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Multiple paternity in reproduction of European eel *Anguilla anguilla* (L. 1758) by artificial mixing of different sperm in equal volumes

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A R T I C L E I N F O

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A B S T R A C T

Because of its complex life cycle and due to multiple stress factors, the European eel is suffering a dramatic decline and has been declared Critically Endangered by the IUCN. A possible way to address this problem could be relieve the pressure on natural stocks by implementing its mass production by artificial breeding protocols. A previous study based on direct observation and parentage assignment underlined the presence of an allegedly hierarchic structure among European eel males in semi-natural mating conditions, with a consequent bias in F1 proportion assigned to each one. The aim of the present study is to attest if a different fertilization protocol based on the artificial mixing of female eggs with different males' milt in equal volume (1 F:4 M) could represent a solution to the disproportion observed in semi-natural conditions. For this purpose, six families of European eels were generated, and 10 species-specific microsatellite loci were used to infer offspring composition by paternity assignment on 280 samples. Due to the asynchronous ovarian development of female European eels, the percentage of fertilized eggs for each female showed a great variability, ranging from 4.70% to 94.50%. A proportion of 94.02% of genotyped offspring were assigned with high confidence to their true parents. As regard males' fertilization pattern, no substantial differences from natural mating were observed: a single male accounted for most offspring, which was just mostly composed of full sibs. Concluding, the obtained results suggest that the admixture of an equal volume of different males' milt seems to contribute to the single-locus genetic variability (observed heterozygosity higher than expected in 7 out 9 loci), but it is not sufficient to ensure all the males the same chance to transmit their gene pool, and new fertilization strategies must be developed.

1. Introduction

European eels (*Anguilla anguilla* L. 1758) have one of the largest migration loops in the ocean, following a million-year-old instinct to reach the Sargasso Sea to reproduce (Tsukamoto et al., 2002, 2009; Inoue et al., 2010; Arai, 2022). But world has changed since the first freshwater eels settled their catadromous migration behavior, and the long way home has become a risky business for their leptocephalus larvae. Oceanic changes in the Sargasso Sea (Friedland et al., 2007; Bonhommeau et al., 2008), the slowdown and northward shift of Gulf Stream (Chi et al., 2021 and references therein) coupled with ocean warming and acidification have modified their dispersal pathway (Baltazar-Soares et al., 2014), survival rate and migratory behavior (Borges et al., 2019). Although they can survive to this new unfriendly environment and are able to reach the European coasts, many other challenges are waiting. Illegal trade (Richards et al., 2020), dammed rivers and hydropower plant pumps (Piper et al., 2015; Anon, 2021), exposure to pollutants (Palstra et al., 2006) and drugs (Capaldo et al., 2018), infection with the swimming bladder parasite *Anguillicola crassus* (Feunteun, 2002; Drouineau et al., 2018) and, of course, fishing nets (Dekker, 2019).

For all these reasons, this species has suffered a dramatic decline throughout its distribution range. Currently only the 10% of the European eel worldwide stock has left (Feunteun, 2002), and according to the International Union for Conservation of Nature (IUCN) *A. anguilla* is just two single steps before the extinction (Jacoby and Gollock, 2014).

In this complicated and multifaceted situation, a multi-level approach should be attempted before the European eel question really

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becomes an "*impossible bargain*" (Feunteun, 2002).

In the short term, based on the panmixia assumption of *A. anguilla* and its wide distribution range (Daemen et al., 2001; Wirth and Bernatchez, 2001; Maes and Volckaert, 2002; Als et al., 2011), EU policy makers should cooperate to define common fishery policies and coordinated management actions to protect the population. Nearby political and ecological actions, conservation aquaculture practices like *ex situ* production of juveniles aiming to close the European eel life cycle, can represent a possible solution, contributing to protect and restore this critically endangered species (Schreier et al., 2012; Epifanio and Waples, 2016; Tancioni et al., 2019).

Several studies suggest that the in vitro fertilization is the technique with the least impact on genetic variability (Beirão et al., 2019; Bartron et al., 2018; Campton, 2011). Nevertheless, as regard the *Anguilla* genus, Di Biase et al. (2016) on European eel, and Tanaka (2015) on Japanese eel, highlighted scarce results with this technique in terms of fertilization rates, and semi-natural mating should be preferred to maximize the yield. On the other hand, in semi-natural conditions, an unbalance distribution in F1 allele frequencies due to behavioral components was observed both in different eel species (Japanese and European, Sudo et al., 2018 and Guarniero et al., 2020 respectively) and in other aquaculture species, with most full- or half-sib juveniles (e.g., Borrell et al., 2008; Duncan et al., 2013; Ma et al., 2017; Ofelio et al., 2020).

Even knowing limitations due to the pool mixing technique, equalizing the semen volume of different males for in vitro fertilization to try to avoid bias in F1 due to behavioral variables in semi-natural mating, currently represents the easiest and simplest technique with a view to a future, and hopefully rapid, closing of European eel life cycle in industrial aquaculture plants,

Parentage assignment trials are simple and essential management expedients in fish farming (Yue and Xia, 2014). Even if tests based on SNP panels are becoming more common thanks to next generation sequencing techniques, those based on microsatellite loci are still one of the most cost-effective available tools (Chistiakov et al., 2006; Guichoux et al., 2011; Gulcher, 2012). They are based on the simple assumption that for each locus, offspring carries an allele form the father and the other from the mother. They have been applied for pedigree tracking (Blouin, 2003; Sudo et al., 2018; Guarniero et al., 2020; Ofelio et al., 2020), and avoidance of inbreeding depression in marine species (Park et al., 2006).

In contrast with other species, for which the artificial reproduction is at an advanced stage of knowledge and practice (e.g., common carp, chinhook salmon or other salmonids; Withler, 1988; Withler and Beacham, 1994; Wedekind et al., 2007; Kaspar et al., 2007; Kaspar et al., 2008), European eel artificial reproduction currently is in exploratory and pioneering situation. Few studies are available on mechanisms underpinning reproduction of this species (Boetius and Boetius, 1980; van Ginneken and Maes, 2005; Guarniero et al., 2020). In this scenario, the aim of this study is to attest if a fertilization protocol based on the artificial mixing of a single female eggs, with an equal volume of four different males' milt instead of spontaneous insemination, should represent a solution to the disproportion observed in semi-natural conditions, rebalancing thus the F1′s allelic composition, obtaining an unbiased distribution of parental contribution to the next generations.

2. Materials and methods

2.1. Ethics

The trials were conducted accordingly the EU regulations on animal research. Sampling and manipulation procedures were formerly approved by the Ethical Committee of the University of Bologna (ID 575/2016).

2.2. Breeders recruiting fertilization procedure

Six females and 16 males were used to produce progeny. All sampled females were wild and caught in the brackish water lagoon of *Valli* di Comacchio (Ferrara, Italy), by a downstream trap (*lavoriero*) that captures eels as the beginning of their migration from the lagoon to the open sea. Twelve out of 16 males were wild and collected near the sluices of the North Adriatic Sea (*Valli* di Comacchio, Ferrara, Italy) using the same traditional 'lavoriero' trap. The remaining four males were selected on a fish farm (Colombo Fish Farming, Milano, Italy). Both wild and farmed eels were then moved to the facilities of the Department of Veterinary Medical Sciences (Cesenatico, Italy). All the animals were weighed (females 629.4 ± 92.03 g; males 141.6 ± 21,1 g) and the initial silvering stage was determined according to the classification system described by Durif et al. (2005). Finally, the animals were individually marked by a fish-tags FLOY TAG Mod Floy T-Bar Anchor. Wild males had an identification code starting with 'R′ (those used to generate families nr. 2, 3, 6, 9, 10) while farmed ones had an identification code starting with 'V′ (family 12 only).

2.3. Reproduction and larval production

The six families generated for the present study were produced according to a polyandry breeding scheme with a sex ratio 1 F: 4 M (Table 1). Males and females were hormonally treated to induce gamete maturation. The females received intramuscular injections once a week with carp pituitary extracts (CPE; 10 mg/kg BW weeks 1–3; 20 mg/kg BW weeks 4–6; 30 mg/kg BW weeks 7–9 and 40 mg/kg BW weeks 10 to final maturation; Mordenti et al., 2014; Mordenti et al., 2018). Males were treated once a week with intramuscular injections 1.0 IU/g human chorionic gonadotrophin (hCG, Corulon, 5000 UI, Intervet, Segrate, Milan, Italy) as described in Asturiano et al. (2006) and Mordenti et al. (2014). Twelve hours before semen collection, males received a booster injection of hCG to reactivate spermation (Buergerhout et al., 2011). To ensure the achievement of the peak in sperm concentration, all males used, both wild and farmed, were treated for 13–14 weeks (Locatello et al., 2018). 24 h after the last CPE injection the females were induced by 17α,20β-dihydroxy-4-pregnen-3-one (henceforth DHP; Palstra et al., 2006; Mordenti et al., 2014) injected in ten different areas of the ovary. To prevent stress due to manipulation, before each injection animals were anaesthetized with phenoxyethanol (400 ppm). After the DHP injection, each female was transferred and kept separately into a 200 L closed recirculating aquaculture system (RAS; Mordenti et al., 2014), in complete darkness condition (−0.04ˆ103 lux at the bottom of the tank without water) and water salinity 31 ± 1 g/L. The temperature was raised to 20 ± 0.5 °C (Dou et al., 2008) and maintained for 12 h, to induce spontaneous ovulation (Mordenti et al., 2018). After an observation period of 9–12 h, if the ovulation did not happen spontaneously, eggs were obtained by dry stripping (Families 6 and 9). In the meantime, the four males destined to each female, were anesthetized with phenoxyethanol 400 ppm, and placed in a dry cloth. Before the sperm collection, the urogenital area was accurately cleaned by Milli-Q water and dried. To avoid any contamination by urine or feces, the first ejaculate was omitted (Sørensen et al., 2013; Butts et al., 2014). The seminal

Table 1
Breeding scheme. Female id, male id and number of larvae collected (l) are reported.

FAMILY	FEMALE	MALE				*l*
code	id	id 1	id 2	id 3	id 4	
2	CP2 VN 35 G	21 R	25 R	24 R	32 R	48
3	CP2 VN 37 G	29 R	22 R	28 R	31 R	48
6	CP2 VN 43 G	29 R	22 R	28 R	31 R	48
9	CP2 VN 48 G	23 R	27 R	30 R	26 R	18
10	CP3 VN 64 G	23 R	27 R	30 R	26 R	48
12	CP3 VN 60 G	4 V	8 V	21 V	32 V	48

fluid was obtained by a delicate pressure on the abdomen and directly stored at + 4 °C into a 1 mL vial. Once the female ovulation was completed, the milt collected from 4 different males (for a total volume of 4 mL) was mixed in a sterile bowl with 2 L of sea water taken from the same recirculating system, and then poured into the females' tank in which the eggs were spontaneously released or stripped out. After one hour, for each family, the fertilized eggs were removed from the spawning tank and kept in an incubation chamber until hatching. Twenty-four hours after hatching, the larvae were randomly picked for paternity assignment. Reproductive results have been calculated in terms of percentage of fertilization. The total fertilization rate (%) for each batch of spawned eggs was observed at 2 h post fertilization and determined by calculating the % of eggs that reached the 8-cell stage; for this purpose, three sub-samples of 1000 eggs were scored and averaged for each batch (Di Biase et al., 2016, 2017).

2.4. Genetic analyses

For each breeder, a fin clip was collected in sterile conditions and preserved at − 20 °C in 96% Ethanol until DNA extraction. Except for a single family in which only 18 larvae were available, for the remaining five families 48 larvae were randomly collected one day post hatching and preserved in the same conditions of the fin clips (Table 1). DNA was extracted using the Promega's SV Wizard Genomic Purification System according to the standard protocol and checked on 1% agarose gel. Genetic profiles were obtained using the ten species-specific polymorphic microsatellite loci already used in Guarniero et al. (2020) in the same amplification conditions. Amplicons were then sent to Macrogen (Korea) for capillary electrophoresis with DS-33 matrix standard (dye set G5) and GeneScan 500 LIZ size standard. Alleles were scored by Peak Scanner (Applied Biosystem). Standard genetic variability analyses (number of alleles per locus, expected heterozygosity and observed heterozygosity), markers' informativeness parameter (PIC) and paternity assignments were obtained with Cervus 3.0 (Kalinowski et al., 2007; percentage of candidate fathers typed 100%; percentage of loci typed from 95.65% -family 7- to 100% -family 2-, rate of mismatching 0.1–10%, 10000 tests, strict confidence level of parentage assignment: 95%, relaxed confidence level: 80%).

For each reproduction event, a chi-square test was performed to evaluate if the differences observed were statistically significant. For the two sets of males used twice (families 3 and 6; families 9 and 10), a chi-square test was also applied to check for significant differences in the contribution of the four males between the two reproduction events.

3. Results

3.1. Zootechnical results

According to the silver index, all the males were categorized as SI-II. As regard the females, two out of six were migrant (SI-V: 35 G, 64 G) and the others pre-migrant (SI-III: 37 G, 43 G, 48 G, 60 G). The percentage of fertilized eggs for each female showed a great variability, ranging from 4.70% to 94.50% (Fig. 1).

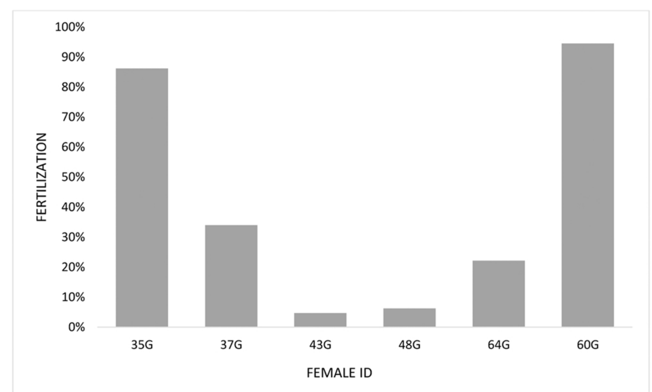

Fig. 1. Bar chart of the percentage of eggs' fertilization for each of the six females used in the present study.

3.2. Genetic variability and paternity assignment

Seven larvae were discarded for technical problems (failed amplification or genotyping). Due to its poor results both in terms of yield and variability (only four alleles detected), locus AAN04 was eliminated from the final data set. Overall mean PIC value was 0.812. Adults' PIC varied from 0.918 (locus 24A09) to 0.765 (locus 42O08), while in larvae ranged from 0.903 (locus 22B09) to 0.685 (locus 26N13) (Table 2). The combined non-exclusion probability for first parent, second parent and parent pair were respectively 8.51E-4, 1.78E-5 and 2.62E-9. The average non-exclusion probability for identity of two unrelated individuals is 2.62E-13 and the average non-exclusion probability for identity of two siblings is 6.87E-5. Mean values of observed heterozygosity in adults and larvae were respectively 0.884 and 0.860, while mean expected heterozygosity were 0.830 (adults) and 0.860 (larvae). Expected null allele frequencies were never significant and not reported in Table 2. 236 out of 251offspring were successfully assigned to the related father (94.02%). Based on paternity assignments, the males' percentage of fertilization success was determined. All the males were able to produce at least one F1 individual, even if the contribution percentages were considerably different. For example, males 21 R, 23 R and 8 V contibuted in absolute terms to F1 with a single individual each, while males 30 R, 31 R, 28 R, 4 V, 32 R were the most productive, being assigned respectively to a total 47, 38, 26, 20 and 19 larvae. In particular, the same males also hold the record of the highest number of F1 assigned for each single family: 30 R with 38 larvae in family 10 and 9 in family 9 (79.2% and 50% of the total respectively), followed by and 31 R with 26 F1 in family 3 (54.2%), 28 R with 22 sons in family 6 (50%), 4 V with 20 larvae in family 12 (44.4%) and finally 32 R with 19 larvae in family 2 (39.6%) (Table 3). These individuals were the best performing males (BPM henceforth). All other males showed contributes varying from 5 to 17 larvae. Eight males were used twice: 22 R, 28 R, 29 R, 31 R in reproductive events nr. 3 and 6, and 23 R, 26 R, 27 R, 30 R in reproductive events 9 and 10. Only male 30 R was the largest contributor in both reproduction events in which it was involved, while other males gave different yields, with no relations to the time of use (first or second time) and a reduction in the second reproduction was not observed. The chi-square values obtained and their related probability ensure that the differences observed in the contribution of each male are always statistically significant (Table 3). For males used twice, results show that their contribution is significantly different between the two reproduction events in which they took part (family 3 vs family 6: chi-square=21, P = 1.1E-04; and family 9 vs family 10: chi-square=9.356, P = 0.02).

4. Discussion

The present study aimed to rebalance the amount of F1 for each male, eliminating the behavioral component of natural mating, directly using an equal amount of males' milt to fertilize female's eggs (sex ratio 1 F:4 M). Results obtained highlight that, despite the new fertilization protocol, which virtually gave all the males the same chance to transmit their gene pool to F1, no substantial differences from natural mating were observed. Even if absolute F1 numbers may vary among families, a general pattern similar to that obtained with semi-natural mating was observed: in each reproductive event a single male contributed alone to the majority of F1, two males contributed to a lesser extent and finally a single male contributed only marginally (Table 3, Fig. 2).

The obtained results suggest that the behavioral component could not be the only reason for the biased distribution of parental alleles in F1, and other causes should be further investigated. A possible

Table 2
Main genetic variability parameters. k: number of alleles per locus; observed Allelic Range (bp); He: expected heterozygosity; Ho: observed heterozygosity; PIC: Polymorphic Information Content.

LOCUS	k	Allelic Range	PARENT			LARVAE		
			Hexp	Hobs	PIC	Hexp	Hobs	PIC
22B09	18	254–304	0.919	0.955	0.890	0.912	0.960	0.903
06E24	16	89–111	0.909	1.000	0.878	0.903	0.894	0.892
24A09	24	163–229	0.944	0.864	0.918	0.901	0.912	0.891
26N13	13	87–153	0.832	0.682	0.793	0.719	0.717	0.685
41E24	9	167–191	0.814	0.955	0.769	0.768	0.827	0.733
42O08	13	169–237	0.808	0.864	0.765	0.763	0.820	0.731
44B22	8	86–106	0.835	1.000	0.792	0.810	0.915	0.782
AAN01	11	219–243	0.819	0.818	0.775	0.803	0.899	0.779
AAN02	18	173–219	0.943	0.818	0.916	0.896	0.802	0.884
Mean	14.4		0.869	0.884	0.833	0.830	0.860	0.809

Table 3
paternity assignment in the six family analyzed. In brackets: female code. Male codes: R animals caught in the wild; V= animals from aquaculture facility. Unassigned: number of larvae for which it was not possible to determine a father. Excluded: larvae which were excluded from dataset since technical problems during amplification or genotipization. In bold: largest contributors to each single family.

FAMILY	2	3	6	9	10	12	*total*
(Female id)	(35 G)	(37 G)	(43 G)	(48 G)	(64 G)	(60 G)	
male id							
21 R	1						*1*
23 R				0	1		*1*
8 V						1	*1*
26 R				4	1		*5*
21 V						7	*7*
25 R	8						*8*
27 R				2	6		*8*
29 R		6	2				*8*
32 V						13	*13*
22 R		11	6				*17*
24 R	17						*17*
32 R	**19**						*19*
4 V						**20**	*20*
28 R		4	**22**				*26*
31 R		**26**	12				*38*
30 R				**9**	**38**		*47*
unassigned	3	1	2	3	2	4	*15*
excluded	0	0	4	0	0	3	*7*
TOTAL	*48*	*48*	*48*	*18*	*48*	48	*258*
Chi-square	*18.556*	*25.255*	*21.619*	*11.267*	*82.870*	*19.390*	
P	* *	* *	* *	* *	* *	* *	

* P ≤ 0.05 < 0.01; * *P ≤ 0.01

explanation could be sought in the intrinsic quality of semen produced by each male (e.g., sperm density, longevity, and motility; Gallego et al., 2014; Asturiano, 2020; Koumpiadis et al., 2021). It is known that these parameters influence the artificial reproduction of species whose reproduction mechanisms in nature are well-known (Withler, 1988; Kaspar et al., 2008). Nevertheless, these parameters must be tested in European eel, which life cycle and biology still remains for many aspects mysterious.

In addition, the males of the single family entirely composed by intensively farmed ones (family 12) showed the same pattern of contribution of wild males (families 2, 3, 6, 9 and 10) regardless their origin, and thus food/nutrients intake during pre-reproductive period seems not to play a key role in the reproductive event. This result should be deepened with further dedicated studies since it consists in a single observation with no replicates. If confirmed, it apparently diverges form result obtained by Locatello et al. (2018) who observed a correlation between different origins of European eel males and the related quality of ejaculates (wild males had greater sperm longevity).

Another necessary consideration must be made on the behavior of the same group of males compared in two consecutive reproductive cycles (13th and 14th hCG treatment): the results show that the allelic distribution in F1 of each male recorded in the first reproduction did not find an adequate correspondence in the second reproductive activity. For example, male 28 R was found to be best performing in breeding with female 43 G while he was the worst in breeding with female 37 G. Having used males during their maximum reproductive potential (Locatello et al., 2018), substantial variation in semen quality within a single week seems to be unlikely. The different responses obtained by each male in the two reproductions could be ascribed to technical reasons (e.g., different egg collection methods in families 6 and 9; or from a lower number of analyzed larvae from female 48 G).

The high variability of fertilization rate obtained over the 6 reproductive events (from 4.70% to 94.5%; Fig. 1) does not seem to depend on the males, but probably on the type of ovarian development of European eel typically asynchronous (Palstra et al., 2005). In fact, the currently adopted artificial spawning technique, based mainly on weekly stimulation by CPE or SPE, although optimized over the years, still leads to some heterogeneity of eggs and consequently fluctuating fertilization rates. In addition, the different egg collection method (stripping vs spontaneous emission) may also have affected the fertilization rate: the worse result was observed in the two families in which eggs were obtained by stripping, in accordance with Di Biase et al. (2016) who has demonstrated that the spontaneous spawning method produces higher quality eggs than those obtained by stripping procedure. Finally, the fertilization rate seems not to be correlated on the different level of silvering of the females used in this study (premigrants and migrants). In

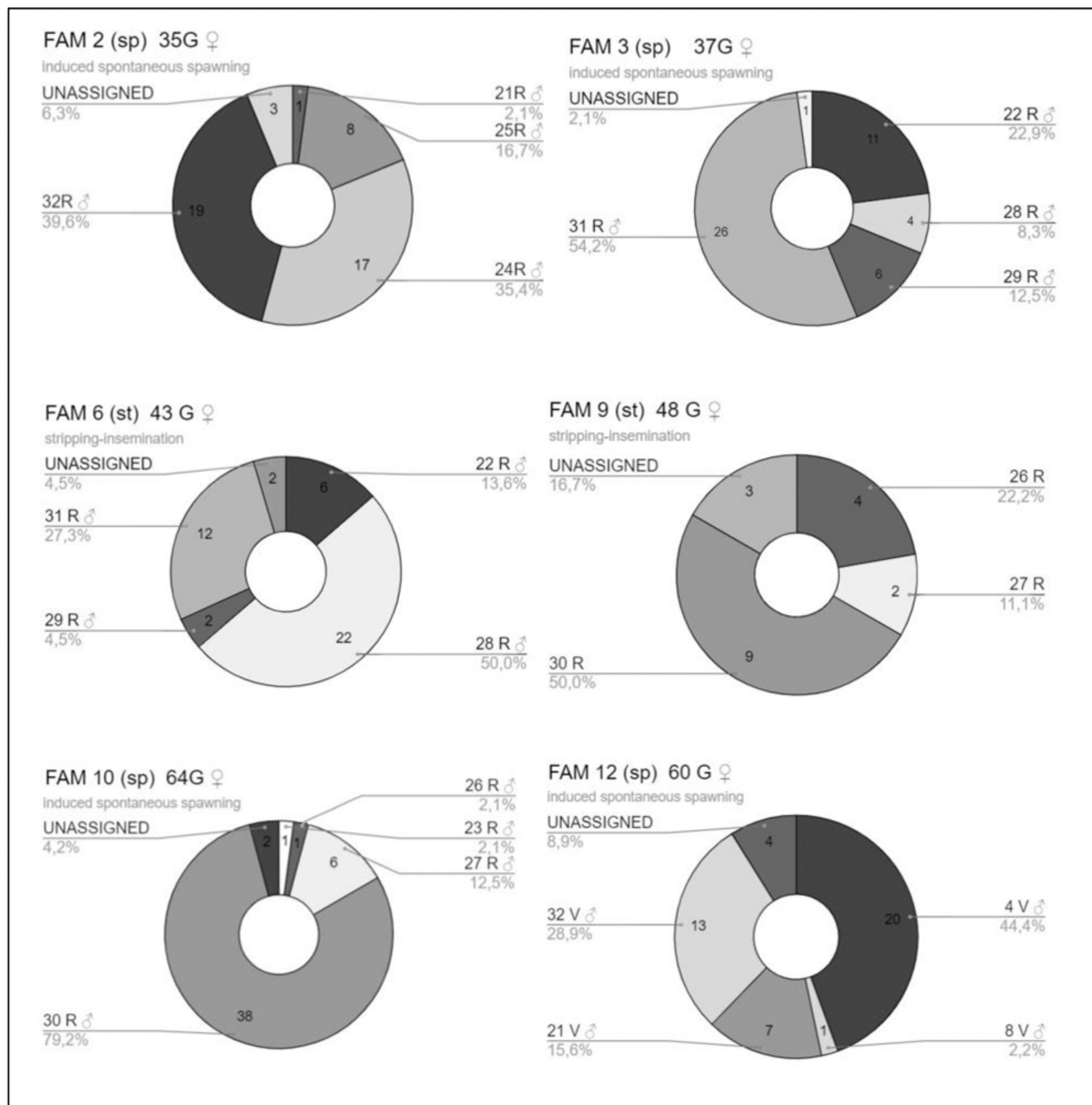

Fig. 2. Male eels' contribution to offspring in each spawning event. sp: eggs obtained by spontaneous emission, st: eggs obtained by the stripping procedure described in Materials and Methods. The absolute numbers represent the number of larvae assigned to each male, while the percentage represent its percentage of contribution to the total F1 analyzed.

this regard, Gentile et al. (2022) pointed out that in the upper Adriatic eel populations, premigrant females have an oocyte maturation level that is superimposable to migrant eels and that reproductive results are similar.

From a strictly molecular point of view, the overall mean PIC and the other parameters associated to errors in genotyping and parental assignment/kinship reconstruction, suggest that the set of microsatellite loci here used is a good compromise in terms of cost-effectiveness, contributing to identify with high confidence true parents in the 94.02% of offspring.

The presence of observed heterozygosity higher than expected in seven out of nine loci used, might suggest that also in *A. anguilla* the forced admixture of males' milt may effectively contribute to the single-locus genetic variability, even if it is not sufficient to ensure all the males the same chance to transmit their gene pool to F1, and parameters like sperm quality must be taken into consideration to optimize future fertilization protocols.

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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.

Data Availability

Data will be made available on request.

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