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Sexual behaviour and reproductive performance of the endangered European eel *Anguilla anguilla* (Linnaeus, 1758) based on direct observations and paternity assignment in semi-natural conditions

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ABSTRACT

European eel *Anguilla anguilla* is among the highly valued species for aquaculture. Since its peculiar biology, it is not yet possible to complete the whole life cycle in artificial conditions and its supply depends entirely on wild catches. In the last 50 years this species has suffered a population reduction of 99 % mainly due to overfishing. In a conservation perspective, it is of fundamental importance to improve the aquaculture production of European eel, to avoid the extinction of this species and preserve its residual genetic variability, allowing at the same time the fulfilling of costumers request without increasing its harvesting pressure.

In this study we aimed to deepen the knowledge about the mechanisms at the basis of reproduction of the European eel in semi-natural conditions, through direct observation of spawning behaviour and through the paternity assignment using microsatellite markers. The systematic and prolonged observation of the reproductive behaviour of European eel and the contextual parentage analyses we carried out for the very first time in this species on 39 adults and 432 F1 randomly collected. We contributed to unravel the sexual behaviour of this species in the most common artificial reproduction conditions (polyandry), and define the precise courtship sequence until the release of gametes, and the male-male hierarchy in courtship. We characterized for the first time three main types of male: dominant (the first who starts the courtship, and the one with the majority of F1 assigned), subordinate (which starts the courtship only in a second time and with a minor percentage of F1 ascribed) and ineffective (which sometime appears totally disinterested to courtship and has few F1 or none).

The evidences here produced represent an important attempt for developing good reproduction practices of the critically endangered European eel, providing a good starting point for its future aquaculture production.

1. Introduction

Currently the European eel *Anguilla anguilla* (L 1758) supply depends mainly on wild glass eels caught in the traditional downstream traps called "lavorieri". However, since the mid 1970s, the recruitment rate of this species has suffered from a rapid and severe decline. Local anthropogenic disturbance (habitat loss and/or degradation, overfishing) and global human-driven environmental changes (climate change and variation in ocean circulation; Pacariz et al., 2014 and references therein) have led to a less than 10 % recruitment rate in the Mediterranean region compared to that before crisis (i.e. before 1970; Aalto et al., 2015). For all these reasons European eel is included in the IUCN Red List of threatened species and is classified as "Critically Endangered" (Jacoby and Gollock, 2014).

Under this scenario, domestication and aquaculture production may represent an effective tool to fulfil customer request and to preserve natural stocks of *A. anguilla* from depletion, providing eels both for food industry and, optimistically, for future restocking projects. Because of its peculiar life cycle, the species represents a true challenge for breeding and production: egg quality, fertilization rate, optimal sex ratio and larval survival are the main challenges. The first two were successfully resolved by the development of breeding protocols based

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on spontaneous spawning in a controlled environment (Di Biase et al., 2016). Nevertheless, this success represents only the very first step to domestication of European eel and further studies are needed to overcome the problem of the larval mortality.

Regarding the spawning behaviour of the European eel in natural conditions, the most accurate information was reported back in the 80's (Boetius and Boetius, 1980), in which five two-hours observations were carried out in five separate tanks, each containing a single female and one to three males. Courtship was observed in four out of five experiments. Nevertheless, as no spawning was observed, the authors concluded that their description must be considered valid for the male behaviour only, and it can hardly be told whether the female has responded to the courtship. Twenty-five years later, van Ginneken and Maes (2005) observed four hormone-treated European eels (sex ratio 1:1) and three types of interaction were documented: male-male, male-female and female-female. As regards the second type of interaction (male-female), they observed the sperm release by both males and identified three different forms of spawning behaviour: males touching the head of the female, the operculum and, to a lesser extent, the urogenital area. The non-sticky pelagic eggs were released, and no parental care was observed. Based on their observations, the authors concluded that the hormone-induced spawning of European eel was collective and simultaneous, probably triggered by pheromones (van Ginneken et al., 2005).

Parentage analysis and genetic surveys represent the basic tools needed to drive breeding protocols to the next-steps: selection by phenotype, by family and marker assisted selection of Quantitative Trait Loci (QTLs). Single Nucleotide Polymorphisms (SNPs) and species-specific hypervariable microsatellite DNA markers (or Short Tandem Repeats, STRs) have been widely used in aquaculture (Abdul-Muneer, 2014; Bylemans et al., 2016). In particular, STRs are popular and versatile markers, which can be successfully applied to different fields such as population genetics, conservation biology, and evolutionary biology. They consist in di-, tri- and tetranucleotide repeats distributed throughout the genome of eukaryotes, inherited in a co-dominant Mendelian fashion, easy to detect, and for these reasons very suitable for parentage analyses. These markers were successfully applied to assess pedigree and kinship in many different cultured fishes, both diploid (Abdul-Muneer, 2014) and polyploid (Guarniero et al., 2017). Parentage assignment studies are useful for aquaculture programs, as well as for fisheries management; because the mixing of captive-bred and wild fish may affect the ecological and genetic integrity of wild fish populations, pedigree analyses may be used to monitor the effects of escapees and/or deliberate releases of aquaculture bred fish on wild populations (Bylemans et al., 2016).

As regards eel species, the only captive bred parentage study available to date is for the Japanese eel *Anguilla japonica* (Sudo et al., 2018). In this study, the authors successfully assigned paternity to 153 larvae of Japanese eel using an array of eight species-specific microsatellite loci with a success rate of 98.7 %, revealing that only two out of the five adult females used contributed to the next generation, whereas 13 out of the 15 adult males produced F1 individuals; different paternity ratios between stages (pre-leptocephalus and leptocephalus) were reported for some males, suggesting a different survival rate of offspring produced by different males. Even if these data cannot be directly applied for breeding programs, they represent a useful baseline to implement knowledge on reproduction of this valuable species.

The main aim of this study is to deepen the knowledge on spawning behaviour and reproduction mechanisms of European eel in semi-natural conditions. Here, we set the groundwork for future marker-assisted artificial reproduction projects by implementing and applying the parental assignment technique. This will allow to optimize future breeding plans aiming to increase both productivity for human consumption and species restocking, after years of fishing pressure worldwide.

2. Materials and methods

2.1. Ethics

All experiments were performed according to European and Italian guidelines on animal experimentation and care. Approval for this study was obtained from the Ethics Committee of Bologna University (protocol no. 19/6912).

2.2. Breeders recruiting

Wild European eels (*Anguilla anguilla*) were caught in 2017 and 2018 during a single day using traditional downstream traps called "*lavoriero*" in a brackish water lagoon near the sluices of the North Adriatic Sea (Val Noghera, Lagoon of Grado, Italy), during their downstream migration (autumn-winter season) and then moved to the aquaculture facility. The wild animals were then measured and sampled to obtain an external indicator of their maturation stage, that is the silver index SI (Durif et al., 2005; Mordenti et al., 2012, 2013). Both in 2017 and 2018, the seven females and 20 males with the maximum SI were selected and then marked individually by inserting fish-tags (FLOY TAG Mod Floy T-Bar Anchor) in the dorsal muscle under anaesthesia with 400 ppm 2-phenoxyethanol and maintained under starvation for the duration of the trial. All eels were kept in a Recirculating Aquaculture System (RAS) consisting of two fish-rearing tanks (1200 L each), one with females and one with males. Fish were maintained in complete darkness (-0.04ˆ10^3 lux at the bottom of the tank without water) in seawater (salinity 31 ± 1 g/L) at the temperature of 15.5 ± 0.5 °C until gonadal maturation was complete (Mordenti et al., 2012, 2013).

2.3. Spawning behaviour, reproduction and larval production

The females (body weight 778.3 ± 132.3 g) received intramuscular injections once a week with carp pituitary extracts (CPE) at a dosage of 10 mg/kg BW (1st–3rd week), 20 mg/kg BW (4th–6th week), 30 mg/kg BW (7th-9th week) and 40 mg/kg BW (10th-final maturation) (Mordenti et al., 2014, 2018).

Males, which were chosen with highly similar characteristics (body weight 116.2 ± 12.3 g, length 40.5 ± 1.6 cm, condition factor *k* 0.175 ± 0.01; SI = II), were induced following standard protocols (Ohta et al., 1996; Palstra et al., 2005; Di Biase et al., 2017) and started spermiation after a 12-week treatment. Just before fertilization, the males received a booster hCG injection to reactivate spermiation (Buergerhout et al., 2011). Sperm motility was checked and only males with at least the 50 % sperm motility (i.e. continuous activity of > 50 % of spermatozoa) were used for the reproduction (Buergerhout et al., 2011).

Twenty-four hours after the last CPE injection (increase in female BW around 120 %; Mordenti et al., 2012, 2013), the ovulation was induced by injecting 17α, 20β-dihydroxy-4-pregnen-3-one (henceforth DHP; Palstra et al., 2005; Mordenti et al., 2014) in ten different areas of the ovary. After the DHP injection, each female was transferred together with three to four spermiating males in a new closed recirculating aquaculture system (Mordenti et al., 2014), where the seawater temperature was raised to 20 ± 0.5 °C (Dou et al., 2008) and maintained for 16 h, in order to obtain spontaneous reproduction (Mordenti et al., 2018).

Each tank was obscured by a dark PVC cover in order to maintain the spawners in near dark-light conditions. The presence of a 200 × 300 mm window in the cover allowed the direct observations of the behaviour of eels inside the spawning chamber. Observations were performed during all the 16 h of permanence of the adults in the reproduction tank and where divided into four periods: phase I post DHP (pDHP) injection (1st–4th hour), phase II (5th–8th hour pDHP), III (9th–12th hour pDHP) and phase IV (13th–16th hour pDHP). After 16 h

Table 1
Spawning behaviour, reproduction and larval production in the 14 reproductive experiments carried out.

	Courtship (Yes/No)				Reproduction (R)				Production of alive F1
Phase	*I*	*II*	*III*	*IV*	*I*	*II*	*III*	*IV*	
2017									
Aa17-1	No	Yes	Yes	No	–	–	R	–	Yes
Aa17-2	No	Yes	Yes	No	–	–	R	–	Yes
Aa17-3	No	No	Yes	No	–	–	R	–	Yes
Aa17-4	No	No	No	No	–	–	–	–	No
Aa17-5	No	Yes	Yes	No	–	–	R	–	Yes
Aa17-6	No	Yes	Yes	No	–	–	R	–	Yes
Aa17-7	No	Yes	Yes	No	–	–	R	–	Yes
2018									
Aa18-1	No	Yes	Yes	No	–	–	R	–	Yes
Aa18-2	No	No	Yes	No	–	–	R	–	No
Aa18-3	No	No	Yes	No	–	–	R	–	Yes
Aa18-4	No	Yes	Yes	No	–	–	R	–	Yes
Aa18-5	No	No	Yes	No	–	–	R	–	No
Aa18-6	No	Yes	Yes	No	–	–	R	–	Yes
Aa18-7	No	No	No	Yes	–	–	–	R	No

all the breeders were removed from the spawning chamber and fertilized eggs were kept in the incubation chamber until hatching.

2.4. Genetic analyses

The sampling for genetic analyses was carried out on hatched F1 eggs during ten successful spawning events (see Table 1). A fin clip was collected under sterile conditions form each adult and preserved at −20 °C in 96 % ethanol. 40–48 larvae (40 for each successful hatching in 2017 and 48 for each successful hatching in 2018) were randomly collected one day post hatching and preserved in the same conditions. DNA was extracted using the Promega's SV Wizard Genomic Purification System Kit according to the producer's protocol and assessed on 0.8 % agarose for successful DNA extraction. Genetic profiles were obtained using ten species-specific polymorphic microsatellite loci selected for their high levels of polymorphism: AAN22B09, AAN06E24, AAN24A09, AAN2613, AAN41E24, AAN42O08, AAN44B22 (Pujolar et al., 2009), AAN01, AAN02, AAN04 (Daemen et al., 2011). Multiplex PCR amplifications were performed in 20 µL using Qiagen Buffer 1X, 1.5 mM $MgCl_2$, 0.8 mM dNTPs, 5 µmol of each primer, 1U of Qiagen HotStarTaq Polymerase, 50 ng of DNA and sterile water to the final volume. Forward primer of each locus was fluorescently tagged with Standard DS-33 GeneScan matrix dye set (6-FAM, PET, NED and VIC, Thermo Fisher Scientific). For a better PCR performance and to avoid genotyping errors due to allele overlap of different sets and/or fluorescent primer and dye interactions, loci were divided into three different sets: S1, loci AAN22B09, AAN06E24, AAN24A09, annealing temperature 57 °C; S2, loci AAN2613, AAN41E24, AAN42O08, AAN44B22, annealing temperature 57 °C; S3, loci AAN01, AAN02, AAN04, annealing temperature 60 °C. The thermal profile consisted in 15′ at 95 °C to activate the HotStarTaq enzyme according to manufacturer's protocol, followed by 35 cycles at 94 °C for 30″, annealing temperature for 90″, 72 °C for 60″, and finally a prolonged extension at 60 °C for 30′. Amplicons were then sent to Macrogen Inc (Korea) for sizing by capillary electrophoresis with Life Technologies GS500LIZ as internal size standard. Alleles were scored using Peak Scanner 1.0 (Life Technologies) and converted to discrete values by manual binning in order to decrease allele-calling error rates linked to standard automated approaches. The *per locus* genetic diversity (number of alleles per locus, k; expected heterozygosity, HE and observed heterozygosity, HO), the marker's informativeness parameter PIC (Polymorphic Information Content), the probability of Hardy-Weinberg Equilibrium corrected by Bonferroni sequential test, the predicted null allele frequencies and the paternity assignment were all obtained by CERVUS 3.0.7 (Kalinowski et al., 2007).

3. Results

3.1. Spawning behaviour, reproduction and larval production

Results on spawning behaviour, reproduction and larval production in the 14 reproductive experiments carried out are summarized in Table 1.

Courtship behaviours were observed in 92.8 % of the 14 reproductive experiments carried out, almost all in phase II and III. Reproduction took place in the same percentage of cases, mostly in phase III. The 71.4 % of successful reproduction lead to the production of alive F1.

The spawning behaviour can be summarized as follow:

1. Phase I: after the arrival in the new tank, males and the female start swimming around without any particular interaction in both sexes.
2. Phase II: the more active male starts chasing the female, followed by other males. In some cases, a single male appeared totally neutral to the activities of other animals, seeking shelter under the tubes.
3. Phase III: the males start approaching with grater verve the female from the bottom, touching with their head the female belly, starting from her urogenital area, moving to the head and dwelling fore few seconds in the throat region of the female obtaining in this way the maximum contact between the male back and the female swollen abdomen. This repetitive behaviour pattern continues until the release of gametes. No twisting of bodies was observed neither in females or in males, the egg emission appears easy and smooth and once stated, the female completes the emission in few minutes.
4. Phase IV: the activity peak and interactions terminate. The animals return quietly to the bottom.

The previously described pattern was observed in 12 cases out of the 14 trials performed, with the exception of Aa17-4 for which courtship nor reproduction occurred and Aa18-7, which showed the above described pattern in phase IV instead of phase III. In two of the 14 trials, courtship and reproduction were observed in phase III but the hatching of eggs failed (Table 1).

3.2. Paternity assignment and genetic variability

The genotyping of the 471 individuals (39 adults and 432 larvae) gave similar results in the two years of experiments, and for this reason they were summarized in a single table (Table 2). Locus 24A09 showed the best performances in terms of variability, with the higher number of alleles (25), the higher values of observed and expected heterozygosity

Table 2
Main parameters of genetic variability. k: number of alleles per locus; Hobs observed heterozygosity; Hexp: expected heterozygosity); PIC: Polymorphic Information Content. HWE: Hardy-Weinberg Equilibrium. NS not significant; ** $P < 0.01$; *** $P < 0.001$. F(Null): predicted null allele frequencies.

Locus	k	Hobs	Hexp	PIC	HWE	F(Null)
22B09	23	0.982	0.928	0.922	**	−0.0307
06E24	13	0.906	0.827	0.807	***	−0.0488
24A09	25	0.921	0.919	0.912	**	−0.0021
26N13	17	0.815	0.800	0.780	***	−0.0080
41E24	11	0.786	0.808	0.787	***	0.0227
42O08	22	0.793	0.900	0.891	***	0.0636
44B22	14	0.904	0.873	0.859	***	−0.0206
AAN01	13	0.802	0.787	0.762	NS	−0.0085
AAN02	24	0.910	0.920	0.914	NS	0.0052
AAN04	7	0.664	0.617	0.543	***	−0.0475
overall	*16,9*	*0.838*	*0.838*	*0.818*	*NS*	

(0.982 and 0.928 respectively) and finally the highest PIC value (0.922). On the contrary, the less variable and less informative locus was AAN04 with seven alleles, lowest values of observed and expected heterozygosity (Hexp = 0.848 and Hobs = 0.617, respectively) and the lowest PIC recorded (0.543).

The combined non-exclusion probability for the 2nd parent was $2.26^{\wedge}10^{-6}$ and the combined non-exclusion probability of identity was $1.99^{\wedge}10^{-12}$.

3.3. Parentage assignment

Since the breeding design considered a single mother for each family, the parentage analysis focused only on paternity assignment. Almost all progeny was assigned to a parental couple (419/432, i.e. the 97 %), with a percentage of success per single experiment ranging from 92.5 % (Aa17-6) to 100 % (Aa18-4 and Aa18-6). The males' percentage of fertilization success was calculated on the basis of paternity assignments. Twenty seven out of the 29 males used were able to produce F1, even if with very different percentage of contribution: in absolute terms J_6 showed the worst performance with three sons attributed, while male J_27 resulted the major contributor to F1 with a total of 50 fingerlings in two different emissions: 20 the first time (50 % of the batch analysed) and 30 the second (75 %). The males' reproductive performances per single reproductive event are given in Fig. 1. In order to easily describe such figure, we introduce the concept of "Best Performing Male" (BPM, that is the male which produced the majority of F1, white slice of the cake graph) and "Worst Performing Male" (WPM, the one which produced the minority of F1, grey slice). The BPM contribution to F1 ranged from 33 % (J_134) to 75 % (J_27), while the WPM contribution ranged from 3 % (J_20 and J_23) to 17 % (J_142). In two spawning events, the difference between BPM and WPM was reduced and three to four males gave similar percentage in fertilization success: Aa17-5 with 4/4 active males, and Aa18-4 with 3/4 active males. All other reproductions showed similar results, with a single dominant male (that is the BPM), generally followed by two males with minor reproductive success, and finally the last male, which contributed marginally to the F1 (i.e. the WPM).

Nine males were used in more than a single reproductive event (that is J_4; J_20; J_23; J_27; J_126; J_126b; J_129; J_147 which were used two times; and J_128 used three times). They all were able to fertilize eggs each time, with a percentage of success per single reproduction ranging from 2.5 % (J_20 and J_23) to 75 % (J_27). Six out of eight males used twice, reduced the production of F1: in three cases the decline was minimal (three to four less F1, males J_23, J_20 and J_126), while in other three cases the drop was more significant, with 11–20 less F1 (males J_4, J_126, J_129). In the remaining two cases, the males increased the production by two units (J_147) and 10 units (J_27). A single male was used three times and showed a fluctuating trend with eight F1 the first time, 16 F1 the second and 14 F1 the 3rd.

4. Discussion

In this study the main aspects concerning the hormonal induced reproduction of European eels in semi-natural conditions by direct observation of spawning behaviour and indirect evidences driven by parentage assignment were considered.

The eel courtship behaviour in captivity has been documented, even if there are some gaps mainly due to experimental conditions, in both *Anguilla anguilla* (Boetius and Boetius, 1980; Van Ginneken et al., 2005) and in *Anguilla japonica* (Dou et al., 2007, 2008). As regards the European eel, the direct observations made in this study clearly demonstrate, using paternity assignment, what was deduced by van Ginneken and Maes (2005): the spawning in this species is collective and possibly triggered by pheromones. However, the reduced experimental conditions of van Ginneken and Maes (a single observation of a single tank in which only four animals were present, sex ratio 1:1, and mostly the absence of paternity tests) did not permit to highlight the detail of courtship and the percentage of success in F1 production of single males. In our study, we evidenced the presence of a sort of hierarchy in the males group, with a single more active male followed by 2–3 less active males and finally by a single male which appeared involved only marginally. Moreover, the courtship described in van Ginneken and Maes (2005) was a linear pattern, without repetition of the sequence. On the contrary, our observations showed that the behavioural pattern of hormonal stimulated males is repetitive and maintained until the sperm release. Boetius and Boetius (1980) based their observations on a more substantial experimental design, even if it has to be underlined that in 1980 the artificial reproduction techniques were at their very infancy, far from a standardization and, thus, from significant results in terms of seed production. The authors carried on five trials in five different moments, with a single female and 1–3 males in each tank. Courtship was observed in four out of the five experiments and partially coincided with our observations: in the very first minutes the males start the exploration of the tank looking for something (probably an escape) until the female is identified. Once detected, they start the courtship loop-sequence: the males start rubbing the female's abdomen in order to obtain the maximum contact between their back and female's belly, partially clinging the female from the bottom. While in the experiment of Boetius and Boetius (1980) all males present in the tank took part into the initial courtship but only one released its sperm, the paternity assignment performed in this study clearly demonstrates that, with the exception of two males, almost all of them were able to fertilize eggs in both years of experiments, even if with very different percentage of success. Moreover, while in the above-mentioned experiment no spawning was observed, making impossible to say if the females responded to courtship, in this study the percentage of success was very high and alive F1 was obtained in ten out of 14 trials.

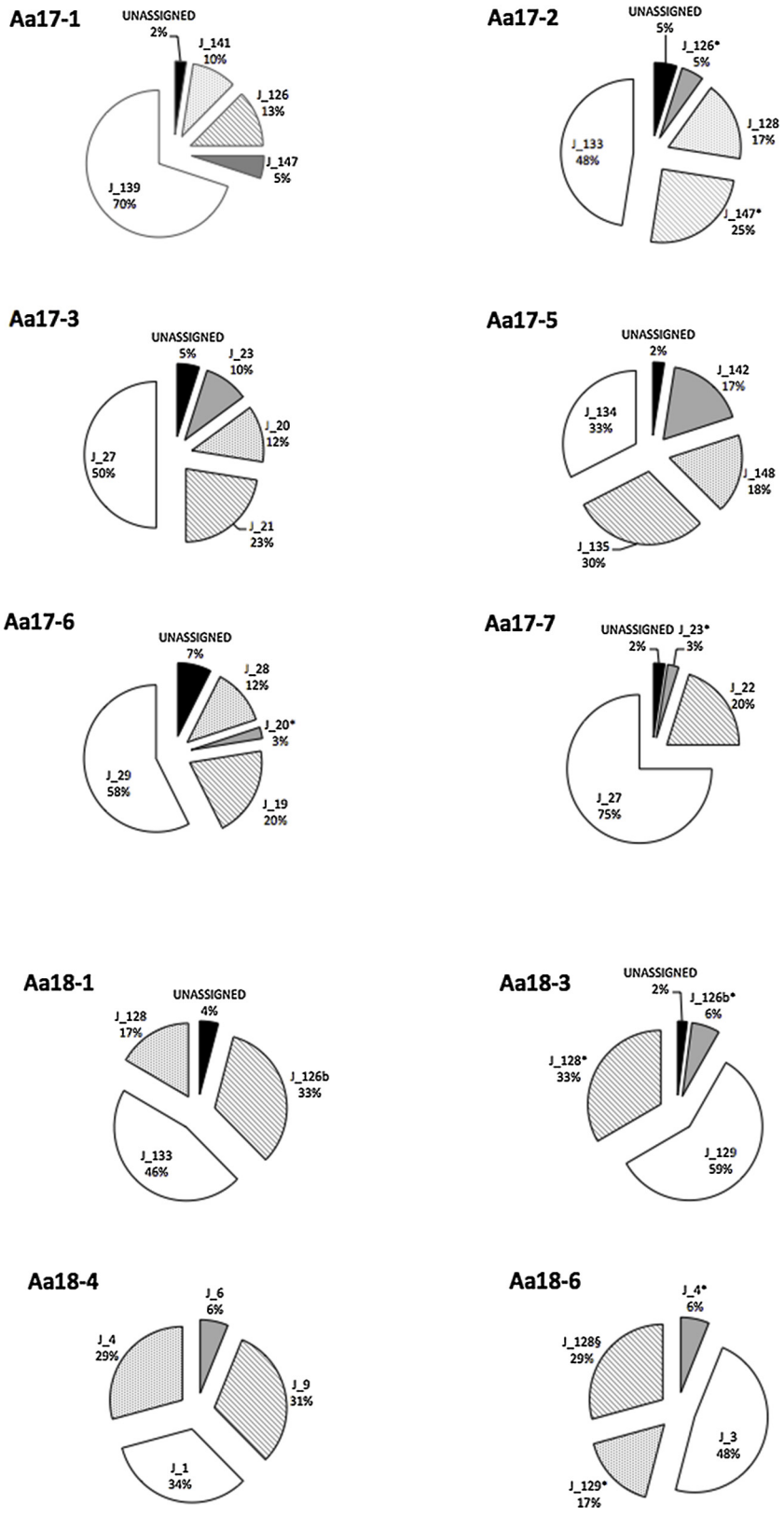
As observed in the Japanese eel (Dou et al., 2007, 2008), the initial quiescence of breeders present in the reproduction tank was interrupted starting from the 8th hour pDHP (phase III), and the males' courtship started probably as a consequence of the release of the female's pheromones, stimulating the sexual response of males, which started the courtship sequence (Sorensen et al., 2005; Huertas et al., 2006).

Regarding the four failed experiments, three different situations were observed, with different possible explanations:

(i) Failure n.1: Aa17-4. The courtship sequence did not start and the animals did not interact for the 16 h of permanence in the tank. A similar case happened also in Boetius and Boetius (1980): in a single experiment no courtship was seen in the two hours of observations and the female died few hours later. In our experiment the female Aa17-4 remained alive, and the reason of this aberrant behaviour might be ascribed to a failure in the hormonal stimulation. This female in fact was probably caught too early, at the pre-migrant stage. Pre-migrant eels are morphologically pretty identical to a migrant (silver) eel but their gonadal maturation is still inadequate to give a satisfactory response to the hormonal stimulation protocol. Perhaps in this experiment the release of female pheromones failed, and males' courtship did not start (Mordenti et al., 2012, 2013).

(ii) Failure n.2 and 3: Aa18-2 and Aa18-5. Even if courtship and reproduction took place in the optimal timing (phase III, 9–12 hours pDHP), in these two trials no alive F1 was obtained. Gametes were released, fecundation occurred, and cell division started but, for some reason, stopped before hatching. This could be due to an inadequate food intake during the period spent into the wild environment and thus to a poor egg quality. Egg quality in fact is strongly affected by nutritional deficiencies and dramatically affects the fertilization success and hatching (Mordenti et al., 2013). A similar scenario was observed in *A. japonica* in similar experimental conditions: a female which did not contribute to the next generation, spawned but the eggs did not survive, probably due to

(a)



(b)

Fig. 1. Contribution of males to progeny in each spawning event that took place in 2017 (a) and 2018 (b).
*: males used for the 2nd time; §: male used for the 3rd time.

the poor quality of her eggs (Sudo et al., 2018).

(iii) Failure n.4: Aa18-7. Unlike all others experiments in which courtship was observed, in this trial the sequence of courtship and reproduction was delayed and took place in phase IV (> 13 h pDHP) instead of phase III. The failure of this experiment may be ascribed to a missed synchronization between pheromones release and gamete emission, triggering a tardive male's courtship, when the eggs were over-ripped due to the DHP treatment and were no more fertilizable. Indeed, the DHP injections lead to acceleration in oocytes maturation and thus reduce the time window for eggs fecundation (Ijiri et al., 2011; Di Biase et al., 2016).

From our observations, the ideal time window for egg fecundation is between the 9th and the 12th hour pDHP, (and more specifically in the last half of this period, i.e. ten to 12 h after the DHP treatment): ten out of 12 trials in which courtship and reproduction were observed during this phase, lead to alive F1.

Thanks to the high-resolution power of the ten species-specific STRs markers used and the absence of genotyping errors and mismatches, the parentage assignment was very efficient and allowed to determine the correct father in 97 % of the fingerlings analysed. Except two, all males used were able to produce at least few fingerlings, confirming that in European eel in polyandry conditions, ejaculation occurred at the same time and is probably triggered by female's pheromones. On the basis of paternity tests, borrowing some terms used for social animals, three main categories of males can be identified: (i) *dominant*: a single male with the majority of F1 ascribed; (ii) *subordinate*: generally two or three less prolific males; and (iii) *ineffective* and/or *sheltered*: a single male which seemed to participate only occasionally into the F1 production (Fig. 2). The dominant male is perhaps the more active in the tank and the one who starts the courtship followed by the subordinate males. These males, which were called "Best Performing Male" in the Result paragraph, were the major F1 contributors (33–75 %). The subordinates have a minor reproductive success and follow the BPMs both in timing terms (they start the courtship sequence only in a second moment) and in percentage of contribution to F1. Finally the ineffective/sheltered contributed only marginally to the F1 and were the males that in some experiments appeared completely disinterested on the on going the activity and sought shelter under the tubes of the tank. Nevertheless, with the exception of two single males which not reproduced at all, this latter category often contributed actively to the F1 production even if with a very small proportion of fingerlings. This contribution might be explained by the greater sperm longevity of the European eel (Locatello et al., 2018), which should have given also to the ineffective/sheltered males the opportunity to fertilize some eggs by chance, adding their alleles to the final F1's genetic variability. As regard the overall sperm quality (longevity, density, spermatocrit, percentage of motile sperm),

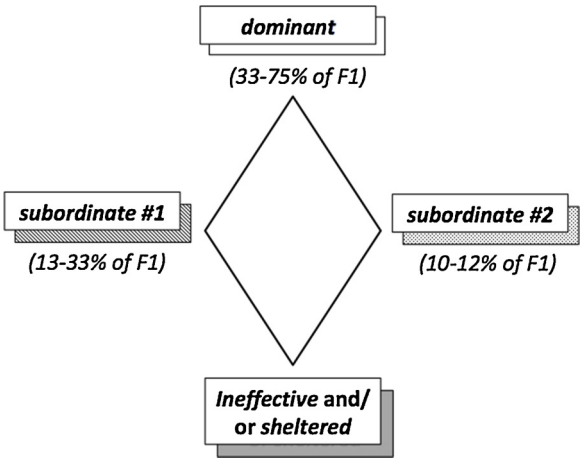


Fig. 2. Males' pattern of reproductive success and related categories.

Locatello et al. (2018) observed a great homogeneity in the six wild Val Noghera silver males of their study. Those males were caught in the same area and in the same period of the males used in the present paper, and were maintained in the same experimental conditions. For this reason, we argued that the differences in fertilization rate here observed might be ascribed to the different behaviour observed (dominance/subordination), rather than a consequence of a real difference in sperm quality.

It is interesting to notice that the nine males used for two or three reproductions, remained alive and were able to produce F1 each time, even if they generally showed a reduced productivity whose entity ranged from slight (–3 to 4 fingerlings) to heavy (–11 to 30 fingerlings), maintaining the sheltered behaviour in a single case, passing from subordinate to ineffective/sheltered in four cases, and from dominant to subordinate in one case. Concerning the two males that showed an increase of production, one maintained the dominant position in both experiments in which was used (J_27: the best performing male in absolute terms), while the other passed from subordinate to dominant. The male used three times (J_128) maintained the subordinate behaviour in all three trials in which was used. This fluctuating scenario does not have any statistical weight and does not allow any kind of advice about the opportunity to use males more than once.

Based on the highly biased results obtained in artificial reproduction of the Japanese eel, in which only two males and two females contributed to next generation, Sudo et al. (2018) state the necessity to select breeders from wild populations with broad genetic diversity before starting selective breeding protocols, and promoted natural mass spawning using at least five females and 15 males to increase the genetic variability. Our results confirm this suggestion: the forced admixture of European eel breeders which genotype was highly differentiated and the overall ability to reproduce of almost all breeders used (10/10 females and 27/29 males), has ensured a very high genetic variability and a general excess of heterozygosity in 7 out of the 10 STRs used. Expected and observed heterozygosity showed only slightly and not significant variations between the two years of experiments and no bottleneck was detected between breeders and their F1.

To conclude, this manuscript has contributed to definitely unravel the sexual behaviour of the European eel in the most common artificial reproduction condition: polyandry. Hormonally stimulated females with the ideal silver index release pheromones which trigger male's courtship with a precise order: the more active (dominant) male starts the sequence of movements, followed by the subordinate males, while a less active male appear disinterested. The male who starts the courtship first is perhaps the most productive and the one whose alleles win the genetic raffle, being transmitted in high proportions to the next generation.

The high percentage of success here obtained in European eel reproduction and hatching suggests that the hormonal induction protocol (Mordenti et al., 2014; Di Biase et al., 2016) and the natural mass spawning of highly genetically variable breeders, with sex ratio 1:3 to 4 (one female, three to four males), should represent an ideal scenario and a good starting point for the implementation of artificial reproduction of this critically endangered species, even if several key points like larval weaning still have to be resolved.

CRediT authorship contribution statement

Ilaria Guarniero: Conceptualization, Methodology, Supervision, Project administration, Formal analysis, Writing - original draft, Writing - review & editing. **Alessia Cariani:** Conceptualization, Supervision, Formal analysis, Writing - review & editing. **Alice Ferrari:** Investigation, Funding acquisition, Writing - review & editing. **Valerio Sulliotti:** Investigation. **Pietro Emmanuele:** Investigation. **Antonio Casalini:** Investigation. **Fausto Tinti:** Funding acquisition. **Oliviero Mordenti:** Project administration, Funding acquisition, Writing - review & editing.

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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.2019.100258.

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