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Subpopulation pattern of eel spermatozoa is affected by post-activation time, hormonal treatment and the thermal regimen

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Abstract. There has been a marked reduction in natural stocks of eels (genus *Anguilla*) over the past 60 years, and the culture of eels is still based on the capture of very large quantities of juveniles. It is necessary to close the life cycle in captivity in order to ease the pressure on wild populations. The aims of the present study were to evaluate sperm subpopulations (through cluster analysis of computer-aided sperm analysis data) in the European eel (*Anguilla anguilla*) and to assess the effects of motility acquisition time after activation (i.e. at 30, 60 and 90 s), the thermal regimen (i.e. 10°C (T10) or 15°C (T15) and up to 20°C, or constant at 20°C (T20)) and hormonal treatments (i.e. human chorionic gonadotropin (hCG), recombinant (r) hCG or pregnant mare serum gonadotropin (PMSG)) on these subpopulations. In all cases, we obtained three subpopulations of spermatozoa: low velocity and linear (S1); high velocity with low linearity (S2); and high velocity and linear (S3; considered high quality). Total motility and S1 were affected by acquisition time; thus, 30 s is recommended as the standard time for motility acquisition. When eels were kept at 20°C (T20), motility data fitted quadratic models, with the highest motility and proportion of S3 between Weeks 8 and 12 after the first injection. Lower temperatures (T10, T15) delayed spermiation and the obtaining of high-quality spermatozoa (S3), but did not seem to alter the spermiation process (similar subpopulation pattern). Conversely, the hormonal treatments altered both the dynamics of the subpopulation pattern and the onset of spermiation (with PMSG delaying it). Total motility and the yield of S3 with the widely used hCG treatment varied throughout the spermiation period. However, using rhCG allowed us to obtain high-quality and constant motility for most of the study (Weeks 7–20), and the S3 yield was also higher overall ($61.8 \pm 1.3\%$; mean ± s.e.m.) and more stable over time than the other hormonal treatments (averaging $53.0 \pm 1.4\%$). Using T20 and rhCG would be more economical and practical, allowing us to obtain a higher number of S3 spermatozoa over an extended time.

Additional keywords: computer-aided sperm analysis, European eel, motility activation.

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Introduction

The genus *Anguilla* contains many species of great commercial importance, but wild stocks have been depleted because of overfishing, parasites, global climate change and other human impacts (Feunteun 2002; Halpin 2007). To these factors we must add the peculiar life cycle of these species: adults spawn in the sea (an event not as yet witnessed) and the leptocephali larvae drift until they reach coastal waters, where they metamorphose into glass eels and move inland while they develop into elver and yellow elver before maturing into silver eels (the entire growth process taking years to decades), which are capable of recognising their way to the spawning areas, where they mature fully, spawn once and die (Ginneken and Maes 2005). The complexity of this cycle has contributed to the difficulty experienced replicating it in captivity. Therefore, although an increasing proportion of eels is now farm raised, the stocks are obtained by capturing very large numbers of glass eels (Halpin 2007). Given the commercial, sociocultural and ecological value of these species, breeding eels in captivity (effectively closing the life cycle in fish farms) is a major focus for researchers. Success would have great benefits not only in terms of the commercial use of the species, but also in terms of easing the pressure on natural populations and could even be applied to restocking natural populations in conservation programs. Some success has been reported in terms of obtaining and conserving gametes, AI and larval rearing

(Tanaka *et al.* 2003; Asturiano *et al.* 2004; Peñaranda *et al.* 2010*a*), but efficient production of glass eels has yet to be achieved (Okamura *et al.* 2007).

Among the many challenges we face in efficiently replicating the eel life cycle in captivity, is the major hurdle of obtaining spermatozoa with high fertility potential at the right time and for an extended period. Currently, the only way of inducing maturation and spermiation in eels is through gonadotropin injections (Miura *et al.* 2002). Although human chorionic gonadotropin (hCG) has been the hormone of choice for many years, the work of Gallego *et al.* (2012) in the European eel (*Anguilla anguilla*) revealed that recombinant (r) hCG yielded better and more economic results. In addition, the authors considered water temperature in that study in an attempt to mimic the temperature changes that adults may undergo before spawning. Water temperature can affect the reproductive biology of fish, at least in temperate climates (Pankhurst and Porter 2003). Because eels migrate considerable distances and possibly at different water depths (Aarestrup *et al.* 2009), Gallego *et al.* (2012) tested three thermal regimens (from 10°C or 15°C to 20°C vs constant 20°C), taking at a more physiological approach to sexual maturation (Pérez *et al.* 2011). The results showed that hormone-treated males could produce spermatozoa only after spending at least 1 week at 20°C (Gallego *et al.* 2012).

Previous studies have focused on production and routine sperm quality parameters. In the present study, we have taken another approach to study eel spermiation. First, we analysed the data using polynomial regression (Quinn and Keough 2002), because previous results suggested that at least part of the experimental data could follow low-order polynomial models (Gallego *et al.* 2012). Our aim was not to obtain a best-fit model to use for interpolation, but rather to find which linear regression model best fit each dataset while making biological sense, thus helping to compare treatments and to obtain information on the evolution of the eel spermiation process. This approach has helped us interpret previous spermatology studies (Fernández-Santos *et al.* 2007; de Paz *et al.* 2012). Second, we wanted to take into account the within-sample heterogeneity that computer-aided sperm analysis (CASA) data conveys, using median values (not mean values, which are very sensitive to extreme values) to study the kinematic parameters more reliably. Moreover, we have taken advantage of the potential of CASA data (Holt *et al.* 2007) and classified the spermatozoa within each sample according to their kinematic characteristics. This approach requires multivariate techniques, such as cluster analysis (Martínez-Pastor *et al.* 2011). Kinematic parameters are used to group spermatozoa into subpopulations, allowing us to characterise the samples not on the basis of average values of CASA parameters, but rather on the basis of the relative proportions of each subpopulation. This approach promises to provide us with a deeper understanding of the inner dynamics of the sperm sample because its intrinsic heterogeneity is taken into account (Holt and Harrison 2002; Martinez-Pastor *et al.* 2005*a*). Subpopulation analysis has been applied in a few studies in fish, including the sole fish (*Solea senegalensis*; Beirão *et al.* 2009; Martínez-Pastor *et al.* 2008), sea bream (*Sparus aurata*; Beirão *et al.* 2011), three-spined stickleback (*Gasterosteus aculeatus*; Le Comber *et al.* 2004) and steelhead (*Oncorhynchus mykiss*; Kanuga *et al.* 2012). In these studies, three to four subpopulations of spermatozoa were identified, one being defined as more desirable (containing fast and linearly motile cells; Beirão *et al.* 2009; Martínez-Pastor *et al.* 2008).

Therefore, we have adapted an unsupervised cluster analysis developed in previous studies on sperm classification (Martinez-Pastor *et al.* 2005*b*, 2008; Domínguez-Rebolledo *et al.* 2011) to discover the subpopulation structure of European eel spermatozoa and to apply this information to improve our knowledge of the effects thermal and hormonal treatments on spermiation and sperm quality in this species. Because there is no prior knowledge about the subpopulation structure of eel spermatozoa, we performed a prior cluster analysis on the sperm samples obtained following a standard protocol at different times after activation. Using this approach, our aim was to test a major hypothesis that the subpopulation pattern of eel spermatozoa is affected by the treatments used to induce spermiation. This kind of study would be of physiological significance, shedding light on the underlying spermatogenic process, which seems to be affected by thermal and hormonal treatments.

Materials and methods

Animal maintenance and handling

Animals were handled in accordance with the European Union regulations concerning the protection of experimental animals (Dir. 86/609/EEC). Male eels were bred at a fish farm (Valenciana de Acuicultura, Puzol, Valencia, Spain) and transported to our facilities in the Aquaculture Laboratory at the Universitat Politècnica de València (Valencia, Spain), where they were gradually acclimatised to sea water over the course of 1 week (salinity 37.0 ± 0.3 g L^{-1}, temperature 20°C). The fish were distributed in 200-L aquaria equipped with separate recirculation systems, thermostats and coolers to strictly control water temperature. No feed was provided during the duration of the experiments, and the eels were kept in the dark. Before the intraperitoneal administration of hormones to induce spermiation (injected 4 cm anterior to the genital pore), the eels were weighed (mean (± s.d.) 100 ± 2 g) and anaesthetised. Anaesthesia was achieved by transferring individual males to water containing benzocaine (Scharlau Chemie, Barcelona, Spain). the benzocaine was prediluted in 70% ethanol and then diluted in saline to a final concentration of 60 ppm.

Experiments

Experiment 1: changes in sperm motility patterns after activation

Males ($n = 9$) received weekly intraperitoneal injections of hCG (1.5 IU g^{-1}; Argent Chemical Laboratories, Redmond, WA, USA) diluted in saline solution (0.9% NaCl). Spermatozoa recovered between Weeks 8 and 11 after the first injection (the highest quality according to previous studies; Asturiano *et al.* 2006; Gallego *et al.* 2012) were used in this experiment. In total, 19 samples were recovered and subsequently analysed for motility. In the motility analysis, image sequences were acquired 30, 60 and 90 s after activation. Data were analysed to determine the effect of post-activation time on motility parameters and subpopulation patterns.

Experiment 2: effects of tank water temperature on sperm motility patterns

In all, 317 adult male eels (mean bodyweight 100 ± 2 g; mean length 40 ± 5 cm) were equally and randomly distributed in six 200-L aquaria (~100 males in each treatment) and subjected to one of three thermal regimens: (1) T10, which consisted of 10°C for the first 6 weeks, 15°C for the next 3 weeks and 20°C for the last 6 weeks; (2) T15, which consisted of 15°C for the first 6 weeks and 20°C for the last 9 weeks; and (3) T20, which consisted of 20°C throughout the entire experimental period. All males were hormonally treated to induce maturation and spermiation with weekly intraperitoneal injections of hCG ($1.5\ IU\ g^{-1}$) for 13 weeks.

Experiment 3: effects of hormonal treatment on sperm motility patterns

Male eels ($n = 18$ per group) were assigned to one of three hormonal treatment groups in different 200-L tanks at 20°C: (1) hCG; (2) rhCG (Ovitrelle, Madrid, Spain); and (3) pregnant mare's serum gonadotropin (PMSG; Sincropart; Laboratory CEVA, Barcelona, Spain). Every week, all males were injected with $1.5\ IU\ g^{-1}$, with all hormones having been diluted in the same volume of saline (0.9% NaCl). This experiment was performed over 20 weeks.

Sperm collection

Sperm samples were collected weekly 24 h after administration of the hormonal treatment in order to achieve the highest sperm quality (Pérez *et al.* 2000). The fish were anaesthetised and the genital area was cleaned with freshwater and thoroughly dried to avoid contamination with faeces, urine or sea water. Spermatozoa were forced out by abdominal pressure. A modified aquarium air pump provided a vacuum for to collect the spermatozoa in a clean tube. Samples were individually treated and kept undiluted at 4°C until analysis. The sperm concentration was measured using a Thoma haemocytometer after diluting the samples 1 : 200 in P1 medium (125 mM NaCl, 20 mM $NaHCO_3$, 30 mM KCl, 2.5 mM $MgCl_2$, 1 mM $CaCl_2$, pH 8.5; Peñaranda *et al.* 2010*a*).

CASA analysis

Sperm motility was analysed according to standardised conditions for European eel spermatozoa (Gallego *et al.* 2013*a*), as described in the Supplementary Material available for this paper. In Experiment 1, motility was determined 30, 60 and 90 s after activation, whereas in Experiments 2 and 3, motility was determined 30 s after activation.

Subpopulation and statistical analyses

Subpopulation and statistical analyses were performed using the R statistical environment (R Core Team 2013). The methodology was based on previous studies (Martinez-Pastor *et al.* 2005*b*; Domínguez-Rebolledo *et al.* 2009; de Paz *et al.* 2012) and is described in detail in the Supplementary Material. In short, CASA data were cleaned up and processed into a single dataset. Total motility and the median values of the kinematic variables were calculated for each individual sample. Subpopulation analysis was performed separately for each experiment by undertaking a hierarchical clustering in each sample and then a second clustering on the median values of the first set of clusters.

Hypothesis testing on motility and clustering results was conducted by using linear mixed-effects models for data from Experiment 1, with acquisition time or treatment as a fixed effect (factor), and the sample and week as the grouping factors in the random part of the model. Data from Experiments 2 and 3 were analysed using linear models and ANCOVA, with the week considered a covariate and either temperature or hormonal treatment as fixed factors. In the case of the week, a polynomial effect was suspected, and therefore polynomial quartic, cubic and quadratic models were tested. When needed, pairwise comparisons between the levels of fixed factors were performed using Tukey's correction. Unless stated otherwise, results are presented as the mean ± s.e.m.

Economic analysis of the hormonal treatments (Experiment 3)

Each hormonal treatment has a different cost, depending on the price of the hormone, the number of doses required and the volume of hormone injected (which depends on the weight of the male; data shown in Gallego *et al.* (2012)). In the present study, we focused on the results of the subpopulation analysis, estimating the cost of producing 10^9 spermatozoa belonging to the highest-quality subpopulation. We have to take into account that male eels must be treated for several weeks before they start spermiating. That offset period was taken into account by calculating the total cost for each male within each treatment and then estimating a corrected cost only for the weeks they were spermiating. Therefore, we obtained an estimated price for the high-quality spermatozoa for each male and each week, which was used to relate the level of investment of each hormonal treatment with the amount of good-quality sperm obtained.

Results

Changes in sperm motility patterns after activation (Experiment 1)

The mean ± (s.d.) motility of the eel spermatozoon was characterised (30 s after activation) by being fast (curvilinear velocity (VCL) $149.4 \pm 33.3\ \mu m\ s^{-1}$), curvilinear (linearity (LIN) $43.6 \pm 7.2\%$) and with limited lateral deviation along the main path (straightness (STR) $71.3 \pm 10.1\%$; wobble (WOB) $62.8 \pm 2.8\%$). Motility decreased at subsequent times, although the change was moderate (Fig. 1). Total motility (Fig. 1*a*) reached a mean (± s.e.m.) value of $63.2 \pm 2.3\%$ for the first measurement at 30 s, decreasing gradually thereafter ($P < 0.05$). The variables related to active motility, such as velocity (VCL, Fig. 1*b*), amplitude of lateral head displacement (ALH; Fig. 1*e*) and dancing (DNC; Fig. 1*f*), followed this downward trend, which slowed down between 60 and 90 s, resulting in no significant differences between these two time points. The variables related to track shape (e.g. LIN and WOB; Fig. 1*c*, *d*) were not affected by acquisition time ($P < 0.05$).

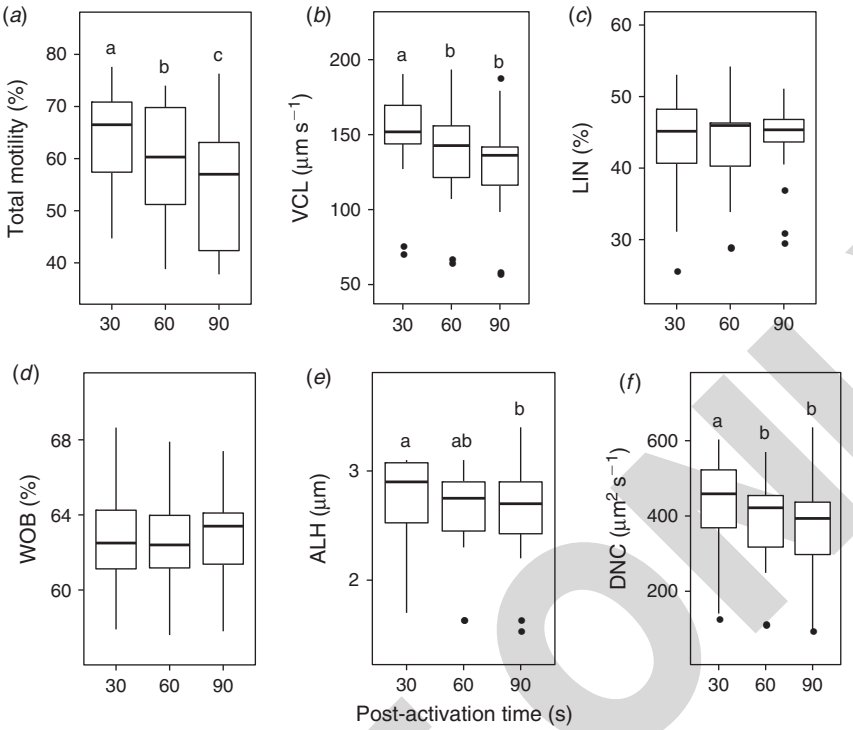

Fig. 1. Motility variables from the study of the effect of acquisition time on eel sperm motility (Experiment 1). Box plots are drawn so that boxes span from the 1st to the 3rd quartile, with the inner line showing the median, whereas whiskers span up to the extreme observations within 1.5 times the interquartile range. Observations beyond this range are drawn as dots. Different letters indicate significant difference between acquisition time groups ($P < 0.05$). VCL, curvilinear velocity; LIN, linearity; WOB, wobble; ALH, amplitude of lateral head displacement; DNC, dance.

Table 1. Subpopulations obtained from the computer-aided sperm analysis dataset at different times after activation (Experiment 1)

Data are the mean ± s.d. of several kinetic parameters. In all, 35 739 motile spermatozoa obtained from 84 samples were used in the clustering analysis. VCL, curvilinear velocity; LIN, linearity (=velocity according to the straight path (VSL)/VCL); WOB, wobble (=velocity according to the smoothed path (VAP)/VCL); ALH, amplitude of the lateral movement of the sperm head; DNC, dance (VCL × ALH)

Subpopulation	VCL (μm s^{-1})	LIN (%)	WOB (%)	ALH (μm)	DNC (μm^2 s^{-1})
S1	46.2 ± 27.9	28.0 ± 16.3	46.6 ± 22.4	1.3 ± 0.4	59.1 ± 52.2
S2	137.0 ± 71.3	17.3 ± 14.5	49.5 ± 15.7	3.0 ± 1.2	427.8 ± 365.3
S3	180.6 ± 48.2	51.8 ± 13.8	64.0 ± 7.6	3.2 ± 0.7	569.2 ± 234.3

The subpopulation analysis yielded three subpopulations, as summarised in Table 1. Subpopulation 1 (S1) was defined as a subset of slow spermatozoa, with circular but regular trajectories. Conversely, Subpopulation 2 (S2) grouped together fast spermatozoa with circular or erratic trajectories and Subpopulation 3 (S3) contained fast and active spermatozoa, but with linear tracks. The mean ± (s.d.) proportion of S1, S2 and S3 at 30 s was 26.0 ± 15.5%, 12.0 ± 14.3% and 62.0 ± 17.3%, respectively, which changed little with post-activation time (Fig. 2). Interestingly, the proportion of S3 ('fast swimmers') was positively correlated with the proportion of motile spermatozoa ($r = 0.32$, $P = 0.016$), whereas the proportion of S1 ('slow swimmers') was negatively correlated with the proportion of motile spermatozoa (not reaching statistical significance in this experiment; $r = -0.23$, $P = 0.084$). The same cluster pattern was obtained in the other two experiments. The proportion of S1 was significantly higher at 60 s, whereas the proportion of S2 followed this trend in reverse ($P > 0.05$). Because it accounted for most spermatozoa, the S3 ('good swimmers') subpopulation had the biggest impact when defining the average characteristics of sperm motility described previously. The proportion of this subpopulation changed little with time, although it was lower at 60 and 90 s (58.2 ± 2.6%) than at 30 s, reflecting the average VCL, ALH and DNC at these times.

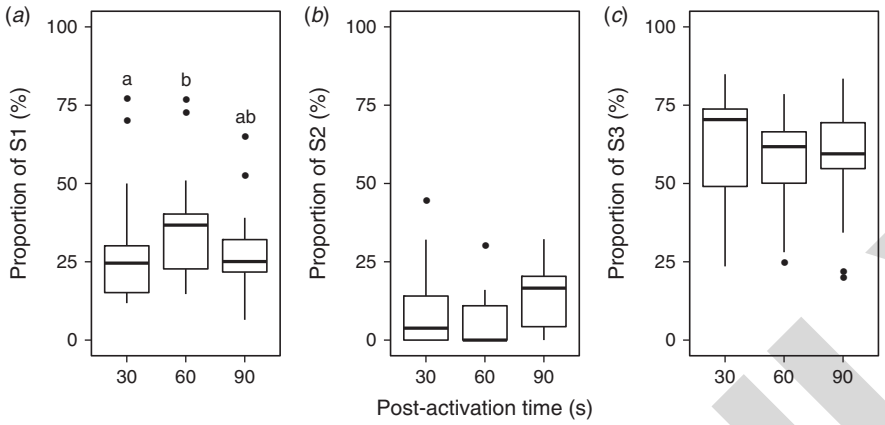

Fig. 2. Proportion of each sperm subpopulation (S1–S3; see Table 1) at each acquisition time (Experiment 1). Box plots are drawn so that boxes span from the 1st to the 3rd quartile, with the inner line showing the median, whereas whiskers span up to the extreme observations within 1.5 times the interquartile range. Observations beyond this range are drawn as dots. Different letters indicate significant difference between acquisition time groups ($P < 0.05$). The proportions of the three subpopulations differed significantly ($P < 0.05$).

Effects of thermal treatments on sperm motility and subpopulations (Experiment 2)

The onset of spermiation at each temperature occurred at different weeks after the beginning of the experiment, and thus conditioned the analysis of sperm motility. In general, the motility data yielded by T10 and T15 fitted a first-grade polynomial (simple linear models), whereas data yielded by T20 fitted a second-grade polynomial (quadratic model), with T10 and T15 delaying the onset of spermiation compared with T20.

The models analysed in this experiment displayed significant interactions between time (week) and thermal treatment. Therefore, these effects were analysed separately. The proportion of motile spermatozoa (Fig. 3*a–c*) was very low at the beginning of spermiation (overall mean ± (s.d.) 2.5 ± 4.8%; onset at Week 10 for T10 and Week 5 for T15 and T20). In the case of T20 (quadratic model with $R^2 = 0.54$, $F_{2,64} = 40.14$, $P < 0.001$), maximum values were reached between Weeks 8 and 11 (predicted maximum at Week 10), whereas the maximum for groups T10 (linear model with $R^2 = 0.70$, $F_{1,21} = 52.33$, $P < 0.001$) and T15 (linear model with $R^2 = 0.28$, $F_{1,52} = 21.67$, $P < 0.001$) were reached at Week 13, at the end of the study (overall mean (± s.d.) 53.0 ± 22.6%). Although T20 showed a downward trend after Week 11, T10 rose quickly from Week 10 to Week 13, reaching a mean value of 65.6 ± 6.6%. This value is similar to the highest one recorded for T20 (66.8 ± 3.3% at Week 11), showing that the peak of the T10 treatment could be near Week 13, and that in this group the motility peak was reached very quickly (at 4 weeks compared with 6 weeks in T20). A linear random-effects model (using the week as the grouping factor in the random part of the model) confirmed that the overall total motility was significantly higher for T20 compared with T10 and T15 (37.3 ± 3.1% vs 29.0 ± 5.3% and 25.3 ± 3.3%, respectively; $P < 0.001$).

The kinematic parameters followed a similar trend. VCL is shown in Fig. 3*d–f*. In this case, no model significantly fitted the data for T10 (due to lack of weeks with data), whereas T15 data increased linearly ($R^2 = 0.07$, $F_{1,45} = 4.59$, $P = 0.038$) and T20 followed a quadratic model ($R^2 = 0.27$, $F_{2,59} = 11.02$, $P < 0.001$), with maxima between Weeks 9 and 10 (predicted Week 9.6). The other velocity parameters and ALH showed similar trends. In terms of the parameters defining the shape of the trajectory (LIN shown in Fig. 3*g–i*) T20 data ($R^2 = 0.19$, $F_{2,59} = 8.05$, $P < 0.001$) fitted a quadratic model (maximum by Week 10), indicating that sperm tracks became more linear in the middle of the treatment. Nevertheless, the variation over time was low, as opposed to the wider range showed by the other variables. The overall values of motility variables were not significantly different between temperatures, although there were significant differences between treatments over the weeks.

The cluster analysis produced three subpopulations from the thermal experiment data (Table 2). S1 grouped together slow spermatozoa ('slow swimmers'), although the linearity parameters were between those of S2 and S3; S2 included relatively fast spermatozoa, with circular trajectories ('circular swimmers'); and S3 contained fast spermatozoa, following more linear tracks ('fast swimmers'). As with Experiment 1, S1 and S3 were correlated with total motility ($r = -0.46$ and $r = 0.41$, respectively; $P < 0.001$).

Very much alike the median motility parameters, the proportion of each subpopulation was highly affected by the week within the spermiation period. We could not detect a valid fit in T10 for any cluster (Fig. 4) due to the between-male variability and the short spermiating period (mean (± s.d.) 17.0 ± 10.7%, 30.5 ± 15.4% and 52.5 ± 11.9% for S1, S2 and S3, respectively). In terms of S1 (Fig. 4*a–c*), the data significantly fit a negative quadratic model for T15 ($R^2 = 0.29$, $F_{2,29} = 7{,}29$, $P = 0.003$) and T20 ($R^2 = 0.23$, $F_{2,48} = 8{,}63$, $P < 0.001$), with minima around Week 11 for T15 and Week 9 for T20. That is, S1 ('slow swimmers') tended to predominate by the beginning and end of the spermiation period, whereas their presence decreased around the middle of that period. The S3 ('fast swimmers') trend seemed to be the opposite (Fig. 4*h*), following a positive lineal

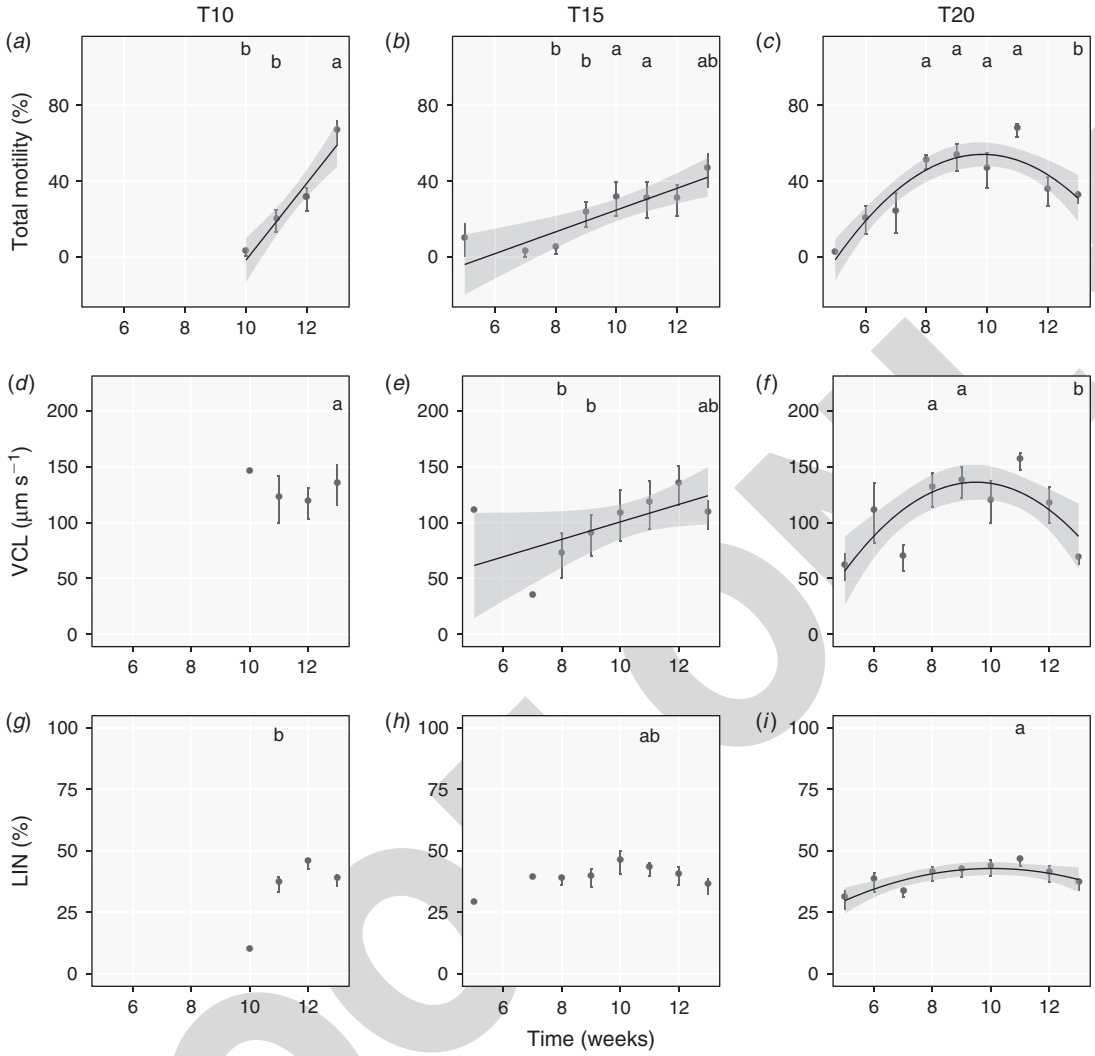

Fig. 3. Summary of the computer-aided sperm analysis (CASA) analysis for Experiment 2 (water temperature). Median data for total motility, curvilinear velocity (VCL) and linearity (LIN) over time and within each treatment group. T10, 10°C for 6 weeks, 15°C for 3 weeks and 20°C for 6 weeks; T15, 15°C for 6 weeks and 20°C for 9 weeks; T20, 20°C for the entire experimental period. Data were fitted to linear models (1st- to 4th-order polynomials). The plots show the mean ± s.e.m., the fitted model and its 95% confidence intervals for the models (confidence interval = shaded area). Different letters indicate significant differences within the same week between different treatments. For T10, total motility followed a positive linear model, with no fitted model for VCL and LIN. Data from T15 followed positive linear models for total motility and VCL, following a quadratic model (highest values by Week 10) for LIN. T20 data fitted quadratic models in all cases.

Table 2. Subpopulations obtained from the computer-aided sperm analysis dataset obtained analysing motility data from the thermal treatments experiment (Experiment 2)

Data are the mean ± s.d. of several kinetic parameters. In all, 27 668 motile spermatozoa obtained from 94 samples were used in the clustering analysis. VCL, curvilinear velocity; LIN, linearity (=velocity according to the straight path (VSL)/VCL); WOB, wobble (=velocity according to the smoothed path (VAP)/VCL); ALH, amplitude of the lateral movement of the sperm head; DNC, dance (VCL × ALH)

Subpopulation	VCL (μm s^{-1})	LIN (%)	WOB (%)	ALH (μm)	DNC (μm^2 s^{-1})
S1	39.8 ± 20.6	31.7 ± 13.8	63.8 ± 13.5	1.2 ± 0.4	47.3 ± 35.9
S2	117.6 ± 72.8	12.9 ± 9.6	53.1 ± 15.0	2.7 ± 1.3	328.0 ± 335.8
S3	169.0 ± 58.3	50.2 ± 13.9	62.8 ± 8.7	3.0 ± 0.7	520.0 ± 254.8

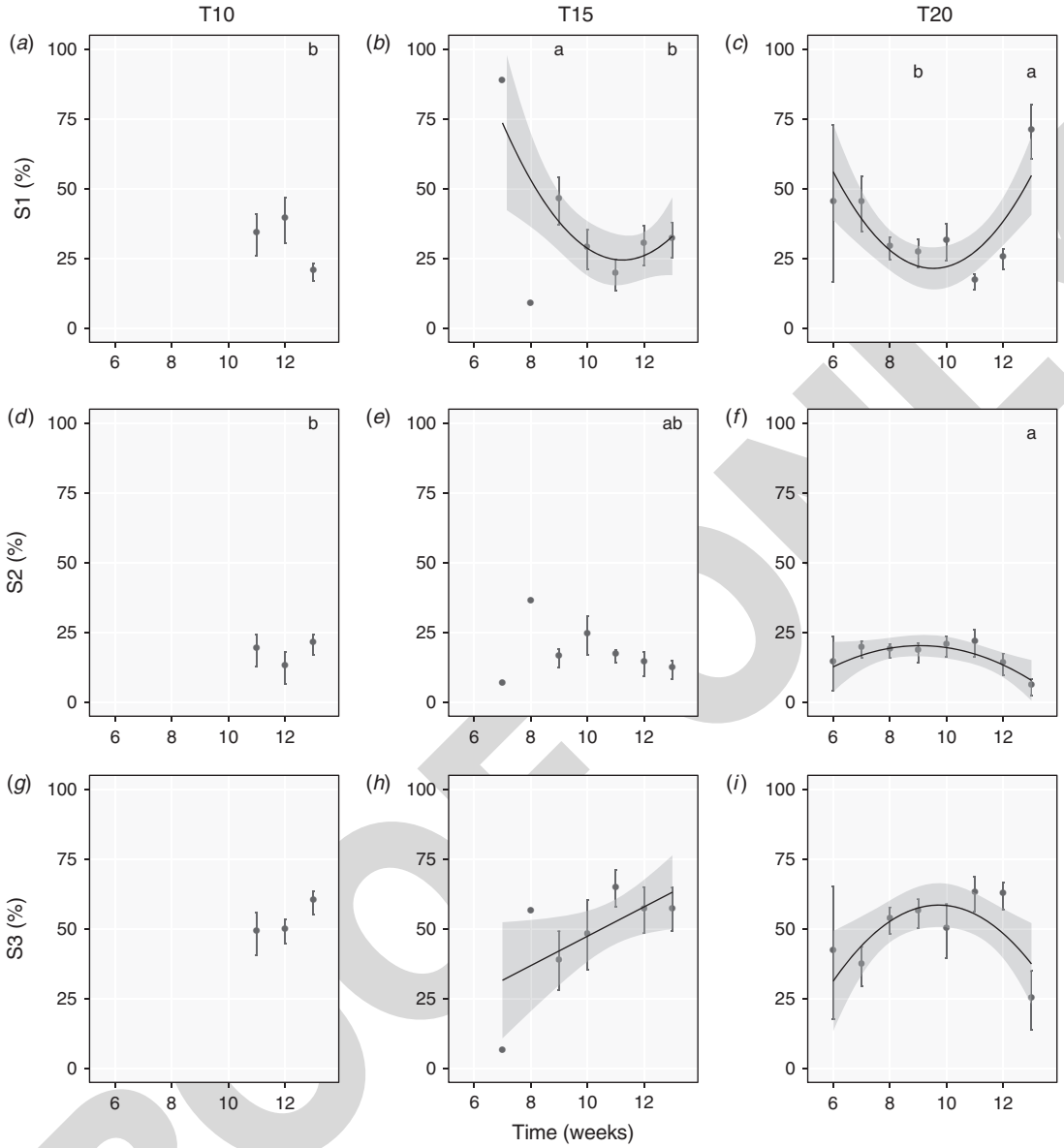

Fig. 4. Summary of the clustering analysis of Experiment 2 (water temperature), showing the proportions of subpopulation (S) 1 ('slow swimmers'), S2 ('circular swimmers') and S3 ('fast swimmers'; see Table 2) with time and within each treatment group. T10, 10°C for 6 weeks, 15°C for 3 weeks and 20°C for 6 weeks; T15, 15°C for 6 weeks and 20°C for 9 weeks; T20, 20°C for the entire experimental period. The plots show the mean ± s.e.m., the fitted model and its 95% confidence interval (confidence interval = shaded area). Different letters indicate significant differences within the same week between different treatments. For T15, the proportion of S1 fitted a negative quadratic model, whereas S3 data fitted a positive linear model. For T20, S1 data fitted a negative quadratic model, and S2 and S3 fitted positive quadratic models.

model in T15 ($R^2 = 0.12$, $F_{1,31} = 5.35$, $P = 0.027$) and a positive quadratic model in T20 ($R^2 = 0.15$, $F_{2,48} = 5.52$, $P = 0.007$). S2 ('circular swimmers') were always present in a lower proportion, and data followed a positive quadratic model in T20 ($R^2 = 0.15$, $F_{2,48} = 5.49$, $P = 0.007$; maximum by Week 9). The overall proportions of each subpopulation (32.0 ± 20.7%, 16.6 ± 10.1% and 51.4 ± 20.9% for S1, S2 and S3, respectively) did not differ significantly between temperatures.

Effects of hormonal treatments on sperm motility and subpopulations (Experiment 3)

Sperm motility developed quickly from Week 5 in the hCG and rhCG treatment groups (Fig. 5*a*, *b*), whereas eels treated with PMSG spermiated later (around Week 10) and motility increased more steeply (Fig. 5). However, there were great differences between hCG and rhCG. The hCG data fitted a cubic

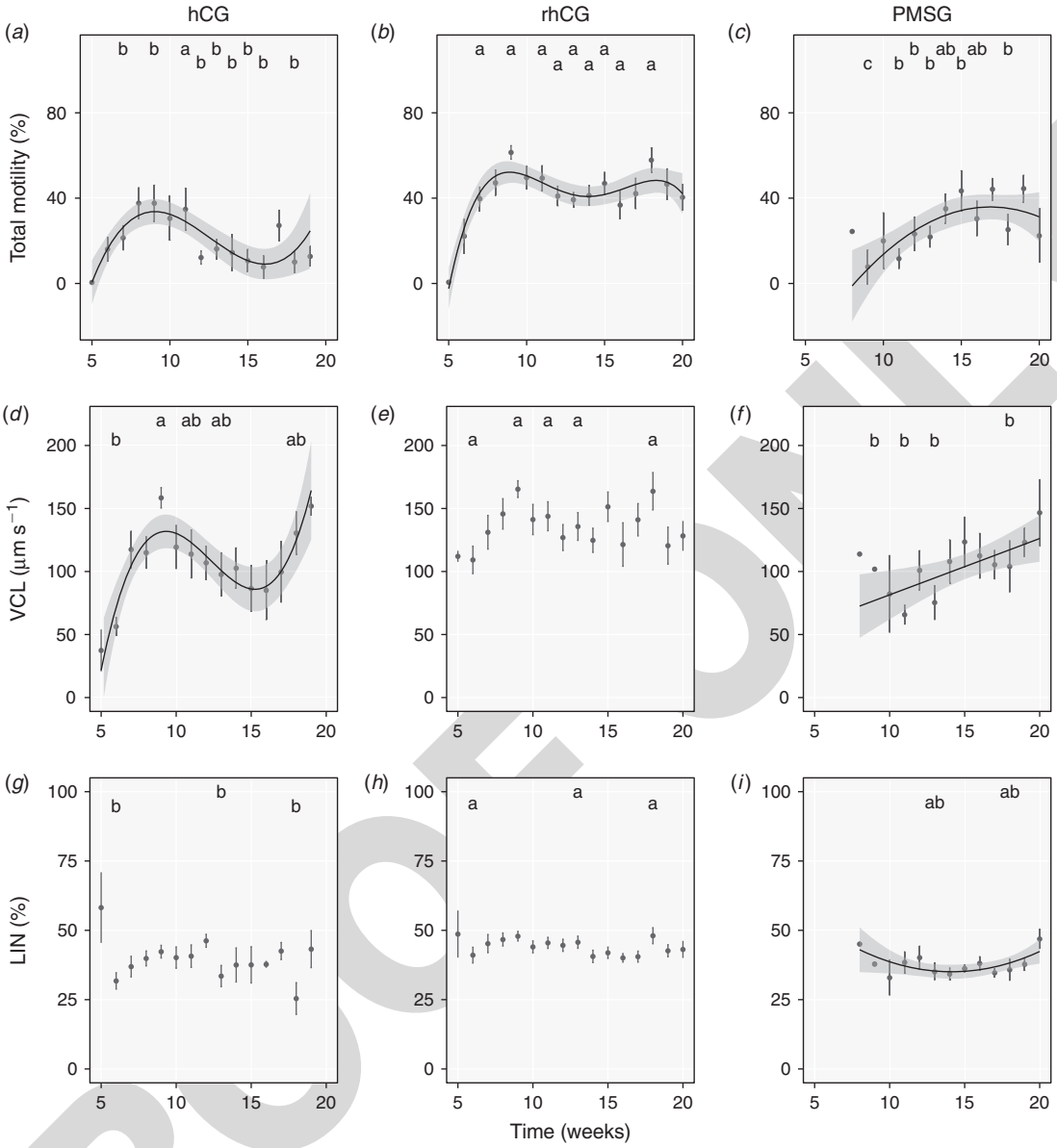

Fig. 5. Summary of the computer-aided sperm analysis (CASA) analysis for Experiment 3 (hormonal treatments; median data for total motility, curvilinear velocity (VCL) and linearity (LIN) are shown) with time and within each treatment group. The plots show the mean ± s.e.m., the fitted model and its 95% confidence interval (confidence interval = shaded area). Different letters indicate significant differences within the same week between different treatments. Human chorionic gonadotrophin (hCG) total motility and VCL data were fitted to a cubic model. Recombinant (r) hCG total motility was fitted to a quartic model. Pregnant mare's serum gonadotropin (PMSG) total motility was fitted to a quadratic model, VCL to a linear model and LIN to a quadratic model.

model ($R^2 = 0.21$, $F_{3,121} = 12.18$, $P < 0.001$), first increasing (peaking by Week 9; mean (± s.d.) 37.7 ± 25.1%) and decreasing until Week 16 (mean (± s.d.) 7.8 ± 11.6%). In contrast, in the rhCG-treated group, after an initial sharp increase (peaking by Week 9; mean (± s.d.) 61.4 ± 11.9%; $P = 0.029$ vs hCG), sperm motility stabilised. Data fitted a quartic model ($R^2 = 0.38$, $F_{4,220} = 36.03$, $P < 0.001$), with a local minimum by Week 14 (mean (± s.d.) 41.2 ± 17.8%) and a second peak by Week 18 (mean (± s.d.) 57.8 ± 20.5%; $P < 0.001$ vs hCG). Moreover, many males treated with hCG produced spermatozoa with little or no motility at all, even during the motility peak around Week 9 (25% of samples yielded <5% of total motility between Weeks 7 and 11). In contrast, only 6% of samples from the rhCG treatment group yielded <5% total motility. PMSG not only delayed spermiation, but also yielded a lower average motility than the rhCG treatment, and the variability was much higher (mean (± s.d.) 40.0 ± 24.6%; % CV 62.1% for Weeks 15–18; quadratic model $R^2 = 0.16$, $F_{2,94} = 10.46$, $P < 0.001$, with a maximum at 16.7 weeks). A linear random-effects model (using the week as a

Table 3. Subpopulations obtained from the computer-aided sperm analysis dataset obtained analysing motility data from the hormonal treatments experiment (Experiment 3)

Data are the mean ± s.d. of several kinetic parameters. In all, 98 666 motile spermatozoa obtained from 334 samples were used in the clustering analysis. VCL, curvilinear velocity; LIN, linearity (=velocity according to the straight path (VSL)/VCL); WOB, wobble (=velocity according to the smoothed path (VAP)/VCL); ALH, amplitude of the lateral movement of the sperm head; DNC, dance (VCL × ALH)

Subpopulation	VCL (μm s^{-1})	LIN (%)	WOB (%)	ALH (μm)	DNC ($\mu m^2 s^{-1}$)
S1	39.7 ± 20.0	30.5 ± 14.4	65.0 ± 12.5	1.2 ± 0.4	46.0 ± 34.5
S2	132.7 ± 86.0	15.4 ± 11.1	56.8 ± 12.2	3.0 ± 1.5	405.9 ± 425.7
S3	180.7 ± 52.6	51.5 ± 12.8	63.8 ± 8.0	3.2 ± 0.9	593.6 ± 265.7

grouping factor in the random part of the model) confirmed that the overall total motility was significantly higher for rhCG (discarding the first 2 weeks as onset of spermiation) compared with hCG and PMSG (48.9 ± 1.4% vs 37.1 ± 2.6% and 37.6 ± 2.4, respectively; $P < 0.001$).

The VCL dynamics in hCG samples (Fig. 5*d*) were similar to total motility (Figs 5*a*), fitting a cubic model with maximum at Weeks 9.5 and 15.3 ($R^2 = 0.24$, $F_{3,97} = 11.33$, $P < 0.001$). The data from the rhCG treatment (Fig. 5*e*) could not be fitted to any model, verifying the stability of these samples throughout the spermiation period and in contrast with the higher week-to-week variability in the other two treatments. The PMSG data (Fig. 5*f*) fitted a positive linear model ($R^2 = 0.09$, $F_{3,81} = 3.11$, $P = 0.020$). rhCG showed the highest overall VCL values (137.1 ± 3.2 μm s^{-1}; $P < 0.001$ vs hCG (108.5 ± 4.7 μm s^{-1}) and PMSG (106.0 ± 5.3 μm s^{-1})). Moreover, rhCG showed the highest mean (± s.d.) values at Weeks 9 and 18 (165.6 ± 6.6 and 163.7 ± 14.7 μm s^{-1}, respectively). The highest mean (± s.d.) values for hCG were 158.5 ± 8.1 and 151.9 ± 7.0 μm s^{-1} by Weeks 9 and 19, respectively, whereas that for PMSG was 146.7 ± 26.4 μm s^{-1} by Week 20. The linearity variables (LIN in Fig. 5*g–i*) behaved similarly in the different treatments (only PMSG data were fitted to a negative quadratic model, with $R^2 = 0.05$, $F_{2,83} = 3.20$, $P = 0.046$). However, rhCG also showed the highest average values compared with hCH and PMSG (44.0 ± 0.6% vs 38.7 ± 1.1% and 37.4 ± 0.9%, respectively; $P < 0.001$).

Subpopulation analysis yielded a solution very similar to the other found in previous experiments (Table 3). Again, we found a 'slow swimmer' subpopulation (S1), a 'circular swimmer' subpopulation (S2) and a 'fast swimmer' subpopulation, and the proportion of S1 and S3 was correlated with the proportion of motile spermatozoa ($r = -0.43$ ($P < 0.001$) and $r = 0.40$ ($P < 0.001$), respectively).

The dynamics of the proportion of S1 in the hCG (Fig. 6*a*) and rhCG (Fig. 6*b*) treatments resembled the inverse of the models found for total motility, fitting a cubic model ($R^2 = 0.12$, $F_{3,64} = 3.97$, $P = 0.012$) and a quadratic model ($R^2 = 0.03$, $F_{4,184} = 8.63$, $P = 0.035$), respectively. Data from PMSG (Fig. 6*c*) could not be fitted satisfactorily. On average, the rhCG samples had the lowest proportion of S1 compared with hCH and PMSG samples (24.0 ± 1.0% vs 29.9 ± 1.8% and 29.5% ± 1.9%, respectively; $P < 0.001$). Moreover, whereas the proportion of S1 in the hCG samples varied widely over the course of the sampling period (mean (± s.d.) 24.3 ± 7.0% by Week 9 to 44.2 ± 17.8% by Week 15), the changes in the rhCG samples were smaller (mean (± s.d.) 13.4 ± 12.5% by Week 8, 27.0 ± 15.1% by Week 14, 15.8 ± 10.6% by Week 18). The proportion of S2 in all treatments was low (Fig. 6*d–f*), much like in the other experiments and, except for hCG (cubic model, with $R^2 = 0.09$, $F_{3,58} = 3.01$, $P = 0.037$), the data could not be fitted to any model. Overall, the presence of this cluster was higher in PMSG (18.7 ± 1.3%) than in rhCG (14.5 ± 0.7%; $P = 0.011$), with hCG being in between (16.9 ± 1.1%). The 'fast swimmers' S3 followed a cubic model in the hCG treatment (Fig. 6*g*; $R^2 = 0.13$, $F_{3,64} = 4.33$, $P = 0.008$), mirroring the one fitted for S1, with maximum values at Week 8.9 and minimum values at Week 15.4 (the minimum and maximum values for S1 were 8.8 and 15.2, respectively). The same was observed for rhCG (Fig. 6*g*), which fitted a negative quartic model ($R^2 = 0.03$, $F_{4,180} = 3.41$, $P = 0.035$), with a predicted minimum at Week 15.1, near of the S1 predicted maximum at Week 13.9. The PMSG data for S3 (Fig. 6*i*) could not be significantly fitted. The rhCG data yielded a higher proportion of S3 overall than in the hCG and PMSG treatment groups (61.8 ± 1.3% vs 53.2 ± 1.9% and 51.7 ± 1.9%, respectively; $P < 0.001$).

Economic analysis of hormonal treatments (Experiment 3)

We calculated the cost of the hormonal treatments following Gallego *et al.* (2012; data on male weight and sperm production per week are available as Supplementary Material to this paper). In terms of the treatment as a whole, the cost per g male eel was 0.003, 0.008 and 0.004 € for hCG, rhCG and PMSG, respectively. We calculated the absolute number of SP3 spermatozoa produced in each collection attempt and used this to estimate the cost in € per 10^9 SP3 spermatozoa obtained. The distribution of the cost per week and male is shown in Fig. 7. In general, eel weight was similarly distributed in the three groups (mean (± s.d.) 80.6 ± 16.8 g), with a mean (± s.d.) weekly hormonal dose of 120.8 ± 25.3 IU per male. The total cost of the hormonal treatment for the entire experiment (21 weeks) was 97.53, 323.09 and 173.68 € for hCG, rhCG and PMSG, respectively). However, the number of SP3 spermatozoa produced in the rhCG group was much higher than in the hCG and PMSG treatment groups (mean (± s.d.) $9.52 \pm 10.95 \times 10^9$ vs $5.69 \pm 7.39 \times 10^9$ and $6.04 \pm 9.18 \times 10^9$ per sperm sample,

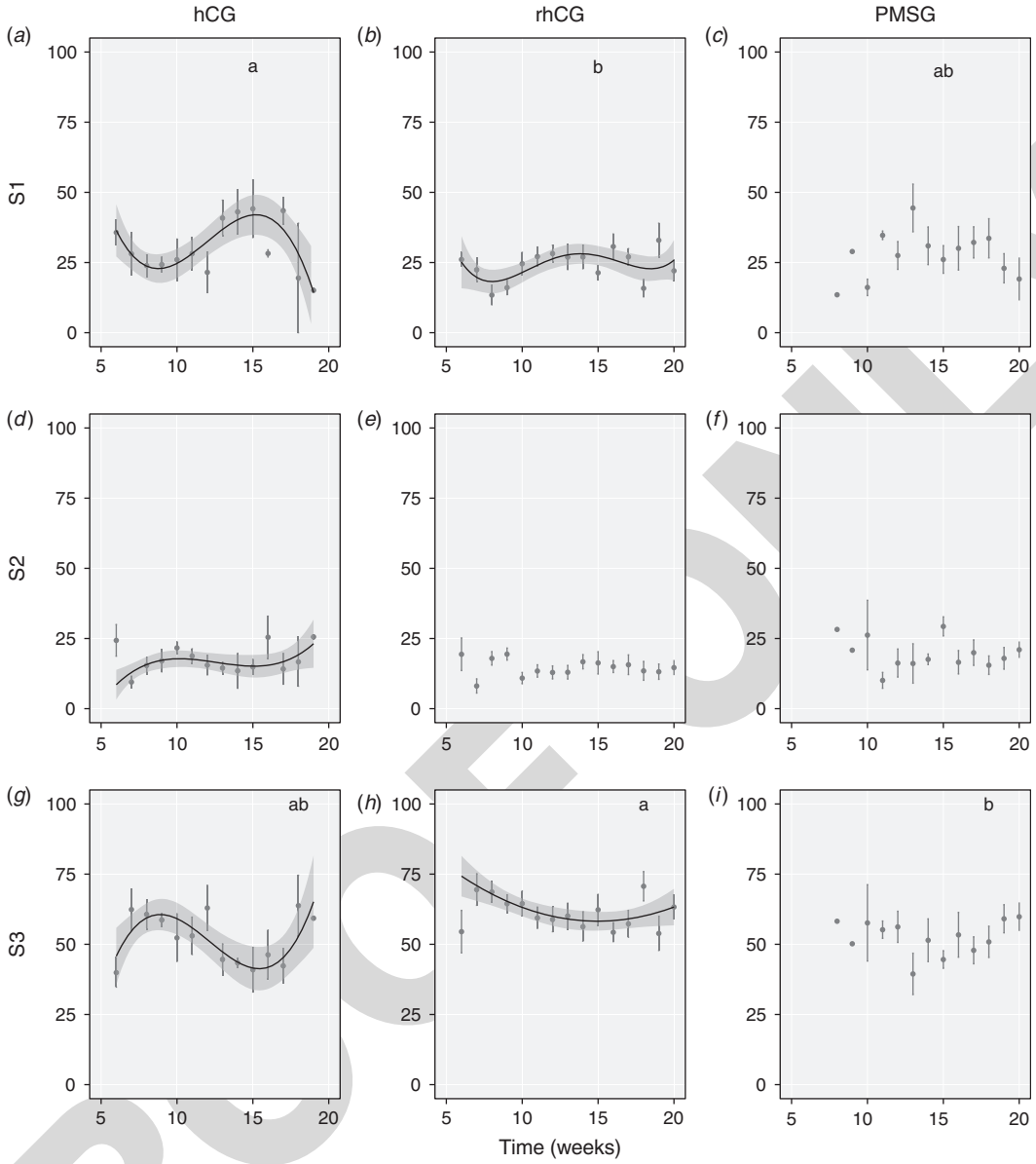

Fig. 6. Summary of the clustering analysis of Experiment 3 (hormonal treatments), showing the proportions of subpopulation (S) 1 ('slow swimmers'), S2 ('circular swimmers') and S3 ('fast swimmers') (see Table 3) with time and within each treatment group. The plots show the mean ± s.e.m., the fitted model and its 95% confidence interval (confidence interval = shaded area). Different letters indicate significant differences within the same week between different treatments. Human chorionic gonadotrophin (hCG) data fitted cubic models, whereas recombinant (r) hCG data fitted quartic and quadratic models (S1 and S3), with no fit for pregnant mare's serum gonadotropin (PMSG) data.

respectively). Thus, the return of investment was higher in the rhCG group, resulting in a lower production cost for 10^9 SP3 spermatozoa (mean (± s.d.) costs of 1.52 ± 4.78, 2.69 ± 6.93 and 3.67 ± 6.21 € for the rhCG, hCG and PMSG groups, respectively). Analysis using a linear mixed-effects model indicated that the cost of the PMSG treatment was significantly higher than that of rhCG treatment ($P < 0.001$). Differences tended to be significant when comparing PMSG and hCG ($P = 0.057$), as well as hCG and rhCG ($P = 0.091$).

Discussion

Subpopulation analysis and changes in sperm motility patterns after activation (Experiment 1)

The motility of the eel spermatozoon has been studied in detail due to the peculiar kinematics of its flagellum (Gibbons *et al.* 1985; Woolley 1998*a*). However, although several studies have used CASA to track eel spermatozoa (Asturiano *et al.* 2004, 2005; Gallego *et al.* 2012), no reports have focused on

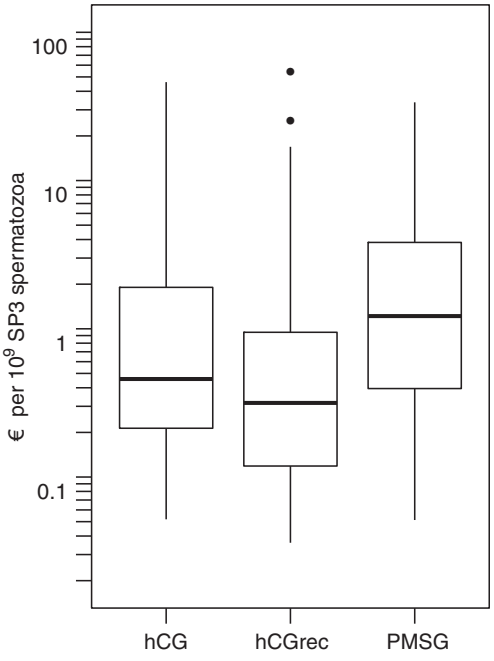

Fig. 7. Distribution of the cost of 10^9 S3 (good motility) spermatozoa in each hormonal treatment. Box plots are drawn so that boxes span from the 1st to the 3rd quartile, with the inner line showing the median, whereas whiskers span up to the extreme observations within 1.5 times the interquartile range. Observations beyond this range are drawn as dots. The box plots show the distribution of the estimated cost for individual sperm samples obtained during the spermiation period. A comparison of the three distributions show a significant difference between recombinant human chorionic gonadotrophin (rhCG) and pregnant mare's serum gonadotropin (PMSG) groups ($P < 0.001$ and $P < 0.1$ for rhCG vs human chorionic gonadotrophin (hCG) and PMSG vs hCG, respectively).

classifying the spermatozoa according to their kinematic patterns. In the present study, we found three subpopulations: 'slow and non-linear', 'fast and non-linear' and 'fast and linear'. This pattern resembles the subpopulations found in sea bream (Beirão *et al.* 2011) and sole fish (Martínez-Pastor *et al.* 2008; Beirão *et al.* 2009), with some differences regarding the 'slow' subpopulation ('slow linear' in sea bream, and 'linear' and 'non-linear' in sole two populations). A study in the three-spined stickleback reported three populations, all with a relatively high velocity (mean $>130\ \mu m\ s^{-1}$). Nevertheless, all the studies had a 'fast and linear' subpopulation and a 'fast and non-linear' subpopulation in common. It is possible that this 'fast linear' subpopulation (S3 in the present study) groups the best-quality spermatozoa, as has been suggested previously (Martínez-Pastor *et al.* 2008; Beirão *et al.* 2009). In the present study, S3 was positively correlated with total motility, implying that the samples with the highest proportion of S3 spermatozoa tended to show highest motility. With S1, the 'slow and non-linear' subpopulation, the opposite was true, being related to sperm samples with the lowest motility. As per previous studies (Woolley 1998*a*, 1998*b*), our S1 subpopulation could be connected to exhausted spermatozoa, about to stop swimming. Therefore, S1 spermatozoa would be unable to fertilise an egg (Gallego *et al.* 2012). They may also correspond to immature cells, forced out during the stripping process (Marco-Jiménez *et al.* 2006). Immature spermatozoa can present not only lower motility, but also lower resistance, losing motility earlier. S2 was the less abundant subpopulation in the three experiments. It is not clear whether the motility of S2 is an intermediate state between the S3 and S1 patterns, or it could just be a transient stage of S3 spermatozoa. Another possibility is that S2 spermatozoa were defective or immature spermatozoa, a hypothesis already posed previously (Martínez-Pastor *et al.* 2008). Unfortunately, these hypotheses could not be tested with our experimental design. We would need to follow a single S3 spermatozoon for a long time, studying changes in motility over time. However, this would require a very different CASA system, with customised hardware and software. In addition, the analysis of sperm physiology using flow cytometry and specific labels (e.g. to assess concentrations of intracellular ions relevant to motility; Gallego *et al.* 2013*b*) could help us understand sperm motility changes.

Eel spermatozoa present considerable longevity (post-activation swimming time) compared with other species. Woolley (1998*a*) and Gallego *et al.* (2013*c*) reported that eel spermatozoa showed a steady decrease in total motility after activation, and that motile spermatozoa could be obtained many minutes after activation. This contrasts with longevity in salmonids (typically <1 min), sole fish (1–2 min; Martínez-Pastor *et al.* 2008), pipefish (<5 min; Dzyuba *et al.* 2008) and sea bream (3–6 min; Zilli *et al.* 2009). Currently, we are unaware of how spawning occurs in eels, but the long duration of motility may provide clues to the biology of the spawning process. In fact, studies in other species have associated some mating strategies with the need for spermatozoa with a long period of motility (Le Comber *et al.* 2004).

We observed a slow decrease in total motility and velocity in the first 90 s of motility, in agreement with previous reports (Woolley 1998*a*; Gallego *et al.* 2013*c*, 2013*a*). Acquiring motility images at 30 s seems to be a good compromise in order to allow all the viable spermatozoa to be fully activated and providing enough time to adjust the microscope, while at the same time preventing significant changes in sperm motility relative to the 'peak' just after activation. Indeed, at 30 s we found the lowest proportion of S1 spermatozoa and the highest proportion of S3 spermatozoa. Oddly, the proportion of S1 spermatozoa increased at 60 s and decreased at 90 s, whereas that of the S2 subpopulation seemed to increase. According to our previous interpretation of subpopulation roles, S1 spermatozoa could be short lived or at least be less resilient than S3 spermatozoa. During the first 60 s, the weakest spermatozoa in the sperm sample could change their motility pattern to S1, explaining the increase in the proportion of this subpopulation from 30 to 60 s. Therefore, a subset of the S1 spermatozoa that become immotile could account for the decrease in total motility noted from 30 to 90 s, concomitantly resulting in a decrease in S1 at 90 s.

A more extensive experiment is required to confirm these changes in patterns. Our experiment was designed to test wether the subpopulation pattern of European eel spermatozoa varied significantly within the first seconds after activation to recommend an acquisition time for subsequent experiments with eel

spermatozoa. Nevertheless, follow-up research would need to study the motility patterns until a high proportion of spermatozoa have become immotile to find the best post-activation time to study eel sperm motility. Furthermore, we have not studied other important factors that could influence sperm motility and endurance. One of these factors is the activation medium, the composition of which (i.e. pH, osmolality and several cations) can enhance motility or increase the motile period (Alavi and Cosson 2005, 2006). In fact, the subpopulation pattern was altered in *Solea* spermatozoa when using an activation medium composed of salts or sucrose (Martínez-Pastor *et al.* 2008), even though osmolality was the same.

Effects of thermal treatments on sperm motility and subpopulations (Experiment 2)

The effects of thermal treatments on European eel spermiation have been discussed by Gallego *et al.* (2012). These authors highlighted that T20 not only promoted spermiation, but also that it appeared necessary for the males for remain at 20°C for least 1 week for spermiation to be induced. We wondered whether the thermal treatments could modify the subpopulation pattern. Our results suggest that T10 and T15 did not alter motility, only the onset of spermiation. In these two treatments, when the spermiation period started the sperm characteristics were similar to those of the samples obtained in T20 during its optimal period (Weeks 8–12). Our study goes deeper into that analysis by using the subpopulation data. We found that the T20 data yielded models that forecast the highest proportion of S3 and S2 spermatozoa between Weeks 9 and 10 and, consequently, the lowest proportion of S1 in that period. In the other thermal treatments, the experiment finished before enough data were obtained to fit the models satisfactorily, but our results suggest that the subpopulation dynamics would follow a similar trend to that of T20, only delayed. If we assume that the fish testicles do not produce a homogeneous sperm population (thus the presence of discernible subpopulations), then it is reasonable to propose that alterations in the spermatogenic process would result in a deeply altered subpopulational structure. Following this hypothesis, the subpopulation analysis supports our suggestion that subjecting male eels to lower temperatures in the T10 and T15 treatment groups did not alter the spermatogenesis process, but rather arrested it, even when an inductor of spermiation (hCG) was applied. It seems that the spermiation process resumed as normal when the water temperature reached 20°C.

Our results shed some light on the reproductive biology of the European eel. This species does not appear to require a previous low temperature period to activate spermatogenesis, as opposed to other fish from temperate climates (Breton and Billard 1977). The European eel seems to follow a spermiation model similar to that of species such as the Nile tilapia (*Oreochromis niloticus*). With the exception that the two species spawn in very different habitats (the Nile tilapia requires temperatures >24°C during spermiation), the Nile tilapia does not require temperature changes to trigger spermiation and spermatocyte meiosis is arrested at relatively low temperatures (Vilela *et al.* 2003). However, it would be necessary to undertake histological studies to find out the degree of similarity between the spermiation process of the tilapia and the eel. In fact, Vilela *et al.* (2003) could not confirm whether the stagnation of tilapia spermatogenesis (at 20°C) would be reversed by increasing the temperature back to above 24°C, whereas this does seem to be the case for *Anguilla*.

Effects of hormonal treatments on sperm motility and subpopulations (Experiment 3)

The choice of hormonal treatment is critical in the induction of spermiation in the eel. We found interesting patterns regarding sperm quality in the hCG- and rhCG-treated groups from the onset of spermiation (Week 5) to Week 20, when the study finished. rhCG provided a constant number of high-motility samples for most of the sampling period, and the kinematic parameters were high and mostly stable throughout the study. This contrasts with the dynamics of CASA parameters for hCG, with total motility and velocity varying much more sharply and decreasing in the last third of the study (hence the cubic model obtained for this treatment vs the quadratic model for rhCG). The reason behind the stability of the rhCG samples was the consistently low presence of S1 and S2, resulting in a high and stable S3 (the putative 'good quality' subpopulation). In contrast, in the hCG samples, S1 and S3 followed 'rollercoaster' dynamics, with S1 increasing noticeably in the second third of the experiment. Several studies have compared the efficiency of hCG and rhCG in assisted reproduction programs in humans, finding no differences between the two hormonal sources in inducing follicular maturation (Hugues 2004; Al-Inany *et al.* 2005). Nevertheless, some authors have found rhCG to be more effective in fertility programs (Papanikolaou *et al.* 2010). rhCG can be produced in high purity, with low variability between batches and with a high consistency of composition (Hugues 2004). Conversely, hCG, although cheaper, is purified from the urine of pregnant women. Not only it is more difficult to maintain batch-to-batch homogeneity, but the purified product is also actually a mixture of five isoforms (Crochet *et al.* 2012). These isoforms may have different biological activities, possibly related to the degree of glycosylation of the protein subunits. In fact, differences in the ability of hCG, rhCG and PMSG to promote spermiation in eel have been attributed to the differences in their glycosylation levels (Gallego *et al.* 2012).

Although eels have been considered synchronous spawners (Murua and Saborido-Rey 2003), the ability of artificially induced animals to produce eggs and spermatozoa for several weeks suggests that they may be group synchronous spawners. Our results with hCG and rhCG, which allowed us to obtain spermatozoa for as long as 14 weeks, support this hypothesis. The hormonal profile of the European eel during hCG-induced spermiation has been studied recently (Peñaranda *et al.* 2010*b*), and the results indicate that hCG induces the production of both 11-ketotestosterone, the major androgen in male eels, and 17,20β-dihydroxy-4-pregnen-3-one (17,20β-P), a maturation-inducing steroid (MIS). The effectiveness of gonadotropins in inducing spermiation seems to be due to their LH-like effect and the modulation of the hypothalamic–pituitary–gonadal axis. Several studies have shown that the onset of spermiation depends on a peak in LH plasma levels, which causes consecutive increases in androgen synthesis and a shift to MIS

production (Asturiano *et al.* 2000, 2002). MIS have important effects in the final phase of sperm maturation, causing sperm hydration and therefore an increase in sperm volume and testicular size (Asturiano *et al.* 2002, 2004; Peñaranda *et al.* 2010*b*). An alteration in this process may hamper spermatogenesis or hydration, resulting in the motility differences observed in the present study.

In agreement with previous studies (Asturiano *et al.* 2006, 2005; Gallego *et al.* 2012), the results of the present study indicate that hCG effectively induces spermiation, but the lower motility and changing quality observed during the spermiation period suggest that it may be less effective at sustaining spermatogenesis or sperm maturation. The heterogeneity of the composition of hCG (Hugues 2004; Crochet *et al.* 2012) could explain these observations. In fact, spermatozoa from hCG-treated males have thicker sperm heads at the beginning of spermiation, becoming thinner and longer over the course of the spermiation period (Asturiano *et al.* 2006; Peñaranda *et al.* 2010*b*). Changes in head size are related to the development of spermatogenic function, and may have important consequences on the swimming ability of the spermatozoa and their fertility (Maroto-Morales *et al.* 2010). These results could be related to the variations in the motility subpopulations detected in the present study, and especially to variations in S3. Peñaranda *et al.* (2010*b*) studied the induction of spermiation up to Week 13, observing that 17,20β-P values, which peaked at Week 5, were stable and sevenfold higher than in untreated males during Weeks 7–13, when motility and viability were at highest. This highlights the importance of MIS in achieving good sperm motility, and coincides with the lower S1 and higher S3 achieved in this experiment in the same period. However, the S1 : S3 pattern inverted after Week 13 in our hCG-treated males. We lack endocrinology data for that period, but we hypothesise that MIS synthesis may fail in the second half of the spermiation period. Conversely, rhCG may modulate the production of androgens and MIS more efficiently, maintaining levels that would allow sperm maturation and good sperm motility for the whole spermiation period. In fact, rhCG yielded 'high-quality' spermatozoa (predominance of S3) from the very beginning of spermiation, which could be due to a faster shift to MIS synthesis. These hypotheses should be confirmed by studying hormonal levels in both treatment groups and for the entire duration of the spermiation period.

Gallego *et al.* (2012) showed that PMSG was less effective than hCG or rhCG, because it delayed the onset of spermiation and resulted in fewer spermatozoa collected overall. These authors attributed their results to different rhythms of gonadal development induced by these hormones. In the present study, we found that PMSG modified the motility patterns of sperm samples. If we consider only the CASA parameters, we could interpret the PMSG models as delayed versions of the models obtained for hCG. However, the dynamics of the subpopulation patterns were more similar (at least for S3) to rhCG, although PMSG resulted in higher between-sample variability. In equids, PMSG acts as an analogue of LH, similar to hCG, but in non-equid species PMSG has a dual activity, behaving like both LH and FSH (Gordon 2004). Although we do not know the actual effects of PMSG on eels, its dual purpose in other species suggests that it could be less efficient at promoting both androgen synthesis (delaying spermiation) and MIS synthesis (resulting in a low-quality subpopulation pattern).

Economic significance of sperm subpopulation patterns

The findings of the present study could have an important impact on economic decisions. With regard to the thermal treatments, T20 is the obvious choice when it comes to obtaining the highest-quality S3 spermatozoa. The T10 and T15 treatments delayed spermiation, and thus the peak of S3, making them economically unsuitable. In terms of hormonal treatments, even though rhCG costs more than hCG or PMSG, the yield of S3 spermatozoa was clearly superior with this treatment. Our calculations demonstrate that rhCG was the most profitable option for obtaining good-quality spermatozoa (SP3). In fact, using rhCG would be even more suitable in practice, because it would allow for a higher and more stable production of good-quality spermatozoa for an extended period. All these properties are desirable in the development of reproductive programs to be applied in eel farms in the future.

Conclusions

In the present study we distinguished three subpopulations from European eel sperm samples. One of them, S3, grouped fast and mostly linear spermatozoa, and their presence may be related to good-quality samples. In addition, eel sperm motility varies with post-activation time, likely affecting the subpopulation pattern. This means that it would be wise to set a fixed time to acquire motility data, preferably 30 s after activation.

Regarding the induction of spermiation, we have confirmed that a water temperature $<20^{\circ}C$ delays the onset of spermiation, but it may not affect the subpopulation structure once spermiation has started. Nevertheless, the choice of hormonal treatment to induce spermiation affected the subpopulation pattern and its dynamics throughout the spermiation period. rhCG allowed both sustained high motility and a high proportion of S3 spermatozoa. It may be the most economical option, although it would depend on the development of egg fertilisation protocols, allowing us to fully take advantage of the availability of high-quality samples obtained after rhCG treatments.

Acknowledgements

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