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Title: The subpopulation pattern of eel sperm is affected by post-activation time, hormonal treatment and thermal regime

Running head: Subpopulations in eel sperm

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Abstract

Natural stocks of eels (genus *Anguilla*) have suffered a dramatic reduction in the last 60 years, and aquaculture is based in the capture of huge quantities of juveniles. It is necessary closing the life cycle in captivity to lift the pressure on wild populations. We have aimed at the evaluation of sperm subpopulations (cluster analysis of computer-assisted sperm analysis —CASA— data) in European eel (*Anguilla anguilla*), assessing the effects of acquisition time (30, 60 and 90 s post-activation), thermal regimes (variable: T10 and T15, constant: T20) and hormonal treatments (hCG, hCG_{rec} or PMSG). In all cases, we obtained three subpopulations: low velocity and linearity (S1), high velocity and low linearity (S2) and high velocity and linearity (S3, considered high-quality). Total motility and S1 were affected by acquisition time, thus recommending 30 s. T20 data fitted quadratic models, with the highest motility and S3 between weeks 8 and 12 after the first injection. T10 and T15 delayed spermiation and the obtention of high-quality sperm (S3), but did not seem to alter the spermiation process (similar subpopulation pattern). Hormonal treatments differed greatly both on the onset of spermiation (PMSG delaying it) and on the dynamics of the subpopulation pattern. Motility and S3 yield of the widely used hCG were very variable. However, hCG_{rec} allowed to obtain good motility for most of the study (weeks 7 to 20), and S3 yield was overall higher (61.8%$\pm$1.3) and more stable along time than the other hormonal treatments (averaging 53.0%$\pm$1.4). Economically, T20 and hCG_{rec} were more effective, allowing to obtain a higher number of S3 spermatozoa for an extended time.

Keywords: European eel, sperm, motility activation, CASA, subpopulation analysis

Introduction

The genus *Anguilla* contains many species of great commercial importance, but wild stocks have been depleted. This has been due to overfishing (both of glass eels and reproductive eels) and other factors such as 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 witnessed yet); leptocephali larvae drift until they reach coastal waters; they metamorphose into glass eels and move inland while they develop into elver and yellow elver stages; after that, they mature to silver eels (the whole growth process could take years to decades), which are capable to recognize its way to the spawning areas, where they fully mature, spawn once and die (Ginneken and Maes, 2005). The complexity of this cycle has contributed to the difficulty of replicating it in captivity. Therefore, although an increasing proportion of eels are farm-raised, the stocks are obtained by capturing huge numbers of glass eels, which are then cultured until they reach commercial size (Feunteun, 2002; Halpin, 2007). Given the commercial, socio-cultural and ecological value of these species, breeding eels in captivity —effectively closing its life cycle in the fish farms— represents a major objective for researchers. Achieving it would greatly benefit not only the commercial use of the species, but it would also lift the pressure on natural populations, and it could even be applied to their restocking within conservation programs. Some success have been reported regarding the obtention and conservation of gametes, artificial fertilization and larval rearing (Tanaka et al., 2003; Asturiano et al., 2004; Peñaranda et al., 2010a), but efficient production of glass eels is still unattained (Okamura et al., 2007).

Among the many challenges to achieve in order to efficiently replicate the eel life cycle in captivity, a major milestone is obtaining spermatozoa with high fertility potential at the right time and for an extended period (Mañanós et al., 2008). Given the complexity of factors affecting eel spawning and the lack of knowledge about it, the only option for artificially inducing maturation and spermiation in eel is to applying hormonal treatments based in gonadotropins (Miura et al., 2002). Whereas human corionic gonadotropin has been the chosen hormone for many years, Gallego et al. (2012), working with European eel (*Anguilla anguilla*), showed that the recombinant hormone (hCG_{rec}) yielded better results and, even though its price is higher, it could represent a more profitable option. They also considered water temperature in their study, in an attempt to mimic the temperature changes that adults might undergo before spawning. Water temperature can affect the reproductive biology of fishes, at least in temperate climates (Pankhurst and Porter, 2003). Since eels migrate considerable distances and they seem to carry out this migration at different depths (Aarestrup et al., 2009), Gallego et al. (2012) tested three themal regimes (from 10 °C or 15 °C to 20 °C vs. constant 20 °C), aiming at a more physiological approach to sexual maturation (Pérez et al., 2011). Results showed that hormone-treated males could produce sperm

only after spending at least 1 week at 20 °C.

Gallego et al. (2012) focused on the proportion of spermiating males along the treatments, sperm volume and average motility parameters provided by CASA (Computer Assisted Sperm Analyzer). Here we present another approach to study the eel sperm motility. First, we analyzed the data using polynomial regression (Quinn and Keough, 2002), since previous results suggested that at least part of the experimental data could follow low-order polynomial models. Our aim was not to obtain a best-fit model to use it for interpolation, but rather to find which kind of linear regression model could fit better each case while making biological sense, helping to compare treatments and to obtain information on the evolution of eel spermiation process. This approach has been successful to interpret results in previous studies on spermatology (Fernández-Santos et al., 2007; de Paz et al., 2012). Second, we aimed at taking into account the within-sample heterogeneity that CASA data conveys, using the median values (not the means, very sensitive to extreme values) for studying more reliably the kinematic parameters.

Moreover, we have taken advantage of the potential of CASA data (Holt et al., 2007), classifying the spermatozoa within each sample according to their kinematic characteristics. This requires multivariate techniques such as cluster analysis (Martínez-Pastor et al., 2011). Kinematic parameters are used to group spermatozoa into subpopulations, allowing to characterize the samples not anymore by the average values of CASA parameters, but by the relative proportions of each subpopulation. This approach promises a deeper understanding of the inner dynamics of the sperm sample, since its intrinsic heterogeneity is taken into account (Holt and Harrison, 2002; Martinez-Pastor et al., 2005a). Subpopulation analysis has been applied in a few studies in fishes: 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, 3–4 subpopulations of spermatozoa were identified, one of them being defined as more desirable (containing fast and linearly motile cells) (Beirão et al., 2009; Martínez-Pastor et al., 2008).

In the present study, we have adapted an unsupervised cluster analysis from previous studies on sperm classification (Martinez-Pastor et al., 2005b; Martínez-Pastor et al., 2008; Domínguez-Rebolledo et al., 2011), in order to discover the subpopulation structure of European eel sperm, and to apply this information to improve our knowledge on the effect of thermal and hormonal treatments on the spermatogenesis and sperm quality in this species. Since there is no prior knowledge about the subpopulational structure of eel spermatozoa, we performed a previous cluster analysis on sperm samples obtained following a standard protocol, at different times after activation. With this approach, we aimed at testing 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 the 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 in the fish farm Valenciana de Acuicultura, S. A. (Puzol, Valencia, Spain) and transported to our facilities in the Aquaculture Laboratory at the Universitat Politècnica de València (Valencia, Spain), where they were gradually acclimatized to sea water over the course of 1 week (salinity 37.0±0.3 g/L, temperature at 20 °C). The fish were distributed in 200-L aquaria equipped with separate recirculation systems and thermostats and coolers to strictly control water temperature. No feeding was provided during the duration of the experiments. Before the intraperitoneal administration of hormones to induce spermiation, the animals were weighed and anesthetized with benzocaine (60 ppm).

Experiments

Experiment 1:Changes in sperm motility patterns after activation

Males (n=9) received weekly intraperitoneal injections of human chorionic gonadotropin (hCG; 1.5 IU/g b.w.; Argent Chemical Laboratories, USA) diluted in saline solution (0.9% NaCl) . Sperm recovered between weeks 8–11 after the first injection (higher quality according to previous studies (Asturiano et al., 2006; Gallego et al., 2012)) was used in this experiment. In total, 19 samples were recovered and subsequently analyzed for motility. In the motility analysis, image sequences were acquired at 30, 60 and 90 s after activation. Data were analyzed to determine the effect of post-activation time on motility parameters and subpopulation patterns.

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

A total of 317 adult male eels (mean body weight 100±2 g; mean length 40±5 cm) were equally and randomly distributed in six 200-L aquaria around 100 males in each treatment) and subjected to three thermal regimes: T10, 10 °C (first 6 weeks), 15 °C (next 3 weeks) and 20 °C (last 6 weeks); T15, 15 °C (first 6 weeks) and 20 °C (last 9 weeks); and T20, 20 °C during the whole experimental period. All the males were hormonally treated for the induction of maturation and spermiation with weekly intraperitoneal injections of human chorionic gonadotropin (hCG; 1.5 IU/g b.w.) for 13 weeks.

Experiment 3: Effect of hormonal treatment on sperm motility patterns

Three groups of males (18 males per treatment) were assigned to three hormonal treatments in different 200-L tanks at 20 °C: hCG, hCG_{rec} (recombinant hCG; Ovitrelle, Madrid) and PSMG (pregnant mare's serum gonadotropin; Sincropart, Lab CEVA, Barcelona). Every week, all males received 1.5 IU/g b.w., all hormones being diluted with the same volume of saline (0.9% NaCl). This experiment was carried out for 20 weeks.

Sperm collection

Sperm samples were collected weekly 24 h after administering the hormonal treatment, in order to achieve the highest sperm quality (Pérez et al., 2000). Fish were anesthetized and the genital area was cleaned with freshwater and thoroughly dried to avoid the contamination with feces, urine or sea water. Sperm was forced out by abdominal pressure. A modified aquarium air pump allowed to obtain a vacuum to collect the sperm in a clean tube. Samples were kept at 4 °C until analysis. Sperm concentration was measured with a Thoma hemocytometer after diluting the samples 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., 2010a).

CASA analysis

Sperm motility was analyzed according to standardized conditions for European eel spermatozoa (Gallego et al., 2013). The CASA system was composed by a triocular optical phase contrast microscope (Eclipse E-400; Nikon, Tokio, Japan), with a $\times$10 negative contrast phase lens and an ISAS 782M camera at 60 fps, connected to a computer by an IEEE 1394 interface. For activating the motility, 2 μL of sperm were diluted in 200 μL of artificial sea water (Aqua Medic Meersalz, 37 g/L, 2% BSA (w/v), pH 8.2 Peñaranda et al. (2010a)), and 4 μL of this dilution were charged in a SpermTrack-10® chamber (Proiser R+D S.L., Paterna, Spain). At exactly 30 s post-activation, images were acquired during 1 s at 60 frames/s using the ISAS v. 1.2 software (Proiser, Paterna, Spain). The software was configured with 2 to 20 μm^2 for head area and VCL $>$ 10 μm/s to classify a spermatozoon as motile. The software yielded the following parameters for each spermatozoa: three velocity parameters (VCL: velocity according to the actual path; VSL: velocity according to the straight path; VAP: velocity according to the smoothed path), three track linearity parameters (LIN: linearity; STR: straightness: WOB: wobble), the ALH (amplitude of the lateral displacement of the sperm head), the BCF (head beat-cross frequency), Dance and Dance Mean (measurements of the pattern of sperm motion) (Boyers et al., 1989). In Experiment 1, images were acquired at 30, 60 and 90 s.

Subpopulation and statistical analyses

Subpopulation and statistical analyses were carried out using the R statistical environment (R Development Core Team, 2012). First, motility data were processed to remove events appearing in less than 50 consecutive frames (broken or lost tracks, or tracks resulting from spermatozoa entering or leaving the field while acquiring). Samples with too few total spermatozoa or too few motile spermatozoa (less than 30) were removed from the subpopulation analysis to prevent the apparition of spurious clusters. Data were processed to obtain the total motility of each sample, defined as the relation between motile spermatozoa (VCL>10 μm/s) and the total number of spermatozoa $\times$100, as well as the median values of each of the motility variables. These data were used for conventional motility analysis.

Subpopulation analysis was carried out separately in each of the three datasets resulting from the three experiments. The variables used in the clustering steps were chosen by perforimng a hierarchical clustering of the motility variables (more similar variables, conveying similar information and thus being redundant, were clustered together), using the Hoeffding D statistic as a measure of similarity. The selected variables were transformed and standardized before starting the actual clustering process.

This procedure was a modification of a two-step methods proposed previously (Martinez-Pastor et al., 2005b; Domínguez-Rebolledo et al., 2009). Two hierarchical clustering steps were used consecutively, and the reliability and stability of the solutions were checked as recommended (Martínez-Pastor et al., 2011). In the first step, the observations (spermatozoa) belonging to each individual sample were classified using an algorithm for agglomerative nesting processing (AGNES) (Kaufman and Rousseeuw, 1990), a kind of hierarchical clustering algorithm (using euclidean metric and Ward's clustering method). The number of clusters (k) was decided based on the Silhouette information for each $k = [2, 8]$, choosing a k such that the Silhouette average width was maximized. These clusters were used as observations for a second step using the same clustering method. The cluster assignments obtained in the second step were lined up with the original clustering. Finally, each sample was characterized according to the relative proportion of each cluster (subpopulations).

Hypothesis testing on motility and clustering results were 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 analyzed by using linear models and ANCOVA, considering week as a covariate and either temperature or hormonal treatment as fixed factors. In the case of week, a polynomial effect was suspected, and therefore quadratic, cubic or quartic models were tested. When needed, pairwise comparison among the levels of fixed factors were carried out by using Tukey's correction. Results are presented as mean$\pm$SEM except if otherwise stated.

193 *Economical analysis of the hormonal treatments (Experiment 3)*

194 Each hormonal treatment has a different cost, depending on the hormone price, the number of doses
195 required and the volume of hormone injected (which depends on the weight of the male) (Gallego et al.,
196 2012). In the present study, we focused on the results of the subpopulation analysis, estimating the cost of
197 producing 10^9 spermatozoa belonging to the highest-quality subpopulation. We have to take into account
198 that male eels must be treated for several weeks before they start spermiating. That offset period was
199 taken into account, calculating the total cost for each male within each treatment, and then estimating a
200 corrected cost only for the weeks they were spermiating. Therefore, we obtained a relative price of the
201 high-quality spermatozoa for each male and each week, which was used to relate the level of investment
202 of each hormonal treatment with the amount of good quality sperm obtained.

203 **Results**

204 *Changes in sperm motility patterns after activation (Experiment 1)*

205 The average motility of the eel spermatozoon (mean±SD) was characterized (30 s post-activation) by
206 being fast (VCL: 149.4±33.3 μm/s), slightly circular (LIN: 43.6%±7.2), and little erratic (STR:
207 71.3%±10.1; WOB: 62.8%±2.8). Motility decreased at subsequent times, although the change was not
208 dramatic (Fig. 1). Total motility (Subfigure 1a) reached a mean value of 63.2%±2.3 for the first
209 measurement at 30 s, decreasing gradually afterwards ($P<0.05$). The variables related to motility vigor,
210 such as velocity (VCL, Subfigure 1b), ALH (Subfigure 1e) and Dance (Subfigure 1f) followed the same
211 trend, although the decrease slowed down between 60 s and 90 s, resulting in no significant differences
212 between these two times. The variables related to track shape (as LIN and WOB, subfigures 1c and 1d)
213 were not affected by acquisition time ($P<0.05$). These results suggest not only a decreasing proportion of
214 motile spermatozoa with time, but also a decreasing ability to maintain vigorous motility. Trajectory
215 shape would not be affected, though.

216 Subpopulation analysis of raw data were carried out successfully, resulting in three
217 subpopulations. Table 1 summarizes the subpopulation structure. Subpopulation 1 (S1) was defined as a
218 subset of slow spermatozoa, with circular but regular trajectories and low vigor. Contrarily, subpopulation
219 2 (S2) grouped fast spermatozoa, with circular or erratic trajectories and with high vigor. Subpopulation 3
220 (S3) also contained fast and vigorous spermatozoa, with rather linear tracks. The average proportions of
221 these subpopulations at 30 s (mean±SD) were 26.0%±15.5 for S1, 12.0%±14.3 for S2 and 62.0%±17.3
222 for S3. These proportions changed little during the acquisition process (Fig. 2). Interestingly, the
223 proportions of S3 ("fast swimmers") correlated positively with the proportion of motile spermatozoa
224 ($r=0.32$, $P=0.016$), whereas S1 ("slow swimmers") correlated negatively, although not reaching

225 signification in this experiment (r=-0.23, P=0.084). This cluster pattern reappeared when we analyzed
226 data from the other experiments.
227 S1 proportion was significantly higher at 60 s than at 30 s, decreasing 90 s, while S2 followed this
228 trend in reverse (with no significant differences between times). S3 ("good swimmers"), due to its highest
229 proportion, had the highest impact defining the average characteristics of sperm motility described
230 previously. The proportion of this subpopulation changed little with time, although its proportion was
231 lower at 60 and 90 s (58.2%±2.6), reflecting on the average VCL, ALH and DNC at these times.

232 *Effect of the thermal treatments on sperm motility and subpopulations (Experiment 2)*

233 The onset of spermiation at each temperature occurred at different weeks after the beginning of the
234 experiment, conditioning the analysis of sperm motility. Each experimental group behaved differently
235 regarding the kind of model fitting the data. In general, motility data yielded by T10 and T15 fitted a
236 first-grade polynomial (simple linear models), while data yielded by T20 fitted a second-grade
237 polynomial (quadratic model). Nevertheless, the data suggests that T10 and T15 might actually follow
238 quadratic models (motility decreasing after week 13), but it could not be confirmed because this
239 experiment could not be continued beyond week 13.
240 The models analyzed in this experiment included interactions between time (week) and thermal
241 treatment, which were significant in most of the cases. Therefore, the effect of week and treatment were
242 analyzed separately. The proportion of motile spermatozoa (subfigures 3a, 3b, 3c) was very low at the
243 beginning of the spermiation (2.5%±4.8, overall mean±SD; onset at week 10 for T10 and week 5 for
244 T15 and T20), but reached an overall mean±SD of 53.0%±22.6 at the peak of each treatment. T20
245 reached its maximum between weeks 8 and 11 (overall motility: 54%±3.2; predicted maximum at week
246 10), whereas the maxima for groups T10 and T15 were reached only at week 13, at the end of the study.
247 T20 motility was significantly higher than T15 at weeks 8 and 10. While T20 showed a decreasing trend
248 after week 11, T10 rose quickly from week 10 to week 13, reaching a mean value of 65.6%±6.6. This
249 value is similar to the highest one of T20 (66.8%±3.3 at week 11), indicating that the peak of the T10
250 treatment could be near week 13, and that in this group the motility peak was reached very quickly —4
251 weeks—, comparing to the 6 weeks of T20. A linear random-effects model (using the week as grouping
252 factor in the random part of the model) confirmed that the overall total motility was significantly higher
253 for T20 (37.3%±3.1 vs. T10: 29.0%±5.3 and T15: 25.3%±3.3; P<0.001).
254 Kinematic parameters followed a similar trend. VCL is displayed in subfigures 3d, 3e and 3f. In
255 this case, no model fitted significantly the data for T10 (due to lack of weeks with data), while T15 data
256 increased linearly, whereas T20 followed a quadratic model, with maximum between weeks 9 and 10
257 (predicted: 9.6). The other velocity parameters and ALH showed a similar trend. For parameters defining

the shape of the trajectory (LIN displayed in subfigures 3g, 3h, 3i) both T15 and T20 data fitted a quadratic model (maximum by week 10), indicating that sperm tracks became more lineal by the middle of the treatment. Nevertheless, the variation along time was low, contrarily to the wider range showed by other variables. The overall values of motility variables were not significantly different between temperatures, although there were significant differences between treatments in several weeks. These data suggest that thermal treatments caused an offset of spermiation onset, T10 and T15 delaying it respect to T20.

The cluster analysis produced three subpopulations from the thermal experiment data (Table 2), resembling those obtained in Experiment 1. Subpopulation 1 (S1) grouped slow spermatozoa with low vigor ("slow swimmers"), albeit linearity parameters were intermediate between those of S2 and S3. Subpopulation 2 (S2) included relatively fast and vigorous spermatozoa, with circular trajectories ("circular swimmers"). Subpopulation 3 (S3) contained fast and vigorous spermatozoa, following more linear tracks ("fast swimmers"). Moreover, similarly to Experiment 1, S1 and S3 correlated with total motility (S1: r=-0.46, S3: r=0.41; P<0.001).

Very much alike the median motility parameters, the proportion of each subpopulation was highly affected by the week in the spermiation period. We could no detect a valid fit in T10 for any cluster (Figure 4), due to the between-male variability and the low number of spermiating weeks in the studied period (mean±SD of each subpopulation in T10 were S1: 17.0%±10.7, S2: 30.5%±15.4; S3: 52.5%±11.9). Concerning S1 (subfigures 4a, 4b,4c), the data were significantly fitted to a negative quadratic model for T15 and T20, 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, while it presence decreased by the middle of that period. S3 ("fast swimmers") trend seemed opposite (Subfigure 4h), which followed a positive lineal model in T15 and a positive quadratic model in T20. S2 ("circular swimmers") was always present in a lower proportion, and data followed a positive quadratic model in T20 (maximum by week 9). The overall proportions of each subpopulation (S1: 32.0%±20.7; S2: 16.6%±10.1; S3: 51.4%±20.9) were not significantly different between temperatures.

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

Sperm motility developed quickly after week 5 in the hCG and hCG_{rec} treatments (subfigures 5a and 5b), whereas eels treated with PMSG spermiated later (around week 10) and motility increased more steeply (Fig. 5). However, there were great differences among hCG and hCG_{rec}. hCG data fitted a cubic model (P<0.001), following with a first increase (peaking by week 9, 37.7%±25.1, SD), and decreasing up to week 16 (7.8%±11.6, SD). Contrarily, hCG_{rec}, after an initial sharp increase from week 5 to week 7 (peaking by week 9, 61.4%±11.9, SD; P=0.029 comparing to hCG), followed a more stable trend, with

data fitting a cuartic model (P<0.001), predicting a local minimum by week 14 (decreasing to 41.2%±17.8, SD), and a second peak by week 18 (57.8%±20.5, SD; P<0.001 comparing to hCG). We must take into account that, while many males treated with hCG produced sperm with little or no motility at all, even during the motility peak around week 9 (25% of samples yielded less than 5% of total motility between weeks 7 and 11), only 6% of samples from males treated with hCG_{rec} yielded less than 5% of total motility. PMSG not only caused a later spermiation, but also yielded a lower average motility than the hCG_{rec} treatment, and the variability was much higher (mean±SD of 40.0%±24.6, SD, and %CV of 62.1% for weeks 15–18; quadratic model, P<0.001, predicting a maximum at 16.7 weeks) A linear random-effects model (using the week as grouping factor in the random part of the model) confirmed that the overall total motility was significantly higher for hCG_{rec} (discarding the first two weeks as onset of spermiation: 48.9%±1.4 vs. hCG 37.1%±2.6 and PMSG 37.6%±2.4, P<0.001).

Kinematic parameters were similar among treatments, but significantly higher for hCG_{rec} overall. VCL in hCG samples (Subfig. 5d) followed a dynamics similar to total motility (Subfigure 5a), fitting a cubic model with predicted maxima at weeks 9.5 and 15.3. The samples from the hCG_{rec} treatment showed a high dispersion (Subfigure 5e), and no model could be fitted to the data, whereas for PMSG (Subfigure 5f), data were fitted to a positive linear model. Despite the high variability, hCG_{rec} showed the highest average VCL values (137.1±3.2 μm/s, P<0.001 comparing to hCG with 108.5±4.7 μm/s and PMSG with 106.0±5.3 μm/s). Moreover, hCG_{rec} showed the highest average values at weeks 9 (165.6±6.6 μm/s, SD) and 18 (163.7±14.7 μm/s, SD). The highest values for hCG were 158.5±8.1 μm/s (SD) by week 9 and 151.9±7.0 μm/s (SD) by week 19, and for PMSG was 146.7±26.4 μm/s (SD) by week 20. Linearity variables (LIN in subfigures 5g, 5h and 5i) were much more alike throughout the study (only PMSG data could be fitted, yielding a negative quadratic model for LIN). Nevertheless, hCG_{rec} showed again the highest average values (44.0%±0.6 vs. hCG 38.7±1.1 and PMSG 37.4%±0.9, P<0.001).

Subpopulation analysis yielded a solution very similar to the one found for the thermal treatments experiment (Table 3). Again, we found a “slow swimmers” subpopulation (S1), a “circular swimmers” subpopulation (S2) and a “fast swimmers” subpopulation. Again, the proportions of S1 and S3 correlated with the proportion of motile spermatozoa (S1: r=-0.43, P<0.001; S3: r=0.40, P<0.001).

The dynamics of the proportion of S1 in samples from hCG (Subfig. 6a) and hCGrec (Subfig. 6b) males resembled the inverse of the models found for total motility, fitting a cubic model and a quadratic model, respectively. Data from PMSG males (Subfig. 6c) could not be fitted satisfactorily to a low-order polynomial. In average, samples in the hCG_{rec} group showed a lower proportion of S1 (24.0%±1.0 vs. hCG 29.9%±1.8 and PMSG 29.5%±1.9, P<0.001). Moreover, whereas the proportion of S1 in hCG samples varied widely throughout the sampling period (24.3%±7.0 by week 9 to 44.2%±17.8 by week

15; SD), the changes within hCG_{rec} samples were smaller (13.4%±12.5 by week 8, 27.0%±15.1 by week 14, 15.8%±10.6 by week 18; SD). S2 proportion in all treatments was low (subfigures 6d, 6e and 6f), much like in the other experiments and, except for hCG (cubic model), the data could not be fitted to any model significantly, due to the between-male variability and that differences among weeks and treatments were small. Overall, the presence of this cluster was higher in PMSG samples (18.7%±1.3) than in hCG_{rec} (14.5%±0.7, P=0.011), being hCG in between (16.9%±1.1). The "fast swimmers" S3 followed a cubic model in hCG (Subfig 6g), mirroring the one fitted for S1 , with predicted maximum at week 8.9 and minimum at week 15.4 (predicted minimum and maximum for S1 were 8.8 and 15.2, respectively). This inverse relationship was also suggested for hCG_{rec} (Subfig. 6g), which was fitted to a negative quadratic model , with a predicted minimum by week 15.1, near of the S1 predicted maxima by week 13.9. PMSG data for S3 (Subfig. 6i) was fitted to a quadratic negative model , with a minimum by week 14.7. Samples in the hCG_{rec} group presented a higher S3 proportion overall (61.8%±1.3 vs. hCG 53.2%±1.9 and PMSG 51.7%±1.9, P<0.001).

Economical analysis of the hormonal treatments (Experiment 3)

We calculated the cost of the hormonal treatments following Gallego et al. (2012). Considering the full treatment, the cost per gram of male eel was 0.003 € for hCG, 0.008 € for hCGrec and 0.004 € for PMSG. We calculated the absolute number of SP3 spermatozoa produced in each collection attempt, using it to estimate the cost in € per 10^9 SP3 spermatozoa obtained. The distribution of the cost per week and male is shown in the Figure 7. In general, eel weight was similarly distributed in the three groups (mean±SD: 80.6±16.8 g), with an average weekly hormonal dose of 120.8±25.3 IU (mean±SD) per male. The total cost of the hormonal treatment for the whole experiment (21 weeks) were: 97.53 € for hCG, 323.09 € for hCG_{rec} and 173.68 € for PMSG. However, the number of SP3 spermatozoa produced in the hCG_{rec} group was much higher than in the other treatments: 9.52±10.95 $\times 10^9$ per sperm sample, vs. 5.69±7.39 $\times 10^9$ in hCG and 6.04±9.18 $\times 10^9$ in PMSG (mean±SD). Thus, the investment return was higher in the hCG_{rec} group, resulting in a lower cost for producing 10^9 SP3 spermatozoa: 1.52±4.78 € for hCG_{rec} (mean±SD) vs. 2.69±6.93 € for hCG and 3.67±6.21 € for PMSG. An analysis using linear mixed-effects model indicated that the cost was higher for PMSG comparing to hCG_{rec} with P<0.001. Differences tended to be significant when comparing PMSG vs. hCG (P=0.057) and hCG vs. hCG_{rec} (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, 1998a). However, although several studies have used CASA to track eel spermatozoa (Asturiano et al., 2004, 2005; Gallego et al., 2012), no reports have aimed at classifying the spermatozoa according to their kinematic patterns. In this study, we have found three subpopulations: the "slow and non-linear", "fast and non-linear" and "fast and linear". This structure resembles the subpopulations found in seabream (Beirão et al., 2011) and sole fish (Beirão et al., 2009; Martínez-Pastor et al., 2008), with some differences regarding the "slow" subpopulation ("slow-linear" in sea bream, and in sole two populations were obtained: "linear" and "non-linear"). A study with three-spined stickleback reported three populations, all of them of relatively high motility (mean higher than 130 μm/s). Nevertheless, all the studies have in common a "fast and linear" subpopulation and a "fast and non-linear" one. This "fast-linear" subpopulation (S3 in our study) seems to group the best quality spermatozoa. This has been suggested previously (Beirão et al., 2009; Martínez-Pastor et al., 2008), and in our study S3 correlated positively with total motility. That is, the sperm samples with the highest proportion of motile spermatozoa tended to have the highest proportion of S3 spermatozoa. The opposite happened with S1, the "slow and non-linear" subpopulation, which was related to the sperm samples with the lowest motility. Indeed, agreeing to previous studies (Woolley, 1998a,b), our S1 subpopulation seems to be related to exhausted spermatozoa, which would be about to lose motility, and therefore they would be unable to fertilize the egg due to their low velocity and linearity (Gallego et al., 2012). S1 spermatozoa might also correspond to immature ones, forced out during the stripping (Marco-Jiménez et al., 2006). Immature spermatozoa might present not only lower motility, but also lower resistance, losing motility earlier.

S2 was the less abundant in the three experiments. We propose that S2 motility pattern could be an intermediate state between S3 and S1 patterns. S3 spermatozoa might skip to a S2 pattern, still fast moving but with altered trajectories, when their energy stocks deplete or they undergo degeneration (for instance, axonemal damage). However, S2 could be just a transient stage of S3 spermatozoa. According to this second hypothesis, some spermatozoa might experiment a shift in their motility pattern, from a linear to a circular motion. This phenomenon could be caused by changes in molecular signaling pathways, as proposed in studies on mammals (Chang and Suarez, 2011). Confirming these hypothesis require molecular studies, which are indispensable to understand the cause of motility patterns.

Eel spermatozoa present a considerable longevity (post-activation swimming time), comparing to other species that have been used for the study of sperm subpopulations. Woolley (1998a) indicates that

eel sperm could remain motile nearly 30 min after activation, showing a steady decrease in total motility. This author reports that just after activation most spermatozoa were very fast (maximum velocity of 160 μm/s, within the values obtained in our study) and linear, slowing down and leveling off by 90 μm/s at 5 min post-activation. This contrasts with longevity in salmonids (typically less than one minute), sole fish (1–2 min) (Martínez-Pastor et al., 2008), pipefish (Dzyuba et al., 2008) (<5 min), or seabream (3–6 min) (Zilli et al., 2009). We have obtained a slow decrease of total motility and velocity in the first 90 s of motility, agreeing to previous reports (Woolley, 1998a; Gallego et al., 2013). Acquiring motility images at 30 s seems to be a good compromise for allowing all the viable spermatozoa to be fully activated and giving enough time to adjust the microscope, while preventing significant changes to the sperm motility relative to its "peak" nearly after activation. Indeed, at 30 s we found the lowest proportion of S1 spermatozoa and the highest proportion of S3 spermatozoa. Oddly, S1 increased at 60 s and decreased at 90 s, while S2 seemed to increase. According to our previous interpretation of subpopulation roles, S1 spermatozoa could be short-lived or —at the least— be less resistant than S3 spermatozoa. During the first 60 s, the weakest spermatozoa in the sperm sample would change their motility pattern to S1, explaining the relatively high proportion of this subpopulation by 30 s and its increase by 60 s. Therefore, the decrease in total motility noted from 30 to 90 s could be accounted for this excess of S1 spermatozoa becoming immotile, which at the same time would result in a decrease of S1 by 90 s.

A more extensive experiment is required to confirm these pattern changes. Our experiment was designed to test if the subpopulation pattern of European eel spermatozoa varied significantly within the first seconds after the activation, in order to define an acquisition time for the rest of experiments with eel spermatozoa. However, we consider that future research should study the motility patterns for the whole duration of motility. In fact, it could be that eel sperm quality could be even better defined at a later time after activation. Currently, we ignore how the spawning occurs in this species (Tsukamoto et al., 2011), but the long duration of motility may provide hints about the biology of the spawning process. In fact, the stabilization of the motility parameters by 5 min post-activation described by Woolley (1998a) could indicate that fertilization might take place at a relatively later time after ejaculation. Studies in other species have associated some mating strategies with the need of a long-motility spermatozoon (Le Comber et al., 2004).

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

The effect of thermal treatments on European eel spermiation was discussed in detail by Gallego et al. (2012). These authors highlighted that not only T20 promoted spermiation, but also that it seemed to be necessary that the males remained at least 1 week at 20 °C to initiate it. In that study, sperm volume, density and motility were higher for T20 for most of the studied period (weeks 7–11). We wondered if

the thermal treatments could alter the subpopulation patterns too. The models obtained in this study analyzing CASA parameters suggest that the delay produced by T10 and T15 did not cause a large modification in the motility of the obtained samples, after the onset of spermiation. In these two treatments, when the spermiation period set up, sperm characteristics were similar to the samples obtained with T20 during its optimal period (weeks 8–12). Our study goes deeper in that analysis, by using the subpopulation data. We have found that T20 data yielded models predicting 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 obtaining enough data for fitting the models satisfactorily, but our results suggest that the subpopulation dynamics would follow a similar trend than for T20, only delayed in time. If we accept that the fish testicles do not produce an 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 support our suggestion than submitting the eel males to lower temperatures in the T10 and T15 treatments did not alter the spermatogenesis process, but rather arrested it even in presence of an inductor of spermiation (hCG). Apparently, the spermiation process was resumed normally when the water temperature reached 20 °C.

Our results shed some light on the reproductive biology of the European eel. This species would not require a previous low-temperature period to activate spermatogenesis, contrarily to other fishes from temperate climates (Breton and Billard, 1977). The European eel would follow a spermiation model of the same type as species such as the Nile tilapia (*Oreochromis niloticus*). Saving that both species spawn in very different habitats (the Nile tilapia require temperatures above 24 °C during the spermiation), the Nile tilapia do not require temperature changes to trigger spermiation, and the spermatocyte meiosis is arrested at relatively low temperatures (Vilela et al., 2003). However, it would be necessary to undergo 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 if the stagnation of tilapia spermatogenesis (at 20 °C) would be reversed by increasing temperature back to above 24 °C, whereas it seems to be the case for the eel.

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

The choice of hormonal treatment is critical for the induction of spermiation in eel. We have found interesting patterns regarding sperm quality in the hCG and hCG_{rec}-treated groups, from the beginning of the spermiation (week 5) to week 20, when the study finished. hCG_{rec} provided a constant number of high-motility samples for most of the sampling period, with only small fluctuations. The kinematic parameters were also 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 abruptly and a tendency to decrease by the last third of the study (thus the cubic model obtained vs. the quadratic one for hCG_{rec}). The reason for the stability of hCG_{rec} 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 hCG samples, S1 and S3 followed a "rollercoaster" dynamics, with S1 increasing noticeably by the second third of the experiment. Several studies have compared the efficiency of hCG and hCG_{rec} in assisted reproduction programs in humans, finding no differences between the two hormonal sources for inducing follicular maturation (Hugues, 2004; Al-Inany et al., 2005). Nevertheless, some authors have found hCG_{rec} to be more effective in fertility programs (Papanikolaou et al., 2010). hCG_{rec} can be produced in high purity, with low variability between batches and a high consistency in composition (Hugues, 2004). Contrarily, hCG, albeit cheaper, is purified from the urine of pregnant women. Not only it is more difficult to maintain batch-to-batch homogeneity, but also the purified product is actually a mixture of five isoforms (Crochet et al., 2012). These isoforms may have different biological activity, possibly motivated by the degree of glycosylation of the protein subunits. In fact, the differences between the ability of the three hormones in promoting spermiation in eel have been attributed to 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 sperm for several weeks suggests that they might be group-synchronous spawners. Our results with hCG and hCG_{rec}, which allowed to obtain sperm 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., 2010b), indicating that hCG induces the production of both 11-ketotestosterone, the major androgen in male eel (Ohta and Tanaka, 1997), and 17,20β-dihydroxy-4-pregnen-3-one (17,20β-P), a maturation-inducing steroid (MIS). The effectivity of gonadotropins to induce spermiation seems to be due to their LH-like effect and the modulation of 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 of its volume and testicular size (Asturiano et al., 2002, 2004; Peñaranda et al., 2010b). An alteration in this process may hamper spermatogenesis or hydration, resulting in the motility differences observed in this study.

Agreeing to previous studies (Asturiano et al., 2006, 2005; Gallego et al., 2012), our results indicate that hCG is an effective inductor of spermiation, but the lower motility and changing quality observed during the spermiation period suggest that it might be less effective sustaining spermatogenesis or sperm maturation. The heterogeneity of hCG composition (Hugues, 2004; Crochet et al., 2012) could

be the cause. In fact, spermatozoa from hCG-treated males have thicker sperm heads by the beginning of spermiation, becoming thinner and longer with the advancement of the spermiation period (Asturiano et al., 2006; Peñaranda et al., 2010b). Changes in head size are related to the development of the spermatogenic function, and may have important consequences on the swimming ability of the spermatozoa and on 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 specially to the variations in S3 presence. Peñaranda et al. (2010b) studied the induction of spermiation up to week 13, observing that 17,20β-P values, which peaked by week 5, were stable and 7-fold higher than in non-treated males during weeks 7–13, when motility and viability were highest. This highlights the importance of MIS in achieving good sperm motility, and coincides with the lower S1 and higher S3 values achieved in this experiment in the same period. However, the S1/S3 pattern inverted after week 13 in our hCG-treated males. We lack hormone data for that period, but we hypothetise that MIS synthesis could fail by the second half of the spermiation period. Contrarily, hCG_{rec} might 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, hCG_{rec} yielded "high quality" spermatozoa (predominance of S3) from the very beginning of the spermiation, which could be due to a faster shift of MIS synthesis. These hypotheses must be confirmed studying hormonal levels in both treatments and for all the length of the spermiation.

Gallego et al. (2012) showed that PMSG was less effective than hCG or hCG_{rec}, since it delayed the onset of spermiation and resulted in overall less sperm collected. However, the quality of motility increased and became similar to hCG_{rec} several weeks after the spermiation was established. These authors attributed their results to different rhythms of gonadal development induced by these hormones. We have found that PMSG modified the motility patterns of sperm samples. Considering only the CASA parameters, we could interpret the fitted models as delayed versions of the models obtained for hCG samples. However, the dynamics of the subpopulation patterns were more similar (at least for S3) to hCG_{rec}, although PMSG resulted in higher between-sample variability. In equids, PMSG acts as an analogue of the luteinizing hormone (LH) —similarly to hCG— but in non-equid species PMSG has a dual activity, behaving both like LH and like FSH (follicle-stimulating hormone) (Gordon, 2004). Although we ignore the actual action of PMSG on eel, its dual activity in other species suggests that it could be less efficient promoting both androgen synthesis (delaying spermiation) and MIS synthesis (resulting in a low-quality supbopulation pattern).

Economical significance of sperm subpopulation patterns

The findings in this study might have an important impact in economical decisions. In regards to the thermal treatments, even though they might not alter the subpopulation pattern, T20 is the obvious choice concerning the obtention of the highest-quality S3 spermatozoa. T10 and T15 delayed the spermiation, and thus the peak of S3, which would not be economically convenient. Considering the hormonal treatments, even though hCG_{rec} has a higher price than hCG or PSMG, the yield of S3 spermatozoa was clearly superior using this treatment. Our calculations demonstrate that hCG_{rec} was the most profitable option for obtaining good-quality spermatozoa (SP3). In fact, using hCG_{rec} would be even more convenient in the practice, since it would allow for higher and more stable production of good-quality sperm for an extended period. All these properties are desirable in the design of reproductive programs to be applied to eel farms in the future.

Conclusions

In this study we have been able to distinguish three subpopulations from European eel sperm samples. One of them, S3, grouped fast and mostly linear spermatozoa, and its presence might be related to good-quality samples. We have also concluded that eel sperm motility varies with advancing post-activation time, likely affecting the subpopulation pattern. This makes advisable to set a fixed time to acquire motility data, preferably 30 s post-activation.

Concerning the induction of the spermiation, we had confirmed that thermal treatments that submit the males to temperatures lower than 20 °C delay the onset of spermiation, but might not affect the subpopulation structure once the spermiation has started. Contrarily, the choice of hormonal treatment for inducing spermiation affected the subpopulation pattern and its dynamics throughout the spermiation period. hCG_{rec} allowed both sustained high motility and high proportion of S3 spermatozoa. It might be the most economical option, although it would depend on the design of egg fertilization protocols, allowing to fully take advantage of the availability of high-quality samples obtained after hCG_{rec} treatments.

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FIGURE LEGENDS

Figure 1.

Motility variables from the study of the effect of acquisition time (x-axes) on eel sperm motility (Experiment 1). Boxplots represent the distribution of data, with the boxes enclosing 50% of the data, and the vertical lines ("whiskers") spreading 1.5 times the length of the boxes up to the farther data point. The horizontal line is the median. Different letters indicate that groups (acquisition times) differ $P<0.05$.

Figure 2.

Proportion of each sperm subpopulation (Table 1) in each acquisition time (x-axes) (Experiment 1). Boxplots represent the distribution of data (showed as points), with the boxes enclosing 50% of the data, and the vertical lines ("whiskers") spreading 1.5 times the length of the boxes up to the farther data point. Different letters indicate that groups (acquisition times) differ $P<0.05$. The proportions of the three subpopulations differed significantly ($P<0.05$).

Figure 3.

Summary of the CASA analysis for Experiment 2 (water temperature). Median data for total motility, VCL and LIN along time (weeks, x-axis) and within each treatment (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 whole experimental period). Data were fitted to linear models (1^{st} to 4^{th} order polynomials). The plots show mean±SEM, the fitted model and its 95% confidence intervals for the models (C.I., shaded area). Letters show 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, with maximum values by week 10. Overall, T20 showed the highest average values for total motility ($P<0.001$).

Figure 4.

Summary of the clustering analysis of Experiment 2 (water temperature), showing the proportions of subpopulation 1 ("slow swimmers"), 2 ("circular swimmers") and 3 ("fast swimmers") (Table 2), along time (weeks, x-axis) and within each treatment (see Fig. 3 for the description of treatments and plot elements). T10 could not be fitted to a linear model, due to the lack of data points (spermiation starting by week 11). For T15, the proportion of subpopulation 1 fitted a negative quadratic model (Subfig. 4b), whereas subpopulation 3 data fitted a positive linear model (Subfig. 4h), with no clear trend for subpopulation 2. For T20, subpopulation 1 data fitted a negative quadratic model (Subfig. 4c), clearly

showing the lowest proportion by week 9, whereas both subpopulation 2 and 3 (subfigures 4f and 4i) fitted positive quadratic models, following an inverse trend.

Figure 5.

Summary of the CASA analysis for Experiment 3 (hormonal treatments; median data for total motility, VCL and LIN are shown) along time (weeks, x-axis) and within each treatment (see Fig. 3 for the description of plot elements). hCG total motility and VCL data were fitted to a cubic model and hCG_{rec} total motility to a quartic model, whereas PMSG total motility was fitted to a quadratic model, VCL to a linear model and LIN to a quadratic model. Note the local maxima and/or minima in the polynomial models. hCG_{rec} total motility increased since week 5, tending to stabilize within 40–50% motility, unlike hCG (which also yielded a large number of samples with low motility). Overall, hCG_{rec} showed the highest average total motility (41.6%$\pm$1.6 vs. hCG: 20.9%$\pm$2.0 and PMSG: 28.5%$\pm$3.4, P<0.001), VCL (137.1$\pm$3.2 μm/s vs. hCG: 108.5$\pm$4.7 μm/s; PMSG: 106.0$\pm$5.3 μm/s) and LIN (44.0%$\pm$0.6 vs. hCG: 38.7%$\pm$1.1; PMSG: 37.4%$\pm$0.9).

Figure 6.

Summary of the clustering analysis of Experiment 3 (hormonal treatments), showing the proportions of subpopulation 1 (“slow swimmers”), 2 (“circular swimmers”) and 3 (“fast swimmers”) (Table 3), along time (weeks, x-axis) and within each treatment (see Fig. 3 for the description of treatments and plot elements). hCG data fitted cubic models, whereas hCG_{rec} data fitted quartic and quadratic models (subpopulations 1 and 3), with no fit for PMSG data. Overall, hCG_{rec} data showed a more stable trend, with a clear predominance of S3 for all the spermiation period.

Figure 7.

Distribution of the cost of 10^9 SP3 (good motility) spermatozoa in each hormonal treatment (description of boxplot elements in Figure 7). Boxplots 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 hCG_{rec} and PMSG groups (P<000.1; P<0.1 for the other two comparisons).

Table 1
Subpopulations obtained from the CASA dataset obtained analyzing motility at different times post-activation (Experiment 1). The table shows average values of several kinetic parameters (mean±SD). A total of 35739 motile spermatoza obtained from 84 samples were used in the clustering analysis.

Subpopulation	VCL (μm/s)	LIN (%)	WOB (%)	ALH (μm)	DNC (μm^2/s)
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

Table 2
Subpopulations obtained from the CASA dataset obtained analyzing motility data from the thermal treatments experiment (Experiment 2). The table shows average values of several kinetic parameters (mean±SD). A total of 27668 motile spermatozoa obtained from 94 samples were used in the clustering analysis.

Subpopulation	VCL (μm/s)	LIN (%)	WOB (%)	ALH (μm)	DNC (μm^2/s)
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

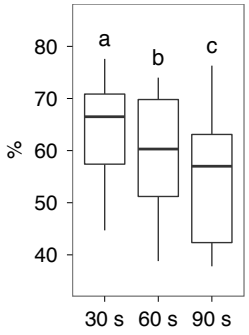
Table 3
Subpopulations obtained from the CASA dataset obtained analyzing motility data from the hormonal treatments experiment (Experiment 3). The table shows average values of several kinetic parameters (mean±SD). A total of 98666 motile spermatoza obtained from 334 samples were used in the clustering analysis.

Subpopulation	VCL (μm/s)	LIN (%)	WOB (%)	ALH (μm)	DNC (μm^2/s)
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

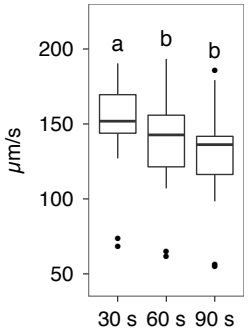
FIGURE 1

las variables están descritas en cada encabezado, sería repetitivo ponerlas en los ejes también

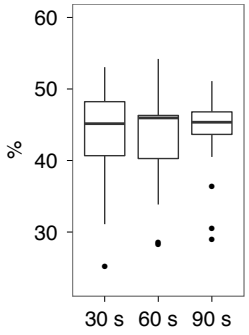
(a) Total motility



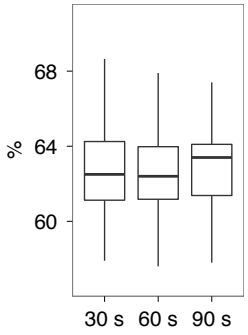
(b) VCL (curvilinear velocity)



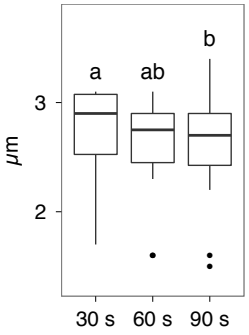
(c) LIN (linearity)



(d) WOB (wobble)



(e) ALH (lateral head displacement)



(f) DNC (Dance)

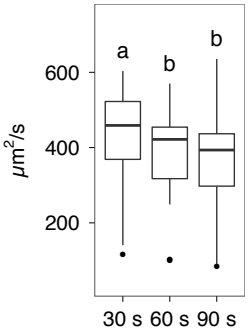


FIGURE 2

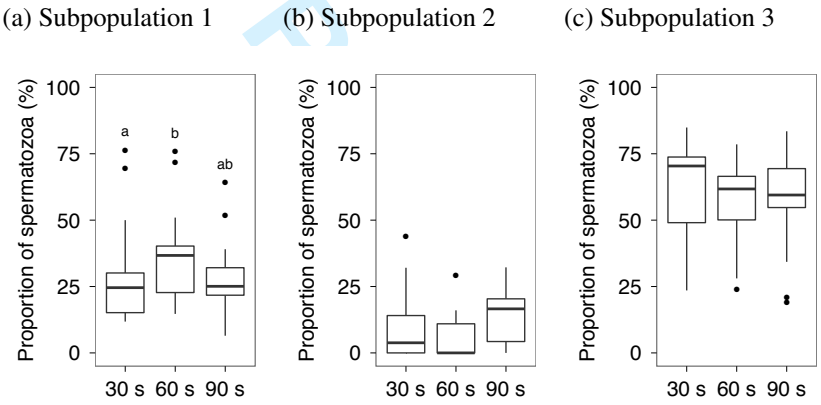


FIGURE 3

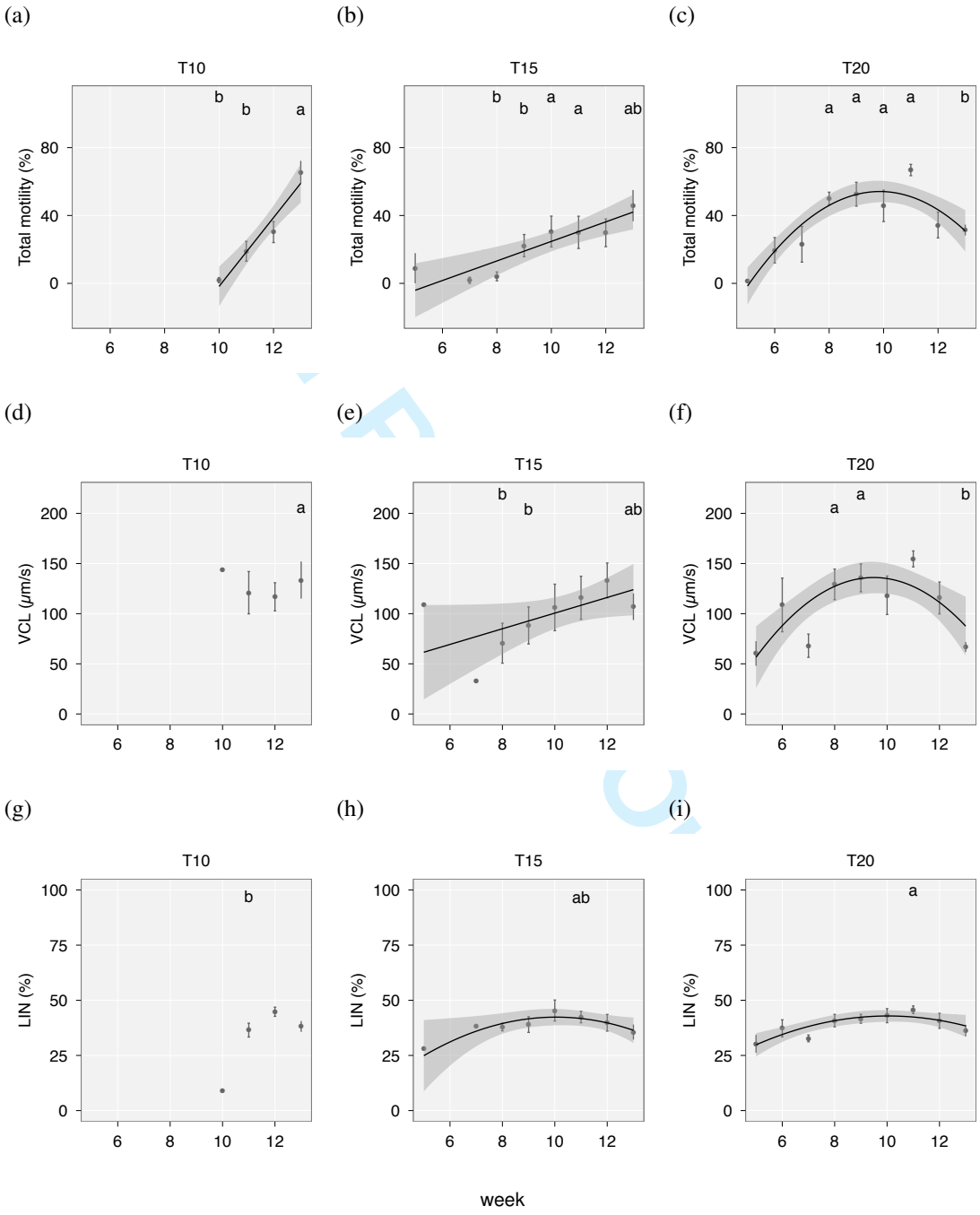

(a)
(b)
(c)
T10
T15
T20
Total motility (%)
b
b
a
b
b
a
ab
a
a
a
a
b
0
40
80
(d)
(e)
(f)
VCL (μm/s)
a
b
b
ab
a
a
b
0
50
100
150
200
(g)
(h)
(i)
LIN (%)
b
ab
a
0
25
50
75
100
6
8
10
12
week

FIGURE 4

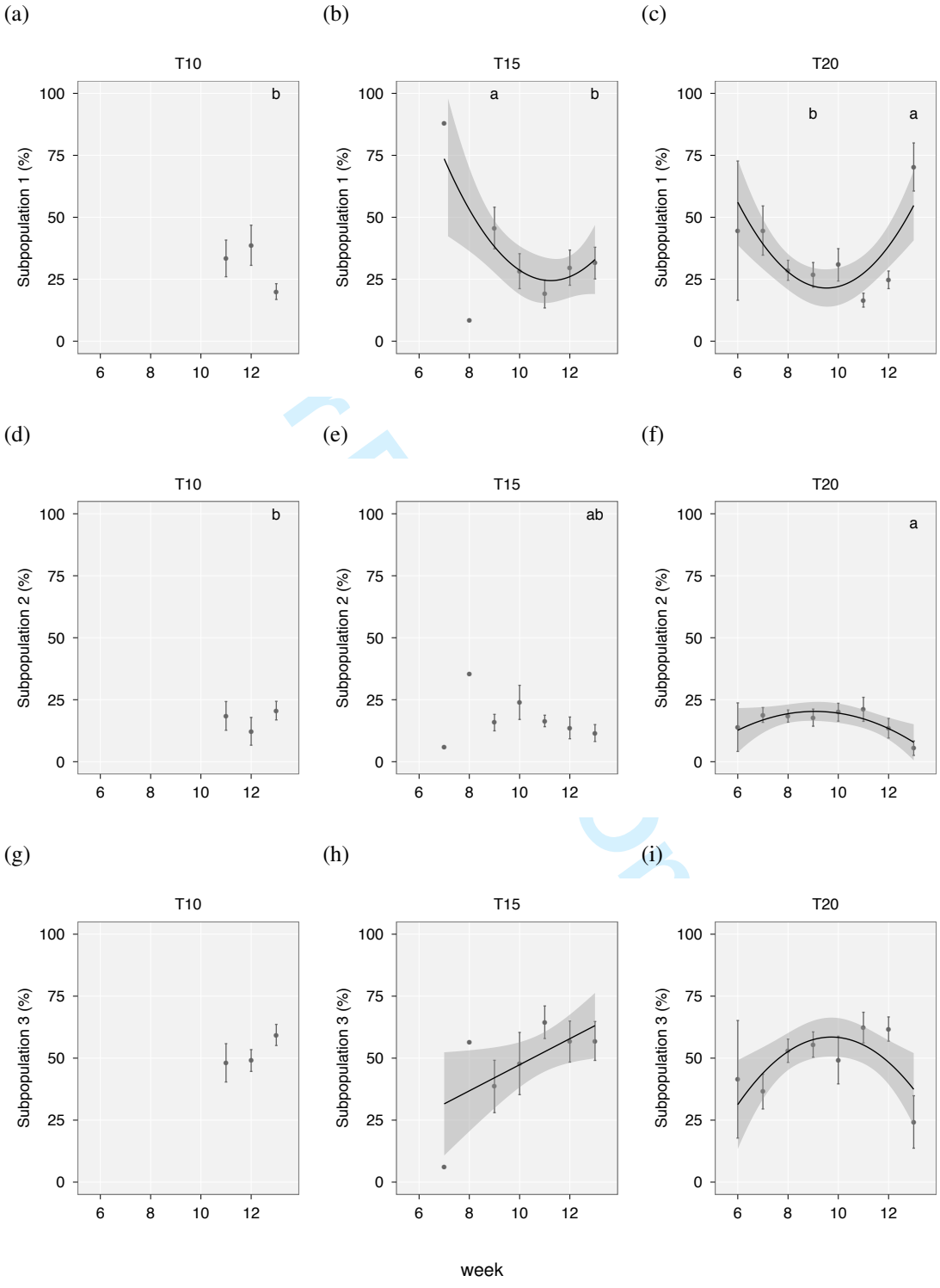

(a)
(b)
(c)
T10
T15
T20
b
a
b
b
a
a
(d)
(e)
(f)
T10
T15
T20
b
ab
a
(g)
(h)
(i)
T10
T15
T20
Subpopulation 1 (%)
Subpopulation 2 (%)
Subpopulation 3 (%)
0
25
50
75
100
6
8
10
12
week

FIGURE 5

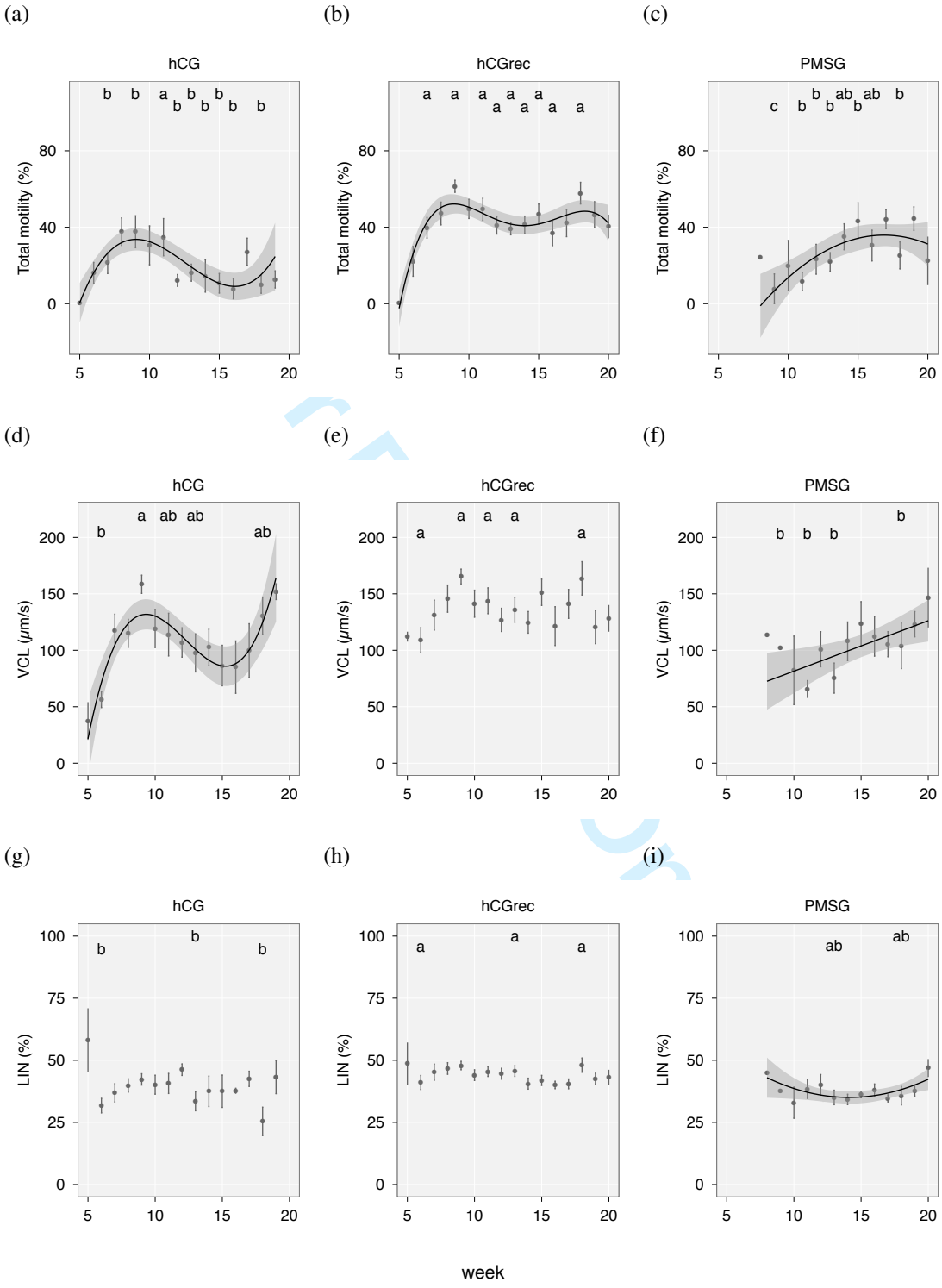

(a)
(b)
(c)
hCG
hCGrec
PMSG
Total motility (%)
b b a b b b b b
a a a a a a a a
c b b ab ab b
(d)
(e)
(f)
VCL (μm/s)
b a ab ab ab
a a a a a
b b b b
(g)
(h)
(i)
LIN (%)
b b b
a a a
ab ab
week

FIGURE 6

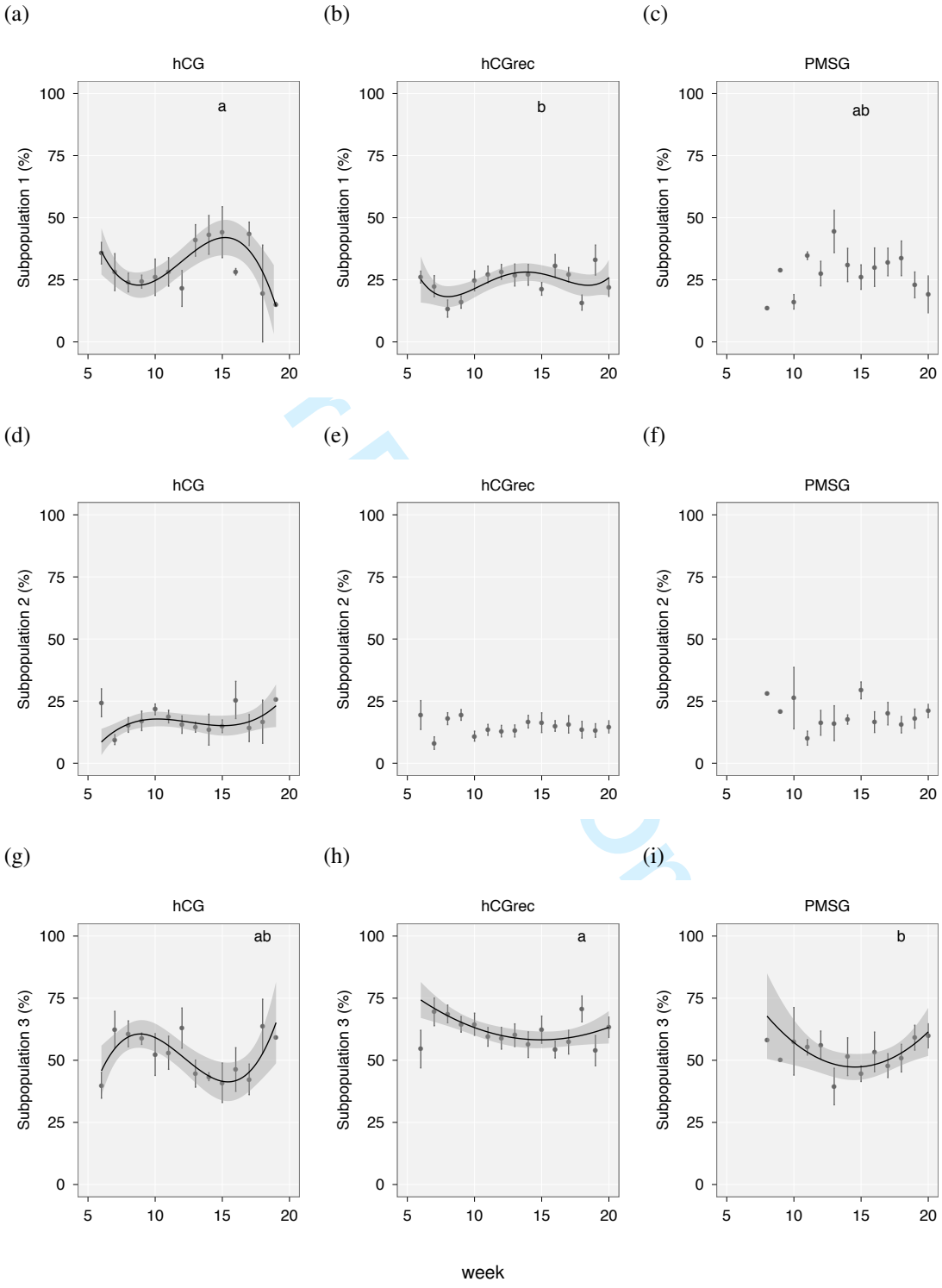

(a)
(b)
(c)
hCG
hCGrec
PMSG
a
b
ab
Subpopulation 1 (%)
(d)
(e)
(f)
Subpopulation 2 (%)
(g)
(h)
(i)
ab
a
b
Subpopulation 3 (%)
0
25
50
75
100
5
10
15
20
week

FIGURE 7