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Spawning kinetics and parentage contribution of European sea bass (*Dicentrarchus labrax*) broodstocks, and influence of GnRHa-induced spawning

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ABSTRACT

Increasing parentage contribution in aquaculture broodstocks is important, in order to take full advantage of the available genetic makeup of the chosen fish, and to avoid inbreeding and loss of allele diversity over subsequent production generations. European sea bass (*Dicentrarchus labrax*) broodstocks were evaluated over two reproductive seasons to examine spawning kinetics, egg production, and parentage contribution during spontaneous/volitional spawning. In addition, we obtained preliminary results on the potential of a hormonal therapy to synchronize spawning and increase parentage contribution. Spawning lasted between 25 and 66 days in January-March and consisted of 12–21 daily spawns per broodstock, with individual females spawning 1–5 times and males participating in 1–8 spawns during each reproductive season. Daily fecundity was variable during the season, without any trend, and so were all the examined egg/larval quality parameters. Parentage assignment of the produced families indicated that the majority of progeny from a whole season may belong to a very small number of breeders, with four females producing up to 80 % of the analyzed eggs, while a single male may sire up to 57 % of the fertilized eggs. No significant improvement in the overall parentage contribution was obtained with the hormonal treatment, using gonadotropin releasing hormone agonist (GnRHa). Nevertheless, the daily fecundity was higher, and parentage of the eggs from the first spawn after GnRHa treatment was more equally distributed to multiple males/females, compared to any volitional spawns. The study demonstrates the need to further improve parentage contribution in European sea bass aquaculture, through synchronization of maturation and spawning. Although the GnRHa induction experiment was not replicated in the present preliminary study, the results suggest that hormonally-induced synchronization of maturation may have the potential of producing a larger number of progenies from more families, from where to select the next generation of breeders for a breeding program.

1. Introduction

The European sea bass (*Dicentrarchus labrax*) is a gonochoristic and highly fecund fish with a multiple-batch group-synchronous ovarian development, ovulating 2–4 times during the reproductive season (Asturiano et al., 2000; Mylonas et al., 2003). Together with the gilthead seabream (*Sparus aurata*), they constitute the vast majority of marine aquaculture production in the Mediterranean Sea (Chatziplis et al., 2020), and around 50 % of the farmed production of European sea bass consists of individuals from selective breeding programs (Janssen et al., 2017; Vandeputte et al., 2019).

Currently, almost all commercial gilthead seabream and European sea bass hatcheries rely on spontaneous mass spawning and communal rearing of the produced progeny for their production purposes, and the same approach is used by most hatcheries in order to implement breeding selection programs (Chavanne et al., 2016; Vandeputte et al., 2019). This scheme is less costly and requires less management and dedicated infrastructures compared to single pair mating that relies on *in*

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vitro fertilization (Gjerde, 2005). However, the mating patterns and reproductive success of each breeder in a mass spawning event are difficult to quantify (Gruenthal and Drawbridge, 2012), and a successful breeding program requires a significant number of parents to participate in each spawn, in order to produce progeny of multiple families (Vandeputte and Haffray, 2014). Broodstock performance can be evaluated only when progenies are assigned to their parents (Mirimin and Roodt-Wilding, 2015; Vandeputte et al., 2011), and not all individuals in a breeding stock contribute to the fertilized eggs of a day's spawn. As a result, the number of produced families may be limited, and the produced progeny is often heavily skewed towards one or only a few families, since even participating breeders do not contribute equally. Likewise, there is often a limited variation in the progeny (Loukovitis et al., 2015), which restricts the implementation of a proper selection program and may ultimately lead to inbreeding depression (Rhody et al., 2014). Meanwhile, the application of molecular techniques has facilitated the implementation of selective breeding projects through the use of genetic markers (*e.g.* microsatellite markers) to trace parentage and relatedness among individuals and, eventually, determine and assess broodstock contribution even at very early life stages of the cultured organism (Jones et al., 2010). Currently, numerous microsatellite markers are available for the European sea bass (Chistiakov et al., 2004; Guinand et al., 2015, 2008; Tsigenopoulos et al., 2003) and may be used to assign parentage contribution to a batch of embryonated eggs, before hatching.

As of now, there is few published information on the parentage contribution of European sea bass during volitional mass spawning in aquaculture facilities (Lončar et al., 2014) and no publications have focused in parentage contribution over the course of a reproductive season. In other studies, parentage is inferred as part of QTL studies from mass spawning events (Chatziplis et al., 2007; Volckaert et al., 2012) or artificial fertilization and factorial matings (Palaiokostas et al., 2018, 2015; Saillant et al., 2009, 2006). Therefore, further knowledge of parentage contribution in European sea bass broodstocks is imperative in order to evaluate the feasibility of using volitional spawning to produce families for a breeding selection program. Furthermore, such information is useful for farmers to know the anticipated genetic variation of the produced fingerlings from the spawns obtained during the reproductive season, especially since European sea bass exhibits inconsistent spawning and reduced egg production/quality (Forniés et al., 2001), as well as low milt volumes (Mañanós et al., 2002; Sorbera et al., 1996).

One possible way to overcome spawning dysfunctions in fish is through the administration of gonadotropin-releasing hormone agonist (GnRHa) in fully vitellogenic and spermiating fish (Mylonas et al., 2017, 2010). In the European sea bass, the use of multiple GnRHa injections was considered as the most appropriate method when females were selected from a broodstock and were induced to spawn individually in small tanks (Forniés et al., 2001; Mylonas et al., 2003). However, the usual practice in the industry involves communal spawning of large broodstocks where the females may be in slightly different stages of gonadal development on a specific day. Under these industry conditions, GnRHa controlled-release implants may be more appropriate to induce/synchronize spawning, in order to increase female parentage contribution and fecundity in a single day's spawn. Furthermore, GnRHa implants have been demonstrated to be the best method for enhancing sperm production in European sea bass (Mañanós et al., 2002; Rainis et al., 2003) and other aquaculture fishes (Fakriadis et al., 2020; Mylonas et al., 2017). Therefore, we hypothesized that GnRHa implants may also improve male parentage contribution, as it has been shown in the yellowtail kingfish (*Seriola lalandi*) (Setiawan et al., 2016).

The objectives of the present two-year study were first to examine the spawning kinetics and egg production/quality characteristics of European sea bass broodstocks spawning spontaneously under aquaculture conditions, and to describe the resulting parentage contribution on a spawning-day basis. Secondly, based on the established effectiveness of GnRHa implants in inducing maturation and synchronizing spawning in many fishes, our study examined their potential in increasing both male and female parentage contribution, as a tool for mass spawning breeding selection programs in European sea bass.

2. Materials and methods

2.1. Broodstock management and hormonal induction of spawning

The experiment was undertaken at the broodstock facilities of the Institute of Marine Biology, Biotechnology and Aquaculture (IMBBC) of the Hellenic Centre for Marine Research (HCMR), Heraklion, Crete, Greece. Reproductively mature hatchery-produced European sea bass breeders of two different ages and from different commercial hatcheries (9-year old, ARGO and 6-year old, HELFISH) were utilized during the spawning seasons (January to March) of 2019 and 2020 (Table 1).

The broodstock tanks (15 m^3, 2-m deep) were supplied with well seawater from a recirculating aquaculture system (RAS), and were exposed to simulated natural photoperiod and a temperature of 16.4 ± 0.7°C (mean ± SD) during the spawning period. The fish were fed twice daily with a commercially available broodstock diet (Vitalis CAL XL, 9 mm, Skretting, Norway) until apparent satiation. Measurements of dissolved oxygen (% saturation), salinity, and water quality (NH_3-N and NO_2-N) were conducted once per week and water renewal in the RAS was maintained at 100–200 % d^{-1}. All experimental animals were individually tagged with a passive integrated transponder (PIT) tag (AVID, Uckfield, East Sussex, UK).

Both broodstocks were allowed to spawn spontaneously in Year 1 (2019). In Year 2 (2020), broodstock G1 was induced to spawn with GnRHa-loaded implants after the first spawn of the broostock was observed (16th of January), while broodstock G2 was again allowed to spawn spontaneously. Unfortunately, the GnRHa-induction trial could not be replicated due to limitations in the availability of (a) broodstocks of the same origin and age, and (b) broodstock tanks where all environmental conditions could be maintained similar over the period of two years.

For fin clip collection from all fish and for implanting broodstock G1 (Year 2, 2020) with GnRHa, fish were sedated in their tank with the use of clove oil (0.01 mL l^{-1}) dissolved in absolute ethanol at a 1:5 ratio and then transferred to a smaller tank for complete anesthesia using a higher concentration of clove oil (0.03 mL l^{-1}) (Mylonas et al., 2005; Wagner et al., 2003). Oocyte development was assessed by taking ovarian biopsies through the insertion of a catheter (Pipelle de Cornier®, Laboratoire C.C.D., France) into the ovarian cavity and viewing the oocytes under a compound light microscope (40x and 100x). The mean diameter

Table 1
Biometric data (mean ± SD) of the breeders used in the experiment (January to March of 2019 and 2020). The fish were reproductively mature hatchery-produced European sea bass (*Dicentrarchus labrax*) of two different ages and from different commercial hatcheries (9-year old, ARGO and 6-year old, HELFISH). Individuals used in broodstock G2 in Year 2 were identical to Year 1, but they were not weighed (n/w) due to the experimental set-up.

	Broodstock G1		Broodstock G2	
Year 1 (2019)	Females	Males	Females	Males
ARGO (n)	6	8	7	8
body weight (kg)	5.2 ± 0.7	3.7 ± 0.4	5.1 ± 0.7	3.3 ± 0.3
HELFISH (n)	4	6	4	6
body weight (kg)	2.7 ± 0.7	1.7 ± 0.4	2.8 ± 0.6	2.0 ± 0.6
	Broodstock G1		Broodstock G2	
Year 2 (2020)	Females	Males	Females	Males
ARGO (n)	5	8	7	8
body weight (kg)	5.5 ± 0.7	3.8 ± 0.3	n/w	n/w
HELFISH (n)	4	6	4	6
body weight (kg)	3.5 ± 0.5	2.4 ± 0.4	n/w	n/w

of the largest and most advanced vitellogenic oocytes (n = 10) was measured. All examined females had in their ovaries, oocytes with a mean diameter of >660 μm with very few atretic oocytes present (Corriero et al., 2021), and were induced to spawn using GnRHa implants with a mean dosage (± SD) of 121 ± 28 μg GnRHa kg^{-1}·BW. The males were also treated with GnRHa implants at an effective mean GnRHa dose of 45 ± 11 μg GnRHa kg^{-1}·BW.

The experimental protocol was approved by the appropriate national authority, which is the National Veterinary Service (PN 255356). All methods followed the "Guidelines for the treatment of animals in behavioral research and teaching" (Rollin and Kessel, 1998), the Ethical justification for the use and treatment of fishes in research: an update (Metcalfe and Craig, 2011) and the "Directive 2010/63/EU of the European Parliament and the council of 22 September 2010 on the protection of animals used for scientific purposes" (EU, 2010).

2.2. Egg quality and larval survival evaluation

Each tank was provided with an in-line passive egg collector, which was fitted in the water tank overflow. The egg collectors were examined twice a day, and egg removal was done approximately 12 h after spawning, in order to evaluate fecundity, egg and larval quality, and parentage participation to the eggs produced after volitional or induced spawning of the breeders. From the egg collector, the eggs were first placed in a 10-l bucket provided with gentle aeration, and total fecundity was estimated by counting each egg obtained in a 10-ml sub-sample collected with a pipette after vigorous agitation. At the same time, fertilization success was evaluated through examination of each egg in the subsample under a stereoscope for the presence of a viable embryo (Mylonas et al., 2004).

To monitor embryo and larval survival to yolk sack absorption (7 days after spawning), viable eggs from each spawn were placed individually in 96-well microtiter plates (in duplicates) adapting the procedure by Panini et al. (2001). Briefly, viable eggs were concentrated in a 250 μm mesh filter and were washed and placed in a 2-l beaker with sterilized seawater. Approximately 100–200 eggs were then scooped from the beaker, placed in a Petri dish with seawater, which was then viewed under a stereoscope. Fertilized eggs were taken individually using a pipette with a cut tip set to 200 μl and transferred to each well of the microtiter plates. The microtiter plates were then covered with a plastic lid, placed in an incubator (16 ± 1.0°C), and monitored for 7 days. The number of viable embryos 24-h after the egg collection, hatched larvae (~72 h after spawning), and live larvae on the 5th and 7th-day post-egg collection were recorded. The estimation of percentage survival utilizing the number of individuals that survived in the previous developmental stage as the denominator was considered as a more objective evaluation of survival within the specific developmental stages, eliminating the potential of a masking effect of the previous stage (Mylonas et al., 2015, 2004, 1992).

After the sample collection for fecundity estimation and egg quality assessments, the bulk of the eggs was placed in a conical incubator provided with gentle aeration and water flow at 16 ± 1.0°C. The next day (~24 h), samples were obtained for parentage analysis. Briefly, viable eggs (~1 mL of eggs) were scooped from the water surface using a dip net. The samples were washed thoroughly and preserved in a 7 mL tube containing absolute ethanol. All the collected samples were kept at 4°C until analysis.

2.3. Parentage analysis

2.3.1. DNA extraction, multiplex PCR, and genotyping

Broodstock genomic DNA was extracted from fin clips following the protocol adapted from Miller et al. (1988) (*see* Loukovitis et al., 2015). On the other hand, the DNA extraction protocol in eggs was based on the use of cetyltrimethylammonium bromide (CTAB) (*see* Somarakis et al., 2013). The obtained DNA pellet was eluted with 100 μL (fin clips) and 40 μL (eggs) 1X TE buffer and stored at -20°C until analysis. The detailed protocol of DNA extractions for both the broodstock fin clips and eggs is given in the supplementary material.

Fourteen European sea bass microsatellite loci based on previous studies (Guinand et al., 2015, 2008) were used in the parentage analysis of individual eggs. The Qiagen multiplex PCR kit was used in the amplification of the microsatellites. Each sample contained 4.5 μl Multiplex PCR Master Mix, 0.5 μl Q-Solution, 3.0 μl primer mix, and 1 μl diluted DNA template at approximately 20 ng/μl. Reactions were performed using a T100™ Thermal Cycler (Bio-Rad, USA) with the following conditions: 15 min initial denaturation at 95C, followed by 30 cycles 30 s denaturation at 94C, 90 s annealing at 59°C and 60 s extension at 72°C, and 30 min final extension at 60°C. Genotyping of each sample by allele sizing was performed by dilution of the PCR products in 100 μl (for eggs) and 200 μl (for broodstock fin clips) of distilled water. The diluted products were then loaded and analyzed on an ABI PRISM® 3700 DNA Analyzer (Applied Biosystems, USA), using HiDi formamide and the GeneScanTM-500 LIZ® size standard (Applied Biosystems, USA) as an internal size standard. Alleles were obtained and sized using the software STRand v. 2.4.110 (https://vgl.ucdavis.edu/STRand).

2.3.2. Parentage assignment

The assignment of parentage for individual eggs was carried out using Vitassign software (Vandeputte et al., 2006). The software uses an exclusion-based computation method following the Mendelian principle of allele segregation. Simulations were run to determine the assignment power of the microsatellite markers set, with all the parents genotyped and using the mating scheme declared (see below for each batch and year). Parentage assignment was run for each batch separately by gradually increasing the number of mismatch alleles accepted, starting from perfect matches (no mismatch) up to 8 mismatches (in the G2 offspring, see Results) and to the point at which the assignment rate seems to have reached a plateau. Ten dams and fourteen sires in broodstock G1, while eleven dams and fourteen sires in broodstock G2 were crossed in each group in 2019. In Year 2, one female died in broodstock G1 leaving nine dams and fourteen sires, while the number of breeders utilized in broodstock G2 remained the same.

Lastly, the effective population size (N_e) for each year batch was calculated using the formula adapted from Caballero (1994). Briefly, the effective number of individuals for each parental sex *s* (N_{es}) was calculated (Eq. (1)) and then, these values were combined to determine the overall N_e (Eq. (2)),

$$N_{es} = \frac{N_s \cdot \mu_s - 1}{\mu_s - 1 + \frac{\sigma^2_s}{\mu_s}} \quad (1)$$

$$\frac{1}{N_e} = \frac{1}{4N_{em}} + \frac{1}{4N_{ef}} \quad (2)$$

where *N* is the number of parents, *s* is the parental sex (male, *m* or female, *f*), μ is the mean number of offspring, and σ^2 is the variance of the offspring.

2.4. Statistical analysis

Differences in mean daily relative fecundity, fertilization success, 24-h embryo survival, hatching, and 7-d larval survival were tested using a two-way (year and broodstock) analysis of variance (ANOVA). A one-way ANOVA was used to test the mean differences in the parental contributions of male and female breeders. In addition, the difference between the total relative fecundities between the two broodstocks in both years (together) was tested using a one-sample *t*-test. Data were transformed accordingly to satisfy the ANOVA assumptions, if not normally distributed. Statistical analyses were performed utilizing statistical software (IBM SPSS Statistics 23) at a minimum significance level of

$P \leq 0.05$. Results are presented as means ± standard error (SEM) unless otherwise stated.

3. Results

3.1. Spawning kinetics and egg-larval quality

Spawning began in the middle of January for the two broodstocks for both years. Between 12 and 21 spontaneous spawns were obtained during the two reproductive seasons, with variable daily fecundity and fertilization success in both broodstocks (Fig. 1). Mean daily fecundity was around 1.5×10^5 eggs kg^{-1} female body weight and total fecundity was around 3.1×10^5 eggs kg^{-1} female body weight (Fig. 2**A-B**), without any significant differences between the two stocks or reproductive seasons. In response to the GnRHa implant in 2020, the G1 broodstock exhibited its highest daily fecundity 3 days post-GnRHa treatment (Fig. 1D) with a value that was markedly higher than in 2019 and in any following spawns, but not very different from maximum values obtained in broodstock G2 that was spawning spontaneously for both years. The total relative fecundity of broodstock G1 in response to the GnRHa treatment was 3.25×10^5 eggs kg^{-1} in 2020, compared to 2.33×10^5 eggs kg^{-1} in 2019, showing an increasing trend (Fig. 2**A-B**).

In terms of egg quality parameters, both fertilization success and 24-h embryo survival were higher in 2019 compared to 2020 (two-way ANOVA, $P = 0.01$ and $P = 0.04$, respectively), without any difference between spawning broodstocks (Fig. 2**C** and **D**). No significant difference was observed in hatching success between broodstocks or reproductive season (Fig. 2E). Finally, 7-d larval survival increased from 2019 to 2020 in the GnRHa-implanted G1 broodstock, whereas it was reduced in the spontaneous spawning G2 broodstock, and was lower than in broodstock G1, opposite to what was observed in the previous year (Fig. 2F).

3.2. Parentage analysis

3.2.1. Families and parental contribution

Approximately 34 ± 4 progenies (mean ± SD) per spawn were genotyped using fourteen microsatellite markers, and missing data were scarce for 12 out of 14 loci ranging from 0.06 to 0.78 %; it was higher for locus DLA0234 (2.0 %) and DLA0049 (3.5 %) (Supplementary Table 2). Simulation results show that the microsatellite marker set provides high assignment power in all four datasets (98.75–99.9%) and single assignment rates increase when a greater number of mismatch alleles is allowed (Supplementary Table 1). For the G1 tank, the maximum rate for single assignments was reached at 4 (year 2019) and 5 (year 2020) mismatch alleles; however, for the G2 tank the maximum rate was reached for 7 and 8 mismatch alleles, respectively, for years 2019 and 2020. Not surprisingly, new "single correct" offspring that are added in our catalogue are samples that in their majority (>95 %) enlarge the most populated families rather than indicating new ones.

In broodstock G2, the parental assignment rate was 94.8 % with 525 progenies from 16 spawns allocated to 44 families, out of 154 theoretically projected families (11 females by 14 males) (Table 2). Nine females (82 %) participated in spawning, while 12 out of 14 (86 %) males have fertilized the analyzed eggs (Fig. 3A), with three females (65 % of the analyzed eggs) and four males (81 % of the analyzed eggs) being the main contributors to the spawning events in 2019. The following year, the assignment rate was 92.3 % with 214 offspring from seven spawns assigned to 30 families using an identical number of broodstocks (Table 2). Nine females (82 %) participated in spawning, while 11 out of

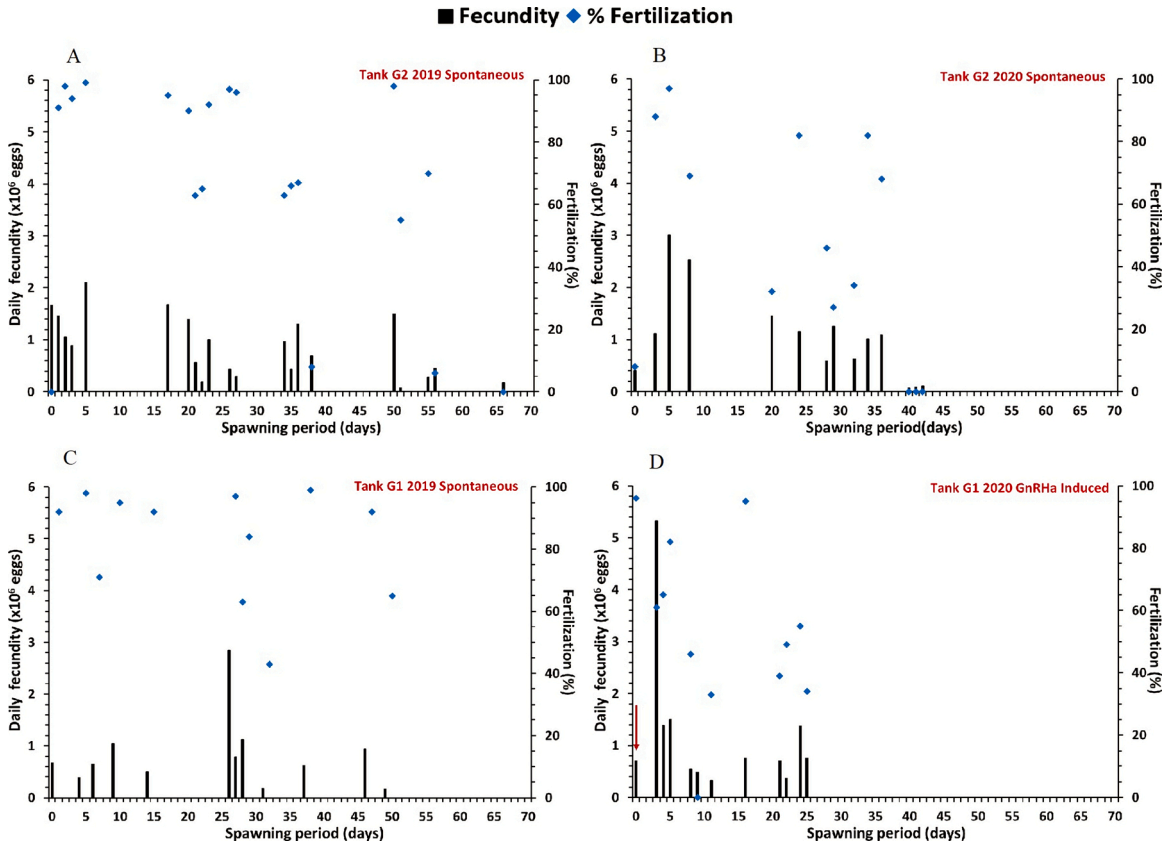

Fig. 1. Daily fecundity (bars) and fertilization success (diamonds) of two European sea bass (*Dicentrarchus labrax*) broodstocks over two consecutive reproductive seasons. In 2019 (A and C), both broodstocks were allowed to spawn spontaneously. In 2020 (B and D), broodstock G2 was again allowed to spawn spontaneously, whereas broodstock G1 was induced using a single GnRHa implantation (red arrow above the day 0 bar in graph D). Day 0 was the first day of spawning each year for each tank (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.).

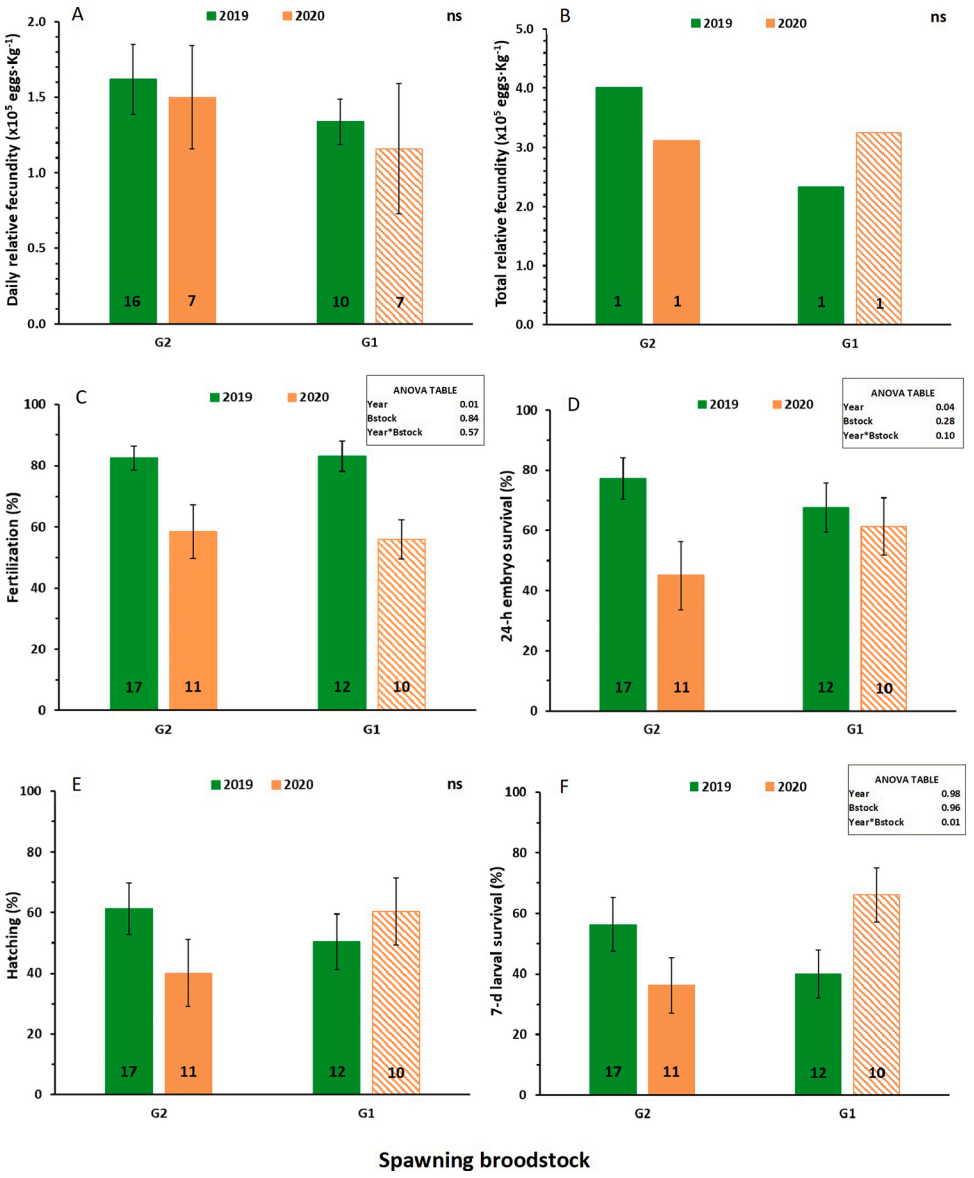

Fig. 2. Mean (± S.E.M) daily relative fecundity (A), total relative fecundity (B), and mean fertilization success (C), 24-h embryo survival (D), hatching, (E) and 7-d larval survival (F) of two European sea bass (*Dicentrarchus labrax*) broodstocks (G2 and G1) during two reproductive seasons (2019 and 2020). In broodstock G2, fish spawned spontaneously in both years, whereas in broodstock G1 the fish spawned spontaneously in 2019, while they were induced with a GnRHa implant in 2020 (striped bars). The numbers inside the bars indicate the n values of the means. A two-way ANOVA was used to examine differences between years and broodstocks (Bstock) for all parameters, except for total fecundity (one-sample *t*-test). Absence of statistical significance ($P > 0.05$) is indicated by "ns".

14 (79 %) males have fertilized the analyzed eggs (Fig. 3B), with four females (80 % of the analyzed eggs) and one male (57 % of the analyzed eggs) being the main contributors to the spawning events in 2020.

In the G1 broodstock, offspring were assigned to their parents with a successful assignment rate of 95.2 % in 2019. A total of 403 eggs from 10 spawns were allocated to 44 families, out of the 140 theoretically expected families (10 females by 14 males) (Table 3). All females from broodstock G1 spawned, while 11 out of 14 (79 %) males have fertilized the analyzed eggs (Fig. 4A), with two females (50 % of the analyzed eggs) and one male (44 % of the analyzed eggs) being the main contributors to the spawning events in 2019. When the same G1 broodstock, was induced to spawn using a GnRHa implant in 2020, the successful assignment was 92.9 %. In total, 221 eggs from seven spawns were assigned to 37 families (Table 3). Eight females (89 %) participated in spawning, while 12 out of 14 (86 %) males have fertilized the analyzed eggs (Fig. 4B), with three females (88 % of the analyzed eggs) and three males (64 % of the analyzed eggs) being the main contributors to the spawning events in 2020. Overall, 97 % of the analyzed eggs in the first two spawns post-GnRHa treatment (Days 3 and 4) were from the three females (F3, F5, F9) that contributed to the majority of the eggs in 2020. These females had a mean oocyte diameter of 730 μm (F3), 740 μm (F5), and 700 μm (F9) before the hormone induction. Regarding male participation, almost 70 % of the analyzed eggs during the first two spawning days after the GnRHa treatment were fertilized by three males (M6, M8, M11) (Table 4) who also participated in most of the spawning events in 2020. These same males contributed significantly to the spontaneous spawns of the previous year (2019), siring ~75 % of the total progeny analyzed for the whole season.

If we consider the effective number of male (N_{em}) and female (N_{ef}) breeders estimated by (1), these values are consistently lower than census number in the breeding stocks; this holds true also for the values of the effective number (N_e) of breeders which range from 6.62 to 10.77 (G2 in 2020 and 2019, respectively). Interestingly, for the GnRHa-induced (G1) batch in 2020, values for N_{em} and N_{ef} are inversed with that for males being the higher (5.93) and for females the lower (3.53). However, in the former induced batch, the estimated N_e (8.85) is close to the one encountered in 2019 (8.93) from spontaneous spawning (Supplementary Table 3).

Regarding the number of breeders contributing to each spawn, in the spontaneously spawning broodstock G2, 1–3 females and 1–5 males participated in each spawn in 2019 (Fig. 3A). In nine out of the 16 spawning events, the eggs were produced by a single female. In the

Table 2
Percentage distribution of broodstock G2 during the spawning seasons of 2019 and 2020. Fish were allowed to spawn spontaneously in both years. The percentage contribution of females and males that contributed greatly in the analyzed egg samples are indicated in bold.

Broodstock G2, 2019 Spontaneous spawning	Males														
Females	M1	M2	M3	M4	M5	M6	M7	M8	M9	M10	M11	M12	M13	M14	Sum
F1															0.00
F2	0.19	2.10			6.48	3.62	0.19							5.14	**17.71**
F3	0.19			0.38	1.52	1.90	2.67		0.19				0.19	3.43	10.48
F4					0.57									0.57	1.14
F5					0.19										0.19
F6	0.38	9.52			6.10	6.10				1.52			0.19	3.62	**27.43**
F7	0.38	0.58			3.81	0.38	0.19							3.05	8.39
F8					0.19	3.24								2.48	5.90
F9						3.24				3.62		0.19		1.90	8.95
F10	0.19				1.90	0.95				11.43	1.14	0.38		3.81	**19.81**
F11															0.00
Sum	1.33	12.20	0.00	0.38	**20.76**	**19.43**	3.05	0.00	0.19	**16.57**	1.14	0.57	0.38	**24.00**	**100.00**

Broodstock G2, 2020 Spontaneous spawning	Males														
Females	M1	M2	M3	M4	M5	M6	M7	M8	M9	M10	M11	M12	M13	M14	Sum
F1														0.47	0.47
F2					6.54	0.47	0.47		0.47					1.40	9.34
F3													0.47		0.47
F4					6.07									15.89	**21.96**
F5		0.93	0.47	0.47	6.07	4.67	0.47							3.74	**16.82**
F6	0.47				5.61	5.14					0.47			11.21	**22.90**
F7	0.47	0.47				0.47	0.47						0.93	15.89	**18.69**
F8											0.47			8.41	8.88
F9															0.00
F10															0.00
F11													0.47		0.47
Sum	0.93	1.40	0.47	0.47	**24.29**	10.75	1.41	0.00	0.47	0.00	0.93	0.00	1.87	**57.01**	**100.00**

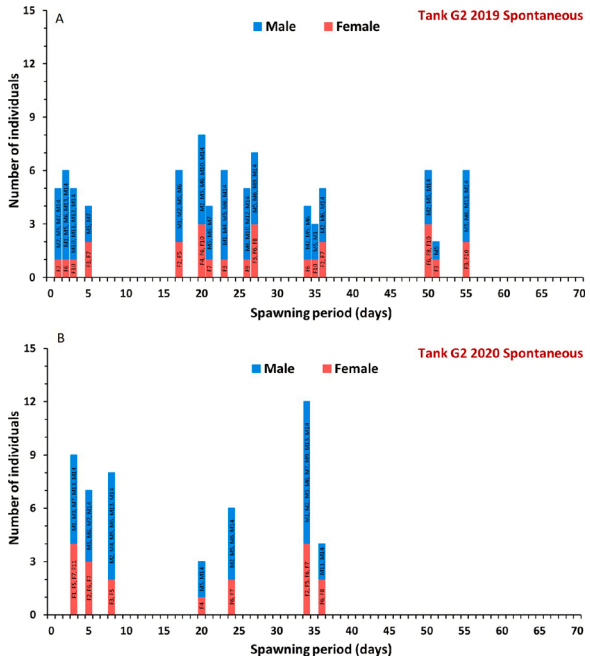

Fig. 3. The number of female and male European seabass (*Dicentrarchus labrax*) breeders in broodstock G2 that contributed to the progeny of each spontaneous spawn in 2019 (A) and 2020 (B). The alphanumeric codes inside the bars indicate the individual breeders that participated in each daily spawn.

following year, 1–4 females and 2–8 males participated in each spawn, with only one spawn consisting of eggs from a single female (Fig. 3B). Each female spawned 1–5 times in 2019 and 1–4 times in 2020 with an interval of every 2–3 weeks and 0.5–3 weeks, respectively. In the case of males, the number of spawning days varied among individuals in both years.

In broodstock G1, 1–3 females and 2–8 males participated in the spontaneous spawns in 2019 (Fig. 4A). In five out of 10 spawning events, the eggs obtained were from a single female. In response to the GnRHa implants, 1–5 females and 4–7 males of the same broodstock participated in each spawn, with only one spawn consisting of eggs from a single female in 2020 (Fig. 4B). Each female spawned spontaneously 1–3 times in 2019 and 1–4 times when induced with GnRHa in 2020, with an interval of every 2–3 weeks and 0.5–2 weeks, respectively. In the case of males, individual participation varied between 1–10 times in the 2019 season, and between 1 and 7 times in 2020, with no noticeable trend.

The mean number of females who contributed to each spawn increased significantly from 2019 to 2020 in both broodstocks (one-way ANOVA, $P < 0.03–04$), regardless of the administration of GnRHa implants (Fig. 5). On the contrary, no change was observed in the mean number of males participating in each spawn between years or spawning broodstocks (one-way ANOVA, $P > 0.05$).

3.2.2. *Breeding program scenario*

Considering a commercial scenario where a farm is interested in rearing together eggs, larvae, and juveniles obtained from a maximum number of families, this can be done by stocking together eggs produced from consecutive spawns of a broodstock, with a maximum time difference of 48 h. The second (younger batch) is usually maintained for a few days at 2°C higher, so that they will progress a bit faster, and then they are incorporated with the first batch. Therefore, we examined the potential (a) number of families produced, and (b) the progeny contribution (%) of these different families, in two consecutive spawns from either spontaneous spawns or in response to a GnRHa induction of spawning. To run this comparison, we looked at two consecutive spawns of adequate fecundity and fertilization success from the spontaneously spawning broodstock G2 and the GnRHa-induced broodstock G1 in 2020 (Table 4). In broodstock G2, six families from four females and five males were identified on Day 3 of spawning in 2020, but one family contributed 86.5 % of the progeny. On day 5, eight families were produced from three females and four males, and one family produced 40.6

Table 3
Percentage distribution of broodstock G1 during the spawning season of 2019 (spontaneous spawning, upper part) and 2020 (GnRHa-induced spawning, lower part). The percentage contribution of females and males that contributed greatly in the analysed egg samples are indicated in bold.

Broodstock G1, 2019 Spontaneous spawning	Males														
Females	M1	M2	M3	M4	M5	M6	M7	M8	M9	M10	M11	M12	M13	M14	Sum
F1		0.99	0.50	1.74		6.20	0.99	2.98			5.96		0.99		**20.35**
F2								0.25			0.25				0.50
F3		1.74		2.23			0.25	4.71			0.50				9.43
F4		0.50				2.23	2.73	0.25			1.24		0.25		7.20
F5		0.25	4.22		0.25	5.96	0.25	3.97	0.25		14.14				**29.28**
F6						1.24	0.25	0.99			8.44				10.92
F7	0.25														0.25
F8						1.74					2.23				3.97
F9						0.25			1.74		2.48				4.46
F10				0.74		0.25	2.48		1.49		8.68				13.64
Sum	0.25	3.47	4.71	4.71	0.25	**17.87**	6.95	13.15	3.48	0.00	**43.92**	0.00	1.24	0.00	**100.00**
Broodstock G1, 2020 GnRHa Induced spawning	Males														
Females	M1	M2	M3	M4	M5	M6	M7	M8	M9	M10	M11	M12	M13	M14	Sum
F1	1.81						0.45	0.45			0.90		0.45		4.07
F2				0.90	0.45	0.45									1.81
F3	5.43		0.90	3.62	0.45	10.86	0.45	7.69							**29.41**
F4		0.45		0.90											1.35
F5		4.52		2.26		1.36	0.45	7.24			8.14		0.90		**24.89**
F6	(this female died prior to the onset of the 2020 reproductive season)														
F7				0.90		0.45	0.45						1.36		3.17
F8	0.45									0.45				0.45	1.36
F9	2.71	4.07		0.90		7.24		7.24			11.76				**33.94**
F10															0.00
Sum	10.41	9.05	0.90	9.50	0.90	**20.37**	1.81	**22.63**	0.00	0.45	**20.81**	0.00	2.72	0.45	**100.00**

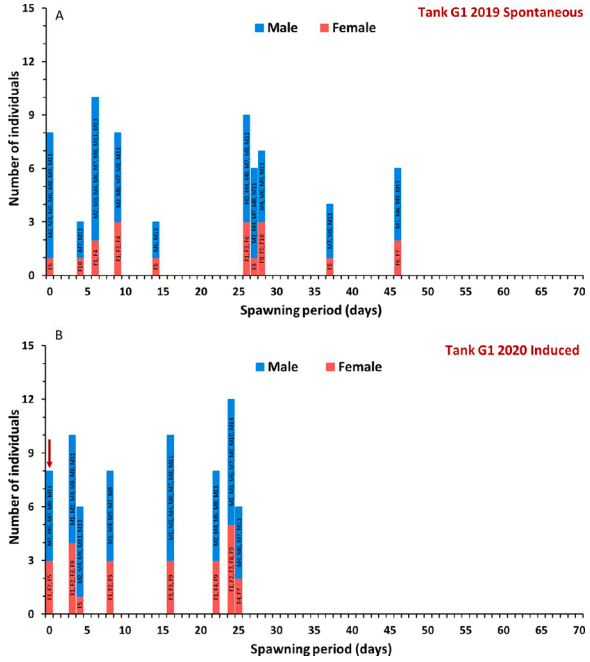

Fig. 4. The number of female and male European seabass (*Dicentrarchus labrax*) breeders in broodstock G1 that contributed to the progeny of each spawn in 2019 (spontaneous) and 2020 (in response to GnRHa). The red arrow above the Day 0 bar (Graph B) indicates the time of GnRHa implantation. The alpha-numeric codes inside the bars indicate the individual breeders that participated in each daily spawn (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.).

% of the progeny. In the GnRHa-induced broodstock G1, eight families were identified from four females and six males on Day 3 post-GnRHa treatment. More than half of the progenies were produced by two families with contributions of 32.5 % and 22.5 %. On Day 4 post-GnRHa treatment, five families were observed from a single female and five males, with one family having a contribution of 60.7 %.

Table 4
The percentage (%) progeny distribution in families produced in two consecutive spontaneous spawns on days 3 and 5 of broodstock G2 (Year 2020), and in two GnRHa induced spawns on days 3 and 4 of broodstock G1 (Year 2020).

Broodstock G2, spontaneous	Males					
Day 3	Females	M1	M3	M7	M13	M14
	F1					2.7
	F5		2.7			
	F7	2.7		2.7		86.5
	F11				2.7	
Day 5		M5	M6	M7		M14
	F2	40.6	3.1	3.1		9.4
	F6	21.9	3.1			15.6
	F7					3.1
Broodstock G1, GnRHa induced	Males						
Day 3	Females	M1	M2	M4	M6	M8	M11
	F1						2.5
	F2			2.5			
	F3	7.5		5.0	15.0		
	F9		12.5			32.5	22.5
Day 4			M2	M4	M6	M13	M11
	F5		10.7	17.9	3.6	7.1	60.7

Considering the effective number of breeders (N_e), we see an increase for the G1 broodstock when using GnRHa (from 5.11 to 6.53 in 2020); however, for the spontaneous spawning in the G2 broodstock, N_e drops from 6.21 to 4.66 in year 2020 (Supplementary Table 4).

4. Discussion

The European sea bass has been proposed to spawn 2–4 times during the reproductive season between January and March (Asturiano et al.,

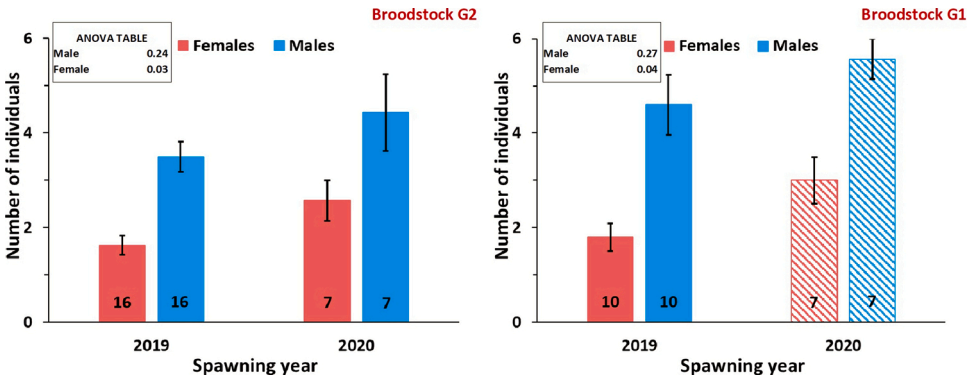

Fig. 5. Mean (± S.E.M) number of male and female European seabass (*Dicentrarchus labrax*) participating in the different spawns during each reproductive season (2019 and 2020). In broodstock G2, fish spawned spontaneously in both years, whereas in broodstock G1 the fish spawned spontaneously in 2019, while they were induced with a GnRHa implant in 2020 (striped bars). The numbers inside the bars indicate the n values of the means (daily spawns).

2000) with an interval between spontaneous or GnRHa-induced spawns of 1–2 weeks (Asturiano et al., 2000; Mylonas et al., 2003). The present study provides the first confirming data on the spontaneous/volitional spawning kinetics of European seabass in aquaculture facilities over two consecutive reproductive seasons. Information is also provided on the number of spawns produced and the periodicity of spawning of each fish in a broodstock, as well as egg production data such as fecundity, fertilization and embryonic/larval survival. Over the two years of the study, spontaneous spawns were obtained with a highly variable periodicity, ranging from 2 to 23 days. The monitored females in both broodstocks spawned spontaneously for a minimum of one to a maximum of five spawns, and a mean (± SD) of 2.2 ± 1.3 spawns·season^{-1}. Daily batch fecundity and fertilization success were also variable during the season, without a trend in either broodstock. The total relative fecundities obtained were comparable to those reported in earlier studies (Barnabé, 1995; Carrillo et al., 1989; Mañanós et al., 1997; Prat et al., 1999, 1990), as were the mean fertilization values obtained, ranging from 66 to 84 % (Carrillo et al., 1989; Prat et al., 1999, 1990). The 24-h embryo survival and hatching success were similar or lower compared to the numbers recorded in earlier studies (Carrillo et al., 1989; Forniés et al., 2001; Mañanós et al., 1997). Therefore, one cannot predict the time of the reproductive season that maximum fecundity -and thus, female participation and/or egg quality may be expected in European sea bass broodstocks- making the decision of when to collect eggs for producing offspring for a breeding selection program very difficult to make.

To look at parentage contribution in the spontaneous spawns produced over the whole reproductive season, 14 polymorphic microsatellite markers were validated and demonstrated to successfully infer parentage in the present study. Up to four mismatches were accepted to identify unique allocations, although the assignment rates plateaued, and a minor change was observed up to eight mismatches (Supplementary Fig. 1). This study is the first to examine the parentage contribution of each European sea bass breeder used in the progeny composition for two consecutive reproductive seasons in spontaneous and GnRHa-induced spawning. The parentage assignment rates differed slightly in both broodstocks in the two reproductive seasons. The assignment rates were greatly higher than the 34.8 % obtained by Chatziplis et al. (2007) in the European sea bass using 13 microsatellite loci, but slightly lower than the rate of 98.9 % reported by Vandeputte et al. (2006) using six microsatellites. Nevertheless, the results should be interpreted with caution since any comparison with previous studies needs to consider the total number of possible parents and the non-sampled broodstock (as in Chatziplis et al., 2007), because obviously it is easier to assign when there are only few possible breeders. Moreover, the values obtained in the present study were comparable to the results from other species, which varied between 73 and 100 % (Bright et al., 2016; Dettleff et al., 2020; Liu et al., 2012a, b; Loukovitis et al., 2011; Mirimin and Roodt-Wilding, 2015; Nousias et al., 2020; Setiawan et al., 2016).

Almost all commercial hatcheries producing European sea bass rely on spontaneous mass spawning to produce eggs for their larval rearing operations. Spontaneous mass spawning is also the main method used to produce families for later selection in mass spawning-based breeding programs (Chavanne et al., 2016; Vandeputte et al., 2019). In theory, each breeder has the same chance to contribute genes in the progeny in a communal spawn. However, this does not happen in practice since female spawning is not synchronous, and also because there are differences in the quality/characteristics of the produced gametes (Bardon-Albaret and Saillant, 2017; Bobe and Labbé, 2010) affecting viable fertilized egg production. Furthermore, sperm competition may exist (Gasparini et al., 2010; Ottesen et al., 2009) and dominance hierarchies develop among male breeders, preventing equal participation. In the present study, a pronounced dominant role of some breeders was observed in the broodstocks spawning volitionally in two reproductive seasons, with some females contributing each up to 30 % of the progeny produced in the whole reproductive season. Similarly, one male contributed up to 50 % of the progeny analyzed in the whole reproductive season and a few others sired about 20 % each, suggesting a strong hierarchy among males in the breeding stock. Such highly skewed parentage contribution was reported in earlier studies in the European sea bass (Lončar et al., 2014), and in an extreme situation, only one dam was the parent of nearly 95 % of the correctly assigned progenies, and one male sired 50 % of them (Chatziplis et al. (2007). Overall, the distribution of families in each tank over the course of two reproductive seasons implied that some parents had larger contribution compared to others, something that has been reported in various studies of other species (Antonello et al., 2009; Bright et al., 2016; Brown et al., 2005; Chavanne et al., 2014; Dettleff et al., 2020; García-Fernández et al., 2018; Hara and Sekino, 2003; Liu et al., 2012a; Sekino et al., 2003). For example, in the Atlantic cod (*Gadus morhua*), the parentage contributions were also skewed towards few individuals, where up to 50 % of the cohort from a single day's mass spawning was sired by a single male and 45 % by one female (Herlin et al., 2008). This highly skewed spawning pattern was also described in mass spawning of brown sole (*Pleuronectes herzensteini*) throughout the spawning period, where parental participation was as high as 73 % by one male and 42 % by one female (Kim et al., 2007). Finally, in a grow-out cage of meagre (*Argyrosomus regius*), sampled juveniles from one volitional spawning event also revealed a skewed parental distribution, where 40 % of the offspring came from two parental pairs, and one male sired almost 50 % of the progenies (Nousias et al., 2020). Highly skewed distribution of parental success in aquaculture may be a factor contributing to the low effective population size or census population size ratios (Bekkevold et al., 2002). As follows, such dominance may result in small numbers of families and a limited effective population size (Falconer and Mackay, 1996; Nousias et al.,

2020), thus causing a negative effect on genetic variability in the hatchery (Loukovitis et al., 2015; Sudo et al., 2018). Eventually, this low number of breeders participating in a communal spawning population may lead to higher levels of inbreeding (Brown et al., 2005), especially in the absence of external manipulations or the introduction of additional broodstock (Trippel et al., 2009). The parentage contribution findings from the present study would be valuable for better broodstock management in commercial hatcheries and should also serve as baseline data for subsequent studies.

Implants loaded with GnRHa have been shown to be very effective for the induction of oocyte maturation and ovulation, enhancement of sperm production and synchronization of spawning in quite a number of fishes (Mylonas et al., 2010; Zohar and Mylonas, 2001). Therefore, in the present study we examined the potential of these implants in increasing the number of the produced families in a single spawning event, by increasing the number of females spawning (Forniés et al., 2001) and by enhancing the sperm production of the males in the broodstock (Rainis et al., 2003; Sorbera et al., 1996). In response to the GnRHa implant, the first spawning was obtained after 3 days, as expected (Forniés et al., 2001), with more spawns obtained in the following three weeks. Although the spawning kinetics in response to the GnRHa treatment did not seem to differ greatly from the spontaneous spawns of the same broodstock (G1) in the previous year, or from the spontaneously spawning broodstock G2 in both years, consecutive spawns from individual GnRHa-implanted females were obtained with a smaller interval, ranging from 1 to 5 days apart. Also, the resulting fecundity in the first spawn after GnRHa treatment (Day 3) was markedly higher than for any spawn from the two broodstocks over the two reproductive seasons, although the values obtained were lower than when females were allocated individually to spawning tanks (Forniés et al., 2001). On the contrary, all the egg quality parameters reported in the latter study were lower than in the present study, possibly due to the separation of females in individual tanks and the stress associated with the smaller enclosures. Unfortunately, the GnRHa-induction study could not be replicated, due to broodstock and facility limitations, so the present results should be considered as preliminary. In general, egg quality parameters were similar between spontaneous and GnRHa induced spawns, suggesting that the hormonal treatment neither improved nor decreased the quality of the obtained gametes, as shown also in other fishes (Mylonas et al., 2010). Additionally, for the GnRHa-induced broodstock in 2020, the overall effective number of breeders (N_e) showed practically no changes when compared to the spontaneous spawning in 2019 (8.85 and 8.93, respectively). Nevertheless, an increase in the effective number of breeders in response to the GnRHa treatment was observed when the two consecutive spawns were compared to the spontaneous spawning in the previous year (6.53 and 5.11, respectively).

In terms of parental participation in the commercial scenario of using eggs from two consecutive spawns produced within 48 h, we did not observe the expected significant increase in the number of females being synchronized to mature and spawn after the GnRHa implantation, nor the expected increase in the number of males participating in a single day's spawning. However, the parentage contribution in the obtained eggs was more equally distributed to multiple breeders of both sexes in response to the GnRHa treatment, compared to any spontaneously produced spawns. As a result, using eggs obtained during the first two spawns after a GnRHa implantation would result in larger numbers of progeny from more families, from where to select the next generation of breeders for a breeding program. These improved results were probably due to (a) the increase in fecundity of the females that matured and spawned, and (b) the enhanced sperm production of the induced males. Both events resulted in the production of larger numbers of fertilized eggs from the different breeders involved in the spawns of Days 3 and 4. In future studies, we should examine ways to optimize the GnRHa treatment in order to synchronize more females to spawn. This can be done by using alternative hormonal therapies (*e.g.* single injections) and/or by delaying the treatment time towards the middle of the reproductive season, as opposed to the present study that it was done in the beginning. This would allow more females to complete vitellogenesis and even undergo one or two spontaneous spawns before the GnRHa treatment. In the present study, GnRHa implants were used, even though repeated GnRHa injections given at intervals of 7–14 days produced better results in European sea bass, in terms of multiple spawns of high fecundity and quality eggs (Mylonas et al., 2003). The same results were also reported in meagre, where repeated weekly GnRHa injections produced consistently eggs of high fecundity and quality parameters (Mylonas et al., 2016, 2015). The reason we chose to use GnRHa implants here, was because the current industry practice necessitates the communal spawning of multiple broodstocks with inclusion of females of varying gonadal development stages and we treated the fish at the very beginning of the spawning season. The controlled-release action of GnRHa implants – with up to 8 weeks elevations in plasma Luteinizing Hormone levels (Zohar and Mylonas, 2001) - was considered to be more suitable to induce maturation and synchronize spawning, under these conditions. In future studies, where fish could be treated during the middle of the reproductive season, perhaps a GnRHa injection may prove to be more effective in synchronizing maturation and inducing spawning of a larger percentage of the females (Mylonas et al., 2003), while the males should be induced again with GnRHa implants to enhance their sperm production. A similar industrial production protocol has been proposed for the meagre (Mylonas et al., 2016).

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.aqrep.2021.100766.

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