Intrinsic Post-Ejaculation Sperm Ageing Does Not Affect Offspring Fitness in Atlantic salmon

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Abstract

Postmeiotic sperm ageing, both before and after ejaculation, has been shown to negatively affect offspring fitness by lowering the rate of embryonic development, reducing embryonic viability, and decreasing offspring condition. These negative effects are thought to be caused by intrinsic factors such as oxidative stress and ATP depletion or extrinsic factors such as temperature and osmosis. Effects of post-ejaculation sperm ageing on offspring fitness have so far almost exclusively been tested in internal fertilisers. Here, we tested whether intrinsic post-ejaculation sperm ageing affects offspring performance in an external fertiliser, the Atlantic salmon *Salmo salar*. We performed *in vitro* fertilisations with a split clutch design where sperm were subjected to four post-ejaculation ageing treatments. We varied the duration between sperm activation and fertilisation while minimising extrinsic stress factors and tested how this affected offspring fitness. We found no evidence for an effect of our treatments on embryo survival, hatching time, larval standard length, early larval survival or larval growth rate, indicating that intrinsic post-ejaculation sperm ageing may not occur in Atlantic salmon. One reason may be the short lifespan of salmon sperm after ejaculation. Whether our findings are true in other external fertilisers with extended sperm activity remains to be tested.

1. Introduction

Sperm of increased age not only exhibit reduced performance during fertilisation compared to younger sperm but are also thought to impair the fitness of the resulting offspring (Reinhardt, 2007; Pizzari *et al.*, 2008). Ageing processes that affect sperm quality may occur both, at the pre-meiotic and the post-meiotic stages of sperm development. Pre-meiotic ageing processes affecting sperm are directly related to the age of the male. Older males have been shown to produce less sperm, sperm of inferior quality (Ford, 2000; Kidd *et al.*, 2001; Kuhnert, 2004; Lewis & Aitken, 2005) and offspring that are more likely to carry genetic disorders due to accumulation of mutations in the germline (de La Rochebrochard & Thonneau, 2002; Lewis & Aitken, 2005; Zhu *et al.*, 2005; Velando *et al.*, 2011; but see also Johnson & Gemmell, 2012). Post-meiotic sperm ageing is independent of male age (Pizzari *et al.*, 2008) (although it may have a stronger effect on sperm of older males (Zubkova & Robaire, 2006)) and is thought to be the result of both intrinsic factors, including oxidative stress and ATP depletion, and extrinsic factors, such as temperature and osmosis (Reinhardt, 2007). Sperm are particularly vulnerable to oxidative stress because of their high metabolic activity, large amount of polyunsaturated fatty acids, and small cytosol (Reinhardt, 2007; Cabrita *et al.*, 2014). Oxidative stress is caused by the accumulation of reactive oxygen species (ROS) within the sperm, which not only lowers sperm motility and decreases the fertilisation capacity of sperm, but also disrupts the genome integrity and thus affects offspring viability and fitness (Reinhardt, 2007; Aitken *et al.*, 2012; Cabrita *et al.*, 2014).

Post-meiotic sperm ageing can be split into two phases, pre- and post-ejaculation. The former takes place within the male's sperm storage organs, while the latter occurs after ejaculation either within the female in internal fertilisers or in the environment in external fertilisers. Both pre- and post-ejaculation sperm ageing have been shown to negatively affect offspring performance. In the black-legged kittiwake *Rissa tridactyla* for example, pre-ejaculation

sperm ageing has been shown to adversely affect sperm fertilisation potential, rate of embryonic development, embryonic mortality and chick condition at hatching (White *et al.*, 2008), while post-ejaculation sperm ageing has been shown to lead to higher hatching failure and poor chick condition (Wagner *et al.*, 2004). To date, most studies on sperm ageing focused on internal fertilisers (e.g.de La Rochebrochard & Thonneau, 2002; Lewis & Aitken, 2005; Zhu *et al.*, 2005; Zubkova & Robaire, 2006; Velando *et al.*, 2011; Johnson & Gemmell, 2012; Wagner *et al.*, 2004; Reinhardt & Siva-Jothy, 2005; White *et al.*, 2008; den Boer *et al.*, 2009; Firman *et al.*, 2015) and very little is known about its effects on fertilisation and offspring performance in external fertilisers.

The aim of our study was to assess the potential for intrinsic post-ejaculation sperm ageing in an external fertiliser, the Atlantic salmon *Salmo salar*. We performed *in vitro* fertilisation (IVF) assays with four different post-ejaculation sperm ageing treatments and assessed the performance of the resulting offspring by measuring embryo survival, embryo hatching time, larval standard length, early larval survival and larval growth rate across our treatments. However, we found no evidence for effects of intrinsic post-ejaculation sperm ageing on offspring fitness.

2. Material and Methods

Gamete collection

Sperm and eggs for the *in vitro* fertilisations (IVFs) were obtained during stripping of wild caught Atlantic salmon, *Salmo salar*, at Älvkarleby, Sweden. All fish were caught three to four weeks before the start of the natural spawning season and were maintained in compartments separating the two sexes until stripping. The fish used for the experiment therefore had no prior spawning experiences. For gamete collection, 38 males and 35 females

were anaesthetised in a tank with Tricaine Methanesulfonate (MS 222, Sigma-Aldrich). Twelve pairs were used for IVFs in a first block three weeks after capture, and the remaining pairs were used in two more blocks one week later. The ejaculates – about 6 ml per male measured by volume indication on the collection tube – were collected into dry, clean 15 ml Falcon tubes. The eggs – about 800 eggs per female scooped up with the help of a calibrated measuring beaker from the whole stripped clutch – were collected into clean, dry plastic containers with lid. To prevent activation of the gametes before the IVFs, we carefully avoided contact of the eggs and sperm with water, urine and faeces. After collection, we stored the sample containers on ice until the experiment. Freezing of gametes was carefully avoided by placing a towel on the ice to keep the samples cool but without direct contact with the ice. The time between gamete collection and fertilisation was between 0.5 and 4.5 hours for the sperm and between 1.0 and 5.0 hours for the eggs.

Sperm activation

Prior to the IVFs, we assessed the optimal ratio of Hank's buffer (HBSS) to water in order to activate the sperm with minimal osmotic stress in order to keep the sperm motile for up to 90 second, which goes beyond their natural lifespan. To do so, we tried a series of different Hank's buffer to water ratios and observed sperm activity under a light microscope. Salmon sperm get activated in an all-or-nothing manner triggered by a change in osmolality that activates all sperm at once (Alavi and Cosson 2006). These observations were made by eye under a microscope. For each of these trials, we placed a small subsample of 500 µl of the ejaculate into an Eppendorf tube, diluted it in 1000 µl Hank's buffer and activated it with different quantities of cold river water (~4.5 °C). After thoroughly mixing the ejaculate-Hank's-water mix, we placed 30 µl of the mix on a microscope slide that was prepared with two stripes of nail polish that elevated the cover slip, so that the sperm could move freely. The

microscope slide was cooled down to the temperature of the river water in a bucket filled with cold river water before each trial. We repeated the procedure with the ejaculates of three to five different males for each of the tested dilution ratios to ensure our results were constant across males. We started with 500 µl of river water and moved down in steps of 100 µl of water at first until reaching 100 µl, then at 10 µl of water. The final ratio used in the experiments was 500 µl ejaculate and 1000 µl Hank's buffer to 80 µl of river water (the optimal water volume thus corresponded to ~5% of the volume of the ejaculate-Hank's mix). Using this Hank's to water ratio, the sperm were fully activated and motile for at least seven minutes. This means that by applying this Hank's buffer to water ratio, all sperm survived until fertilisation in our IVF trials and that we were able to avoid selecting against sperm cohorts that are particularly sensitive to osmosis.

Sperm motility

Sperm motility was assessed by estimating the percentage of forward motile sperm in the ejaculate by eye for each individual male right before its ejaculate was used for IVF to ensure sperm were fully motile. To do so, we activated 0.3 µl of ejaculate with 30 µl of cold river water directly on the microscope slide prepared in the same way as described above. Males whose sperm showed less than 50% motile sperm when activated were excluded from the experiment (n = 3).

IVFs

We performed IVFs with a total of 35 males and 35 females. To account for parental effects, we applied a full-sibling split design for the IVFs. We divided the egg clutch of each female and the ejaculate of each male into four equal parts (hereafter called subclutches) to be able to apply all four treatments to each parental pair. Eggs were sub-divided by the help of a

calibrated measuring beaker as counting was not possible due to the fragility of unfertilised eggs. The sperm ageing treatments were conducted as follows. For each male, we filled four 15 ml Falcon tubes with 500 µl ejaculate and 1000 µl Hank's buffer. Again, due to time limitations, we did not count sperm numbers for each male. Since we use a split design, the sperm density for each male will be identical across all treatments and hence variation across males in sperm density will be taken into account by including pair IDs in the statistical analyses. To ensure equal sperm densities, ejaculates were carefully mixed by gently pipetting the entire ejaculate 5-6 times before distributing the samples into Falcon tubes. We then activated the sperm in the ejaculate-Hank's mix within the Falcon tubes with 80 µl of cold river water. Ejaculate and water were carefully mixed to ensure the activation of all sperm. Activation of sperm samples was timed according to each treatment (0 s, 20 s, 40 s and 60 s) and all fertilisations were performed simultaneously across the four treatments per pair by two people (two for each treatment changing in combination for each pair). Since under natural conditions, the sperm of Atlantic salmon are only motile for about one minute post activation (Yeates, 2005), one minute was considered to be a biologically relevant experimental post-ejaculation sperm ageing maximum. Fertilisation was initiated by simultaneously adding the activated sperm and 100 ml of cold river water (~4.5 °C) to the eggs. Caution was taken that the IVFs for all four sub-clutches of each parental pair were performed simultaneously for the eggs to be treated equally across the four treatments.

About five minutes after fertilisation, the eggs were rinsed to get rid of dead sperm and bleached using a poly vinyl pyrrolidone iodine solution following a standard procedure to minimise the risk of fungal growth (Frantsi & Whitey 1972). As during the IVFs, care was taken to perform the antifungal treatment on all four sub-clutches of each replicate pair simultaneously to avoid differential egg treatment across the experimental treatments.

Incubation and fertilisation success

After the antifungal treatment, the eggs were transferred into compartments of a run-through river water system with an average temperature of 3.3°C. Egg condition was controlled about once a week throughout the experiment. Damaged eggs (i.e. eggs that changed from orange to white or started to mould) were removed. Three months later, when the eyes of the embryos could be seen, we assessed fertilisation success twice by taking pictures of the eggs in their compartments and counting the number of fertilised and unfertilised eggs using ImageJ's cell counter plug-in. Two weeks later, we removed all unfertilised eggs and reduced the number of embryos to 50 embryos per sublcutch to ensure larval densities were the same across the treatments and subclutches (if we had less than 50 embryos in one subclutch, we only kept 30 embryos for all treatments of that replicate pair). This step is necessary as variation in larval density will differentially affect larval activity and development and hence growth rate. Of the total 35 replicate pairs, we removed all subclutches of three pairs because the fertilisation success was too low in all subclutches resulting in too few offspring (<10 fertilised eggs). We therefore had a total of four subclutches for each of 32 pairs (32 males and 32 females).

Hatching, offspring survival, standard length and growth rate

During the hatching period, we checked the number of newly hatched embryos of a subset of 22 replicate pairs (22 males and 22 females) on a daily basis. The reason for using this subset was that larvae from the first block (10 families) started hatching unexpectedly early and at the time of first observation, most larvae had already hatched. Exact hatching dates are necessary for estimating hatching rate over time and the calculation of a mean hatching time within each sub-clutch, which we therefore did for the remaining 22 families. Embryo and larval survival, standard length and growth rate were measured for all 32 replicate pairs (32 males and 32 females). Embryo and larval survival were assessed on a daily basis until

152 days post fertilisation and dead larvae and dead embryos were removed. Hatched larvae were briefly placed into a Petri dish containing a little water to take pictures for subsequent standard length measurements. To measure the growth rate of the offspring, we took another series of pictures three weeks after hatching, and again measured 15 larvae in each sub-clutch. For the growth rate measurements, we excluded one of the 32 replicates because pictures taken three weeks after hatching were of too poor quality for measuring larvae reliably. Standard length was measured from the tip of the head to the base of the tail fin. We measured 15 haphazardly chosen larvae of each sub-clutch using ImageJ. The reason why we limited our sample size for measuring standard length to 15 was the difficulty to obtain suitable images of all larvae within a reasonable amount of time due to the constant movements. In order to ensure our measurements were accurate, we only chose larvae that were lying still at the moment of taking an image. Each larva was measured twice, and only larvae that were lying on their side, straight and in focus were included in the measurements. To be able to convert the length measurements taken by ImageJ into centimetres, we placed a ruler next to the Petri dish containing the larvae while we photographed them as a size indicator.

Statistical analyses

All statistical analyses were performed with R (version 3.3.2) (R Core Team, 2016). Fertilisation success (fertilised versus unfertilised eggs), egg condition (good versus damaged eggs), relative hatching rate over time (hatched versus unhatched eggs over time), embryo survival (dead versus live embryos), larval survival (dead versus live larvae), i.e. data with binomial error distribution were analysed running generalised linear mixed effect models using the *cbind* function and a *logit* link function (function glmer, *lme4* package (Bates *et al.*, 2015). We tested all models for overdispersion, and if necessary, introduced an ID as random

factor for each individual datapoint to control for it. To avoid convergence errors, we scaled all fixed effects where necessary. For relative hatching rate over time we added time at hatching (in hours) as a continuous fixed effect and the interaction with treatment was also considered. Larval standard length and growth rate (i.e. data with Gaussian error distribution) were analysed using linear mixed effect models (function lmer, *lme4* package). In all models, we controlled for gamete age at the moment of IVF as the time in hours between egg (egg age) and sperm (sperm age) collection respectively and their use in IVFs as fixed continuous variables. Treatment was added as a continuous fixed effect with four time points. Family ID and Block (we performed the IVFs in three blocks) were included as random effects, unless they were close to 0 (which was true Block in several models). We estimated the significance of the fixed variables using an ANOVA with type III sums of squares and tested them with an analysis of deviance on a chi-square distribution (function Anova, *car* package (Fox & Weisberg, 2011)).

3. Results

We found no effects of intrinsic post-ejaculation sperm ageing on offspring fitness (Figure 1). This was true for all traits measured, i.e. fertilisation success rate (Table 1; Figure 1A), egg condition (Table 2, Figure 1B), embryo survival from the eyed stage until hatching (Table 3, Figure 1C), early larval survival from hatching until 152 days post fertilisation (Table 4, Figure 1D), relative hatching rate over time (Table 5, Figure 1E), larval standard length (Table 6, Figure 1F) and relative larval growth rate (Table 7, Figure 1G).

4. Discussion

We found no effect of intrinsic post-ejaculation sperm ageing on offspring fitness in Atlantic salmon. None of the fitness traits we measured in the offspring showed any effect in response to our sperm ageing treatments. The absence of any effect suggests that the accumulation of and the damage by ROS are likely to take longer than the short active lifespan of salmon sperm. Below we discuss our results in a wider context and assess the possible implications.

Prior research has shown that ROS damage is one of the main culprits in intrinsic sperm ageing, as it reduces sperm motility and fertility and decreases offspring viability and performance due to its negative effects on cell performance, sperm motility, DNA integrity and RNA stability (Reinhardt, 2007; Aitken *et al.*, 2012; Cabrita *et al.*, 2014). Atlantic salmon sperm are expected to contain particularly high concentrations of ROS during their active phase when their metabolism is tremendously boosted by activation of the sperm (Vladic & Petersson, 2016); at the same time they are thought – like sperm in general – to be very sensitive to ROS damage because they provide plenty of substrate for ROS attacks on the plasma membrane, the mitochondria, the genome, and RNA, while having very limited antioxidant protection (Vladic & Petersson, 2016). The lack of an effect of our treatments suggests that the brief active lifespan of salmon sperm may be too short for ROS damage to occur but this needs further investigation.

In theory, the short time between ejaculation and fertilisation in Atlantic salmon and other externally fertilising fish could be a mechanism to reduce sperm ageing. However, a more likely explanation for the evolution of this brief time window is the harsh fertilisation circumstances. Atlantic salmon spawn in rivers with strong currents, so immediate fertilisation is crucial as sperm are washed away quickly (Yeates 2005). Atlantic salmon sperm are therefore not adapted to fertilisation at 60 seconds post-ejaculation, and sperm ageing effects should become evident if there were any, especially in our 60 s treatment. It would also be interesting to study intrinsic post-ejaculation sperm ageing effects in external

fertilisers with longer fertilisation windows to understand how long it takes for ROS damage to show a significant effect..

While there is extensive research on ROS accumulation dynamics and its effects on sperm quality, male fertility and offspring performance in externally fertilising fish in the context of assisted fertilisation and cryopreservation for commercial farming (Cabrita *et al.*, 2014), we know little about the role of intrinsic post-ejaculation sperm ageing under natural conditions in external fertilisers. This is surprising, as studying post-ejaculation sperm ageing in external fertilisers has several practical advantages over studies in internal fertilisers, such as the possibility of IVFs and the application of split-clutch designs, which allow to separate intrinsic sperm ageing processes from those inflicted by the female. The main reason for the lack of studies in external fertilisers is probably that the time between ejaculation and fertilisation in external fertilisers is generally shorter than in internal fertilisers (Han, 2014). Our results indicate that the brief fertilisation window in some external fertilisers may indeed be too short for intrinsic sperm ageing effects to occur. However, studies assessing the potential for intrinsic sperm ageing in external fertilisers with extended sperm activation prior to fertilisation will help understanding the dynamics of the processes involved.

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Data accessibility. Data available from the dryad digital repository: doi: 10.5061/dryad.3n5tb2rcz

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Tables

Table 1: Non-linear mixed effect model (*glmer* in R) for fertilisation success with response variable *cbind*(fertilised eggs, unfertilised eggs). A random factor ID at the datapoint level to control for overdispersion was included. Random factor Block was removed due to variation close to zero (N = 32 replicate pairs with 182 ± 45 eggs (mean ± S.D.) per subclutch for four subclutches per pair). Estimates are provided with standard error (S.E.) and confidence intervals (C.I).

Variable						
Random	**Variance**					
ID	0.255					
Family ID	2.531					
Fixed	**Estimate**	**S.E.**	**C.I.**	***z***	**D.F.**	**P**
Intercept	2.31	0.54	0.27, 4.35	4.26	1	<0.0001
Treatment	-0.003	0.002	-2.04, 2.04	1.15	1	0.25
Sperm age	-2.50	1.05	-4.53, -0.46	2.38	1	0.017
Egg age	1.73	0.97	-3.77, 0.31	1.77	1	0.077

Table 2. Non-linear mixed effect model (*glmer* in R) for egg condition with response variable *cbind*(damaged eggs, good eggs). A random factor ID at the datapoint level to control for overdispersion was included. Random factor Block was removed due to variation close to zero (N = 32 replicate pairs with 210 ± 70 eggs (mean ± S.D.) per subclutch for four subclutches per pair). Estimates are provided with standard error (S.E.) and confidence intervals (C.I).

Variable						
Random	**Variance**					
ID	0.388					
Family ID	1.158					
Fixed	**Estimate**	**S.E.**	**C.I.**	***z***	**D.F.**	**P**
Intercept	-2.60	0.39	-4.64, -0.56	6.73	1	<0.0001
Treatment	0.0003	0.002	-2.04, 2.04	0.14	1	0.89
Sperm age	0.67	0.74	-1.37, 2.71	0.91	1	0.36
Egg age	-0.73	0.69	-2.77, 1.31	1.07	1	0.29

385 **Table 3.** Non-linear mixed effect model (*glmer* in R) for embryo survival with response variable *cbind*(dead embryos, live embryos). A random factor ID at the datapoint level to control for overdispersion was included. Random factors Block and Family ID were removed due to variation close to zero (N = 32 replicate pairs with 48 ± 7 eggs per subclutch for four subclutches per pair). Estimates are provided with standard error (S.E.) and confidence
390 intervals (C.I).

Variable						
Random	**Variance**					
Family ID	2.961					
Fixed	**Estimate**	**S.E.**	**C.I.**	***z***	**D.F.**	**P**
Intercept	-5.13	0.79	-7.17, -3.09	6.72	1	<0.0001
Treatment	0.007	0.005	-2.03, 2.04	1.38	1	0.17
Sperm age	-0.076	0.74	-2.11, 1.96	0.10	1	1.92
Egg age	-0.42	0.72	-2.46, 1.62	0.58	1	0.56

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Table 4: Non-linear mixed effect model (*glmer* in R) for larval survival with response variable *cbind*(dead larvae, live larvae). A random factor ID at the datapoint level to control for overdispersion was included. Random factor for family ID was removed due to variation close to zero (N = 32 replicate pairs with 48 ± 7 eggs per subclutch for four subclutches per pair). Estimates are provided with standard error (S.E.) and confidence intervals (C.I).

Variable						
Random	**Variance**					
ID	0.00					
Block ID	0.413					
Fixed	**Estimate**	**S.E.**	**C.I.**	***z***	**D.F.**	**P**
Intercept	-6.58	1.14	-8.62, -4.54	5.76	1	<0.0001
Treatment	0.011	0.01	-2.03, 2.05	0.81	1	0.42
Sperm age	-0.34	1.00	-2.38, 1.70	0.34	1	0.73
Egg age	0.053	0.97	-1.98, 2.09	0.97	1	0.96

Table 5: Non-linear mixed effect model (*glmer* in R) for hatching rate over time with response variable *cbind*(hatched eggs, unhatched eggs). A random factor ID at the datapoint level to control for overdispersion was included (N = 22 replicate pairs with 47 ± 7 eggs per subclutch for four subclutches per pair). Estimates are provided with standard error (S.E.) and confidence intervals (C.I).

Variable						
Random	**Variance**					
ID	4.214					
Family ID	3.069					
Block ID	2.825					
Fixed	**Estimate**	**S.E.**	**C.I.**	***z***	**D.F.**	**P**
Intercept	0.36	1.25	-1.71, 2.43	0.29	1	0.77
Treatment	-0.042	0.10	-2.12, 2.03	0.10	1	0.64
Hatch time	-7.64	0.21	-9.71, -5.57	0.21	1	> 0.0001
Sperm age	0.11	0.42	-1.96, 2.18	0.42	1	0.80
Egg age	0.69	0.48	-1.38, 2.76	0.48	1	0.15
Treatment * HT	-0.27	0.16	-2.34, 1.80	0.16	1	0.09

Table 6: Linear mixed effect model (*lmer* in R) for larval standard length. A random factor ID at the datapoint level to control for overdispersion was included. Random factor Block was removed due to variation close to zero (measurements for N = 32 replicate pairs and 15 larvae for each of the four subclutches per pair). Estimates are provided with standard error (S.E.) and confidence intervals (C.I).

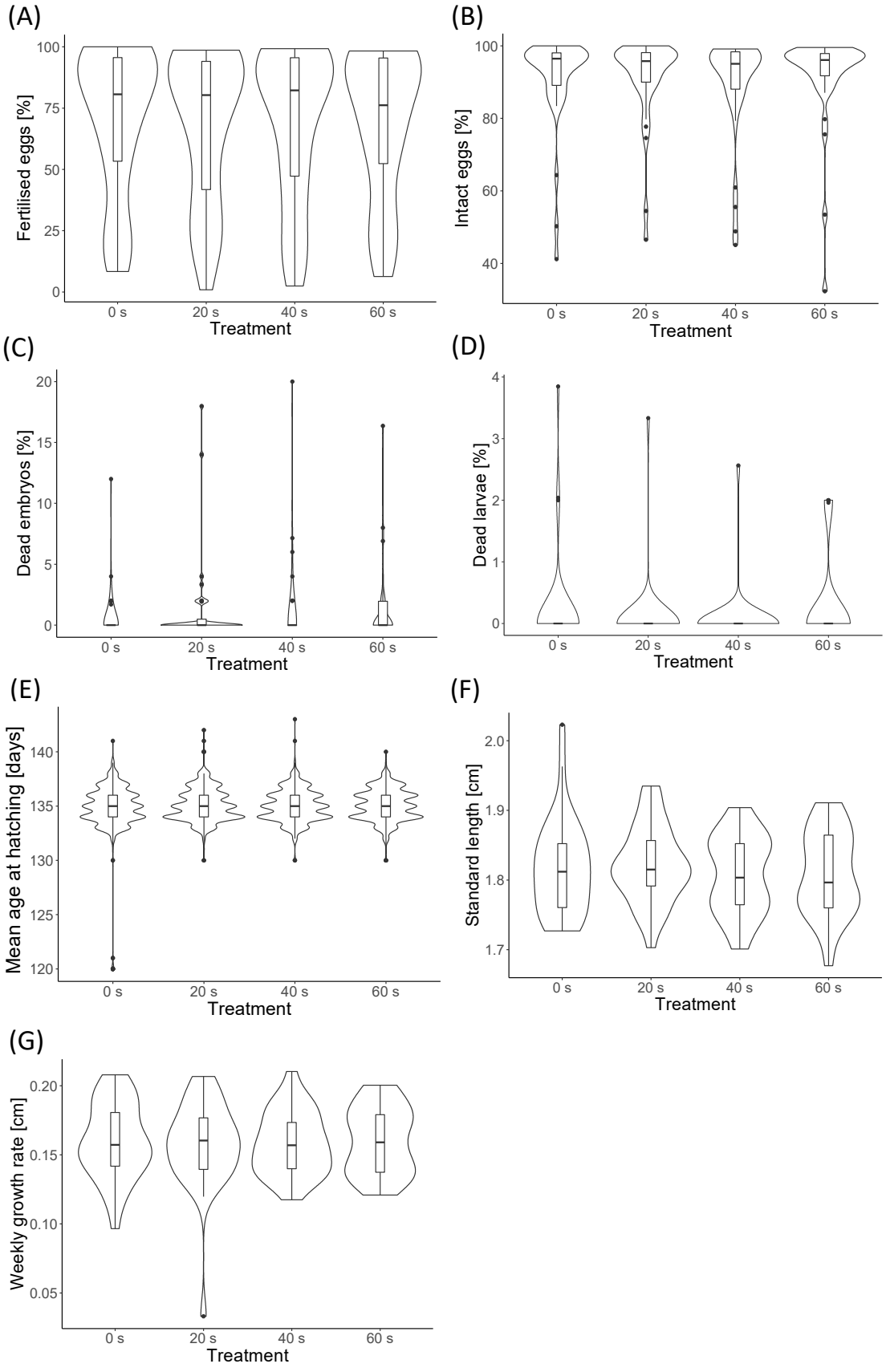
Variable						
Random	**Variance**					
Family ID	0.002					
Block ID	0.0006					
Fixed	**Estimate**	**S.E.**	**C.I.**	***t***	**D.F.**	**P**
Intercept	1.78	0.03	-0.26, 3.82	62.03	1	0.02
Treatment	-0.0001	0.0001	-2.04, 2.04	1.56	1	0.12
Sperm age	0.039	0.03	-1.99, 2.07	1.38	1	0.17
Egg age	-0.018	0.03	-2.06, 2.02	0.66	1	0.51

Table 7: Linear mixed effect model (*lmer* in R) for larval growth rate with change in size between two measurement times (measurements for N = 31 replicate pairs and 15 larvae for each of the four subclutches per pair two times for each subclutch**).** Estimates are provided with standard error (S.E.) and confidence intervals (C.I).

Variable						
Random	**Variance**					
Family ID	> 0.0001					
Block ID	0.0006					
Fixed	**Estimate**	**S.E.**	**C.I.**	***t***	**D.F.**	**P**
Intercept	0.16	0.02	-2.20, 1.88	8.35	1	0.85
Treatment	0.000003	> 0.0001	-2.04, 2.04	0.08	1	0.94
Sperm age	0.00004	0.006	-2.04, 2.04	0.007	1	0.99
Egg age	0.0014	0.006	-2.04, 2.04	0.23	1	0.82

Figure

Figure 1. Combined violin and boxplots of offspring performance across the four post-ejaculation sperm ageing treatments: Shown are (A) fertilisation success (N = 32 replicate pairs with 182 ± 45 eggs (mean ± S.D.) per subclutch for four subclutches per pair); (B) egg condition (N = 32 replicate pairs with 210 ± 70 eggs (mean ± S.D.) per subclutch for four subclutches per pair); (C) embryo survival (N = 32 replicate pairs with 48 ± 7 eggs per subclutch for four subclutches per pair); (D) larval survival (N = 32 replicate pairs with 48 ± 7 eggs per subclutch for four subclutches per pair); (E) mean age at hatching (N = 22 replicate pairs with 47 ± 7 eggs per subclutch for four subclutches per pair); (F) larval standard length at hatching (measurements for N = 32 replicate pairs and 15 larvae for each of the four subclutches per pair); (G) larval growth rate (measurements for N = 31 replicate pairs and 15 larvae for each of the four subclutches per pair two times for each subclutch).


(A)
Fertilised eggs [%]
100
75
50
25
0
0 s
20 s
40 s
60 s
Treatment
(B)
Intact eggs [%]
100
80
60
40
0 s
20 s
40 s
60 s
Treatment
(C)
Dead embryos [%]
20
15
10
5
0
0 s
20 s
40 s
60 s
Treatment
(D)
Dead larvae [%]
4
3
2
1
0
0 s
20 s
40 s
60 s
Treatment
(E)
Mean age at hatching [days]
140
135
130
125
120
0 s
20 s
40 s
60 s
Treatment
(F)
Standard length [cm]
2.0
1.9
1.8
1.7
0 s
20 s
40 s
60 s
Treatment
(G)
Weekly growth rate [cm]
0.20
0.15
0.10
0.05
0 s
20 s
40 s
60 s
Treatment

455