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Evaluation of the Reproductive Performance of Females of *Anguilla anguilla* Characterized by Different Levels of Silvering

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Abstract: The European eel, *Anguilla anguilla* (Linnaeus, 1758), is a species of high conservation and commercial value. Also, with a high aquacultural value, it was one of the most farmed species in Europe before its decline. Conservation measures for this species are currently being implemented, some of which are the release of adults to allow them to migrate to spawning grounds and artificial reproduction to minimise the impact of overfishing. Much progress has been made regarding the closing of the life cycle, but several problems remain to be solved, such as the weaning and growth of larvae. In this regard, the study of local populations and the identification of the best spawners could be a good way forward as it would help to identify spawners with high reproductive potential and good offspring quality. In this study, we compared the reproductive performance of female eels from migratory areas of the Northern Adriatic Sea at different maturation stages (10 pre-migrant stage; 10 migrant stage), treating both groups with the same hormonal protocol (weekly carp pituitary extract, injection of 17α,20β-Dihydroxy-4-pregnen-3-one at complete oocyte maturation). The research showed that eels at the beginning of metamorphosis (SI III), i.e., eels not yet ready to undertake migration, achieve reproductive performance equal to that of eels ready for migration (SI IV). Their performance was on par in both qualitative and quantitative terms. The optimal results in hatching (with values of 65.8 ± 3.2% for pre-migrant and 68.2 ± 4.1% for migrant) and survival rates (with value of 25.7 ± 5.4% pre-migrants; 27.2 ± 3.7% migrants), as well as the results about the time to reach full gonadal maturation, may have positive implications when considering release and restocking measures.

Keywords: European eel; artificial reproduction; egg production; egg quality; aquaculture

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1. Introduction

The aquaculture sector is increasingly recognised as a key contributor to global food security and nutrition in the 21st century [1]. One of the most important features of aquaculture is to help reduce pressure on natural marine fish resources. In this respect, to ensure the reliable production of offspring to support commercial farming, closing the life cycle of captive fish is the basis for the development of successful aquaculture [2]. The European Eel, *Anguilla anguilla* (Linnaeus, 1758), is a species with high social, cultural, and economic value and also has great aquaculture potential. However, wild juveniles, known as 'glass eels', caught in targeted fisheries are the sole source of eel aquaculture. In the last five decades, the European eel has gone from being a significant resource for fisheries in Europe and North Africa to being threatened with extinction. The species has been subject to extensive scientific inquiry due to its enigmatic natural ecology and social and economic importance.

The stock has declined severely in most of its distribution areas and the population is at a historical minimum and classified as "critically endangered" [3,4]. In view of the

precarious state of the natural population, the European Commission recognised the urgent need to establish an action plan for the recovery of *A. anguilla*. Among the different actions there are the following: EC Reg. No. 1100/2007, which establishes measures for the recovery of the European eel stock and the inclusion in Annex B of EC Reg. 338/1997, which establishes the CITES certificate requirement for marketing. Subsequently, the adoption of EU Regs. Nos. 2019/124 and 2023/194 prohibit any fishing activity during all stages of the life cycle of eels for a period of three months and six consecutive months, respectively, based on what each Member State determines. Additional protection measures relate to Directive 92/43/EEC and Directive 2000/60/EC, which aim, inter alia, to protect, conserve, and improve the aquatic environment in which eels spend part of their life cycle.

A. anguilla follows the same complex catadromous life cycle as other eels [5–7], during which it undergoes morphological and physiological changes in preparation for long migration and reproduction [8,9]. During its life phase in inland waters, it undergoes a process known as silvering, a metamorphosis that initiates puberty and sets in motion the physiological and morphological changes necessary for reproductive migration at sea. Currently, sexually mature eels cannot be obtained in a controlled environment without hormonal treatment. For female eels, this means weekly repeated injection of carp (CPE) or salmon (SPE) [10] pituitary extract and a final injection of 17,20b-dihydroxy-4-pregnen-3-one (DHP) [11–13]; the routine for males is a protocol of weekly human chorionic gonadotropin (hCG) injections [14]. New protocols have been developed, and there is a continuous effort to optimize the induction of maturity and artificial reproduction [15–19].

Fish recruitment may be significantly affected by egg quality, as reproductive success depends on the quantity and quality of eggs produced at spawning. In recent years, efforts have been made to develop reliable parameters to identify good quality eggs. According to Kjørsvik et al. [20], egg quality is defined as the ability of eggs to produce viable fry, while Bromage et al. [21] defines good quality eggs for aquaculture as those with low mortality during fertilisation, hatch, and first feed.

Other than the morphometric parameters, the reproductive performance (quantity and quality of the eggs/spermatozoa) is an important marker often used to determine the quality of the protocols used in artificial reproduction and aquaculture [16,17,22,23] and to evaluate the quality of broodstock for the restocking efforts of the natural populations [14,24,25]. In the case of female eels, the quantity aspect of the reproductive performance is usually determined by the relative fecundity (total egg per female/body weight, expressed in %), and absolute fecundity (total egg production per female) [16,26] while the quality aspect often requires further physiological tests to determine the differential effect of the specific diet or hormone treatment [15–19,26,27]. A quality indicator for Japanese eel (*A. japonica*) has been approximated by the buoyancy rate expressed as a percentage, as it has been shown to highlight differences in biochemical content [26]. Egg buoyancy was utilised to identify high-quality pelagic eggs, as poor-quality eggs usually sank in the water column [20,28–30]. A positive correlation was observed between buoyant eggs and hatching rates.

Alternatively, the liver weight of the animal can be used as a highly correlated measure of gonadal development and vitellogenin levels [31].

Before commencing reproduction, the selection of spawners is a crucial parameter. Due to the challenge of determining the eel's maturity, different indices have been created that, based on external morphometric measurements [8,32], provide insight into the level of maturity.

The impact of the maturation stage on reproductive performance has been examined in the Japanese eel [33]. Both this investigation and the research of Durif et al. [31] suggest that there is a favourable association between the maturation stage and the individual's reproductive performance.

The maturity level of European eels is impacted by various environmental factors within their habitat. Additionally, specific trophic conditions in particular areas can result in distinctions in population structure. Molecular evidence has revealed the existence of small yet significant population structures [34–39]. A study with greater geographic sampling

similarly revealed divergences among various sampling locations, these potentially being linked to temporal fluctuations in allele frequency [36]. The process of silvering is also more flexible than generally assumed [37] and can be influenced by various trophic and environmental factors [38,39].

Significantly, the reproductive success of individuals can also vary according to location [40], a phenomenon likely influenced by the silvering process. The sampling for this study took place in typical areas of the North Adriatic. These *valli* comprise lagoon areas that are entirely dammed. They are vast wetlands of ecological importance, safeguarded by the National Natura 2000 Directive. Particularly rich in biodiversity, these areas are characterised by a very abundant trophic component. The major economic activity is fish farming, also known as *Vallicoltura*. This ancient system of fish breeding exploits the mass migrations of certain species of fish, such as mullet and eel, as they leave the inland basins.

Considering the social, economic, and ecological importance of eel, measures must be taken to monitor and restock the remaining populations. Whatever action is taken, it is necessary to measure current conditions, and this can only be achieved through a better understanding of the biological mechanisms that control population dynamics, e.g., the metamorphosis from the resident to the migratory phase. Continuous methodological development and the monitoring of inland waters for potential broodstock to be included is required to obtain an effective program capable of impacting the conservation of the stock. The objective of this study is therefore to evaluate the reproductive potential of female eels (*A. anguilla*), characterized by different stages of silvering, for potential inclusion in artificial reproduction and/or restocking programs.

2. Materials and Methods

2.1. Animals

Wild eels were caught using traditional "lavoriero" (downstream trap) during the October–December downstream migration period in *Valle Bertuzzi* (44°48′12″ N 12°15′16″ E), a typically closed lagoon (named "Valle") near the sluices of the North Adriatic Sea (Italy) (Figure 1). *Valle Bertuzzi* is a brackish *valle* with an extension of about 1000 ha, is one of the best-preserved lagoons in Emilia-Romagna from an ecological-environmental point of view, and is used for extensive aquaculture. A number of 250 female eels were randomly selected and anaesthetised with a bath of clove oil (0.2 mL/L), measured, and sampled to obtain an external indicator of their maturation status (Silver index, SI) and their level of stimulation of migration (resident, pre-migrant, migrant) [28,34]. Morphometric parameters were measured for each individual: body length (BL, cm), body weight (BW, g), horizontal eye diameter (EDh, mm), vertical eye diameter (EDv, mm), and pectoral fin length (PFL, mm). The following indices were calculated according to the formulae below:

$$\text{Condition factor (K)} = (\text{BW} \times \text{BL}^{-3}) \times 103$$

$$\text{Eye index (EI)} = 100 \times (((\text{EDh} + \text{EDv}) \times 0.25)2\,\pi \times (10 \times \text{BL})^{-1})$$

$$\text{Pectoral fin leght index (PFI)} = 100 \times \text{PFL BL}^{-1}$$

All the eels were kept in unfiltered natural seawater in two 700 L tanks (one with females and one with males) connected to a recirculation system and maintained in indoor conditions for the duration of the experiment. During the trial, total ammonia nitrogen (TAN) was maintained at 0.06 ± 0.2 mg/L, NO_2–N remained at 0.07 ± 0.03 mg/L, NO_3–N remained at 1.5 ± 0.5 mg/L, and pH at 8.2 ± 0.15. The animals were marked individually by inserting fish-tags (Mod Floy T-Bar Anchor- Floy Tag & Mfg., Inc., Washington, DC, USA) and maintained in starvation for the duration of the experiment.

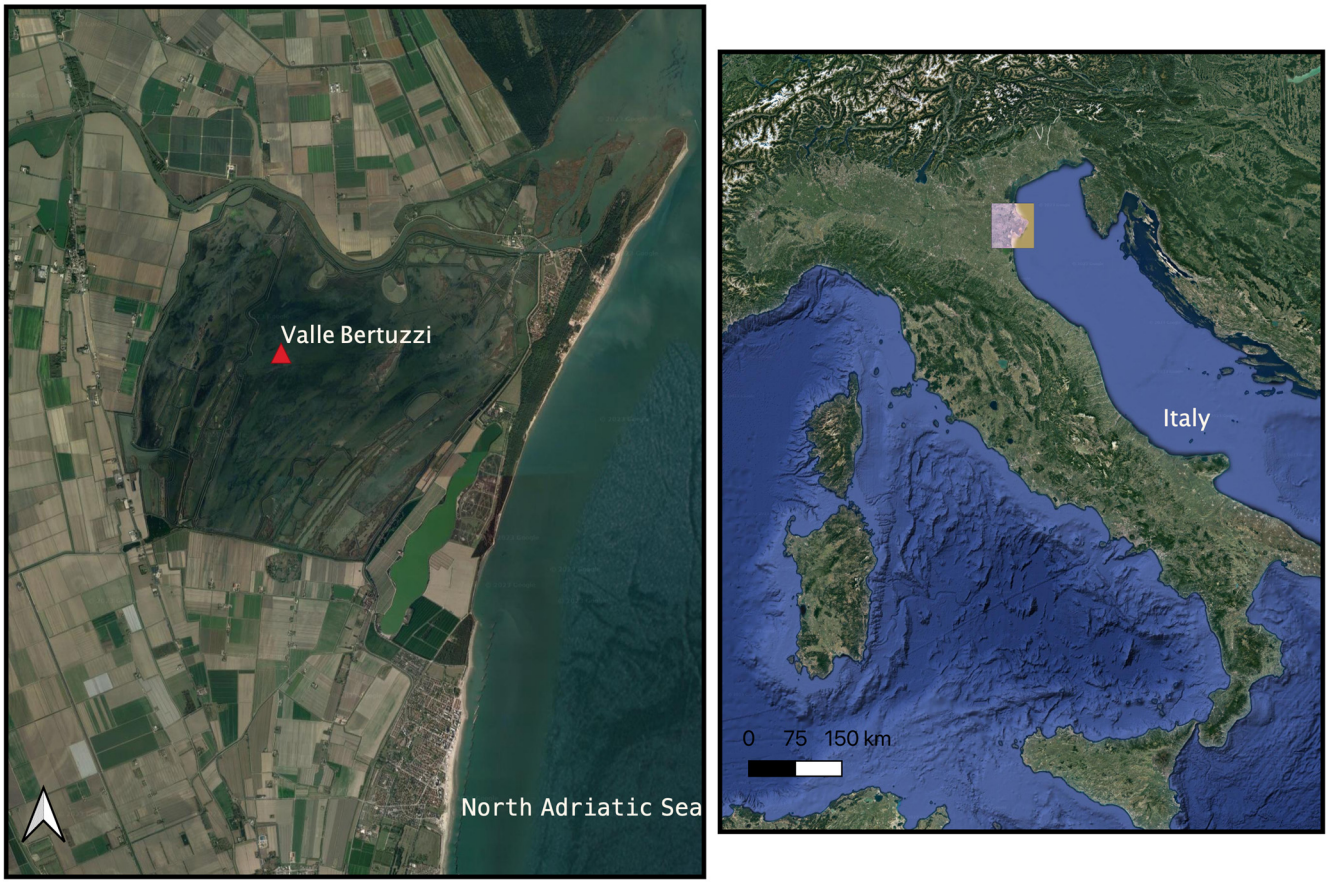

Figure 1. Map of the sampling area in a closed lagoon of the Northern Adriatic Sea. The highlighted square indicates the geographic position of *valli*; the triangle shows a zoom on the position of *Valle Bertuzzi*.

2.2. Age and Gonadosomatic Index

To understand the initial stage of gonadal maturation, 10% of female eels investigated (pre-migrants, and migrants, respectively), intended for human consumption, were transported to the laboratories of the department of Veterinary Medical Sciences, University of Bologna, DIMEVET-Cesenatico (Italy). These eels were sacrificed with an overdose of 2-phenoxyethanol (800 mg/L) and, after dissection, the gonads were removed. The gonads were weighed (GW, g) and the gonadosomatic index (GSI) was calculated as follows:

$$\text{Gonadosomatic index (GSI)} = (\text{GW BW} - 1) \times 100.$$

The same animals used for IGS were used for otolith extraction. The age was determined using the otolithometric technique, based on the reading of the sagitta, the largest of the three otoliths of the inner ear of fish species. The protocol for preparing the reading of the annuli was based on the indications of Panfili et al. [41].

2.3. Induction of Maturation

Carp pituitary extracts (CPE) were injected intramuscularly for 20 female eels (10 III, 10 V) weekly, as developed by Mordenti et al. [14,25], while males were induced using weekly injections of 1 IU/g BW hCG [42,43]. Just before fertilization experiments, males received a booster hCG injection (1 IU/g BW) to induce sperm maturation [14] simultaneously with DHP injection of females with high and homogeneous oocyte maturity. The DHP injection timing was optimized for every individual female. To define the correct timing, females were repeatedly anaesthetised in 400 mg/L 2-phenoxyethanol and ovary-biopsied (~0.3 mL, equating to ca. 500 follicles) every 8 h, using a needle and syringe, after

their BW exceeded 110% of the initial body weight, and gravidic appearance indicated that oocyte hydration had probably started. The maturation status of the oocyte was assessed under a stereomicroscope and considered homogeneous and mature when at least 50% of the oocyte where transparent with the nucleus and a few large lipid droplets were visible [26,42,44]. After DHP injection, each female was placed in a closed RAS [45] in 20 ± 0.5 °C seawater [46] with spermiating males, to facilitate spontaneous spawning [47].

2.4. Reproductive Performance Comparison

To compare the reproductive performance of pre-migrant and migrant stage eels, we registered what was necessary to achieve (or not, after 22 weeks of treatment) gonadal maturation and reproduction. For the measurement of relative fecundity, the weight of spawned eggs (%BW) was calculated from the difference of the body weight after spawning and that at the time of the DHP injection divided by the body weight at DHP injection and multiplied by 100 to obtain percentile [26]. The absolute fecundity (numerical estimation of the spawned eggs/female) was calculated considering 1680 eggs/g of ovarian biomass [25]. The quality indicator (buoyancy percentile) was obtained after maintaining egg samples for 30 min in a 500 mL beaker, after which the buoyancy percentile was determined by the number of buoyant eggs divided by the number of total eggs put in the beaker multiplied by 100 [15].

The eggs/embryos were transferred into six 18-litre incubation chambers, two hours post-fertilisation. Each chamber had a cylindrical base and a top tube and was equipped with inlet jets generating a rotating current. There was an outlet screen, measuring 300 µm in diameter, which was replaceable, located on the partition panel between the incuba-tion and outlet chambers through which the inlet jets circulated water. The water flow and current speed within the incubation chamber were adjusted using the hatchery water pipe valve. Each chamber was filled with water taken directly from the North Adriatic Sea and then filtered and treated with UV. If the salinity was lower than under experimental conditions, it was adjusted with sea salt. In general, the experimental conditions were temperature of the water 18–19 °C and salinity 35 PSU. The dissolved oxygen (DO) level during the trial was 8.2 ± 0.1 mg O_2 /L. The water pH was 8 ± 0.15. The TAN in water were 0.05 ± 0.3 mg/L, NO_2–N remained at 0.06 ± 0.02 mg/L, NO_3–N remained at 1.4 ± 0.5 mg/L.

The fertilisation rate (%) was calculated by counting the number of embryos in the 8-cell or 16-cell states divided by the total number of eggs (fertilization success was also considered on the sunken eggs) [15].

From 52 to 60 h of incubation, the hatching rate was calculated by sampling the number of embryos and larvae in 1 L of incubation water according to equation:

$$\text{hatching rate (\%)} = (\text{number of hatched larvae}/250) \times 100$$

where 250 is the number of eggs/L incubated according to the protocol of Di Biase et al. [15].

Survival rates during the starvation phase were assessed at 5 dph (beginning of mouth opening) and 12 dph (end of yolk sac uptake) by taking a 1 L sample of incubation water and calculating the percentage of viable larvae compared with the number of non-viable larvae in the same 1 L sample.

2.5. Statistical Analysis

Morphometric characteristics of the eels were statistically analysed. The normality was checked using a Shapiro–Wilk test. As the data were not normally distributed, non-parametric statistics were used. Statistics were performed using a non-parametric analysis of variance (Kruskall–Wallis test) to compare among silvering stages, followed by Mann–Whitney U. The significance level was set at $p = 0.05$.

Permutational multivariate analysis of variance (PERMANOVA) test, based on the Bray–Curtis and Sørensen–Dice dissimilarities, was used to compare the reproductive performance, using $p > 0.05$ confidence interval in PAST software (Mac, version 4.11).

3. Results

3.1. Measurements

The morphometric characteristics of the eels present in Valle Bertuzzi in December are shown in Table 1. This stock was characterized by a very strong presence of pre-migrant eels (73.6% in stage III) and a moderate presence of resident eels (5.6% in stage II), while only the category with the highest level of silvering (20.8% in stage V) was present in the migrant eel group (Table 1). There is a significant increase in both body weight and total length from stage II to stage III, and there are no differences from stage III to stage V. Fulton's Condition Factor was similar for pre-migrant and migrant eels. While the eye index and pectoral fin leght rising as the Silver Index rises (Table 1).

Table 1. Morphometric parameters investigated in *Valle Bertuzzi.* Data are given as the mean ± SD. Letters represent statistical differences, for $p < 0.05$.

Silver Index			II	III	IV	V
Eels (n. 250)		n.	14	184	-	52
		%	5.6	73.6	-	20.8
Body Weight (BW)		g	431.16 ± 120.31 ^{b}	552.34 ± 67.05 ^{a}	-	555.44 ± 110.73 ^{a}
Body Lenght (BL)		mm	622.44 ± 58.36 ^{b}	674.48 ± 56.82 ^{a}	-	671.74 ± 45.25 ^{a}
Pectoral Fin Length(PFL)		mm	28.79 ± 3.4 ^{b}	32.81 ± 2.60 ^{a}	-	35.47 ± 2.12 ^{a}
Fulton's Condition Factor			1.73 ± 0.21 ^{b}	1.81 ± 0.17 ^{a}	-	1.83 ± 0.28 ^{a}
Eye Index (EI)			3.82 ± 0.81 ^{c}	6.26 ± 1.02 ^{b}	-	9.28 ± 1.86 ^{a}
Pectoral Fin Lenght Index(PFI)			4.64 ± 0.24 ^{c}	4.87 ± 0.33 ^{b}	-	5.29 ± 0.39 ^{a}
Gonadosomaic Index (GSI)			0.64 ± 0.31 ^{c}	1.65 ± 0.21 ^{b}	-	1.98 ± 0.14 ^{a}
Age	<6	-	-	-	-	-
	6–6+	5.3%	20	5.7	-	-
	7–7+	62.1%	80	64.1		50
	8–8+	20.8%	-	18.9	-	33.3
	9–9+	10.4%	-	9.4		16.7
	10–10+	1.4%	-	1.9		-
	>10+	-	-	-		-

3.2. Age and Gonadosomatic Index

The most representative age group was 7–7+ with almost two-thirds of the population. At the same time, the 8–8+ age group was the most representative in-migrant eels. The increase in silvering stages corresponds to an increase in the gonadosomatic index.

3.3. Reproductive Performance

All females responded to the hormone treatment, with weight gain exceeding 120% in completely comparable times (Table 2). Comparing pre-migratnt and migrant females show no statistically significant differences ($p > 0.05$) in weight gain and number of weeks required to reach maximum gonadal development (Table 2).

Table 2. Zootechnical performance from artificial reproduction trials. Week (n°): means the week of hormonal treatment; Ovulation n°/total: the number of eels that reached ovulation in relation to the total number testedare given as the mean ± SD.

	Initial Weight (g)	Final Weight (g)	Weight Gain (%)	Ovulation n°/Total	Week (n°)
PRE-MIGRANT III	618.62 ± 62.05	762.72 ± 79.65	123.39 ± 6.92	10/10	20.2 ± 2
MIGRANT V	559.94 ± 112.4	704.72 ± 95.05	126.51 ± 7.65	8/10	20.4 ± 4

No statistically significant differences ($p > 0.05$) were found in the quantity of eggs emitted (347.9 ± 71.9 g pre-migrant; 352.53 ± 18.9 g migrant), floating rate and fertilisation rate (Figure 2).

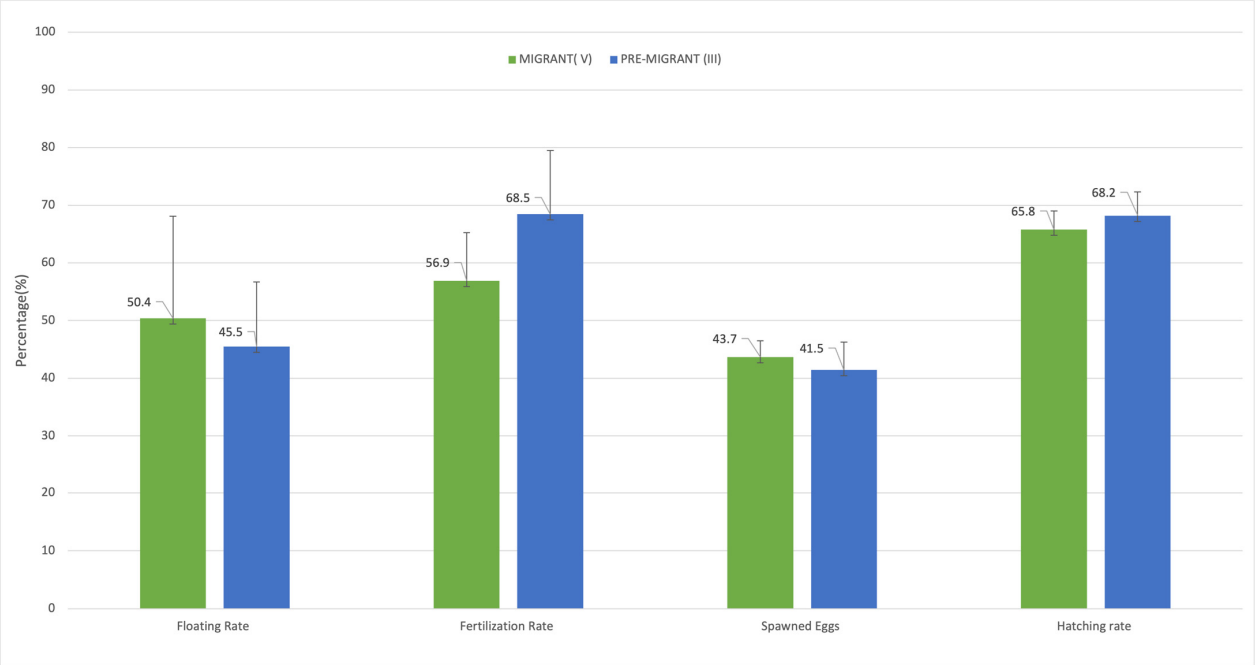

Figure 2. This graph shows all qualitative characteristics expressed in percentages of the two groups (migrant, pre-migrant). Data are given as the mean ± SD.

No statistically significant differences were found in the hatching rate between pre-migrating and migrating eels, with values of 65.8 ± 3.2% and 68.2 ± 4.1%, respectively.

The results of survival rates at 5 days then still in the endogenous feeding stage were similar in the two groups with rates of 46.8 ± 4.1% for eel larvae born from pre-migrants and 48.1 ± 5.6% for larvae born from migrant eels. At 12 days, the survival rate decreased by almost half in both groups but remained similar with no statistically significant differences in the two groups (25.7 ± 5.4% pre-migrants; 27.2 ± 3.7% migrants).

4. Discussion

To date, two approaches are used for the recovery of endangered species such as eel. The main one involves the search for candidates in the local stock, usually individuals at the advanced stage of silvering (SI–IV/V), to release the best quality spawners for migration to the Sargasso Sea [48]; this is one of the actions also present within the LIFEEL project. The second, a tool used for restocking several endangered fish species such as sturgeon, is artificial spawning with the aim of producing juveniles for release into the wild; this action is present within European projects for sturgeon (LIFE11 NAT/IT/188, Be Natur), but also for eel as the LIFEEL(LIFE19 NAT/IT/000851). Based on the results obtained in the weaning of Japanese eel larvae and the first positive results on European eel, this method is gaining ground [49–52]. There are several protocols that often start with breeding animals whose specific silvering status has not been taken into account. It is clear that the wrong choice of female can lead to unsatisfactory reproductive results. However, the method is not fully standardized.

In artificial reproduction techniques, the initial stage of maturation before hormonal induction is important, as several studies have examined the impact of the eel's stage of maturation on reproductive performance [53]. These studies on Japanese eels [33] and European eels [31] report that the quantity and quality of eggs obtained are highly dependent on the initial maturation stage. Okamura et al. [33] tested eels at different stages of maturation and showed that under-mature eels did not respond to hormone treatment.

In this study, the reproductive performance of eels at the beginning of the metamorphosis (SI III) was not statistically different from the eels at the migrating stage (SI V). The positive response of the stage III eels to the hormonal-induced stimulus can be explained

by the overlap of IGS of the pre-migrant eels with that observed by Durif et al. [32] in Atlantic migrating eels (IV). Apparently, the pre-migrant eels of the North Adriatic Sea, if subjected to an artificial reproduction programme, reach sufficient gonadal maturation to give a hormonal response. This is confirmed by the study of Gentile et al. [54], which investigated gonadal maturation at the histological level of different silvering eels from the North Adriatic lagoon area, showing that pre-migrant eels, SI III eels, had overlapping oocyte maturation confronted with eels in later stages of maturation. In addition, this early gametogenic activity was found to be associated with accelerated weight growth in the pre-migrating eels studied, a phenomenon normally seen in Atlantic migrant stage IV eels.

The general picture of the females from Valle Bertuzzi is certainly very peculiar, since the total absence of migratory stage IV eels is compensated for by a high concentration of pre-migratory eels, which represent almost 75% of the total population; a plausible explanation could be that the pre-migratory females from this *valle*, despite having external morphometric values typical of eels at the beginning of metamorphosis, actually seem to have all the characteristics to be considered migratory eels. Identifying the levels of silvering in these environments using the method of Durif et al. [32] would give an ambiguous picture of the state of the local stock and is therefore not sufficient. Possibly, the longer distance to travel during migration, compared with Atlantic eels, on which the silvering index is based, combined with the shallow waters throughout the North Adriatic, may have delayed certain morphological changes such as the dilation of the eyes and the elongation of the pectoral fin. Thus, North Adriatic eels, although morphologically not classified as migrants, are physiologically ready for migration. This consideration is also supported by the analysis of otolith sections, which showed that pre-migratory and migratory eels fall within a narrow two-year interval. Finally, the same response time of SIII and SV females to hormone treatment suggests that the eels in Valle Bertuzzi are sufficiently mature to begin migration. Consequently, Durif's morphometric-based silvering classification, although useful, seems to be insufficiently standardised for stocks in the North Adriatic, providing an unclear picture of the local stock status. This could lead to errors in the approaches used at a regional and national level for the recovery of the European eel stock through adult rearing and/or spawning.

Looking for each characteristic, both groups pre-migrant and migrant showed similar absolute fecundity probably due to the same initial weight, but more importantly, they achieved relative fecundity that exceeded 40% in both groups, favourable and higher safe levels than those obtained by Mordenti et al. [14], who had used broodstock from the same Adriatic area. This condition is probably due to the good trophic conditions in Valle Bertuzzi, which provide a diet with a good lipid and fatty acid composition, which has been identified as an important dietary factor in determining successful reproduction and offspring survival [55]. Furthermore, De Leo and Gatto [56] have shown that the North Adriatic lagoons, characterised by an incredibly large and abundant spectrum of prey (mainly clupeids and engraulids), allow eels to grow well not only for growth but also for gonadal development.

When quantitatively analyzing the results obtained in the artificial reproduction, again there is no difference between the groups. If we compare the results obtained in a study by Di Biase et al. [15], using eels from the same sampling area and applying the same hormone induction protocol, it is evident that the data on floating rate and spawning eggs are comparable between the pre-migrant eels used in this study and the migrant eels used in the Di Biase study.

In terms of egg quality, even in this case considering the hatching values of the two groups, there were no differences. The high hatching values are in line with those obtained by Di Biase et al. [15], always using migrant eels from the North Adriatic. The survival rates were also similar and comparable in quantitative terms with other studies like Politis et al. [57] who, although using different protocols, obtained similar results to those obtained in this study. This result shows that, even in terms of yolk sac energy reserve, there are no differences between larvae from migrating and pre-migrating eels.

Finally, the weight gain recorded during hormonal induction and the timing of reaching full gonadal maturation were superimposable between pre-migrating and migrating eels. The results can be explained by the induction protocol used, in which the low hormone doses applied in the first weeks promote the synchronization of oocyte maturation and similar characteristics of individuals from the same area [14,36,37].

In conclusion, eels with silvering index III, considered not yet ready for migration, had the same reproductive performance as eels with silvering index V when subjected to induced reproduction and, although Valle Bertuzzi is characterised by a high density of stage III eels, seem to have all the characteristics to be able to participate in conservation actions (release/artificial reproduction) of the species.

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Conflicts of Interest: The authors declare no conflict of interest.

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