncan et al., (2012) demonstrated using microsatellite paternity assignment, that a number of spawns from groups of six breeders were from a single pair, indicating that at least when held in a small group a pair had spawned together. Also, Mylonas et al. (2015), set up pairs of breeders and induced the pairs to spawn each week for up to a total of 7 weeks.

The present studies, have advanced these studies twofold. Firstly, paired spawning was achieved repeatedly for up to 17 weeks with no changes in the pair structure. Secondly, male fish were exchanged before each spawning induction to produce a different full or half-sib family with each induced spawning. The efficacy of spawning pairs was high 76% (Experiment 1, 14 pairs spawned from 16 (87%); experiment II, 22 pairs from 37 (59%); experiment III, 25 pairs from 27 (93%)) and across the three experiments a total of 61

families (full and half-sib) were produced that had >200,000 eggs of >80% fertilization success. Obtaining a large number of families with adequate quantities of eggs that can be used on a commercial scale from crosses of selected breeders with desired phenotypes is a prerequisite of a breeding program.

However, not all crosses were successful. In experiment I (section 2), 14 full and half-sib families were achieved when the aim was for 16 (4 x 4 crosses). Two females did not present vitellogenic oocytes after the third induction and could not be spawned a fourth time. In experiment II (section 3), 22 full and half-sib families were produced when the aim was 36 families (6 x 6 crosses) and in experiments IIIa and IIIb (section 4), 25 full and half-sib families were produced when the aim was 32 families (2 x 4 x 4 crosses). It should also be noted that in experiments II and III, this number of families was achieved by also introducing new females when existing females were found not to contain vitellogenic oocytes or did not spawn after consecutive inductions. The pairs not spawning in experiments II and III were also most often associated with females that either did not spawn after a number of inductions or did not present vitellogenic oocytes after a number of inductions and could be induced to spawn again. This was observed after 1 induction (3 females), after two inductions (1 female) and after three inductions (9 females). In addition to this failed spawning or decrease in maturity status after a number of induced spawns, a decline in fecundity with increasing number of induced spawns was observed in some cases. Experiments I and IIIb exhibited a significant decrease in fecundity from the first induction to the second (Exp. IIIb) or third induction (Exp. I). Whilst, experiment II exhibited a slight (not statistically significant) decline to the third induction and experiment IIIb exhibited similar fecundities over the first three inductions. The decrease in fecundities and failed spawning or decline in maturity status represents different spawning kinetics in meagre compared to other studies (for example Section 1 and Mylonas et al., 2013b, 2015; Fernandez-Palacios et al., 2014). Mylonas et al., (2013b; 2015) and Fernandez-Palacios (2014) did not observe this early decline (after 3 inductions or less) in fecundity or maturity status and observed a prolonged period of spawning as long as 17 weeks without an early decline in fecundity or maturity status.

When the experiments in Sections 1 and 2 are compared, the main difference was that in the section 2 experiment the males were rotated before each spawning induction and this suggests that the rotation of the males may have caused a stress in the breeders that resulted in the decline in fecundity and maturity status of the females. This would appear to have had a more pronounced effect in experiments II and III, possibly because of different holding conditions in center P3. IRTA, or that the stock consisted of large, predominantly wild fish that were approximately twice the size / weight of the hatchery produced breeders in centre P1. HCMR (18.4±5.4 to 21.24±3.69 kg in IRTA compared to 9.7 ± 1.0 to 10.2 ± 1.2 kg in HCMR) and may be more prone to stress when transferred to smaller spawning tanks.

Experiment II was conducted with natural water temperatures and the 1st (7 April 2014) and 6th (15 May 2014) spawning induction appeared to have been affected by environmental temperatures. Failed spawning of the 1st spawning induction was when temperatures were 16.6 ± 0.3ºC possibly indicating that this date/temperature when was too early in the spawning season. On the other hand at the 6th spawning induction the temperature was > 20ºC and there was no successful spawning and when the females were assessed for maturity one week after the induction all females had a low level of maturity with <500 µm oocyte diameter and were replaced. Mean latency period was negatively correlated (R=-0.76) with mean temperature ranging from 111 ± 31 hours at 16.6 ± 0.3ºC to 48 hours at 18.2ºC. These observations indicate the importance of temperature control for successful spawning of meagre, with temperatures in the range of 18-20ºC being associated with successful spawning in the present study.

As far as seed production by a commercial hatchery, the reduced fecundities in subsequent spawning inductions with male rotation is considered undesirable, since a hatchery cannot expect to maintain a stable egg production over the course of the reproductive season of meagre. Similar results have also been reported for European seabass, where egg fecundity decreased by ~50% after each subsequent spawning induction with GnRHa injections (Mylonas et al., 2003). So, changing the male for each spawning induction is not a method that should be considered for the general production of a commercial hatchery. However, the method presented in this deliverable is proposed only for the development of multiple families from which to select later the next generation of breeders. The produced fecundity, fertilization success and overall egg/larval quality in the male rotation experiments demonstrated that this method is quite appropriate for the

production a large number of families of adequate number of progeny (eggs and larvae) on which a breeding program could be established.

Therefore, we are confident that this method of pairing male and female meagre with a weekly rotation of the males can be used by commercial hatcheries in order to produce multiple families and build a breeding selection program that can maintain genetic variability. Obviously, in a commercial situation a much larger number of males and females should be used, but the experiment was a successful "proof of concept" for this approach.

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Co-funded by the Seventh Framework Programme of the European Union