Sperm quality and function deteriorate in old males of the naturally short-lived turquoise killifish

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Abstract

Most of the variation in male reproductive aging observed in nature may be explained by different life-history strategies, which reflect trade-offs between growth, reproduction and survival. The disposable soma theory predicts that the energy allocated in reproductive functions is traded-off with soma maintenance. In particular, males are expected to maximise the investment in reproduction at the expense of soma maintenance, due to the strong selection for reproduction. Therefore, according to the disposable soma theory, species showing rapid somatic aging and high actuarial senescence are expected to have evolved high allocation in reproduction, with limited if any reproductive aging. We tested this prediction in turquoise killifish (*Nothobranchius furzeri*), a vertebrate of extremely short natural lifespan. We analysed age-related changes in sperm traits, the proportion of fertilized eggs, as well as embryo survival. Surprisingly, we found that, similar to their extensive actuarial senescence, turquoise killifish males also undergo spontaneous reproductive aging, consisting in the age-dependent decline of sperm quality, decreased proportion of fertilized eggs and lower embryo survival. Our results suggest that male reproductive aging may result from processes that are not necessarily dependent on the adaptive resource allocation between reproduction and somatic maintenance.

Keywords: reproductive aging, male fertility, disposable soma theory, trade-off, *Nothobranchius furzeri*

Introduction

During the aging process, male fertility is subject to dramatic decline. In humans age negatively impacts reproductive function of males, which may lead to potential negative effects on offspring (*1-3*). Despite the increasing evidence of male reproductive aging in humans, there seems to be a weak support for male aging on ejaculate quality across animals (*4*). Most of the variation in male reproductive patters is thought to be explained by the large diversity in life-history strategies (*5*), which are likely to result from trade-offs in resource allocation between growth, reproduction and survival have been proposed to be the main forces shaping life-history strategies (*5, 6*).

The optimal allocation between reproduction and survival is the most well-known trade-off in resource allocation (*7*) and is the core of the disposable soma theory of aging. The disposable soma theory predicts that the energy allocated in reproductive functions is traded-off with soma maintenance (*8, 9*). A great investment in reproduction exposes individuals to a faster rate of somatic deterioration and thus to a shorter lifespan (*10, 11*). Moreover, in most species males experience stronger selection for reproduction compared to females, due to greater competition for mating and fertilization (*12*). Males are thus expected to maximize the investment in reproductive traits at the expense of soma maintenance, often favouring a live fast-die young strategy (*13, 14*).

Accordingly, short-lived species are predicted to maximize the investment in reproduction, with limited if any reproductive aging. For instance, in many insects male reproductive traits, as sperm quality, show an overall negligible age-related decline (*4*, but see *15*), providing support for a constrain in resource allocation. On the contrary, taxa that show continuous growth during life, as fish, are expected to invest more in somatic maintenance than taxa with determinate growth rate (*16, 17*). The investment in somatic maintenance is predicted to lead to a greater reproductive senescence and lower actuarial senescence, which is indeed concordant with evidence reported in many fish species (*16*). However, some fish species, despite showing continuous growth during life, show increased mortality rate with age (*18*), raising the question on how trade-offs in resource allocation shape life-history strategy in these species.

The turquoise killifish (*Nothobranchius furzeri*) is one of the shortest-lived vertebrates (4-8 months, *19*) and has become increasingly used in experimental aging research (*20, 21*). While this species shows continuous growth in body size, contrary to most fish species, it undergoes spontaneous somatic aging, which leads to the expression of many aging-related phenotypes and eventually to a short lifespan (*21, 22*). The turquoise killifish inhabits ephemeral ponds in Zimbabwe and Mozambique in which fish can mate just for a few weeks before dying (*22, 23*). Sexually mature individuals, especially males, are expected to maximize the investment in reproduction throughout their short lifespan. According to the disposable soma theory, low allocation to soma maintenance is predicted to plastically trade-off with high allocation in reproduction. We thus predict to observe negligible reproductive aging in turquoise killifish males.

Work done in several species investigated the effects of age on male fertility mainly by measuring age-related changes in sperm traits (*24, 25*). Sperm quality appears to decline in some species (*26-28*), while increases (*29, 30*) or does not change in other species (*31, 32*), raising the question of whether the age-related change in sperm quality alone reflects male reproductive aging as a whole. To gain a comprehensive insight into whether species with short lifespan and extensive somatic aging display limited reproductive aging, we directly measured the effect of male age on 1) sperm traits that are generally associated with fertilization success in fish, namely sperm viability, sperm motility and sperm DNA integrity, 2) the number of fertilized eggs (hereafter fertilization success) and 3) embryo survival by using *in vitro* fertilizations (IVFs).

Materials and Methods

Fish maintenance

The turquoise killifish used in this study were laboratory-reared fish from GRZ-D strain maintained at the Fish Facility of the Leibniz Institute on Aging (Jena, Germany) under standard laboratory conditions (12:12 light–dark cycle; water temperature 26 ± 1 °C; pH 6.5-7.5). Full details on housing and breeding conditions are reported in (*33*).

Experiment 1: effect of age on sperm quality

We investigated the age-related deterioration in the ejaculate by measuring sperm quality parameters. Before sperm traits analysis, each adult male was maintained under standardized conditions (see **Supplementary material**). After obtaining sperm following an established protocol (*34*), sperm were maintained in a Hank's buffer solution (*35*) and all sperm assays were performed within 1h from sperm collection (**Figure 1**). In total, we performed sperm assays using n=28 adult and n=42 old males (see **Table 1S** for details). The average age of adult males was 9.18 ± 1.87 weeks (mean ± SD) and that of old males was 22.33 ± 3.99 weeks, which corresponds to the median lifespan in this cohort population (see **Figure 2**).

Sperm quality assays

Full details on each sperm quality assay are provided in the **Supplementary material**.

Sperm motility and velocity. After activating the sperm using distilled water, we recorded sperm motility through a digital camera connected to a phase-contrast microscope and then we analysed the videos using a computer-assisted sperm analyser (CEROS, Hamilton-Thorne). We measured sperm motility (i.e., proportion of motile cells over the total cells) and sperm velocity (VCL, μm/s). These parameters are known to be important for fertilization success in many fish species (e.g. zebrafish, *36*).

Sperm viability. We measured sperm viability following established methodologies (*37*). Briefly, we labelled sperm using a membrane-permeant nucleic acid stain (acridine orange), which labelled live sperm in green, and a membrane-impermeant stain (propidium iodide), which labelled dead or damaged sperm in red. We took fluorescent images (×40) and we counted the number of live (green) and dead (red) spermatozoa.

Sperm DNA integrity. We assessed the proportion of sperm with intact DNA using the Halomax-SCD kit (Halotech DNA), which is based on the sperm chromatin dispersion procedure. We visualized sperm DNA by staining with DAPI staining and we took fluorescent images. Sperm presenting fragmented DNA appeared as large spots with a blurred halo of chromatin dispersion while sperm with intact DNA appeared as small spots with a compact halo of chromatin dispersion.

Experiment 2: effect of age on fertilization success and embryo survival

We investigated the impact of male age on fertilization success and embryo survival by performing controlled IVFs in which sperm from adult (n=14) and old (n=14) males were used separately to fertilize the eggs from the same female (**Figure 1**). The average age of adult males was 9.1 ± 1.97 weeks (mean ± SD) and that of old males was 26.15 ± 2.54 weeks. After collecting sperm as previously described, we standardized sperm concentration of males used in the same IVF trial (see **Supplementary material**, **Figure 1S** and **2S**) and we then pooled the sperm from two males of the same age to reduce the potential for male x female effects. We collected eggs from the females and we performed IVFs (**Figure 1**) following an established protocol (*35*). The complete procedure is described in the **Supplementary material**. After IVFs, we assessed fertilization success by checking the eggs after 3.5 hours (**Figure 1**) and we removed the unfertilized eggs. To score embryo survival, we checked fertilized eggs after 24, 72, 96, 120 hours post fertilization (hpf).

Statistical analyses

We conducted data analyses using R v 4.2.0 (*38*). We fitted mixed effect model using 'lme4' package (*39*). We tested sperm viability, sperm DNA integrity, sperm motility, fertilization success and embryo survival by fitting a generalized mixed model ('glmer' function) assuming a binomial error distribution and a logit link function. We instead tested sperm velocity by fitting a linear model with the 'lmer' function. We fitted all models with male age (adult vs old) as a fixed effect, and in the model for embryo survival we also fitted the time (level: 24hpf, 72hpf, 96hpf, 120hpf) as a fixed factor. To control for potential batch effect in sperm traits, we fitted models using the male block as random factor. The models on fertilization success and embryo survival were fitted with female identity, as the eggs from the same female was used in two IVF trials, and with quartet male identity, as sperm from the same set of males were used in multiple IVF trials. We checked the residuals of each model by inspecting residuals vs. fitted values. We tested all the generalized mixed models for overdispersion by using the 'dispersion_glmer' function ('lme4' package). The model on sperm viability was overdispersed (2.10) and we corrected it by adding an Observation-level random effect. We calculated *p*-values of the fixed effect by Type II Wald chi-square tests using the 'Anova' function in the 'car' package (*40*). Effect

sizes (Hedges' *g*) and the associated 95% CIs were calculated using the 'effectsize' package (*41*). Means and SE are reported.

Results

Impact of male age on sperm quality

Sperm from adult males were significantly more viable than sperm from old males (**Table 1**, **Figure 2**). On average, the percentage of live sperm over the total was 85% ± 2 for adult males and 77% ± 2 for old males. We found that adult males had a higher proportion of sperm with intact DNA compared to sperm from old males, 60% ± 1 vs 51% ± 2 (**Table 1**, **Figure 2**). Moreover, after activation with water, the proportion of sperm that were motile was significantly higher in sperm samples from adult males compared to old males (**Table 1**, **Figure 2**), with adult males having the 42% ± 1.8 of motile sperm and old males having the 30% ± 1.9 of motile sperm. However, we found no significant effect of male age on sperm velocity (**Table 1**, **Figure 2**).

Effect of male age on fertilization success and embryo survival

We found a significant effect on male age on fertilization success (**Table 2**, **Figure 2**) with adult males fertilizing the 81% ± 3 of the eggs and old males fertilizing the 65% ± 5 of the eggs. We found a significant effect of male age on embryo survival (**Table 2**, **Figure 2**) and of the time on embryo survival (χ^2= 166.921, p<0.001), meaning that embryos survival decreases over time (from 24h to 120h post fertilization). However, the effect was not significantly different between sperm from adult and old males (male age*time: χ^2= 2.520, p=0.470).

Discussion

The vast variation in the reproductive patterns observed in nature is predicted to result from constraints between development, reproduction, and survival (*5*). Theories predict that the investment in somatic maintenance should lead to a greater reproductive senescence and to lower actuarial senescence. On the contrary, short-lived species are predicted to maximize the investment in reproduction. The turquoise killifish is one of the shortest-living vertebrates, males and females can reach sexual maturation in about

2 weeks and their natural lifespan range between 4 and 8 months, depending on the population (*19*). We thus expected to observe a high investment in reproduction, especially in males, by detecting limited sign of reproductive senescence in old fish. Instead, our results point towards the presence of strong reproductive aging in turquoise killifish males. There are at least three hypotheses that can potentially explain our results.

First, despite the optimal allocation between development, reproduction, and survival is predicted to explain most of the variation in life-history strategies, other processes which are not relying on resource allocation, may explain the emergency of life-history strategies. Mutations with no (or slight) effect on fitness in early life but that negatively affect late-life fitness can accumulate in the populations due to the age-related decline of purifying selection, and eventually shorten lifespan (*42*). In African annual killifishes, deleterious genetic variants have accumulated in the genome due to relaxed selection during the transition to the annual environment. The accumulation of deleterious mutation might have extensively contributed to the evolution of fast somatic aging and short lifespan, both at the species and population level (*43, 44*). Hence relaxed selection for late-life acting genes, instead of adaptive evolution, appears to have largely contribute to shaping late-life phenotypes in the African annual killifishes. It is thus possible that the decline in reproductive performance that we observed in the present study does not result from the adaptive optimization between reproduction and survival but rather results from the non-adaptive accumulation of deleterious mutations. Under this scenario, we hypothesise that longer-lived species of annual killifishes, which have accumulated less deleterious genetic variants compared to shorter-lived species, should show a lower age-related decline in the reproductive performance, compared to shorter-lived species, as the species used in the present study.

Secondly, the apparent lack of trade-off between reproduction and survival that we found at the species-level does not exclude that trade-offs exist at the individual level. Within the same population individuals may display alternative life-history strategies, with some individuals reproducing earlier and dying young, and other individuals starting later to reproduce (or reproducing at a lower rate) and living longer (the so called “pace-of-life”) (*45*). In turquoise killifish, the duration of the diapause (i.e.,

dormancy of the embryos) has may be linked to the emergency of alternative mating strategies. Fish undergoing fast embryonic development tend to mature and die earlier than fish characterized by slow embryonic development (*46*, but see, *47*). However, no differences in fecundity between fast and slow fish were detected, and it may be strain dependent. Whether reproduction-survival trade-offs occur at the individual level, will require future studies.

Thirdly, to successfully reproduce, males need to both mate and fertilize the eggs. Traits associated with mating and fertilization success likely have independent developmental and aging trajectories. In guppies, for instance, it has been shown that traits associated with mating success (i.e., sexual behaviour and coloration) senesce at a faster rate than traits associate with fertilization success (i.e. sperm quality) (*28*), suggesting that different reproductive traits may have different relative senescence rate depending on the biology and ecology of the species. Turquoise killifish males are larger than females, display conspicuous sexual coloration, and intensely court the females, all traits that are supposed to be important to obtain matings. It would be thus important to investigate whether traits associated with mating success show a slower rate of senescence than traits associated with fertilization success.

Overall, our findings show that turquoise killifish males undergo a spontaneous decline in ejaculate quality, which leads to an impairment of their reproductive functions. Old males fertilized fewer eggs and produce fewer viable embryos. To what extent male age can affect offspring fitness via ejaculate-mediated effects remains an important and open question in evolutionary biology. With age, sperm can accumulate genetic and epigenetic changes (*1, 48*). Mutations accumulate due to the high number of germline cell divisions and limited DNA repair of mature sperm. Such mutations can be potentially transferred to the next generation and impact offspring health (*3*). Similarly, the sperm methylome undergoes age-related changes, which have been linked to the reduced lifespan observed in offspring from old fathers compared to young fathers (*49*). To assess the evolutionary consequences of male reproductive aging, future work is needed to investigate male age effects on offspring fitness. Species undergoing spontaneous reproductive aging, such as the turquoise killifish, will help clarify the impact of male reproductive aging on offspring fitness.

Ethics statement

Fish were euthanized following European and German laws under the termination notification O_DV_21-24. Euthanasia procedure was performed by immersion in a 1.5 g/L Tricaine solution.

Data availability

Data generated in this study is uploaded as supplementary material.

Acknowledgements

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Competing interests

The authors declare no competing interests.

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Table 1: Results from the mixed models on the effect of age on sperm traits. All the variables were fitted with a binomial distribution, with the exception of sperm velocity that was fitted with a gaussian distribution.

Trait	Estimates ± SE	χ^2	p	Effect size - Hedges' *g* (95% CIs)
Sperm viability	0.48 ± 0.15	10.991	< 0.001	0.99 (0.34, 1.62)
Sperm DNA integrity	0.31 ± 0.07	21.454	< 0.001	1.77 (0.73, 2.77)
Sperm motility	0.94 ± 0.11	69.327	< 0.001	1.39 (0.48, 2.27)
Sperm velocity	-0.18 ± 1.62	0.0125	0.9108	0.16 (-0.71, 1.01)

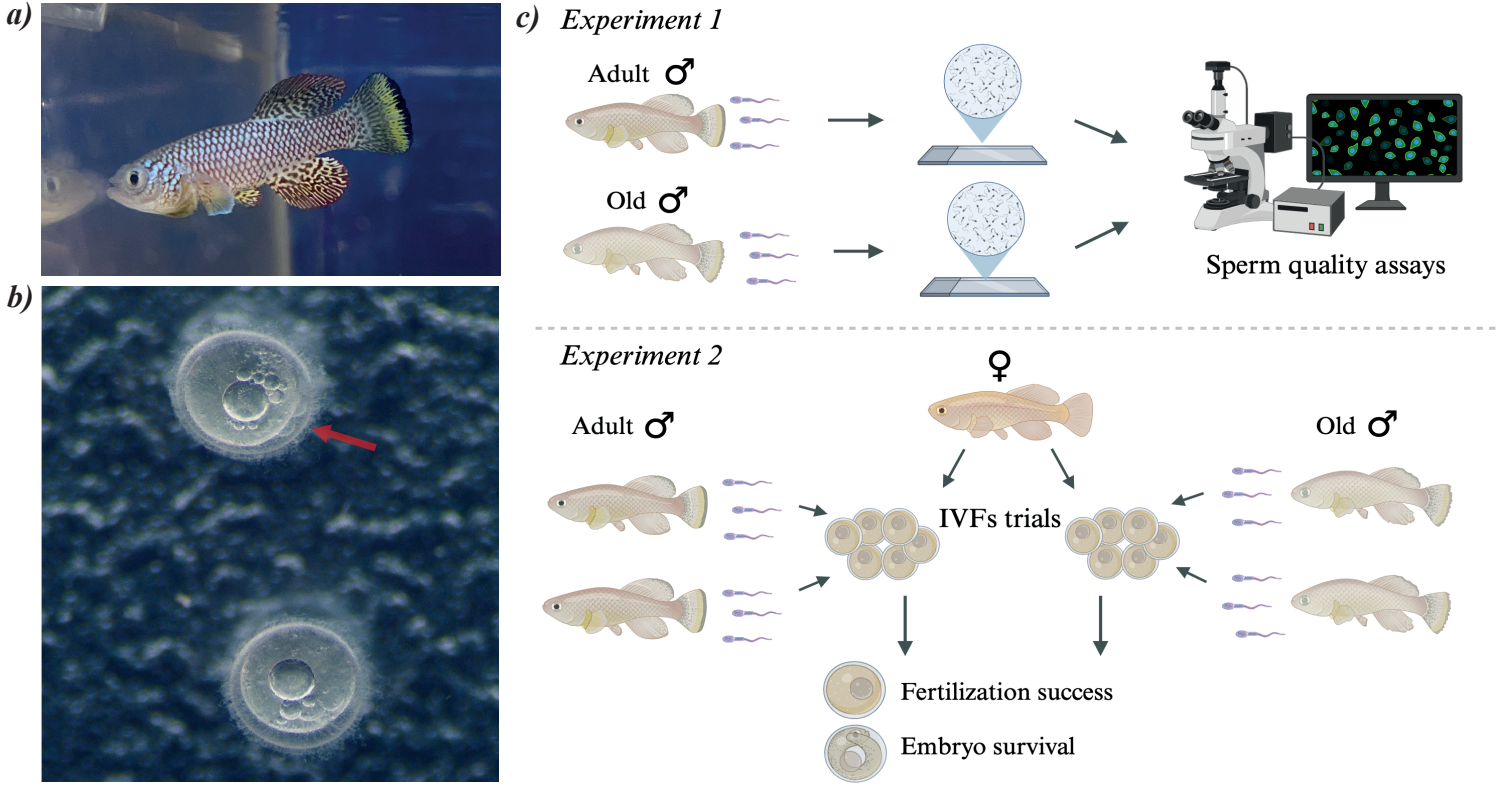
Table 2: Results from the mixed models on the effect of age on fertilization success and embryo survival. The variables were fitted with a binomial distribution.

Trait	Estimates ± SE	χ^2	p	Effect size - Hedges' *g* (95% CIs)
Fertilization success	1.26 ± 0.20	38.556	< 0.001	0.81 (0.17, 1.44)
Embryo survival	0.73 ± 0.13	32.062	< 0.001	0.48 (0.15, 0.81)

Figures legend

Figure 1: ***a)*** Representative GRZ-D male (photo by SC); ***b)*** example of fertilized eggs at 3.5 hours post fertilization. The red arrow indicates the dividing cells; ***c)*** Experimental designs for Experiment 1 (on top) and Experiment 2 (on the bottom).

Figure 2: ***a)*** Box plots depicting differences in sperm quality from adult and old turquoise killifish males. All sperm traits, except sperm velocity ($p=0.918$), are significantly different between adult and old males ($p<0.001$); ***b)*** box plots depicting significant differences ($p<0.001$) between adult and old males in fertilization success and embryo survival over the first five days from fertilization; ***c)*** survival probability of the fish cohort used in the experiments. Median lifespan: 23 weeks (22.82-24.00, 95% CIs). Box plots depicting the difference between adult (~9 weeks) and old males (~26 weeks) in embryo survival at 72hpf are superimposed to the survival curve to indicate the parallel decline in survival probability of the parents and the sperm function of the males. Median embryo survival at 72hpf: 69% (45.88-80.91, 95% CIs) for adult males and 46.43% (26.90-60.00, 95% CIs) for old males. Percentages are used only in graphs for ease of visualization.


a)
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Experiment 1
Adult ♂
Old ♂
Sperm quality assays
Experiment 2
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Adult ♂
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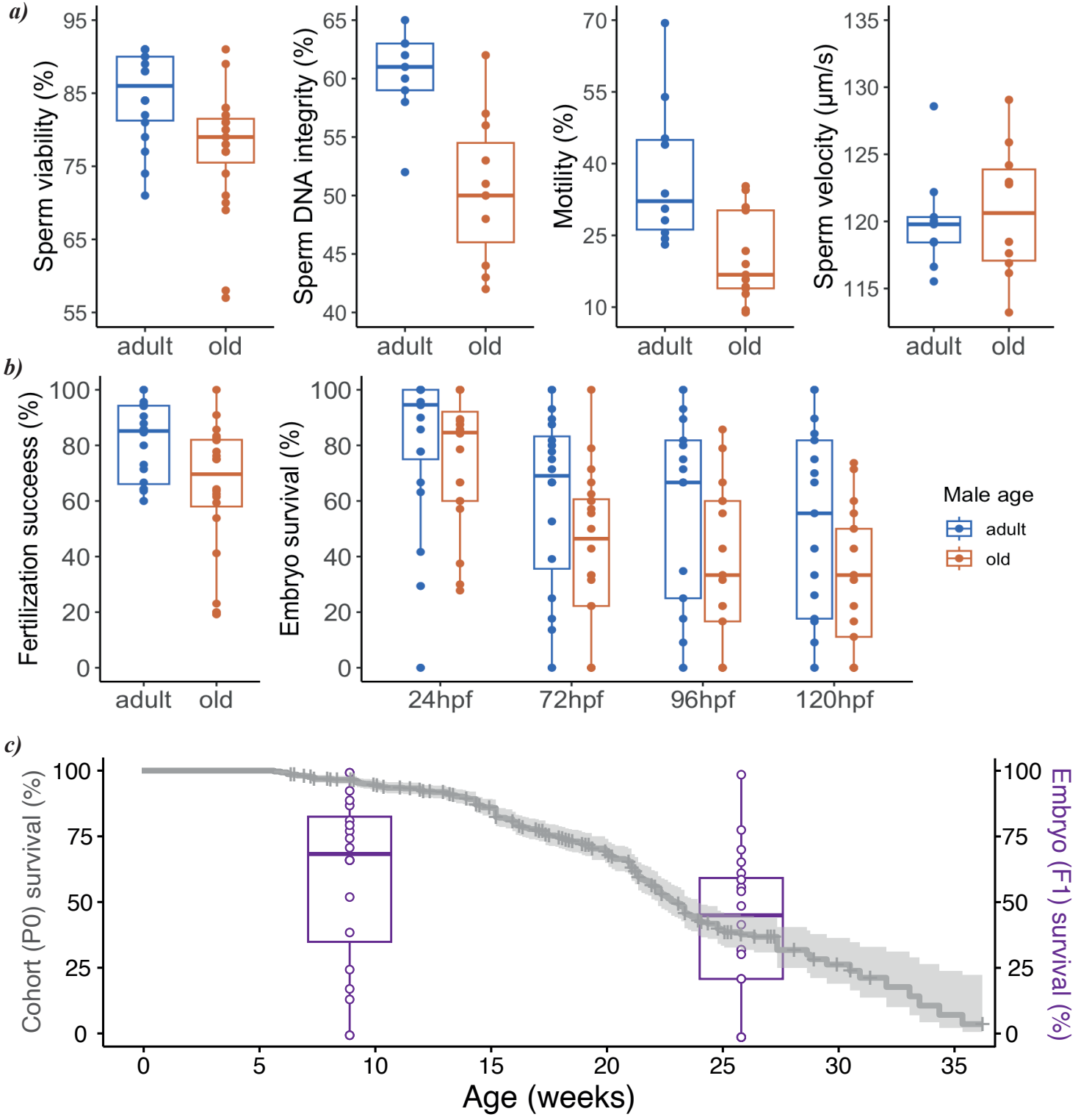
IVFs trials
Fertilization success
Embryo survival


a)
Sperm viability (%)
95
85
75
65
55
adult
old
Sperm DNA integrity (%)
65
60
55
50
45
40
adult
old
Motility (%)
70
55
40
25
10
adult
old
Sperm velocity (µm/s)
135
130
125
120
115
adult
old
b)
Fertilization success (%)
100
80
60
40
20
0
adult
old
Embryo survival (%)
100
80
60
40
20
0
24hpf
72hpf
96hpf
120hpf
Male age
adult
old
c)
Cohort (P0) survival (%)
100
75
50
25
0
Embryo (F1) survival (%)
100
75
50
25
0
0
5
10
15
20
25
30
35
Age (weeks)