Aquaculture Reports 18 (2020) 100495

Contents lists available at ScienceDirect

Aquaculture Reports

journal homepage: www.elsevier.com/locate/aqrep

Monitoring of common sole *Solea Solea* (L) captive broodstock from Northern Adriatic Sea over consecutive spawning seasons

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ARTICLE INFO

Keywords:
Solea solea
Aquaculture
Reproduction
Parentage contribution
STRs
Mediterranean

ABSTRACT

The high nutritional quality of common sole *Solea solea* increases its value for the fishery industry and for the aquaculture sector. To ensure the expansion of its production, it is necessary to implement farming and broodstock management technique to produce high quality eggs and larvae. This work summarizes eight years of study on reproductive performances, growth and parental contribution of a common sole broodstock from the Mediterranean-North Adriatic Sea. The broodstock (11 females and 13 males) reached the peak of fecundity after 5/6 years of captivity, with a production of 296,476 and 376,541 × 10^3 of total eggs kg^{-1} female with a fertilization rate of 31.6 ± 18.3 and 41.9 ± 23.8 %, respectively. Results shows that variations in temperature cycles are pivotal for a successful breeding season, and body condition during the first 3 years of captivity was the most important parameter positively correlated to parental contribution for both males and females. Parental contribution was assessed by eight species-specific microsatellite loci during the first 3 years of reproduction. Although the two sexes displayed different temporal trends, parentage assessment demonstrated an overall increase in the number of active breeders. However, only 13 couples out of 50 produced the 70.4 % of the larvae in year 5, confirming the same reproduction behavioural pattern in Mediterranean common sole than that observed both in Senegalese sole and in common sole of Atlantic origin.

This work provides valuable data needed to understand the mechanisms behind the maturation characteristics of this species and support future strategy for the establishment and management of Mediterranean common sole aquaculture.

1. Introduction

Common sole (*Solea solea* L.) is one of the most important fish species landed in Europe. Particularly appreciated by consumers, it has a high commercial value both for its fisheries and for aquaculture (Bjørndal et al., 2016; Jayasinghe et al., 2017). Most of the recent European research on sole aquaculture has been devoted to the development of the Senegalese sole *Solea senegalensis*, as reviewed by Morais et al. (2016). However, the development of common sole culture has a potential to diversify aquaculture production in Mediterranean countries such as Italy, and to preserve wild stocks (Parisi et al., 2014; Bjørndal et al., 2016). In this context, some advances have been made in *S. solea* aquaculture during the last decades on the determination of eggs and larval quality, larval rearing, early feeding nutrition and flesh quality (Bonaldo et al., 2011; Bonvini et al., 2015; Parma et al., 2013, 2015, 2019) leading to successful implementation of production through the grow-out phase. To guarantee successful breeding programs, it is necessary to improve the reproductive performances of the broodstock and ensure the availability of high-quality larvae over generations (Howell et al., 2011; Duncan et al., 2013; Palstra et al., 2015). In this regard, several studies have examined the reproductive biology of common sole reared in captivity by applying genetic analyses including parentage assignment, pedigree reconstruction, inbreeding estimates and heritability of morphometric traits using wild (Blonk et al., 2009, 2010a, 2010b) and farmed F1 (Palstra et al., 2015) broodstocks. The aforementioned studies have been conducted on *S. solea* broodstocks

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https://doi.org/10.1016/j.aqrep.2020.100495
Received 13 May 2020; Received in revised form 8 September 2020; Accepted 14 September 2020

from the Atlantic, while no information is available on common sole broodstock of Mediterranean origin.

To date, the reproductive dysfunction on F1 specimens has been attributed in *S. senegalensis* to a behavioural reproductive mismatch of cultured males that do not complete the courtship to fertilise the eggs (Fatsini et al., 2020), while in *S. solea* it seem due to the absence of specific natural photo-thermal conditions (Palstra et al., 2015). Although there are encouraging results of research groups aimed to solve the reproductive dysfunction on F1 specimens of both sole species, artificial reproduction still rely on natural spawning of wild broodstock adapted to captivity (Martín et al., 2019). This process (i.e. natural spawning of wild adults) is the result of a combination of several factors, such as environmental conditions, nutritional requirements and success of courtship behaviour (Martín et al., 2014; Vaz et al., 2019). The adaptation to captive condition is time-dependent and can last few years before a standardized reproduction activity can be established (Duncan et al., 2013). Previous studies on Atlantic common sole broodstocks have indicated that, in each spawning season, few parental pairs produced >50 % of the total progeny leading to genetic bottleneck, significant inbreeding rates and loss of genetic variation in F1 (Blonk et al., 2009). However, whether this reproduction pattern is consistent over the years or not, have never been investigated on this species. The microsatellite DNA (or short tandem repeats, STRs) is a particular type of DNA constituted by multiple copies of 2–4 basepairs tandemly repeated and uniformly distributed throughout the genome. Their high mutation rate, the codominance and the Mendelian inheritance, make them particularly suitable for the assessment of population genetic structure, and for parental analyses in aquaculture and in fishery management programs (Guarniero et al., 2002; Chistiakov et al., 2006; Martín et al., 2014; Bylemans et al., 2016; Jansson et al., 2017; Exadactylos et al., 2019; Guarniero et al., 2020).

In order to provide useful information for the establishment of Mediterranean captive common sole broodstocks of wild origin and for the implementation of future breeding programs, the aim of the present study was to evaluate the reproductive performance and parental contribution of a newly settled broodstock during consecutive spawning seasons and to determine relationships with thermal cycles, fecundity, fertility and morphometric parameters.

2. Material and methods

2.1. Broodstock and rearing condition

Twenty-four sub-adults of wild common sole have been caught during winter by fishing trawlers in the Northern Adriatic Sea and transferred to the facilities at the Laboratory of Aquaculture, University of Bologna, Cesenatico, Italy. After two months of quarantine and preliminary adaptation to captive conditions, each individual was anaesthetised with 2-phenoxyethanol (300 mg L^{-1}) and marked with PIT tag microchips (Passive Internal Transponder, 12 mm, Biomark, Inc.). Fish were then kept at the facilities with a male:female sex ratio of 1.2:1 throughout the period of study which lasted eight years. Fish were placed at initial density of 1.0 kg m^{-3} in an indoor elliptical tank (7.0 m^3) covered with 10 cm of marine sand where the fish remained during the whole period. The tank was supplied with natural seawater (36.0 ± 2.0 g L^{-1}) and connected to a recirculating aquaculture system (RAS, total volume 10 m^3). The rearing system consisted of a mechanical sand filter (PTK 1200, Astralpool, Barcelona, Spain), ultraviolet lights (PE 25 mJ/cm^2: 8 m^3 h^{-1}, Blaufish, Barcelona, Spain) and biofilter (PTK 1200, Astralpool, Barcelona, Spain). The water exchange rate in the tank was 20 % h^{-1}, while the overall water renewal in the RAS was 5 % day $^{-1}$. The water temperature was regulated by a chiller unit (York YCSA-08HP, York Italy s.r.l.). The oxygen level was kept constant (> 8.0 ± 1.0 mg L^{-1}). Ammonia (total ammonia nitrogen ≤ 0.1 mg L^{-1}) and nitrite (≤ 0.2 mg L^{-1}) were monitored weekly (Spectroquant Nova 60, Merk, Lab business).

During the pre-spawning season, fish were fed ad libitum 3–5 times a week (at 16:00 h) with semi-moist pellets formulated as reported in Table 1. Ingredients were finely ground, mixed and 4 mm pellets were produced. Feed was produced monthly and stored at -20 °C upon supplied. During the spawning season, fish were fed ad libitum with live polychaetes, *Nereis virens*, (Topsy Baits, Wilhelminadorp, the Netherlands) 2–4 times a week, and with fresh mussels, *Mytilus galloprovincialis*, once a week. Sex was determined by visual observation during the first captive breeding season. Once per year, in summer, individual weight and total length was recorded. At the same time, the sand present in the bottom of the rearing tanks was cleaned with high-pressure tap water and the salt water renewed in the whole system. All experimental procedures were evaluated and approved by the Ethical-Scientific Committee for Animal Experimentation of the University of Bologna, in accordance with European directive 2010/63/UE on the protection of animals used for scientific purposes.

2.2. Temperature, photoperiod manipulation, and association of spawns to thermal cycles

Fish were maintained under controlled water temperature regimes (7.5–18.0 °C) approximating seasonal values recorded on the seabed of the Northern Adriatic Sea (Arpa Emilia Romagna, Cesenatico, Italy). The photoperiod was adjusted by an artificial white light bulb (Philips Softone 40 W; 20 lx at the water surface) once a week to mimic the natural conditions at 44 30 0″ N, 12° 620 0″ W. During spring (March–May), the fish were induced to spawn naturally by increasing the water temperature (from 7.5 °C to 10.5–11.0 °C) over 3 consecutive days. The temperature was then maintained in the range of 10.0–11.2 °C for 10–15 days and thereafter it was reduced to 7.5–8.0 °C (1.5 °C day^{-1}) to suspend the spawning (except during the year 6 when spawning was not suspended).

In order to further investigate the association between temperature induction, egg release, fecundity and fertilization rates we have considered the relative changes of temperatures between consecutive days and transformed the data in weekly observations by taking the average of the daily observations within the same week. Two linear regression models have been estimated to measure how the relative fecundity and the fertilization rates vary as function of the relative change of temperatures.

Table 1
Formulation of the semi-moist broodstock's diet.

Ingredient (g kg^{-1})	
Fishmeal	449
Mussels[1]	449
Fish oil	35
Water	37
Carboxymethylcellulose	11
Vitamin and mineral premix[2]	17
Proximate composition, % on dry matter	
Protein	62.1 ± 1.3
Lipid	16.4 ± 0.3
Ash	15.9 ± 0.3

[1] Boiled and frozen *Mitilus* spp.

[2] Vitamin manufactured to supply the following vitamins (mg or IU kg^{-1} diet): vitamic C (500 mg/kg), vitamin A (7500 IU), vitamin D3 (1125 IU), vitamin E (225 mg), vitamin B1 (12 mg), vitamin B2 (24 mg), vitamin B6 (24 mg), vitamin B12 (0.02 mg), vitamic K3 (7.5 mg), vitamin H (0.2 mg), vitamin PP (90 mg), folic acid (4.5 mg), pantothenic acid (72 mg), BHT (150 mg). Mineral mix to supply the following elements (mg kg^{-1} diet): manganese oxide (22.5 mg), zinc sulphate monohydrate (135 mg), ferrous sulphate (60 mg), copper sulphate pentahydrate (7.5 mg), potassium iodide (3 mg), betain (600 mg).

2.3. Growth and morphometric parameters

The Annual Growth Increment (AGI % year $^{-1}$) was calculated as:

$$AGI = 100*(e^{g} - 1)$$

where g is the instantaneous growth coefficient, obtained by the equation:

$$g = \ln(Wt - W0)/t$$

Where Wt and W0 are the arithmetic means of wet weight (WW, grams) at the end and at the beginning of each sampling, and t is the time.

Average Body Weight (ABW) and length (ABL) were equal to the annual arithmetic mean of wet body weight (g) and body length (cm), respectively.

Fulton's condition factor (K_F):

$$KF = (WW/L\hat{3})*100$$

2.4. Eggs and larval sampling

During each spawning season, eggs were collected daily on a 500-μm fine-mesh sieve placed at the tank outflow, weighed and transferred to a 2-L graduated cylinder to estimate the floating volume rate (%). Floating eggs were transferred to conical tanks (80 L) filled with sea water (salinity 38, T. 12.0 ± 1 °C, flow 50 L h^{-1}) and connected to a RAS including UV lamps, sand filter, and chiller unit. Tanks were gently aerated and sinking eggs were removed daily. To quantify the quality of the spawns, total fertile eggs and daily fertilization success were determined as the number of eggs produced in relation to the total weight of the females, for each year. From each batch, two samples of~ 100 eggs were observed under a microscope and the presence of egg segmentation was assessed to evaluate the fertilization rate. After hatching, the larvae were kept in the same 80-L tanks for at least 3−4 days before sampling for genetic analysis. To determine larval parental allocation, all fertilized spawns, which produced vital larvae, were sampled at an average number of 10 larvae per spawn. After sampling, larvae were rinsed with distillate water and placed in tubes with absolute alcohol for subsequent genetic parentage analysis. Due to the low numbers of fertile eggs and viable larvae obtained during the first two years of spawning; six and seven batches were sampled during year 3 and 4, respectively; while 21 batches were sampled during year 5.

Parental contributions of both females and males were quantified during years 3–5 covering the beginning of natural spawning (year 3) until full broodstock maturation (year 5). Data on growth and reproductive performance of the following years (6, 7 and 8) were included as an indicator of further productivity of broodstock maturation.

2.5. Tissue sampling and genotyping of individuals

Each of the 24 adults was individually anaesthetised with 2-phenoxyethanol and a non-invasive biopsy (fin-clip from caudal fin) was performed in sterile conditions. Hatchery-reared offspring were randomly collected and euthanized. Samples were stored in 96 % ethanol at 4 °C and total genomic DNA extracted according to a cetyltrimethylammonium-bromide (CTAB) based procedure (Doyle and Doyle, 1987; Doyle and Dickson, 1987; Cullings, 1992).

Genetic profiles were obtained using eight species-specific microsatellite loci, selected for their high levels of polymorphism. Primer pairs and amplification conditions were those indicated in Iyengar et al. (2000) for loci F8-ICA9, F8-IGAA7, F8-ITG11, F8-IIGT15, F13-II8/4/7, while for loci Sos(AC)3, Sos(AC)6b and Sos(AC)45 were described in Garoia et al. (2006 and 2007). Specimens were genotyped by assessing allele sizes on an ABI310 Genetic Analyser, using forward primers labelled with Standard DS-33 GeneScan matrix dye set (6-FAM, PET, NED and VIC, ThermoFisher Scientific) and LIZ500 as internal size standard. Allele calling was performed using Peak Scanner™ Software 2 (Life Technologies) and converted to discrete values by manual binning.

Individual genotypes of parents were analysed by CERVUS 3.0.7 (Kalinowski et al., 2007) in order to compute the per locus genetic diversity parameters (number of alleles per locus, k; observed heterozygosity, Hobs; expected heterozygosity, HExp, deviations from the Hardy-Weinberg Equilibrium corrected for multiple comparisons by sequential Bonferroni, Rice, 1989), the Polymorphic Information Content (PIC) of markers and the predicted null allele frequencies. The same tool was employed to perform the paternity assignment of the data from offspring.

2.6. Regression models between zootechnical parameters and parental contribution

To explore any possible correlation between morphometric and growth parameters (ABW, ABL, AGI, and K_F) with the relative parental Contribution Rate (CR) obtained during spawning in years 3, 4 and 5, a regression model was applied. In order to respect the constraint that the response CR is measured in terms of proportions in the interval (0.1), a Beta regression analysis was performed (Ferrari and Cribari-Neto, 2004; Simas et al., 2010). Within this framework, the dependent variable is transformed according to the logistic function and it is calculated in relation to the independent variables of interest under the assumption that it is distributed according to a Beta probability density function. Males and females were analysed separately to prevent any influence of the effect of sex on morphometric parameters.

2.7. Statistical analyses

Growth data and morphometric parameters among years were analysed using one-way ANOVAs. Pair-wise comparisons between males and females within the same year were analysed by Student's T-test. To establish the association between temperature induction, relative fecundity and fertilization rates linear regression models have been performed to measure how the relative fecundity and the fertilization rates vary as function of the relative change of temperatures. The model parameters of the Beta regression analysis were estimated by the package betareg freely implemented in the software R (Cribari-Neto and Zeileis, 2010).

3. Results

3.1. Growth and morphometric parameters

Growth and morphometric parameters increased for both wild males and females over eight years (2006–2013) of captivity (Table 2). Males did not show any significant difference in weight from year 1 to year 4, while significant differences were observed from year 5 onwards. Females increased significantly in weight during the first three years of breeding; afterward no significant differences were detected until the end of the study. Males and females increased in length from years 1–3. No increase was observed from year 4 onward. The overall growth was significantly higher in males during the first year of captivity (year 2, AGI years 1−2). After year 3, males growth slowly but constantly, until the end of the experiment. Similarly in females, the greatest growth was observed during the first two years of captivity (AGI/y1−2, AGI/y2−3). The annual growth increment at year 2 and 8 (AGI/y1−2 and AGI/y7−8) was higher in females than in males. The condition factor was highly different between males and females in the same year.

3.2. Egg production parameters: fecundity and fertilization

Table 3 reports the total egg production parameters obtained from year 3 to year 8 of captivity, while Fig. 1 show the daily relative fecundity and the daily water temperature regime of the broodstock

Table 2
Growth and morphometric parameters of the Northern Adriatic *S. solea* broodstock during 8 consecutive years.

Year	1	2	3	4	5	6	7	8	*P-value*
Males									
ABW (g)	237.2 ± 37.0^{a}	299.2 ± 65.6^{ab}	333.6 ± 73.6^{ab}	354.2 ± 82.1^{ab}	387.4 ± 106.9^{b}	398.6 ± 116.9^{b}	400.2 ± 130.8^{b}	399.6 ± 130.7^{b}	*0.0001*
ABL (cm)	29.5 ± 1.31^{a}	31.6 ± 2.18^{ab}	32.6 ± 2.14^{bc}	33.1 ± 2.19^{bc}	34.0 ± 2.56^{bc}	34.5 ± 2.82^{bc}	34.5 ± 3.04^{bc}	34.6 ± 2.97^{c}	*0.0001*
AGI (%)		25.9 ± 11.6$^{c\ A}$	12.0 ± 10.7^{b}	5.9 ± 6.4^{ab}	8.7 ± 9.12^{ab}	2.5 ± 4.2^{a}	0.2 ± 4.4^{aA}	0.2 ± 3.5^{a}	*0.0001*
K_F	0.92 ± 0.08^{A}	0.94 ± 0.10^{A}	0.95 ± 0.10^{A}	0.96 ± 0.10^{A}	0.96 ± 0.11^{A}	0.94 ± 0.11^{A}	0.94 ± 0.10^{A}	0.94 ± 0.11^{A}	*0.96*
Females									
ABW (g)	348.8 ± 7.2^{a}	483.8 ± 113.5^{ab}	583.1 ± 159.7^{bc}	607.5 ± 157.7^{bc}	683.0 ± 173.1^{bc}	704.1 ± 183.5^{c}	767.4 ± 190.8^{c}	758.7 ± 194.0^{c}	*0.0001*
ABL (cm)	31.9 ± 2.8^{a}	34.4 ± 2.7^{ab}	36.1 ± 2.8^{bc}	37.0 ± 2.8^{bc}	38.0 ± 2.7^{c}	38.7 ± 2.5^{c}	39.2 ± 2.7^{c}	39.4 ± 2.6^{c}	*0.0001*
AGI (%)		39.7 ± 15.5$^{c\ B}$	20.6 ± 18.9^{b}	4.8 ± 6.3^{a}	13.0 ± 8.0^{ab}	2.9 ± 4.2^{a}	9.7 ± 10.9^{ab}	−1.2 ± 3.5^{a}	*0.0001*
K_F	1.06 ± 0.11^{B}	1.17 ± 0.10^{B}	1.21 ± 0.18^{B}	1.18 ± 0.14^{B}	1.22 ± 0.17^{B}	1.19 ± 0.18^{B}	1.26 ± 0.19$^{B,\ B}$	1.22 ± 0.20^{B}	*0.19*

Data are given as the mean value across individuals (n=13, male; n=11 female) ± SD. In each line, different lowercase superscript letters indicate significant differences among years (one−way ANOVA P ≤ 0.05). In each column different uppercase superscript letters for AGI and KF indicate significant differences between male and female for the observed parameters within the same year.
ABW = average body weight, g.
ABL = average body length, cm.
AGI = annual growth increment, %.
KF = condition factor.

Table 3
Annual reproductive output of the *S. solea* broodstock of Northern Adriatic Sea.

Year	Total number of spawns	Number of fertile spawn	Total relative fecundity (10^3 eggs kg^{-1} female)	Daily relative fecundity (10^3 eggs kg^{-1} female)	Total fertile eggs (10^3 eggs kg^{-1} female)	Daily Fertilization success (10^3 eggs kg^{-1} female)	Floating volume rate (%)	Fertilization Rate (%)
1	0						0	
2	0						0	
3	26	12	59.050	21.89 ± 20.75^{a}	21.75	0.80 ± 1.23^{ab}	/	45.0 ± 20.3
4	39	19	200.09	51.32 ± 34.31^{abc}	37.78	0.97 ± 1.17^{ab}	41.5 ± 21.2^{ab}	37.8 ± 20.4
5	66	25	296.48	43.71 ± 35.84^{ab}	57.38	0.86 ± 1.54^{a}	39.6 ± 17.2^{a}	31.6 ± 18.3
6	45	19	376.54	83.68 ± 81.83^{c}	109.12	2.60 ± 4.42^{b}	41.6 ± 20.0^{ab}	41.9 ± 23.8
7	42	25	304.97	70.92 ± 69.28^{bc}	82.24	1.92 ± 2.57^{ab}	48.0 ± 12.0^{ab}	29.7 ± 7.5
8	33	12	256.77	77.81 ± 55.95^{c}	57.56	1.74 ± 2.52^{ab}	61.9 ± 16.0^{b}	37.5 ± 12.0

Different superscript letters indicate significant differences between different years ($p < 0.05$). Standard variation is represented as ±.
Fertilization rate has been calculated on batches showing egg segmentation.

along the studied period. During the first two years, fish were still immature. From year 3 onwards, females started to respond to photothermal cues by spontaneously releasing eggs during the months of February-May (8−12 °C at a photoperiod range between 11–16 hours).

The total relative fecundity increased significantly from year 3 to year 4, then increases progressively until year 6 and slightly decreased over the years 7 and 8. The mean daily relative fecundity followed the same pattern than the total relative fecundity, with highest values on year 6. Total fertile eggs ranged between 21752–109126 egg kg^{-1} female over the year 3–6, respectively, while the mean daily fertilized eggs was lower in year 5 than year 6. The fertilization rate was similar among years, with a slight increase on year 7. Similar proportions of floating eggs were also produced in years 3–7, with the highest value obtained in year 8. Fig. 2, A and B, shows the scatterplot of the daily relative fecundity (A) and fertilization (B) in relation to the relative changes of water temperatures, together with the estimated regression lines. In both cases the linear regression is significant (with R squared = 0.13 A, and 0.21 B). The slopes are both positive (β = 53577.8 A, and 185.213 B) denoting that when the relative temperature changes increased, the relative fecundity and the fertilization increase as well of an average value equal to the slope.

3.3. Paternity assignment and genetic variability

The parental contribution was analysed over three years, from year 3 to year 5. During this time window, 394 specimens (24 adults and 370 larvae) were genotyped with the eight specie-specific loci above described. Since the mandatory presence of a bottleneck effect in F1 obtained in captive conditions, the overall genetic parameters were calculated only in the breeders group and are summarized in Table 4.

The mean PIC value recorded was 0.6076 and the percentage of assignment to at least one single parent was 90.32 % in year 3, 96.15 % in year 4 and 87.11 % in year 5. No match between parents and progeny was observed for a total of 41 cases (six in year 3, two in year 4, and 33 in year 5). The parental contributions of each single male and female of the breeding stock during the three years of observations are detailed in Fig. 2. The number of active females increased from 5 out of 11 in year 3, to 9 out of 11 in year 5. In the same way, the number of active males increased from 9 out of 13 in year 3–12 out of 13 in year 5. Despite this general common tendency of the two sexes, females and males showed different trends if taken separately.

Females contribution in fact showed a homogenous tendency over the three spawning seasons: female 5 was the main overall performer with 68 %, 63 % and 46 % of F1 in year 3, 4 and 5, respectively, followed by female 8 with a lower but fairly constant yield (14 %, 9%, 13 %

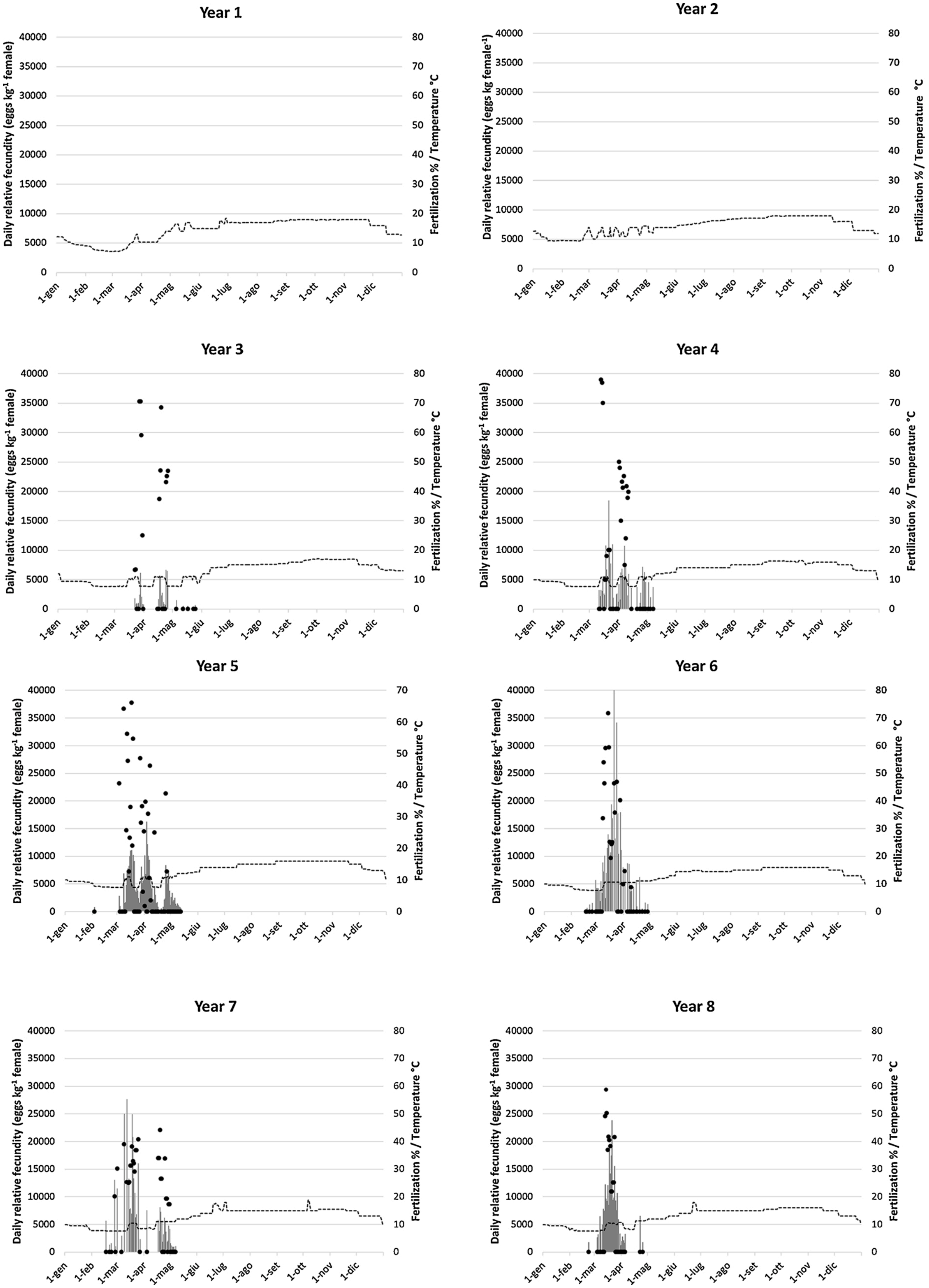

Fig. 1. Water temperature regime, daily relative fecundity (eggs kg female^{-1}), and fertilization rate (%) of the broodstock along the studied period. A-H: year 1-8.

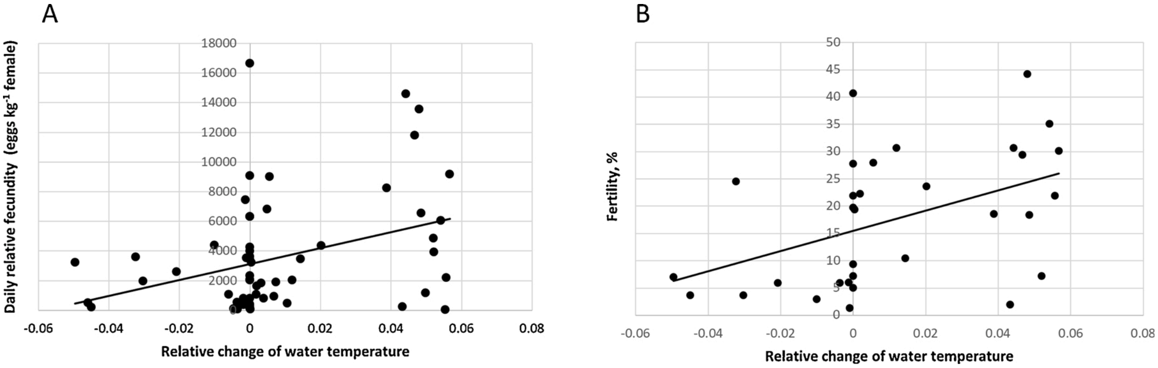

Fig. 2. Linear regression models to measure how the daily relative fecundity (A) and the fertilization rates (B) vary as function of the relative change of water temperatures. The regression coefficients are significant ($p < 0.01$). $R^2 = 0.13$ A, and 0.21 B. $\beta = 53577.8$ A, and 185.213 B).

Table 4
Main genetic parameters of loci used, in the 24 *S. solea* adults of North Adriatic sea used as breeders.

locus	k	HObs	HExp	PIC	F(Null)
F8-ICA9	4	0.625	0.512	0.451	−0.1238
F8-IGAA7	4	0.167	0.160	0.311	−0.0345
F8-ITG11	6	0.917	0.807	0.758	−0.0760
F8-IIGT15	6	0.625	0.652	0.581	0.0124
F13-II8/4/7	4	0.458	0.391	0.355	−0.1182
Sos(AC)3	19	0.542	0.937*	0.912	0.2613
Sos(AC)6b	8	0.667	0.712	0.669	0.0487
Sos(AC)45	9	1.000	0.861	0.824	−0.0880

k: number of alleles per locus; HObs observed heterozygosity; HExp: expected heterozygosity (*= deviation from HWE P ≤ 0.01); PIC: Polymorphic Information Content; F(Null): predicted null allele frequencies.

respectively). The same scenario was also observed in females 3, 4 and 15 once they started the reproduction in year 4. On the contrary, the female 1 never took part to the F1 production, while the female 12, which contributed in the precedent years, did not participate to the spawning season of year 5. The range of contribution of females 13 and 14 to the F1 generation was about 1−2% (Fig. 3A).

Males showed a less homogeneous pattern: the contribute of male 2 for instance changed from 46 % of success in fertilizing eggs in year 3, to 7% in year 4, to return to 31 % in year 5. The male 24 showed the same trend but opposite yields, with a performance peak in year 4 (35 %), compared to 5 % during the previous and the following years. The same situation was observed in male 22, which started the reproduction in year 4 with the 10 % of fingerlings and the 4% in the following year. The male 19 also started the reproduction in year 4 by increasing its reproductive success from 14 % to 26 %. The rest of the males (9, 10 and 17) maintained a constant yield even with different proportion of contribution (14–18 %, 2−3% and 4–7 % for males 9, 10 and 17, respectively). Finally, like in the females group, the range of contribution to the F1 generation of males 11, 16 and 18 was about 1–3% (Fig. 3B).

As regard the couple contribution analyses, focusing in year 5, which was the most representative in terms of number of larvae sampled, 13 couples out of 50 contributed to F1 with the 70.7 % of larvae. In particular, the couples 2:5, 19:7 and 19:5 (male:female), can be considered the most dominant, with 48, 23 and 20 larvae produced, respectively (which represent by themselves the 41.4 % of the total).

3.4. Correlation model

The overall relationship among individual morphology and growth parameters with the relative parental contribution (CR) during three consecutive spawning seasons (year 3, 4 and 5) are reported in Table 5. The Pseudo-R square of the models, range between 0.39 and 0.75. The mean K_F recorded both in males than in females during the first three years of breeding (years 1−3), was positively correlated to the CR of the year 3. For the same year, length (length/y3) was also positively correlated to CR in female, while weight (weight/y3) and the value of AGI of the first year of breeding (AGI/y1−2) were negatively correlated. During the second spawning season (year 4), the probability to increase CR in males was significantly correlated to weight/y4, K_F /y1−3, AGI/y2−3, while was negatively correlated to K_F/y4 and length/y4. For the same year, K_F/y1−3 and AGI/y3−4 was positively correlated in female to CR, while K_F/y4 and AGI/y1−2 was negatively correlated. During the last spawning season analysed (year 5), the probability to increase CR in males was correlated to AGI/y4−5. At the same time, in females CR showed a positive relationship with length/y5, K_F/y1−3, AGI/y2−3 and AGI/y4−5, while a negative relation was obtained with weight/y5.

4. Discussion

Several studies have described the captive reproduction of wild-caught broodstocks of common sole *Solea solea* (Devouchelle et al., 1987; Blonk et al., 2009; Baynes et al., 1993). However, little information is available on how spawning activity of a newly constituted broodstock of *S. solea* changes over consecutive years. This study provides therefore an insight into the mating relationships, which occurred into the breeding tank through the parental assignment of a total of 370 larvae collected during three consecutive spawning seasons. No mortality of any of the 24 breeders was observed over the studied period (8 years), indicating that the management procedures fulfilled the needs of the species. Fish did not undergo into natural reproduction during the first two years of captivity. This could be attributed to the time needed to adapt to captivity, reaching in the meanwhile the size of first maturity. In this regard, females used these two years to increment their body weight and length, indicating that a significant energy demands was still required for body growth instead of gonadal development and reproduction.

The water temperatures supporting the reproduction of this North-Eastern Mediterranean broodstock are similar to those observed in common sole captured from Atlantic waters (Devouchelle et al., 1987; Baynes et al., 1993; Imsland et al., 2004; Blonk et al., 2009; Vaz et al., 2019). In the present study, spawning begins when temperatures increase from 8 °C to 12 °C, with the maximum production peak recorded around 10.5–11.0 °C. These results are in agreement with the spawning temperatures previously reported by Devouchelle (1987) and Palstra et al. (2015), pointing out the importance of keeping long winter periods at temperatures below 8 °C as one of the key feature for the onset of *S. solea* reproduction. In our study, a positive correlation was found between relative change of temperature and daily relative fecundity and fertilization rate, being the variation in temperature regime (from 7.5 °C to 10.5–11.0 °C) occurred in the week preceding the spawning day the most important factor for an efficient reproduction. This trend, has been previously described by Baynes et al. (1993), where the succession of

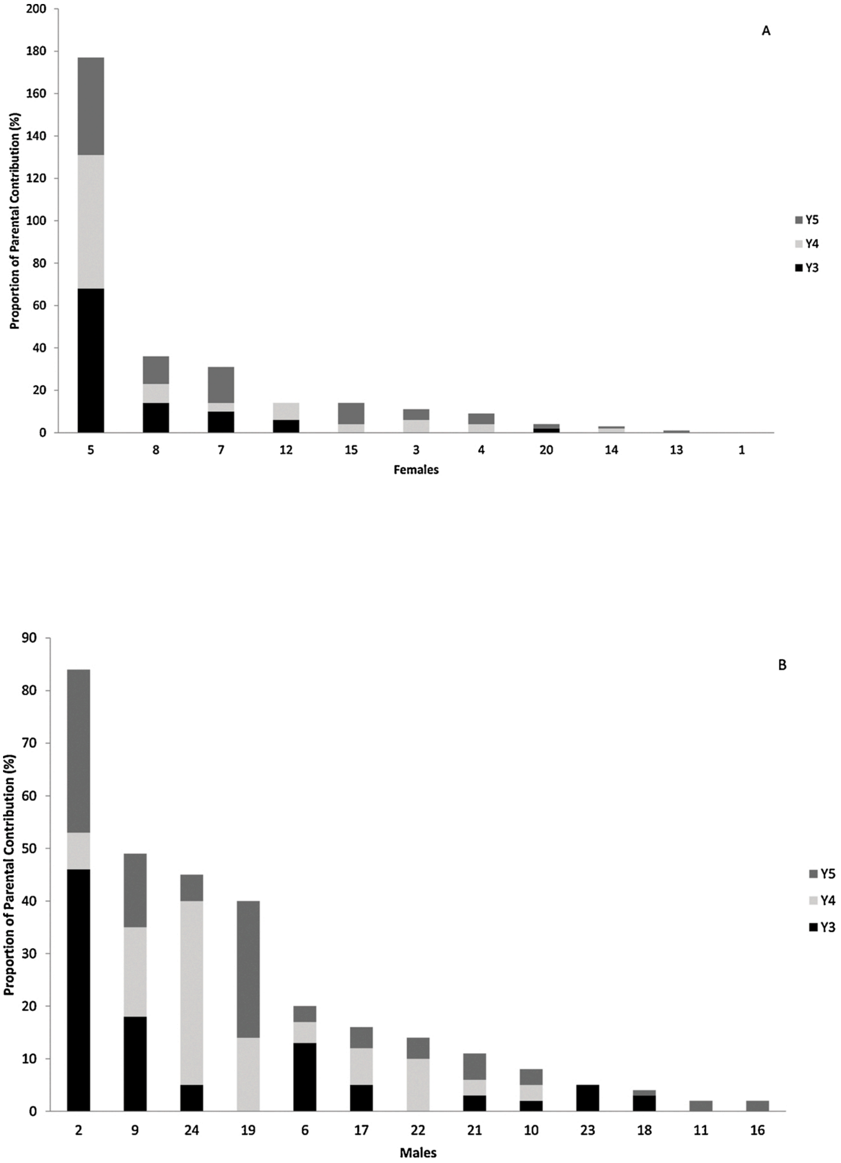

Fig. 3. Parental contribution over years 3, 4 and 5. A: females; B: males. On the left: most contributing parents, on the right: less contributing parents.

short cold periods followed by an increase in temperature may yield in Atlantic common sole higher quality eggs.

In addition, the effect of water temperature variation on reproduction is consistent to the high natural environmental fluctuation of the Northern Adriatic, which has a characteristic water masses and circulation scheme and, due to its shallowness, shows the greatest seasonal thermal gradient in the Mediterranean Sea (Russo and Artegiani, 1996; Grati et al., 2013).

In terms of total egg yield, an increase of total relative fecundity and total fertilized eggs was observed from year 3 to year 5 and 6, when the maximum production capacity was achieved. In addition, no significant differences in the percentage of floating eggs (used as egg batch quality parameter) was observed within the years 4 and 7, indicating that the eggs quality remained quite constant along the studied period, with a slight increase on year 8. The total relative fecundity ranged from 59,050 to 376,541 eggs kg^{-1} female which is in line or slightly higher than previous results reported for this species (Imsland et al., 2003). The proportion of daily relative fecundity has ranged in the present study from 21,890 ± 20,750, to 83,680 ± 81,830 eggs kg^{-1} with daily eggs fertilization ranging from 0 to over 90 %. The overall mean fertilization rate was about 37 ± 18 %. Increases in total fecundity during subsequent spawning seasons have been reported in previous studies on common sole from Atlantic origins, with average values of fertilization rate about 51 % (Houghton et al., 1985), 74 % (Devauchelle et al., 1987), 65 % (Lenzi and Salvatori, 1989) and 60 % (Baynes et al., 1993), suggesting that similar results can be expected and perhaps achieved also for the Mediterranean broodstocks. However, Bertotto et al. (2006) reported inhibited spawning of a broodstock of common sole from Mediterranean Sea after one year. In that study, the broodstock was exposed to warmer summer temperatures (up to 25 °C) compared to this study (17−18 °C), which may explain the different observations.

Regarding the parental contribution, it was possible to assign to at least one single parent a quite high percentage of larvae, obtaining a reliable picture of the parental contribution of each single male and female that founded the breeding stock. Over the three years of parental analyses, we assisted to an overall increase in the number of the active members of the breeding stock, both in the females than in males. Even if the sample size was larger in year 5 (256 larvae), the increase in number of active parents seems not to be ascribed to a sampling bias, since the positive trend was observed also in year 4, when the sample size was similar to year 3 (52 and 62 larvae, respectively). The lower number of active adults registered in year 3 is, in our opinion, due to the fact that it

Table 5
Growth and morphological parameters of male and female showing a significant impact on the relative parental contribution (CR) during 3 different spawning seasons.

Parameters	Male			Female		
	Coefficient	*P*	Pseudo-R square	Coefficient	*P*	Pseudo-R square
	Year 3					
K_F	−16.821	0.506	0.4889			
K_F y1−3	**26.665**	<0.01		**39.1585**	<0.001	0.3928
Weight y3				−0.5121	<0.001	
Length y3				2.5363	<0.001	
AGI y1−2				−0.10037	<0.01	
	Year 4					
Weight y4	0.06125	<0.001	0.7508			
Length y4	−1.9403	<0.001				
K_F y4	−38.7323	<0.001		−15.0474	<0.001	0.45
K_F y1−3	**18.1810**	<0.01		**31.9816**	<0.001	
AGI y2−3	0.1270	<0.001				
AGI y1−2				−0.04963	<0.01	
AGI y3−4				0.25886	<0.001	
	Year 5					
AGI y4−5	−0.05160	0.0535	0.4193			
Weight y5				−0.03659	<0.001	0.667
Length y5				2.0716	<0.001	
K_F y1−3				**22.2302**	<0.001	
AGI y2−3				0.04979	<0.001	
AGI y4−5				0.2165	<0.001	

Y = year.
K_F = condition factor.
K_F y1−3 = mean condition factor of the first 3 years of captivity.
AGI = annual growth increment.

was their very first attempt to reproduce in captive conditions. Despite this positive trend registered over the three years of observation, it is interesting to notice that, while in the females' group there was a quite constant production trend, with the same females (female 5 *in primis*, followed by females 8 and 15) responsible for the majority of larvae produced in all the three years, males showed a less homogeneous

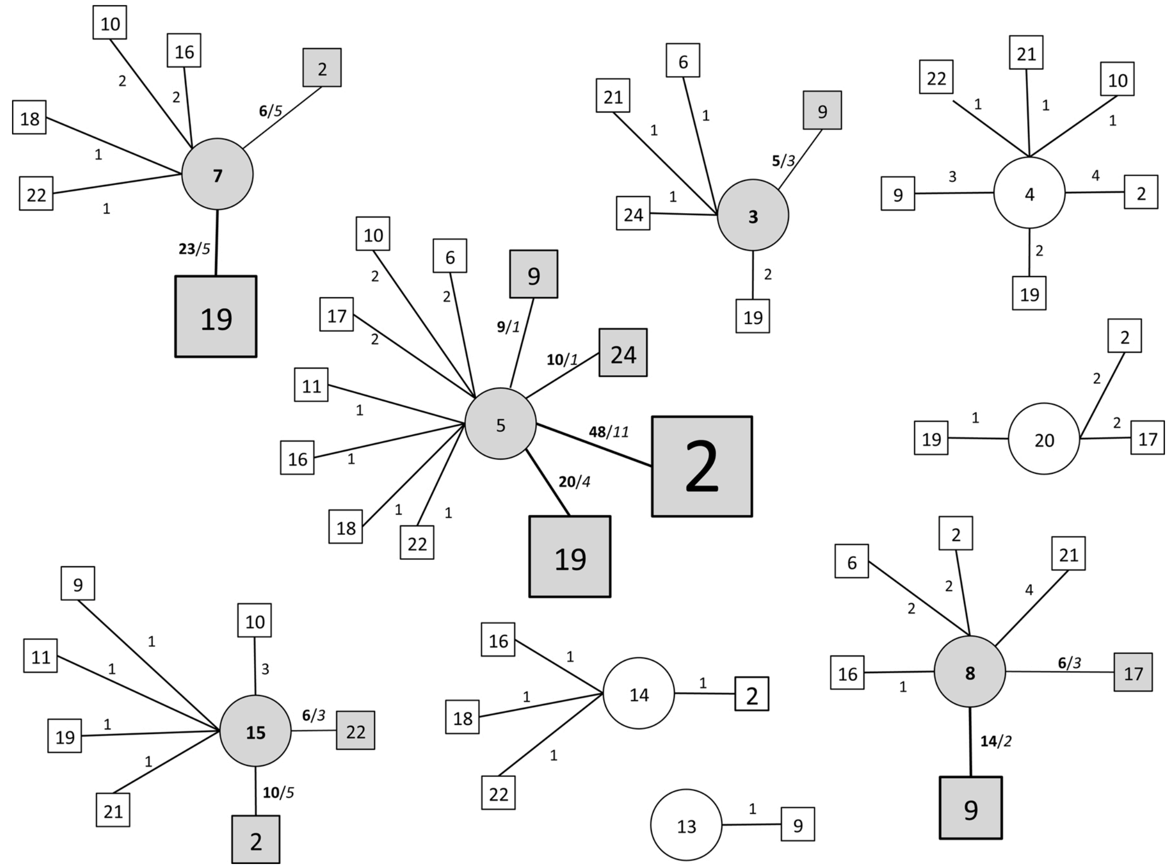

Fig. 4. Contribution of the mating pairs in year 5. Squares = males, rounds = females. In gray: couples which contributed most to F1. Squares are sized proportionally to the male's contribution. On axes: number of larvae produced per each couple (in bold) over the number of spawns in which they participated (in italics).

pattern. Males followed a biannual tendency, so the male that was the best performer in year 3 (i.e. male 2) had to wait until year 5 to return to the performance of the first year. This hypothesis seems to be supported by the behaviour of male 24, which showed the exact opposite trend. Some individuals also seemed to participate only occasionally in the breeding season, as observed in females 13 and 14 and in males 11, 16 and 18. These results agree with those reported by Baynes (1993), who stated that about 70–80% of the females within a stock cannot spawn. These data have been persistently confirmed by more recent studies on the reproductive behaviour both in Atlantic common sole and Senegalese sole in captivity, where may occur that > 50 % of the production is the results of few dominant couples (Blonk et al., 2009; Guarniero et al., 2010; Martín et al., 2014, 2019), or only one female and few males contribute to the entire production (Porta et al., 2006).

The reproductive dominance of only few couples observed both in *S. senegalensis* (Martin et al., 2019; Carazo et al., 2016) and in *S. solea* of Atlantic origins (Baynes et al., 1994) could be the result of mate selection as indicated by "follow behaviour" and/or "coupled swimming". The results obtained in our study (year 5), with 13 couples producing over the 70 % of larvae and, in particular, only three couples that can be considered the most dominant (Fig. 4), seem to support the same behavioural pattern also in *S. solea* from Mediterranean Sea, which represents the southern limit of the biogeographic distribution of this species.

Condition indices including Fulton's condition factor, weight, length and relative body condition, have been linked to fecundity or used to predict maturity and spawning of wild population in several fish species (Morgan, 2004; Morgan and Lilly, 2005; Rideout and Morgan, 2010; Rodgveller, 2019). In the present study, the R square of the models indicated that 39–75 % of the CR variability is explained by the parameters involved. The condition factor in the year of spawning was either weakly or negatively correlated within parental contribution. However, according to Devauchelle et al. (1987) who stressed the importance of feeding during the seasons preceding reproduction in order to satisfy the energy needs for the winter growth of the gonads, in our broodstock, fish that were more able to adapt to captive conditions and showed a higher nutritional status during the first years of captivity, were more prone to enter into natural spawning. In many flatfish, the body carcass appears to be the principal source of energy fuelling gamete production (Rideout and Morgan, 2010). However, the absence of relationship between K_F and parental contribution was likely due to the timing of sampling which occurred after the spawning season and before fish could replenish their somatic energy reserves. The seasonal timing of sampling is an important aspect to be considered when describing the relationship between somatic condition and fecundity. In wild flatfish populations, body condition is a better predictor of fecundity when length and weight measurements are made several months before the spawning season compared to the ongoing spawning season (Kennedy et al., 2007). The role of the body carcass and nutritional adaptation in the reproduction of this species was also reinforced by the growth increment parameters in females and were positively correlated for the year 4 and 5. However, this correlation was not observed for males, being in agreement with the low energy investment in the reproductive biology of males of this (and other fish) species, which produce and release small quantities of gametes (Baynes et al., 1994). In addition, the lack of correlation could also be explained by the earlier cessation in somatic growth of males compared to females observed in this study. Female length was positively correlated with parental contribution at the onset of the reproduction (year 3) and at broodstock maturation (year 5). On the other hand, individual female wet weight was unrelated to parental contribution. In this regard, Millner et al. (1991) reported that fecundity in wild common sole from North Sea and English Channel is best explained by a model including both length and somatic condition, supporting results from the present study where longer and stout individuals have more probability to participate in the breeding season.

Solea solea represent one of the three *Solea* species present in the Mediterranean Sea, together with *S. senegalensis* and *S. aegyptiaca*. These three species show a longitudinal gradient of distribution, with *S. solea* widely distributed in all the Mediterranean sea, *S. senegalensis* in the western area of Mediterranean sea, (She et al., 1987; Goucha et al., 1987) and the cryptic species *S. aegyptiaca*, in the Levantine basin of Mediterranean sea (Fischer et al., 1987; Mehanna, 2007), even if recent data suggests that its distribution may be wider than described up today (Sabatini et al., 2018). This pattern of distribution, with *S. solea* as the only Sole species covering the whole Mediterranean basin, increases the commercial importance of this species in the Mediterranean area.

In conclusion, the present study reports the evolution over years in term of productivity and mating performance of a broodstock group of *Solea solea* of Mediterranean origin settled ex-novo from wild sub-adults individuals with no replicate broodstock fish tanks. Broodstock reached its establishment in terms of growth and egg productivity after 5/6 years of captivity. Variations in temperature cycles have shown to be pivotal for a successful reproduction. Parentage assessment using eight species-specific microsatellite loci demonstrated that only three couples of breeders contributed to the almost half of F1 generation. The use of predicting models has been applied to investigate any possible relationship between parental contribution and morphometric parameters. The mean individual condition factor observed during the first 3 years of captivity was the most important parameter positively correlated to the parental contribution for both males and females, being one of the main factors to take into consideration for the constitution of new broodstocks. In this regard, future works should focus on eggs and larval quality by photo-thermal manipulation studies, sample allocation along the breeding period, mate incompatibility and genetic drift of F1 generation to overcome the critical points of sole aquaculture.

Funding

This work was supported by the Ministry of Agricultural, Food and Forestry Policies (Italy). Grant id. 0042305 under the project title “Studio di fattibilità dell’intero ciclo produttivo della sogliola comune *Solea solea*: analisi e risoluzione dei punti critici e valutazione economica” (A feasibility study on the entire production cycle of the common sole *Solea solea*: analysis and resolution of critical aspects and an economic assessment), invito DG Pemac Segreteria Prot. Uscita 0042305, 25/11/2001.

CRediT authorship contribution statement

Claudia Ofelio: Investigation, Conceptualization, Methodology, Formal analysis, Writing - original draft, Writing - review & editing. **Ilaria Guarniero:** Investigation, Conceptualization, Methodology, Supervision, Project administration, Formal analysis, Writing - original draft, Writing - review & editing. **Alessia Cariani:** Investigation, Methodology, Formal analysis, Writing - review & editing. **Cinzia Viroli:** Formal analysis. **Alessio Bonaldo:** Conceptualization, Project administration, Funding acquisition. **Pier Paolo Gatta:** Conceptualization, Project administration, Funding acquisition. **Luca Parma:** Investigation, Project administration, Methodology, Writing - review & editing.

Declaration of Competing Interest

The authors whose names are listed immediately below certify that they have NO affiliations with or involvement in any organization or entity with any financial interest (such as honoraria; educational grants; participation in speakers' bureaus; membership, employment, consultancies, stock ownership, or other equity interest; and expert testimony or patent-licensing arrangements), or non-financial interest (such as personal or professional relationships, affiliations, knowledge or beliefs) in the subject matter or materials discussed in this manuscript.

Acknowledgements

The authors gratefully thank Prof. Dr. Myron Peck for the scientific contribution and language revision. The authors thank the two anonymous reviewers who substantially helped to improve the manuscript.

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