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Short-term variation in sperm competition causes sperm-mediated epigenetic effects on early offspring performance in the zebrafish

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The inheritance of non-genetic factors is increasingly seen to play a major role in ecology and evolution. While the causes and consequences of epigenetic effects transmitted from the mother to the offspring have received ample attention, much less is known about how variation in the condition of the father affects the offspring. Here, we manipulated the intensity of sperm competition experienced by male zebrafish *Danio rerio* to investigate the potential for sperm-mediated epigenetic effects over a relatively short period of time. We found that the rapid responses of males to varying intensity of sperm competition not only affected sperm traits as shown previously, but also the performance of the resulting offspring. We observed that males exposed to high intensity of sperm competition produced faster swimming and more motile sperm, and sired offspring that hatched over a narrower time frame but exhibited a lower survival rate than males exposed to low intensity of sperm competition. Our results provide striking evidence for short-term paternal effects and the possible fitness consequences of such sperm-mediated non-genetic factors not only for the resulting offspring but also for the female.

1. Introduction

Epigenetic effects are increasingly accepted to play a major role in evolutionary processes and heredity [1–3]. Conditions experienced during early development and growth may affect both the individuals exposed to such conditions and also their offspring and may lead to context-dependent non-genetic inheritance (parental effects, e.g. [4]). Epigenetic effects transmitted from the mother to her offspring through the ovum or placental functions are known to affect early embryo development, which may have fitness consequences for the offspring later in life [5]. Such maternal effects have been extensively studied for example in the context of variation in hormone levels contained in eggs in birds [6], or the maternal transfer of methylation patterns and cytoplasmic molecules such as different types of RNA to the zygote in rodents [7,8]. By contrast, the fitness consequences of paternal effects are much less well understood.

Evidence has been mounting that sperm-mediated epigenetic effects exist and may play an important role in early embryo development. In the nematode *Caenorhabditis elegans* for example, chromatin and histone modifications in the germ line are not only retained in mature sperm but are also largely retained in the early embryo [9]. Similarly, extensive epigenetic marking at loci of high developmental importance has been identified in human sperm [10], and histone methylation across regulatory regions of loci in human and mouse sperm has been shown to play an important role for developmental expression patterns [11], highlighting the potential for epigenetic transfer from sperm to embryo. In fact, two very recent studies of the zebrafish *Danio rerio* have shown that the paternal methylation pattern is inherited in a nearly unaltered fashion by the zygote, whereas the maternal methylation patterns experience substantial reorganization [12–14]. The next important step is now to investigate the

mechanisms causing variation in such paternal epigenetic effects, as well as to study the potential fitness consequences.

Sperm competition is a powerful force driving the evolution of both male and female sexual traits [15,16]. Males invest into sperm production according to the risk and intensity of sperm competition they face, both across species [17–20] and within species [21,22]. Males have been shown to alter ejaculate traits such as sperm numbers, sperm density, sperm swimming velocity and sperm morphometry when facing varying levels of sperm competition [22–25]. Such changes are usually achieved over very short periods of time. In the red jungle fowl *Gallus gallus* for example, males that are forced to change social status exhibited changes in sperm numbers and sperm motility within 14 days after the change in their social status [23]. In the broadcast spawning ascidian *Styela plicata*, sperm traits such as sperm total length and sperm swimming velocity differed significantly after just four weeks of exposure to experimentally manipulated conditions varying in intensity of sperm competition [26]. Similarly, after just three weeks of experiencing varying social environments, male Gouldian finches *Erythrura gouldiae* produced sperm with a significantly different morphometry compared with sperm they produced at the beginning of the experiment and the response differed across different social environments [27]. While the advantage of such changes for increasing the fertilization success under competitive conditions is intuitive, the potential consequences of these changes for the resulting offspring are still poorly understood.

Males that are more successful in sperm competition are known to sire sons that are more successful in sperm competition [28], and offspring that have higher fitness [29]. Such results have been interpreted as evidence supporting the 'good sperm' hypothesis, which creates a direct link between the performance of a male's sperm and the male's genetic quality [30]. However, whether these heritable effects are genetic or rather epigenetic is no longer clear. A recent study of the neriid fly *Telostylinus angusticollis* has shown that males kept in same-sex groups produced larger offspring, but a paternal mixed-sex environment prior to mating led to more viable offspring [31]. Similarly, in the broadcast spawning ascidian *S. plicata*, males exposed to high levels of sperm competition sire offspring which hatch faster and survive better [32]. These results suggest a potential role of paternal epigenetic effects mediated by the social environment.

The aim of our study was to test whether rapid changes in ejaculate traits due to varying intensity of sperm competition has any effects on the resulting offspring. We used the zebrafish *Danio rerio* as a study species, because the use of an external fertilizer allows minimizing the potential effect of the seminal fluid as well as the use of a split clutch design to assess and potentially disentangle paternal and maternal effects. We exposed males to one of two treatments: a high-intensity sperm competition treatment, where two males were kept with one female, and a low-intensity sperm competition treatment, where one male was kept with two females over the duration of just two weeks. This time period is long enough for two spermatogenic cycles to be completed [33]. We then tested for potential effects of variation in intensity of sperm competition on ejaculate traits and early offspring development. We found that males exposed to high intensity of sperm competition not only produced ejaculates containing faster and more motile sperm, but also sired offspring that hatched over a narrower time period. Interestingly, offspring sired by males from high-intensity sperm competition treatments suffered reduced survival compared with offspring sired by males exposed to low intensity of sperm competition. These results suggest that even short-term adjustments to social conditions may be translated into the resulting offspring through paternal epigenetic effects.

2. Material and methods

(a) Study species

The zebrafish used in this experiment are AB wild-type descendants (parental fish purchased at ZIRC: Zebrafish International Resource Center, University of Oregon, Eugene, OR, USA) that have been raised to maturity under standard laboratory conditions in the SciLifeLab facilities at the Evolutionary Ecology Center at Uppsala University. All fish were fed ad libitum twice per day, where dried flake food was given in the morning and live *Artemia* larvae in the afternoon. The Swedish Ethical standards were respected and all experimentation was approved (Jordbruksverket approval no. C341/11).

(b) Experimental set-up

Males were kept in two groups exposed to one of two treatments: in the high sperm competition treatment, two males were kept with one female but only one male was used for the *in vitro* fertilizations (IVFs) ($N = 25$ treatment groups), whereas in the low sperm competition treatment, one male was kept with two females ($N = 29$ treatment groups) for a total of two weeks to ensure the completion of two spermatogenic cycles, which last 6 days (spermiogenic phase: 6 days, [32]). Males were separated from the females 24 h prior to the IVFs in order to ensure sperm replenishment. The main reason for keeping the animals in small groups is that zebrafish are shoaling fish and direct contacts allows them to behave naturally, keeping them in isolation (even with visual and olfactory contact with other fish) prevents them from shoaling, which would lead to significant stress levels [34,35] and could jeopardize the outcome of the experiment. Furthermore, in the zebrafish, both males and females compete for spawnings and females may dominate males as well as other females, which suggests that the difference in mating rates between the treatments is likely to be rather small [36]. Treatment groups were kept in 3 l tanks provided with artificial aquarium plants for spatial heterogeneity and for sheltering and hiding. After two weeks, comprehensive sperm measurements were taken from every focal male (i.e. all males from low treatments and one focal male from each high treatment; total $N = 54$), and males returned to stock. In order to avoid any possible confusion with maternal effects due to treatment differences, for IVFs we used females that had been kept under standard conditions in groups of 16 fish with 1 : 1 sex ratio for two weeks. Owing to handling constraints, the experiment took place in five separate blocks, which are included as a random effect in all statistical analyses.

(c) Sperm measurements

Males were anaesthetized (more than 60 s) in MS-222 solution, briefly washed in tank water and placed ventral side up on a damp sponge cradle placed under a stereomicroscope. Their genital area was gently dried with a clean paper towel before sperm collection. In order to obtain sperm, the sides of the fish were gently stroked several times in a cranio-caudal direction using smooth forceps. The sperm appearing at the genital pore was collected with a microcapillary, the amount was recorded and the sample then deposited in 30 µl of Hank's solution (HBSS) and stored on ice until examination (60–120 min post-collection). Sperm motility was recorded using a Leica DMRXE microscope

Table 1. Sample sizes (means and standard deviations, as well as statistical comparisons across the two different treatments) for the traits investigated. Sperm traits have been measured in the individual focal males' ejaculates, offspring traits are based on the clutches obtained in IVFs. Traits that differ significantly between the 'high' and 'low' sperm competition treatments are highlighted by an asterisk (*).

trait	mean ± s.d. high	mean ± s.d. low	test statistic	*p*-values
sperm longevity (s)	83.61 ± 29.32	80.22 ± 22.77	$F_{1,43} = 0.208$	0.650
ejaculate volume (μl)	3.38 ± 1.82	4.17 ± 1.71	$F_{1,57} = 2.916$	0.931
sperm density	56.83 ± 38.91	51.74 ± 30.14	$F_{1,53} = 1.010$	0.319
% non-motile sperm*	22.68 ± 22.52	37.38 ± 21.21	$\chi^2_1 = 9.690$	0.008*
eggs fertilized	20.01 ± 19.7	22.46 ± 22.11	$\chi^2_1 = 0.125$	0.723
embryo survival (alive at 24 h)	20.00 ± 19.9	22.17 ± 23.00	$\chi^2_1 = 2.430$	0.119
larval survival (80 h)	15.39 ± 18.81	17.74 ± 20.52	$\chi^2_1 = 0.127$	0.721
larval survival* (one week)	10.80 ± 19.9	14.02 ± 23.00	$\chi^2_1 = 4.485$	0.034*

(Leica Microsystems, Germany) fitted with a heating stage (heated to 28°C, Linkam DC95, Surrey, UK), a camera (Hitachi KP-D20BP, Hitachi Kokusai Electric Europe GmbH, Germany) and a DVD recorder (Sony VRD-MC6, Tokyo, Japan). One microlitre of the sperm solution was placed on a 20 μm glass cell counting chamber (2X-CEL Chamber, Hamilton-Thorne), covered with a coverslip and activated with 8 μl of tank water. The motility recording was immediately started upon activation and continued until more than 95% of the sperm had stopped moving. The measurements were repeated for each sample until at least two videos containing motile sperm in adequate numbers had been taken from each ejaculate. The video files were subsequently adjusted to optimize brightness and contrast for the sperm tracking program and cut into 2-s long subfiles at 10, 20, 30 and 40 s post-activation, respectively, using the software Avidemux (v. 2.5.6). The edited videos (qualitatively suitable videos were obtained from $N = 40$ males) were analysed using CASA software (ISAS Proiser, Projectes i Serveis R+D, S.L., Valencia, Spain) with the following settings: frame rate: 50 frames per second, frames used: 50, particle area: 5–50 μm^2, threshold measurements for VCL values ($\mu m\ s^{-1}$): $10 < slow < 45 < medium < 100 < rapid$. For the velocity analyses, slow and static sperm were excluded. The samples used in the analyses contained on average 217.5 motile sperm (range: 23–862; per cent motile: table 1). In addition, we counted the number of sperm on five different frames per sample to get an indication for sperm density within the ejaculates.

(d) *In vitro* fertilization

After the collection of the sperm, we collected eggs from females ($N = 48$) for IVF. To do so, we placed an anaesthetized, rinsed and lightly dry-blotted female into a 35 mm Petri dish. Eggs were obtained by gently squeezing along the sides of the body towards the genital opening with damp fingertips. Any contact of the eggs with water was carefully avoided prior to fertilization in order to avoid premature egg activation. The female was revived in warm tank water straight after the stripping procedure. Clutches containing at least 20 yellowish, translucent eggs (indicating viability) were carefully split into two parts ($N = 17$), which were placed in separate Petri dishes and each sub-clutch fertilized with sperm from a different male, by adding 10 μl of sperm solution and 20 μl of tank water for activation. If less than 20 eggs were gathered, the clutch was not split ($N = 22$), in cases where more than 70 eggs were retrieved, the clutch was split into three parts ($N = 6$) and into four parts ($N = 4$) when the clutch contained more than 90 eggs. Each male's sperm was used to fertilize subclutches from two different females. This 'split-clutch' design allowed us to examine the paternal effects on offspring development while also investigating maternal and paternal compatibility (i.e. male–female interaction) effects. A total of $N = 90$ clutches ($N = 45$ per treatment) of fertilized eggs were obtained.

(e) Offspring measurements

We checked for fertilization success 1 h post-fertilization and recorded embryonic survival at 3 h, 24 h, 80 h and one week post-fertilization. We scored the proportion of hatched larvae within each sub-clutch every 2 h, starting from 48 h after fertilization until either all larvae had hatched or stopped at 80 h post-fertilization, as no further successful hatching events have been observed past this point in time in the study population. Any larvae that had not hatched at that point were assumed to be dead.

(f) Statistical analyses

All analyses were conducted using packages developed for the software R v. 2.15.2 [33] as specified below.

(g) Sperm traits

We conducted ANOVAs to investigate the effect of the social environment on sperm longevity and sperm number. To investigate the effects of treatment on the curvature of the functional decline of sperm velocity over time, we performed linear mixed models (LMMs; function *lmer* in the package *lme4*) [37]. Curvature was modelled as a second degree polynomial of time (in seconds, post-sperm activation). Treatment effects on curvature were evaluated by testing the significance of the interaction terms between treatment and the polynomial terms (linear, quadratic). Tests were performed by removing model terms (backward selection), starting with interaction and highest order terms (e.g. treatment × $time^2$ for the quadratic curvature of the polynomial). This reduced model was tested against the full model, using likelihood-ratio tests, with twice the difference in log-likelihoods assumed to follow a χ^2 distribution. In the final model, only terms whose elimination from the model did not enhance the model fit (i.e. with $p < 0.05$) were retained. All models are listed in the electronic supplementary material, table S1. Male identity was included as a random intercept in all models. We obtained 95% highest posterior density intervals (i.e. Bayesian credible intervals) with the functions *mcmcsamp* and *HPDinterval* of the *lme4* package. The true value of estimated parameters is predicted to be in these confidence regions with a probability of 95% (table 2).

(h) Survival

We investigated treatment effects on larval survival using generalized LMMs (GLMMs; function *glmer* in the package *lme4*) [37] with

Table 2. Parameter estimates for the final models for each of the response variables testing for a difference in sperm traits (VCL: curvilinear velocity; VAP: average path velocity; VSL: straight-line velocity) and offspring performance (survival: hatchling survival to one week of age; hatch: hatching timing). CI represents the 95% credible intervals (VCL; VSL; VSL) or 95% confidence intervals (survival; hatch). Baseline factor is the 'high' sperm competition treatment. Significant explanatory variables are highlighted by an asterisk (*).

			CI	
response	model terms	effect value	lower	upper
VCL	treat	−12.783	−31.690	11.029
	time*	−17.874	−27.328	−8.453
	treat × time	−8.966	−21.405	3.563
	$time^2$	−0.535	−2.335	1.369
	treat × $time^{2}$*	3.122	0.564	5.447
VAP	treat*	−18.098	−32.827	−1.871
	time*	−16.582	−18.149	−15.030
	treat × time*	3.081	1.012	5.147
VSL	time*	−18.991	−24.031	−13.736
	$time^{2}$*	1.553	0.523	2.553
survival	treat*	−0.473	−0.801	−0.147
	time*	2.502	2.185	2.821
	treat × time*	0.232	0.124	0.341
	$time^{2}$*	−0.414	−0.481	−0.348
hatch	treat	0.135	−0.560	0.830
	time*	1.951	1.763	2.139
	$time^{2}$*	−0.138	−0.163	−0.113
	$time^{3}$*	0.004	0.003	0.005
	treat × time*	−0.074	−0.113	−0.035

a binomial error distribution and a logit link function. All models used in the backward selection approach are listed in the electronic supplementary material, table S3. This approach allowed us to take parental effects into account, by including male and female identifiers and the male × female interaction term as random intercepts. We report symmetric confidence intervals as parameter estimates ± s.e. 1.96 (*mcmcsamp* cannot handle generalized model structures, table 2).

(i) Hatching success

Similar to the above, hatching was analysed in GLMMs, using a backward selection approach [37] (see the electronic supplementary material, table S3). However, owing to the extended time series investigated here, we were able to investigate curvature as third degree polynomials of time (linear, quadratic and cubic). In addition to male and female identity and their interaction term, clutch identity, which serves as unique identifier to control for split clutch sizes, was included as a random intercept in all models. Treatment effect was evaluated with likelihood-ratio tests when GLMM were used. Confidence intervals are reported in table 2.

3. Results

(a) Adjustment of sperm traits to intensity of sperm competition

We found no significant differences between the treatments in sperm density, ejaculate volume or sperm longevity (table 1). By contrast, we found that ejaculates from males in the low competition treatment contained a higher fraction of non-motile (static) sperm than ejaculates from males in the high sperm competition treatment (table 1). Furthermore, we found that the decline over time in curvilinear velocity (VCL) was much more pronounced in sperm produced by males from high sperm competition environments compared with males from low sperm competition environments (backward model selection, see the electronic supplementary material, table S1: M1 versus M2, $\chi^2_1 = 6.017$, $p = 0.0142$; M1 versus M3, $\chi^2_2 = 9.722$, $p = 0.0077$; M1 versus M4, $\chi^2_2 = 38.235$, $p < 0.001$; table 1 and figure 1*a*).

Similarly, we found a significant treatment effect on the linear decline of average path velocity (VAP) over time (M7 versus M8, $\chi^2_1 = 8.368$, $p = 0.0038$; but no effect of curvature: M5 versus M6, $\chi^2_1 = 1.720$, $p = 0.1896$; M6 versus M7, $\chi^2_1 = 2.753$, $p = 0.0971$; table 2, electronic supplementary material, table S1), with sperm from the high competition treatment declining more rapidly (figure 1*b*). However, there was no significant treatment effect on the decline in straight-line velocity (VSL, figure 1*c* and table 2; for details on model outcomes, see the electronic supplementary material, tables S1 and S2). Additional analyses on velocity ratios revealed further significant treatment effects on their respective decline over time (electronic supplementary material, tables S1 and S3; linearity: M16 versus M17: $\chi^2_1 = 6.435$, $p = 0.0112$; straightness: M18 versus M19: $\chi^2_1 = 9.0082$, $p = 0.0027$; wobble: M20 versus M21: $\chi^2_1 = 0.8257$, $p = 0.3635$, M21 versus M22: $\chi^2_1 = 15.517$, $p < 0.0001$, M21 versus M23: $\chi^2_1 = 3.7742$,

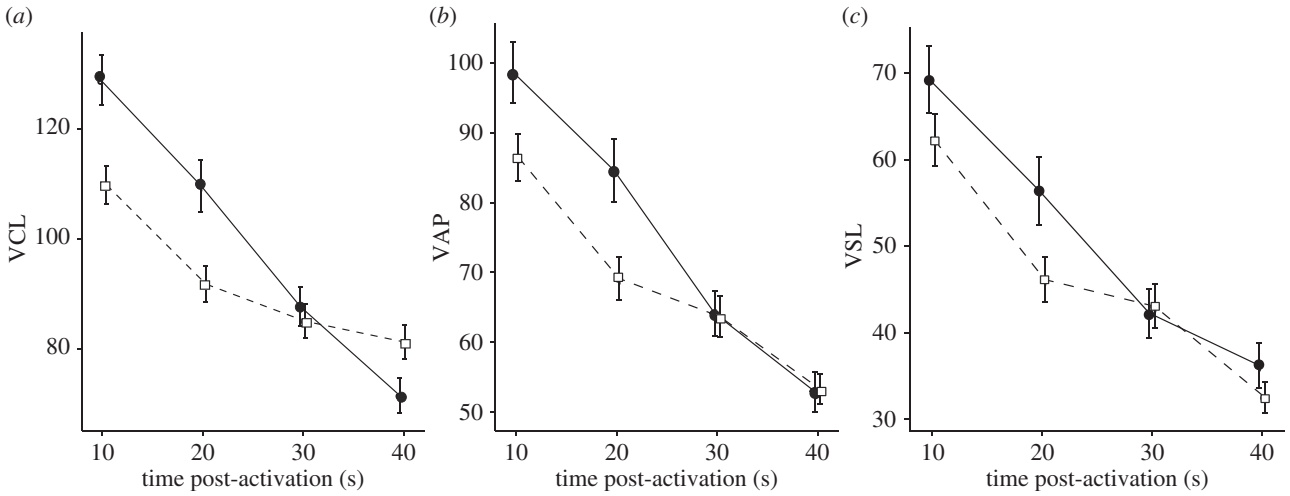

Figure 1. The change of sperm velocity over time (seconds post-activation), showing means and 95% CI. (*a*) VCL: curvilinear velocity (μm s^{-1}), (*b*) VSL: straight-line velocity (μm s^{-1}); (*c*) VAP: average path velocity (μm s^{-1}). In all three panels, the full line and black dots represent the 'high' sperm competition treatment, whereas the 'low' sperm competition treatment is represented by the dotted lines and open squares.

$p = 0.05$). The finding that VSL showed no strong response to varying intensity of sperm competition may be explained by the fact that VAP and VCL are thought to be the main determinants of fertilization success in externally fertilizing species [38,39]. It is important to note that selective pressures on sperm traits are likely to differ considerably between internal and external fertilizers. To allow direct comparison with other studies, we report all three velocity parameters.

(b) Effects of paternal environment on offspring performance

We recorded embryo survival at 3, 24, 80 h and 7 days post-fertilization (table 1). Survival was significantly affected by sperm competition intensity (GLMMs indicated significant effects of treatment due to differences in the decline over time, M25 versus M26, $\chi^2_1 = 30.01$, $p < 0.001$; but no effects of a quadratic treatment interaction term: M25 versus M24, $\chi^2_1 = 0.760$, $p = 0.383$; electronic supplementary material, table S4), as offspring sired by high sperm competition males exhibited higher mortality over time than offspring from low sperm competition males. This effect is also evident in the significantly higher survival numbers of offspring sired by low sperm competition males at one week of age (table 1).

We also recorded the time between fertilization and hatching for all sired offspring and tested for a difference between the treatments. During the critical period of hatching (48 h post-fertilization until 80 h post-fertilization), we scored the number of hatched offspring in each clutch every 2 h. We found that offspring from the high sperm competition regime hatched within a narrower time frame than offspring sired by males under relaxed sperm competition (figure 2; electronic supplementary material, table S4; GLMMs indicated significant effects of treatment due to differences in the linear decline over time, M29 versus M30, $\chi^2_1 = 14.154$, $p < 0.001$; but no effects of quadratic and cubic treatment interactions: M27 versus M28, $\chi^2_1 = 3.374$, $p = 0.065$, M28 versus M29, $\chi^2_1 = 0.809$, $p = 0.368$).

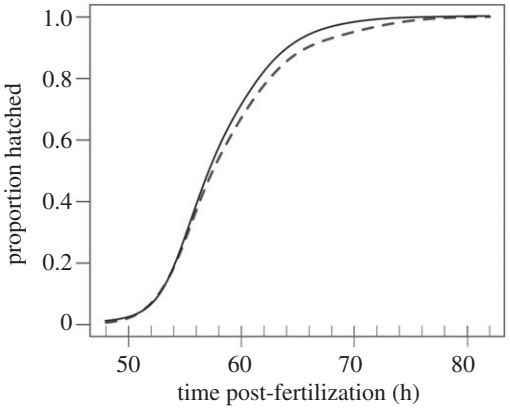

Figure 2. Differences in hatching timing (in hours since fertilization) between offspring sired by males from high (solid line) and low (dashed line) sperm competition environments as shown by curves of hatching success. Shown are the proportions of hatched offspring in the two treatments measured in 2-h intervals between 48 and 80 h post-fertilization.

4. Discussion

Our results suggest that varying intensity of sperm competition not only affects sperm performance but also the resulting offspring through sperm-mediated epigenetic effects. Male zebrafish exposed to high intensity of sperm competition produced faster swimming and more motile sperm and also sired offspring that hatched faster than males exposed to low intensity of sperm competition. However, the survival was better among the offspring sired by males exposed to low intensity of sperm competition. This effect was evident after just two weeks of exposure of target males to varying levels of sperm competition and hence suggests that even short-term changes in a male's environment may create epigenetic effects inherited by the offspring. We note that although differences in mating rates among males between the treatments could partly contribute to the observed results, we believe that this effect is likely to be small (see Material and methods for details). Here, we discuss the potential implications of our findings and provide an overview of possible mechanisms underlying the observed effect.

(a) Paternally inherited epigenetics

Our finding of significant paternal effects caused by variation in the social environment over the course of just two weeks in a vertebrate is rather surprising. We currently can only speculate about the underlying mechanisms, but there are two

main ways by which paternal condition may be transferred into the resulting zygote through sperm in zebrafish: for one, through mRNAs transferred from the sperm into the zygote [40], or through histone pre-marking and gene methylation patterns of developmental genes [41,42]. In humans, sperm exhibiting variation in motility have been found to differ significantly in the profile of specific types of mRNA [40,43,44]. In addition, mRNAs are also known to be transferred into the egg during fertilization and may affect early embryo development [40]. One possible mechanism is that transcription rates in the haploid spermatids of the males may change and affect mRNA content in the sperm affecting performance in sperm as well as in the resulting zygote.

An alternative possibility is that methylation patterns in the male are affected by environmental changes as a result of variation in hormone levels and these patterns may be translated into the sperm and hence into the embryo. Gene expression patterns during early embryo development strongly coincide with activating and repressing marks of chromatin in mature sperm in the zebrafish [41,42]. Many of the genes exhibiting such pre-patterning are coding for basic metabolic processes and developmental genes. Moreover, two recent studies in the zebrafish highlighted the fact that methylation patterns are largely inherited from fathers in the zebrafish [13,14]. How fast these methylation patterns can change over time remains to be tested. In addition, little is known about what affects these methylation patterns and how much variation exists across males. However, our results on the increasingly important influence of paternal influence on offspring survival fit the observed paternal inheritance of methylation patterns [12,13].

While paternal epigenetic effects due to long-term differences in male environmental and nutritional conditions are known [1,45–47], short-term paternal effects are less well understood. A recent study in the neriid fly *T. angusticollis* investigated epigenetic effects caused by variation in the male social environment [31]. In this study of neriid flies, the authors manipulated the diet during larval development as well as the social environment of adult male flies. Males reared on a nutrient-rich diet sired larger male offspring when kept in mixed-sex groups prior to mating, but they produced more viable offspring when kept in same-sex groups. However, effects on sperm performance in the fathers were not investigated. The authors argue that males in mixed-sex groups may be more sperm depleted and hence females may lay more unfertilized eggs but this was not formally tested. Similarly, a study in the broadcast spawning ascidian *S. plicata* reports a paternal effect of the sperm competition environment of males experienced for one month, where offspring sired by low sperm competition males hatched faster and survived better than offspring sired by high sperm competition males [32]. These results contrast somewhat with our results where offspring from high sperm competition treatments hatched over a narrower period of time than offspring sired by males from a low sperm competition environment.

Finally, we cannot entirely rule out the possibility of differential selection among sperm genotypes within an ejaculate of a male when the intensity of sperm competition varies. Under this scenario, one sperm genotype would be more favoured under low intensity of sperm competition, whereas another sperm genotype would be more favoured by the high sperm competition intensity environment. This could be seen as a meiotic drive changing its direction according to male condition—a 'condition-dependent meiotic drive', which is a completely untested concept. However, we believe that this explanation is rather unlikely given the recent advances in the study of the epigenetic mechanisms in the zebrafish (see above).

(b) The fitness consequences of epigenetic effects

The apparent fitness consequences of paternal effects induced by varying levels of sperm competition are two-sided. For one, they affect hatching and survival of the offspring and for the other, the effect on survival of the offspring ultimately affects the fitness of the female. In the zebrafish, the moment of hatching is assumed to be relatively variable compared with steps of early embryonic development including the formation of different vital organs and structures such as the heart, the eye or the somites [48]. The protein product tetraspanin cd63 produced by the hatching gland is known to be vital for successful hatching, as knocking down the gene responsible for the production of this proteolytic enzyme has been shown to make hatching impossible [49]. Whether variation in the levels of expression and translation of cd63 is responsible for the observed differences in offspring hatching time is currently under investigation. In addition, larval activity may contribute to the actual timing of hatching as it causes the chorion to break and the larva to be released [50]. If paternal epigenetic effects such as those described above influence the metabolic rate of the offspring, larval activity levels may be affected, which could explain the differential hatching patterns observed in our experiment. A connection between stress and standard metabolic rate (SMR) has been for example reported in brown trout *Salmo trutta*, where fish that were forced into subordinate roles exhibited markedly higher levels of SMR compared with dominant fish [51]. However, more detailed investigations are needed to confirm this hypothesis on an epigenetic level. Effects on the metabolic rate may also explain the difference in survival of the resulting offspring [52].

Whether the relatively earlier hatching provides a fitness advantage later in life is still unclear, especially in the light of the potential trade-off with larval survival. High intensity of sperm competition may induce higher levels of stress [27], which may accelerate metabolic rates not only in the males themselves, but also the sperm they produce and the resulting offspring. Such an increased metabolic rate could lead to an increased activity within the egg resulting in earlier hatching. Such increased metabolic rates may in turn negatively affect the survival of the offspring. Nonetheless, the timing of hatching relative to competitor broods has been shown to have fitness effects in other externally fertilizing fish such as the Atlantic salmon *Salmo salar*; competition for larval feeding grounds may be strong and larvae arriving early at these feeding grounds appear to have an advantage compared with those arriving even a day later [53]. Also, positive fitness consequences for early offspring emergence have been demonstrated in a range of species, including mole salamanders *Ambystoma talpoideum*, where early hatching individuals were found to have higher survival rates than late hatching conspecifics [54]. Fitness advantages for early hatchers in asynchronously laying bird species are well documented (see for example [55], and references therein), as are the advantages for early hatching in lizards [56]. For insects, early eclosion represents an advantage for mating opportunities and is a strong selection pressure

[57]. It is therefore possible, that also in zebrafish a slight advantage at this early stage in life may have far-reaching consequences later in life.

As mentioned above, the lower survival rate of offspring sired by males exposed to high sperm competition risk compared with that of offspring sired by low sperm competition males may affect female fitness, as eggs fertilized by such males lead to less viable offspring. Females may therefore try to avoid mating with males under such conditions—an aspect that has not been investigated much yet. Whether this initial disadvantage is compensated for later in life needs to be tested. In any case, epigenetic factors may need to be considered more carefully in future investigations of female choice, the evolution of female preference and sexual selection in general [58].

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Data accessibility. All data recorded have been made available online on Dryad at doi:10.5061/dryad.g90hd.

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