Sperm production and quality in European eel (*Anguilla anguilla)* in relation to hormonal treatment

Short title: European eel sperm production and quality

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Abstract

Aquaculture production relies on controlled management of gametogenesis, especially in species where assisted reproduction is needed for obtaining gametes in captivity. The present study used human chorionic gonadotropin (hCG) treatments to induce and sustain spermatogenesis in European eel (*Anguilla anguilla*). The aim was to evaluate effects of strip-spawning timing (12 *vs.* 24 h) after weekly administration of hCG and the necessity of a primer dose (in addition to weekly hormonal treatment) prior to strip-spawning (primer *vs.*

no-primer) on sperm quality parameters. Sperm parameters included milt production (weight), density, and sperm kinematics at Week 9, 11, and 13 after onset of treatment. Spermiation commenced in 11.5% of males in Week 5 and by Week 9, all males produced milt. Male weight, milt production, sperm density, and spermatocrit did not differ among hormonal treatments during the experimental period. Overall, male weight decreased from 106.3 to 93.0 g, milt weight increased from 3.5 to 5.4 g, sperm density counts decreased from 11.7×10^9 to 10.5×10^9 cells/mL, and spermatocrit decreased from 46.5 to 40.5%. Furthermore, spermatocrit was positively related to hemocytometer counts ($R^2 = 0.86$, $P < 0.001$), providing a reliable indicator of sperm density. Differences in sperm kinematics were observed depending on strip-spawning timing after hormonal injection (12 vs. 24 h) but with no consistent pattern. These sperm quality parameters also did not consistently differ between the no-primer and primer treatments. Considering that each male may be stripped 4-5 times over the 2-3 months spawning season, omitting the primer would reduce animal handling, material costs, and labour intensity, while sustaining high quality sperm production.

Key words: *Anguilla anguilla*, CASA, hemocytometer, sperm density, sperm motility, spermatocrit

1. Introduction

Aquaculture is the fastest growing food production sector in the world with global fish production reaching 82 million tonnes in 2018 (FAO, 2020). The growth of the aquaculture sector relies on species, which life cycle has been closed in captivity (Olesen et al., 2003). This involves selection and management of broodstock for efficient hatchery production of high-quality gametes and viable offspring to supply the industry with juveniles for on-growing

(Mylonas et al., 2010). While egg quality dominates offspring quality, an increasing number of studies over the last two decades have shown the importance of sperm quality for fertility, embryonic survival, hatch success, and early larval growth and development (Butts & Litvak, 2007; Bobe & Labbé, 2010; Gallego & Asturiano, 2019).

A number of traits have been used to assess fish gamete production and quality. In practice one of the most frequently used biomarkers is sperm density (Fauvel et al., 2010). Quantification of sperm density is conducted by estimating the number of sperm per milt volume. Here common methods include hemocytometer counting, flow cytometry, spectrophotometry, and spermatocrit measurements (Sørensen et al., 2013). These methods all have advantages and disadvantages. In brief, hemocytometer counting provides high precision and simple equipment, but is a time-consuming method and depends on skilled personnel. Flow cytometry provides precise and accurate results, but requires expensive equipment as well as experienced personnel. On the other hand, spermatocrit and spectrophotometry measurements are fast, require low level training and relatively cheap equipment (Mylonas et al., 2017). Sperm motility and velocity parameters are also widely used quality biomarkers (Gallego & Asturiano, 2019). Here, the development of computer-assisted sperm analysis (CASA) enables objective, rapid, and accurate assessment of various parameters such as total motility (MOT), progressive motility (pMOT), curvilinear velocity (VCL), straight-line velocity (VSL), and average path velocity (VAP), which have been linked to fertilization and hatch success in different fish species (Mylonas et al., 2017; Gallego & Asturiano, 2018, 2019).

The assessment of sperm production and quality is particularly important when developing assisted reproductive techniques and technologies for species that do not spawn naturally in captivity (Mylonas et al., 2017; Tomkiewicz et al., 2011). *Anguillids* (eels) are among these species, due to their complex hormonal control mechanisms inhibiting sexual maturation in continental habitats (Dufour et al., 2003; Vidal et al., 2004). While this mechanism is likely

naturally released when eels approach their oceanic spawning areas (Tesch, 2003), hormonal treatment is required to induce and sustain gametogenesis in captivity. The first successful induction of spermatogenesis in eel (Fontaine, 1936) was based on human chorionic gonadotropin (hCG). Since then, an array of hormonal treatment protocols have been developed and applied, particularly for Japanese eel, *Anguilla japonica* (Ishida & Ishii 1970; Yamamoto & Yamauchi, 1974; Ohta et al., 1997). Although a single dose can lead to spermiation (Miura et al., 2002), common protocols use weekly injections of hCG, allowing for continuous sperm production to match the variability in the timing of egg production of female eels, leading to a spawning season that may span over 2-3 months (Pérez et al., 2000; Tomkiewicz et al., 2011). Moreover, an additional hCG injection is generally applied prior to strip spawning, which is referred to as a "primer" or "booster" to ensure availability of high-quality sperm, when needed between weekly injections (Ohta et al., 1997).

European eel, *A. anguilla*, is a high value species in aquaculture (Nielsen & Prouzet, 2008). However, supply of juveniles for eel farming has remained capture-based, and development of hatchery technology is required to complete the life cycle and enable a self-sustained aquaculture production. State-of-the-art assisted reproduction protocols often lead to successful larval production, however, variability in fertilization and embryonic developmental success still challenge hatch rates (Asturiano 2020). For this species, weekly injection of hCG at a standard dose leads to initiation of spermiation after 4-5 weeks, reaching milt production volumes suitable for *in vitro* fertilization from week 9 (Pérez et al., 2000; Butts et al., 2020). Efforts to enhance hormonal treatment focused on hormone dose (Asturiano et al., 2005) and application of a priming dose before strip spawning (Palstra et al., 2005), strip-spawning timing post hormonal treatment (Pérez et al., 2000), and production of recombinant hormones (Gallego et al., 2012). Over time, reproduction protocols have evolved and production of viable offspring has become feasible (Mordenti et al., 2019; Tomkiewicz et al., 2019). Nevertheless,

these commonly applied assisted reproduction treatment protocols need to be revisited to explore opportunities to reduce animal handling as well as labor and hormone cost. This is expected to establish more cost-efficient production of offspring.

In this context, the objective of this study was to assess milt production and sperm quality applying different assisted reproductive protocols, considering resource requirements. The experiment focused on i) the necessity of a primer injection prior to strip-spawning (primer *vs.* no-primer), and ii) the effect of strip-spawning timing post hormone injection (12 *vs.* 24 h). Sperm quality parameters included sperm density, assessed by a hemocytometer and spermatocrit, as well as sperm motility (MOT, pMOT) and velocity parameters (VCL, VSL, VAP), determined using CASA, at three time points post onset of hormonal treatment (Weeks 9, 11, and 13).

2. Material and methods

2.1 Ethics

All fish were handled in accordance with the directives of the European Union on the protection of animals used for scientific purposes (Dir 2010/63/EU). Experimental protocols were approved by the Animal Experiments Inspectorate (AEI), Danish Ministry of Food, Agriculture and Fisheries (permit-Nr.: 2015-15-0201-00696). Efforts were made to minimize animal handling and stress. All fish were anesthetized using benzocaine (saturated solution of ethyl p-aminobenzoate, Sigma-Aldrich, Denmark) at a concentration of 5 mL/L prior to initial pit-tagging and morphometric measurement.

2.2 Broodstock collection and husbandry

Male broodstock were obtained from Lyksvad Fish Farm K/S (Vamdrup, Denmark), where fish were reared from the glass eel stage on a commercial diet (DAN-EX 2848, BioMar A/S, Brande, Denmark) at ~20 °C, 0.5 PSU, and under constant illumination. Fifty-two fish, farmed for three years, were selected for the experiment (length = 40 ± 0.77 cm; weight = 106 ± 2.36 g) and transferred to a research facility of the Technical University of Denmark (EEL-HATCH, Hirtshals, Denmark). Here, the males were evenly distributed into three of four 450 L tanks connected to a separate recirculating aquaculture system (RAS). The fourth tank was used for rotation in relation to treatments (see below).

The male broodstock were acclimated over a two-week period prior to hormonal induction of gametogenesis. Salinity was stepwise increased from ~10 to ~36 PSU using seawater from the North Sea and sea-salt (Aquaforest, Brzesko, Poland). Light regime was adjusted from constant light to a 12 h light / 12 h dark photoperiod at low intensity of 0.02 μmol $m^{-2}s^{-1}$. Water temperature was kept at ~20°C. All animals fasted during experimentation, as migrating silver eels cease feeding (Tesch, 2003). All fish were tagged with a passive integrated transponder tag (Dorset, The Netherlands) in the dorsal muscle and received weekly intramuscular injections of hCG (Sigma-Aldrich, Denmark) at 1.5 IU/g initial body weight (IBW) to induce spermatogenesis.

2.3 Experimental setup

Milt quality was assessed in relation to hormonal treatment in Week 9, 11, and 13 in terms of hemocytometer counts, spermatocrit, and sperm motility (Fig. 1). Six hormonal treatment schemes were applied in clusters of two groups per tank to test the effect of strip-spawning timing (12 *vs.* 24 h) after injection and the necessity of a priming dose after four days from the

weekly injection. Here, four days post weekly injection was selected to match the most frequent timing of female spawning (Fig. 1a-b). In addition, a priming injection either 12 or 24 h was applied to compare two common sperm production protocols. A placebo treatment was also included, where 0.9% saline water was applied instead of the extra hormonal injection. Each treatment followed the same individuals throughout the experimental period.

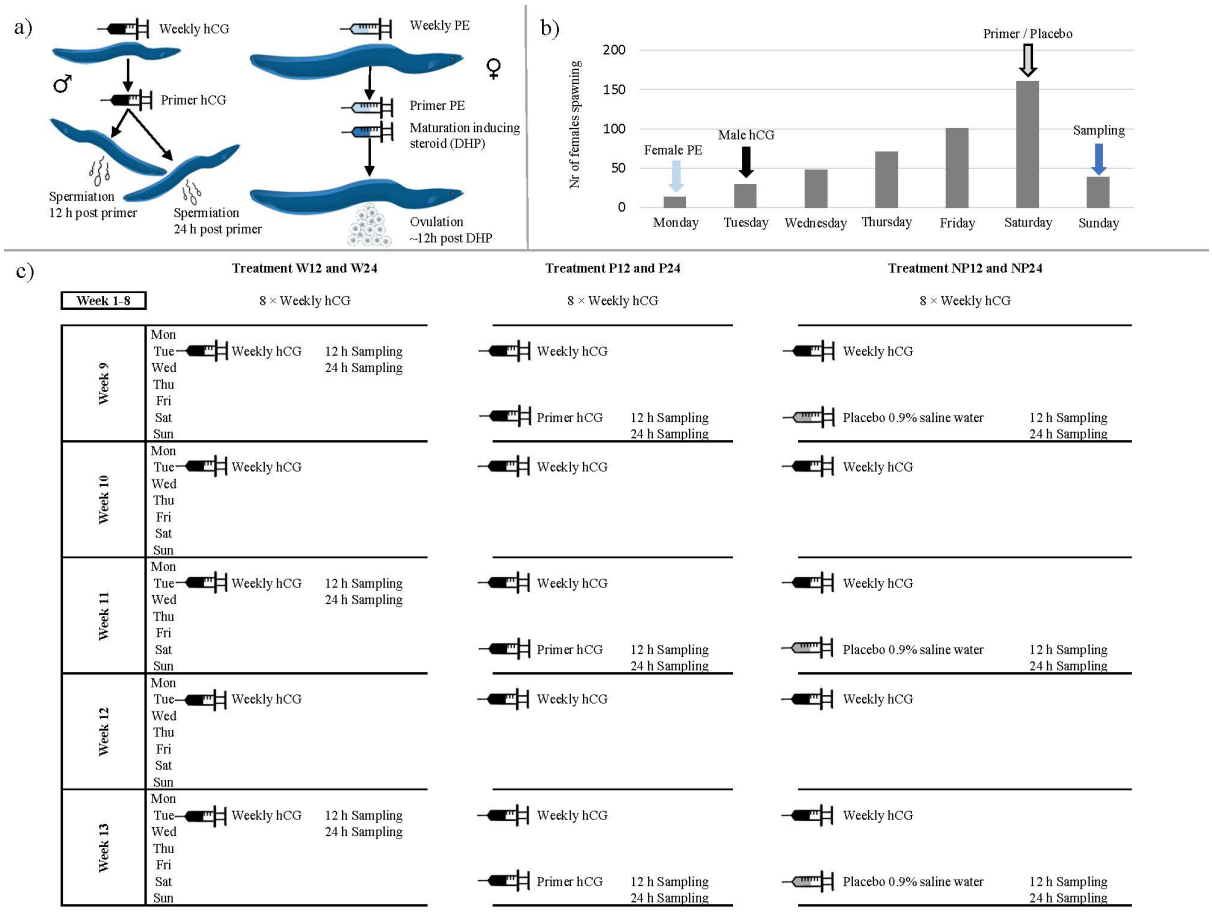

Figure 1:

a) Current protocol for artificial maturation of male European eel (*Anguilla anguilla)* to match female maturation. b) Frequency of European eel female spawning events throughout week in relation to female and male treatment schemes as well as time of sperm sampling. Data summarized from several spawning seasons within the ITS-EEL project. c) Experimental set-up, including different hormonal treatment schemes, using human chorionic gonadotropin (hCG), applied in the current study, on European eel males.

The experimental design is overviewed in Fig. 1c, where:

- Treatment W12 and W24: Stripping following weekly injection (W), where one group of male eels (n = 9) was stripped 12 h after the weekly injection (W12) and a second group (n = 8) after 24 h (W24).
- Treatment P12 and P24: Stripping following primer injection (P), where a priming dose of 1.5 IU/g IBW was given four days after the weekly injection. Here, one group of male eels (n = 9) was stripped 12 h after the priming injection (P12) and another group (n = 8) 24 h (P24) after the priming injection.
- Treatment NP12 and NP24: Stripping following placebo injection [No-Primer (NP)], where males received a 0.9% saline water injection four days after weekly injection. Here, one group of male eels (n = 9) was stripped at 12 h (NP12) after placebo injection and another group (n = 9) at 24 h (NP24).

The groups within the treatments (W, P, and NP) were held in separate tanks to minimise influence across treatments. For each group, males were immediately moved after stripping to a new tank (fourth tank), leaving one tank available for transferring males from the next treatment after stripping.

2.4 Sampling

For milt collection, the genital pore was rinsed using deionized water and wiped dry. Males were stripped by applying gentle pressure on the abdomen. Milt was collected into dry weigh boats (42 × 42 mm). Milt weight was recorded and a milt sample (100 µL) from each male was immediately diluted in 900 µL immobilizing medium (Peñaranda et al., 2010), thereby creating

189 a stock solution for hemocytometer counting and CASA. All sperm analysis took place within
190 2 h after stripping.
191
192 **2.6 Analysis**
193
194 **Hemocytometer counting**: All samples were mixed by vortexing for ~5 s to ensure a
195 homogeneous distribution of sperm. A Neubauer Improved hemocytometer chamber was used
196 for counting sperm under a compound microscope (Nikon Eclipse 55i, Nikon Corporation,
197 Tokyo, Japan) at 40× magnification. Sperm counts were carried out in triplicate for each male
198 from an aliquot of the stock solution. Sperm were counted in 5 squares (0.20 × 0.20 mm) per
199 replicate. Sperm density was assessed according to Butts et al. (2014). The mean of three
200 replicates per male was used for statistical analyses and results are expressed as sperm cells ×
201 10^{-9} mL^{-1}.
202
203 **Spermatocrit**: For each male, samples of milt were drawn directly from the weigh boat into
204 replicated microhematocrit capillary tubes (75 mm × 1.15 mm) and sealed with sigillum wax
205 (Vitrex). The tubes were then centrifuged for 10 min at 6000 × g (Haematokrit 210, Hettich
206 Zentrifugen, Germany). Spermatocrit was determined by using a digital caliper (Cocraft). The
207 mean of three replicate measurements per male was used for statistical analyses.
208
209 **CASA**: From the immobilized stock solution (1:10), 0.2 µL was micropipetted (Gilson SAS,
210 France) into a Hamilton Thorne chamber (80 µm 2X-CEL) and covered with a 22×22 mm
211 coverslip for sperm motility and velocity assessment. Sperm were activated with 12 µL of
212 seawater (36 PSU) with the addition of 1% w/v bovine serum albumin (Sigma-Aldrich,
213 Denmark) to prevent sperm from sticking to the glass slide. For each male, sperm motility was

captured in triplicate at 10, 20, and 30 s post activation (±1 s) using a compound microscope (PROiSER, UB200i) equipped with a negative phase objective (Plan 10x PHN). A digital video camera (ISAS 782M) was connected to a computer where images were captured at 50 frames per second (fps), for 1 s using the Procadi PROiSER 1.4 software (1404 video recordings). MOT, pMOT, VCL, VSL, and VAP were assessed using CASA (ISAS v1; PROiSER R + D, S.L., Paterna, Spain) according to Gallego et al. (2013) after evaluating the trajectories of the different sperm in the image.

2.7 Statistical analysis

All data were analysed using SAS statistical analysis and R software (R Core Team, 2020). Residuals were evaluated for normality (Shapiro–Wilk test) and homogeneity of variances (Levene's test). The significance level was set at 0.05 for main effects and interactions. Treatment means were contrasted using Tukey's Honest Significant Difference test. Data were log(10) or arcsine square root (percentage data) transformed to meet these assumptions when necessary. Male weight, milt weight, hemocytometer counts, spermatocrit, MOT, pMOT, VCL, VSL, and VAP were compared using a repeated measure ANOVA model that contained the Treatment and Week main effects as well as the Treatment × Week interaction. When no Week × Treatment interaction was detected, the main effects were analysed and displayed independently. If a significant Week × Treatment interaction was detected, the model was decomposed into a series of reduced ANOVA models to determine the effect of Treatment for each Week. Moreover, for all sperm CASA parameters, a repeated measure ANOVA was performed for each time post activation (10, 20, 30 s). In addition, a series of regression models were performed to analyse the relationships between spermatocrit and hemocytometer counts, male weight and milt weight, as well as milt weight and hemocytometer counts. These

239 regression models used data for all treatments and weeks (Legendre & Oksanen, 2018; Pinheiro
240 et al., 2021).
241
242 **3. Results**
243
244 **3.1 Milt production and sperm quality**
245
246 Spermiation occurred in 11.5% of males on Week 5 and in 40.4% of males on Week 6 after
247 onset of hCG treatment. The initial volume of milt was low (<0.5 mL) and variable among
248 males, while by Week 9, when the first sampling sperm quality was performed, all males
249 produced milt in sufficient amounts for the analyses.
250 The progression in male weight, milt weight, hemocytometer counts, and spermatocrit is
251 shown in Fig. 2. The statistical model showed no Week × Treatment interaction, thus the main
252 effects were analysed and displayed independently. None of the parameters differed among
253 treatments (Fig. 2a-d). However, male weight significantly ($P < 0.05$) decreased from 106.3 ±
254 2.5 g in Week 9 to 93.0 ± 2.5 g in Week 13 (Fig. 2e), while milt weight significantly ($P < 0.05$)
255 increased from 3.5 ± 0.6 g in Week 9 to 5.4 ± 0.6 g in Week 13 (Fig. 2f). Moreover, sperm
256 density obtained from hemocytometer counts significantly ($P < 0.05$) decreased from 11.7 ×
257 10^9 ± 0.6 cells/mL in Week 9 to 10.5 × 10^9 ± 0.6 cells/mL in Week 13 (Fig. 2g), while
258 spermatocrit significantly ($P < 0.05$) decreased from 46.5 ± 2.3% on Week 9 to 40.5 ± 2.3% on
259 Week 13 (Fig. 2h).
260

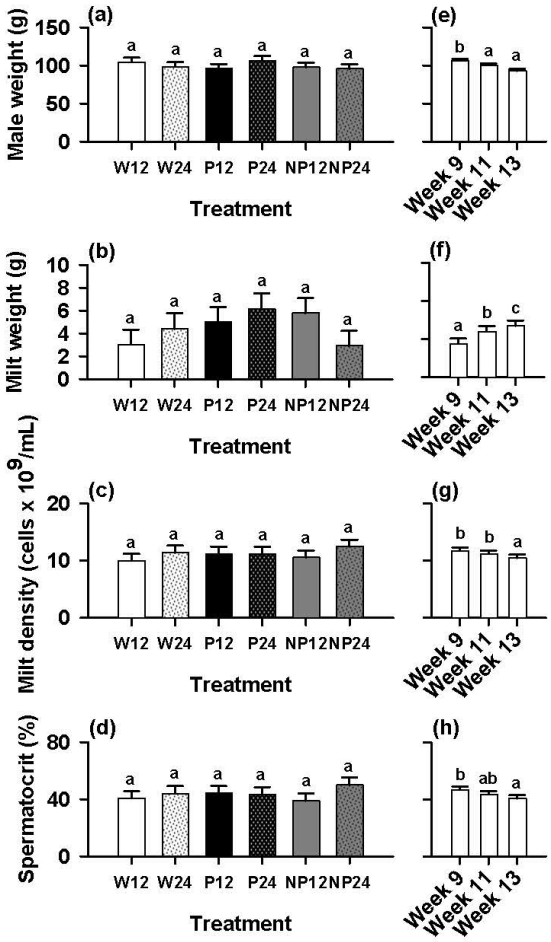

Figure 2:

Male weight (a), milt weight (b), sperm density, (c) and spermatocrit (d) in European eel, *Anguilla anguilla* in relation to hormonal treatment and week. A repeated measure ANOVA model was applied, containing the Treatment and Week main effects as well as the Treatment × Week interaction. All parameters showed no Week × Treatment interaction, thus the main effects were analysed and displayed independently. Results are expressed as mean values ± SEM. Different subscripts show significant differences ($P < 0.05$).

No significant relationships were found between milt weight and male weight (Fig. 3a) nor between milt density and milt weight, when quantified using hemocytometer counting (Fig. 3b). On the other hand, a positive relationship ($R^2 = 0.86$, $P < 0.001$) was detected between spermatocrit and hemocytometer counts (Fig. 3c).

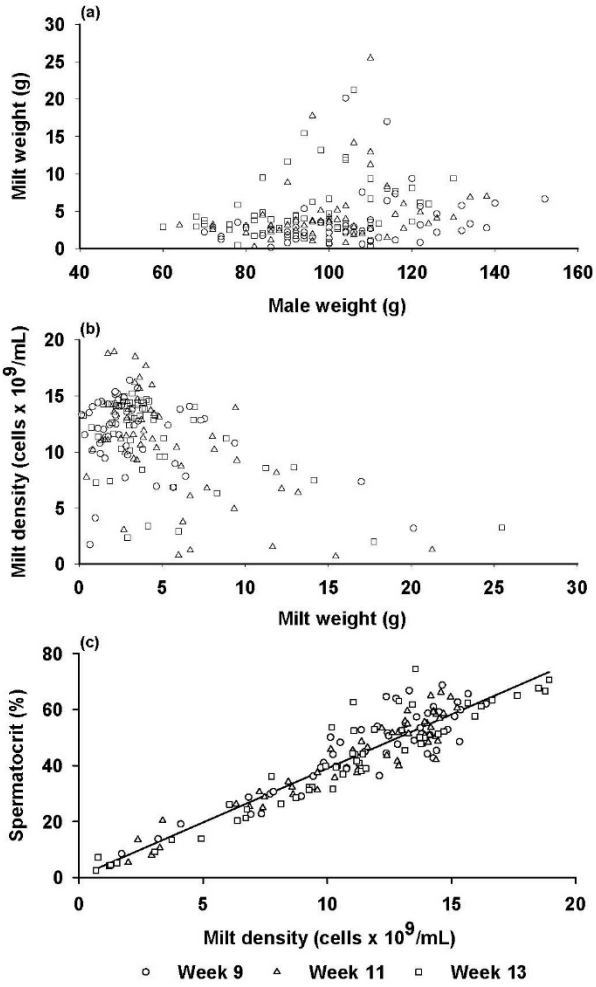

Figure 3: Plots for (a) milt weight *vs* male weight, (b) milt density *vs* milt weight and (c) spermatocrit *vs* hemocytometer counts in European eel, *Anguilla anguilla*. Model II linear regression was used due to variability in both axes. Regression analysis included all males in all weeks (n = 156) and the regression line ($y = 3.86e^{-9}x + 0.42$, $R^2 = 0.86$, $P < 0.001$) is represented as a solid line.

3.2 CASA

Figure 4 shows sperm kinetic parameters and motility at different times post activation (10, 20, 30 s). At 30 s post activation, the Week × Treatment not significant for VCL, thus the main effects were again analysed and displayed independently. Here, no significant differences were

285 detected between hormone treatments (Fig. 4c), while VCL significantly ($P < 0.05$) increased
286 from 94.2 ± 3.2 μm/s on Week 9 to 124.1 ± 3.2 μm/s on Week 13 (Fig 4d). For all other CASA
287 parameters, irrespective of the time post-activation (10, 20, 30 s), a significant Week ×
288 Treatment interaction ($P < 0.05$) was observed (Fig. 4). Therefore, the statistical model was
289 decomposed into a series of reduced ANOVA models to determine the effect of Treatment for
290 each Week. On Week 9, no significant differences were detected between treatments for any
291 of the parameters at all time points post-activation (10, 20, 30 s). On the contrary, on Week
292 11, significant differences among treatments were observed at all time points (10, 20, 30 s),
293 where generally treatment NP12 had significantly lower and NP24 had significantly higher
294 sperm parameter estimates. In Week 13 and at 10 s post activation, VSL was significantly
295 higher in treatment W24 than W12 and NP24, while VCL and VAP were significantly higher
296 in Treatment W24 than W12, P12 and NP24. Moreover, at 20 s, VCL, VSL, and VAP were
297 significantly higher in Treatment W24 than in W12 and NP24, while at 30 s only VAP was
298 significantly higher in W24 than in P12 and NP24.
299

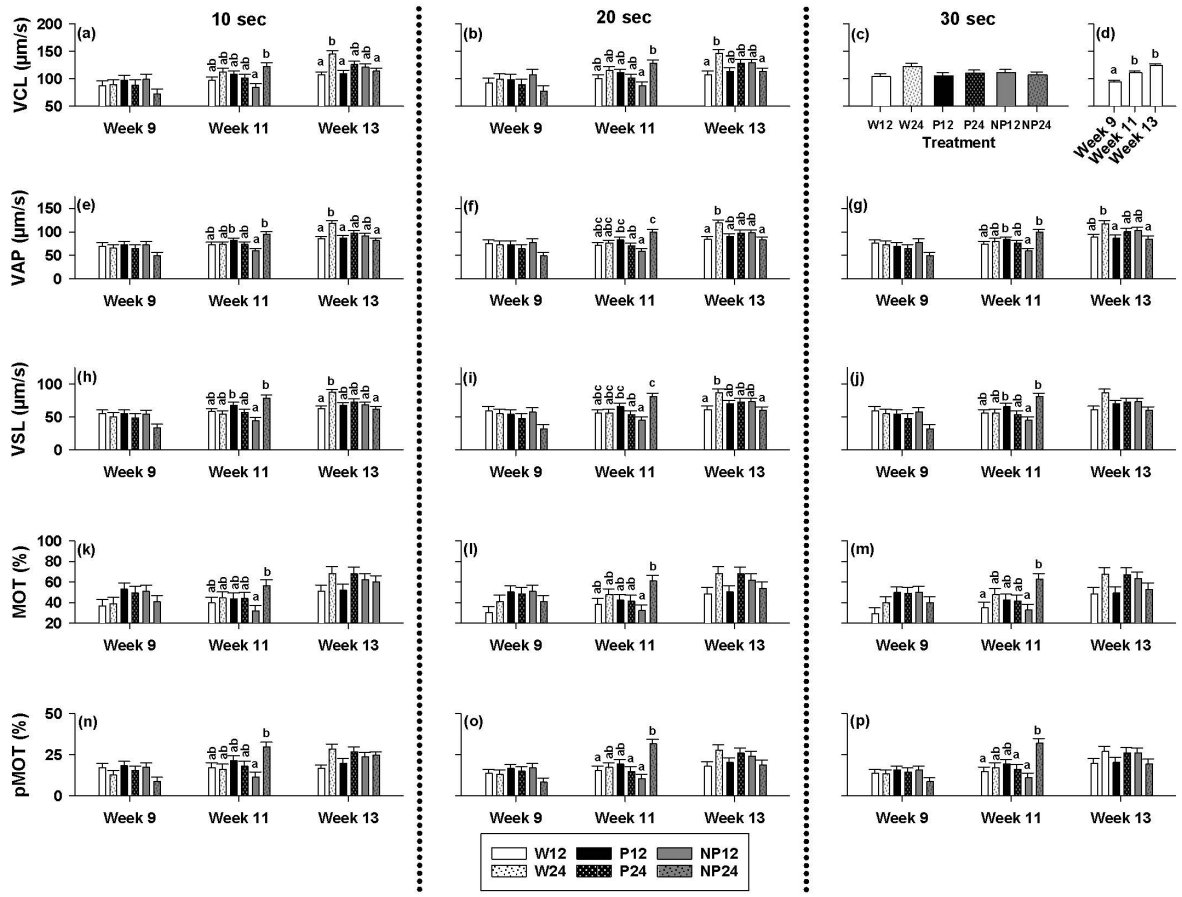

Figure 4: Sperm kinetic parameters and motility in European eel, *Anguilla anguilla* at different times post activation (10, 20, 30 s): (a-d) curvilinear velocity (VCL), (e-g) average path velocity (VAP), (h-j) straight-line velocity (VSL), (k-m) total motility (MOT) and (n-p) progressive motility (pMOT). For all models, significant Week × Treatment interactions were observed, thus the models were decomposed to determine the effect of Treatment for each Week, except for VCL at 30 s (c-d), where no significant interaction was observed, therefore main effects were interpreted independently. Different subscripts represent significant differences. Results are expressed as mean values ± SEM.

4. Discussion

Stable hatchery production of viable offspring relies on controlled management of gametogenesis, for obtaining high-quality gametes. In the case of male fish, parameters such as milt volume, density, and sperm motility/velocity are essential for monitoring reproductive

performance and optimising fertilisation success, especially in species where assisted reproduction is needed (Mylonas et al., 2017). The present study suggests that protocols for sperm production for use in assisted reproduction of European eel can be simplified to reduce animal handling and lower labour costs without compromising sperm quality. Here, assisted reproduction methods rely on availability of high-quality sperm at any time during the week for a period of several months during the female spawning period (Palstra et al., 2005).

In hormonally treated European eels, spermiation generally occurs after four to five weekly hormonal injections, where milt becomes available in small quantities (Pérez et al., 2000; Butts et al., 2020). In accordance, 11.5% of males in the present study started producing sperm in the 5^{th} week of hormonal administration. During the following weeks, milt and sperm production gradually increased, reaching levels "suited for fertilization procedures" by Week 9, similar to Butts et al. (2020). Hereafter, milt production (milt weight) increased, while sperm density decreased. This is a common observation in fish species, where hormonal therapies are applied to enhance sperm production, resulting in reduced sperm density through enhanced production of seminal fluid (Clemens & Grant, 1965; Bobe and Labbé, 2010, Mylonas et al., 2017). This tendency was also observed in the relationship between sperm density and milt weight in our study, where higher sperm density values ($>10 \times 10^9$ cell/mL) were typically present in males producing ~5 g of milt. Furthermore, the sperm density assessment showed a strong positive relationship between spermatocrit values and hemocytometer counts. Spermatocrit can be used as an indicator of sperm density in fish, but applicability varies depending on fish sperm characteristics (Trippel et al., 2003, Mylonas et al., 2017). The ability to use spermatocrit as a reliable indicator of sperm density is an advantage as it allows standardization of the sperm to egg ratio in fertilization protocols for European eel in a cost-efficient way (Butts et al., 2014; Sørensen et al., 2013).

Sperm motility and velocity are additional traits for assessing quality, because sperm with high motility and speed are considered to have better chances to fertilize eggs (Mylonas et al., 2017; Gallego & Asturiano. 2019). Similar to Butts et al. (2020), all sperm motility and kinetic parameters in the present study displayed similar patterns when compared at different times post activation (10, 20, 30 s), except for VCL that showed no interaction on 30 s post activation. Moreover, estimates of sperm motility and velocity increased within the experimental period and peaked on Week 13. In another study on European eel, where male eels similarly were treated with hCG but stripped weekly (Gallego et al., 2012), estimates of sperm motility and kinetics decreased beyond Week 11. This difference in observed sperm kinetic parameters may be due to deviation in time lapse between stripping of males (weekly in Gallego et al., 2012 *vs.* every 2nd week in this study). However, other factors such as size and age or nutritional and physiological condition of males could also be in play.

In terms of strip-spawning timing, Pérez et al. (2000) found that stripping males 6 h post hormonal treatment provided milt with highest sperm density, while stripping males 24 h post hormonal treatment provided sperm with highest motility. However, these differences between stripping at 6 h or 24 h after treatment were not statistically significant. Other studies focusing on offspring production have applied an intermediate procedure, where males are stripped 12 h post hormonal treatment (Butts et al., 2014; Politis et al., 2014; Benini et al., 2018) in order to synchronise the timing of priming with female final maturation treatment (da Silva et al., 2018; Kottmann et al., 2020). The comparison of sperm quality of males stripped at 12 *vs*. 24 h after hormonal induction in the present study did not show any differences between treatments in terms of milt weight or sperm density. For kinetic traits, our results showed that sperm velocity was higher for males stripped after 24 h compared to 12 h post weekly injection (W12 *vs* W24) but only for Week 13. Moreover, and only on Week 11, sperm showed higher motility and velocity when stripping occurred 24 h compared to 12 h post placebo injection

(NP12 *vs.* NP24). It cannot be excluded that the placebo handling procedures applied in the current study affected sperm motility and velocity in those treatments (NP). Previous studies have described that stressors for some species can alter gamete quality (Hajirezaee et al., 2010; Żarski et al., 2020). In this regard, it might be that the fish in treatment NP12 had less time to "recover" from handling "stress" compared to treatment NP24. However, since this pattern was neither universal nor consistent throughout the experimental period, further clarification regarding the stripping time post hormonal treatment is needed. Interestingly though, this pattern never occurred when males were given the additional primer (P12 *vs* P24), possibly showing that the handling effect could have been overshadowed by the hormonal influence in those treatments receiving "booster" injections.

Moreover, the results of the current study revealed that in Week 11, sperm seemed to be performing better in terms of VAP and VSL, when stripping occurred at 12 h after the primer injection (P12) compared to stripping at 12 h post placebo injection (NP12). On Week 9 and 13, however, sperm performed equally well irrespective of males receiving a primer injection (P12, P24) or just a placebo treatment (NP12, NP24). Thus, overall, the results indicate that high quality sperm can be attained also without primer injection, which in turn means no further handling would be required after weekly injection. Such simplification of the procedures would also reduce labour requirements and cost of treatment.

To summarize, the results of the present study showed that all of the applied hormonal treatments, using stripping at two week intervals, resulted in continuous milt production with high sperm quality. Moreover, strip-spawning timing (12 *vs* 24 h post hormone injection) caused variability of sperm motility and velocity in some cases, however, results are not unambiguous. At the same time, the primer treatment did not show a consistently significant positive effect on sperm quality, when given 4 days post weekly injection.

In conclusion, differences were observed in sperm motility and velocity depending on strip-spawning timing after hormonal injection (12 vs. 24 h), however the pattern was not consistent. Furthermore, these parameters also did not differ between the no-primer and primer treatments in an unambiguous way. Considering that each male may be stripped 4-5 times over the 2-3 months spawning season, omitting the primer would reduce animal handling, material costs, and labour intensity, while still sustaining high quality sperm production. However, due to the variability in observations between sampling points, further studies are encouraged to substantiate results. This would include exploring the effects of hormonal treatment over a prolonged period, stripping intervals as well as the relationship between sperm quality parameters and subsequent fertilization success and offspring quality for European eel.

5. Acknowledgments

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6. Author Contribution

PK, SNP, JT, IEB, IAEB conceived and designed the experiment. PK and DES performed the experiment. PK analysed the primary data. VG performed CASA. IAEB and DES performed statistical analysis. PK, SNP, JT interpreted results. JT and SNP obtained primary funding. JT and JFA provided facilities and equipment. PK wrote the original draft of the

manuscript. All authors contributed to review and editing and approved this version for publication.

7. Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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