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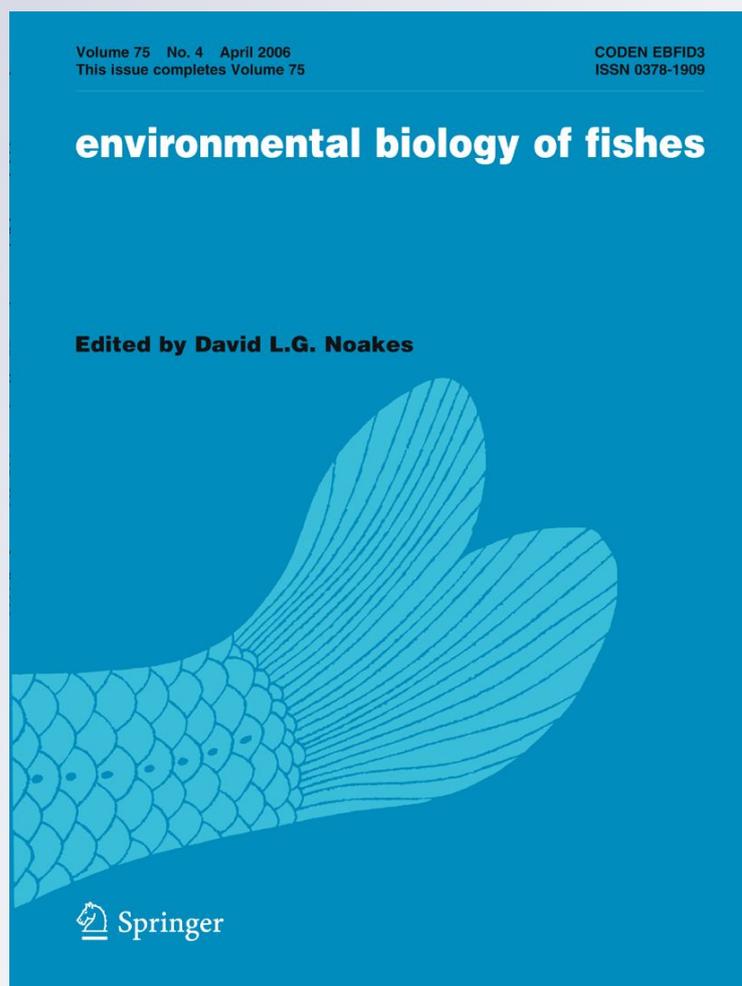
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Paternal body size affects reproductive success in laboratory-held zebrafish (*Danio rerio*)

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Abstract Across many fish species, large females tend to exhibit higher individual reproductive success due to elevated fecundity and the provisioning of better conditioned eggs and offspring compared to small females. By contrast, effects of paternal body size on reproductive success are less well understood. We disentangled the maternal- and paternal-size dependent

effects on reproductive output and early life history in zebrafish (*Danio rerio*). In the laboratory, females and males from four size categories (small, medium-sized, large and very large) were allowed to spawn freely in a full factorial design with 10 replicates per size combination. As expected, larger females produced more eggs and better conditioned offspring compared to smaller females. Male body size further contributed to zebrafish reproductive success: offspring sired by large males exhibited higher hatching probability and these offspring also hatched earlier and larger than offspring fertilized by small males. However, the largest males experienced lower mating success and received fewer eggs than males of the smaller size classes. While male body size substantially affected reproductive success in zebrafish, it remained unclear whether and to what degree direct paternal effects (e.g., related to sperm quality) or indirect paternal effects stemming from differential allocation patterns by females were the mechanism behind our findings. Answering this question constitutes an important future research topic.

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Introduction

Females are known to invest more resources into an embryo relative to males whose contribution is often

confined to sperm only, thus it is commonly believed that a progeny's phenotype is more strongly influenced by the female's phenotype than by the phenotype of the male (Chambers and Leggett 1996; Heath et al. 1999). Any effect of maternal phenotype on the offspring's phenotype is referred to as maternal effect (Bernardo 1996; Mousseau and Fox 1998). The positive relationship between female body size and offspring performance is supported by findings according to which large females of many marine and freshwater fish species spawn greater numbers and often larger eggs and larvae compared to their smaller-sized conspecifics (reviewed in Wootton 1998; Green 2008; Marshall et al. 2008). However, such results should not be prematurely generalized across fish species and ecological contexts (McLean et al. 2004; Kamler 2005; Marshall et al. 2010). In fact, larvae hatching from eggs produced by large females may also be smaller than larvae hatching from eggs produced by small females due to the size-dependent variance in hatching time and differences in larval resource intake (Heath et al. 1999). According to the fundamental life-history trade-offs (e.g., trade-off between egg number and size; Stearns 1989; Roff 2002) it is unlikely that large females can maximize all reproductive traits simultaneously. Thus, the often-cited positive influence of maternal size on offspring performance and reproductive success may not always apply in nature (Marshall et al. 2010).

Relative to maternal-size effects, the effects of paternal body size on reproductive success may be less pronounced and it has also been studied less intensively (Chambers and Leggett 1996). Males' contribution to offspring development has not been assumed to be as distinct as that of females' because males do not provide any extra-nuclear material or nutrition to the developing offspring (Marteinsdottir and Steinarsson 1998; Kennedy et al. 2007). However, paternal-size effects can operate directly, either through genetic contribution to the developing offspring (e.g. 'good genes' –hypothesis; Andersson 1994) or via physiological and energetic pathways. For example, larger males may have larger testis and longer spermatozoa with higher motility, which may elevate fertilization rates (Gage et al. 2004), compared to small males (e.g., Howard et al. 1998; Skinner and Watt 2007a). Furthermore, the effect of male body size on reproductive success may be indirectly expressed by increased female reproductive

investment when mating with a high quality (e.g., large) male (Howard et al. 1998; Kolm 2001). The advantage female gains by investing more reproductive resources towards high quality male (known as differential allocation; Burley 1988) is thought to be associated with the prospects these males offer to the female's fitness, such as better oviposition sites or more intensive parental care in nest-guarding species (e.g., Sabat 1994).

Much of the previous research focused on detecting parental-size dependent effects on reproductive success in fish has been conducted by using artificial fertilization experiments in the laboratory (e.g., Chambers et al. 1989; Marteinsdottir and Steinarsson 1998). This inhibits sexual selection, mate choice and differential resource allocation patterns to be expressed, potentially biasing study findings regarding to maternal and paternal-size effects on reproductive traits (Thériault et al. 2011). To address this issue, a model species, which allows individuals to spawn freely and express mate choice may be useful. We used zebrafish (*Danio rerio*, Hamilton) to investigate maternal and paternal-size dependent effects on reproductive success using natural spawning events without artificial insemination in a full factorial design. We defined reproductive success as a combination of important reproductive traits, such as spawning probability, clutch size, egg and larval size, embryo survival and hatching probability.

Zebrafish is a small-bodied, batch-spawning cyprinid species, which spawn all year round under laboratory conditions (Spence and Smith 2006). Domesticated strains spawn at intervals of 1 to 6 days, and clutch size is known to correlate positively with inter-spawning interval (Spence and Smith 2006), female age (Eaton and Farley 1974) and body size (Spence and Smith 2006; Uusi-Heikkilä et al. 2010). In addition, female reproductive success may correlate with male body size as females have been shown to prefer (Pyron 2003) and differentially allocate eggs towards larger males if exposed to large and small males in a short sequence (Skinner and Watt 2007b). However, other zebrafish studies have not reported female mating preference towards large males (Spence and Smith 2006; Hutter et al. 2010), and ultimate female mate choice may be related to other visual (Hutter et al. 2010) or olfactory cues (Gerlach and Lysiak 2006). In addition to mate preferences, sex-ratio and population density have been shown to

affect zebrafish mating behavior and reproductive success (Spence and Smith 2005; Spence et al. 2006). High density and biased sex-ratio may lead to increased levels of aggressive interactions among males, which can have a negative effect on female egg production (Pritchard 2001; Spence and Smith 2005; Paull et al. 2010). Although parental-size dependent effects on zebrafish mating success, reproductive output and early life-history traits have been demonstrated earlier in trials comparing spawners composed of similarly-sized individuals (Uusi-Heikkilä et al. 2010), the contribution of either maternal or paternal-size effects and their interaction on reproductive success remains obscure. The objectives of the present study were to investigate whether the higher reproductive success of large zebrafish spawners relative to small spawners (Uusi-Heikkilä et al. 2010) is determined mostly by female body size or whether the variation in reproductive success is also related to male body size or the interaction between female and male body size. We hypothesized that both female and male size contribute to the reproductive success in zebrafish but the effect of female body size on early life-history traits was expected to be greater than the effect of male body size.

Materials and methods

Fish holding conditions

Our experimental fish were third generation offspring from a wild zebrafish population captured from a river system 70 km west of Coochbihar (West-Bengal, India, 22.56°N, 87.67°E). Fish were raised in six glass fiber - polyester tanks (diameter: 79 cm, height: 135 cm, volume: 320 l) in a light (14 h light: 10 h dark) and temperature controlled ($26.8 \pm 0.79^\circ\text{C}$, mean \pm S.D.) recirculation facility with an inflow rate of 0.25 l s^{-1} . The recirculation system was run with insipid tap water, and the water quality was controlled weekly for pH (8.4 ± 0.1), nitrite (N-NO_2^- ; $< 0.3 \text{ mg l}^{-1}$), ammonium (N-NH_4^+ ; $< 0.05 \text{ mg l}^{-1}$), and daily for oxygen levels ($7.9 \pm 0.6 \text{ mg l}^{-1}$). The stocking density per holding tank was 0.9 ± 0.2 individuals l^{-1} . We fed fish with freshly hatched *Artemia*-nauplii (Inve Aquaculture NV, www.inve.com) and commercial flake food (TetraMin, Tetra

GmbH, www.tetra.net; 47% protein, 10% fat) ad libitum. Fish were fed five times per day with small amounts of food as it has been shown to result in an efficient feed utilization and to maximize growth and reproductive output (Priestley et al. 2006).

At age 250 days post fertilization (dpf), females and males were caught using a dip net. Zebrafish start maturing at age 90 dpf (Schilling 2002) and at standard length of about 19 mm (Uusi-Heikkilä et al. 2011) so by the time our experiment was initiated all fish were mature. We measured females and males for standard length (SL) to the nearest mm and then assigned them into four different size categories: small (24–25 mm), medium (26–27 mm), large (28–29 mm) and very large (30–31 mm). The size ranges were based on a preliminary experiment, where females below 24 mm were found having an extremely low reproductive success, and fish above 31 mm were rare in our experimental populations. We coupled females and males from different size categories with each other employing a full factorial design and consequently produced 16 different size combinations of females and males, each replicated 10 times (altogether 160 couples). This full factorial design allowed us to disentangle the size-dependent female and male contributions to reproductive success.

By the time the experiment was initiated, spawners (i.e., mature females and males) were transferred into a standalone spawning facility (Aquarien-Bau Schwarz, 37081 Göttingen, Germany, www.aquaschwarz.com; temperature $26.7 \pm 0.64^\circ\text{C}$; pH 8.4 ± 0.1 ; $\text{N-NO}_2^- < 0.3 \text{ mg l}^{-1}$; $\text{N-NH}_4^+ < 0.05 \text{ mg l}^{-1}$; oxygen-level $7.9 \pm 0.4 \text{ mg l}^{-1}$) in spawning boxes designed to prevent egg cannibalism by separating adults from eggs as previously applied by Uusi-Heikkilä et al. (2010). A grid of a mesh size of $2 \times 2 \text{ mm}$ was inserted inside of each spawning box (volume 3 l, length: 21 cm, width: 11 cm, height: 13 cm). Each box was additionally equipped with green plastic filter material serving as a spawning substrate. Spawning boxes were stocked with one female and one male. Reproductive success of the fish from the four different size categories was assessed for four consecutive days. Zebrafish are known to spawn every 1–6 days (Spence and Smith 2006), thus it was likely that each couple willing to spawn reproduced at least once during the 4 days spawning period.

Reproductive traits

Reproductive output

Zebrafish usually spawn within the first few hours after sunrise (Hisaoka and Firlit 1962), thus the assessment of reproductive output took place between 0800 and 1000 h. During the 4 days spawning period, we cleaned the spawning boxes each morning, assessed the occurrence of a spawning event and counted the number of eggs spawned per female per one spawning event. For assessing the egg fertilization probability, we distinguished fertilized eggs from unfertilized eggs. Zebrafish eggs are translucent, and fertilized eggs can be easily identified by the presence of a multi-cellular blastodisc, which is not present in unfertilized eggs (Kimmel et al. 1995). Only clutches larger than 30 eggs were used in the egg fertilization probability estimation to avoid inflated egg fertilization probability estimates due to random egg mortalities in very small clutch sizes.

Egg traits

Egg trait measurements included the assessment of egg size and egg mortality rate. We measured egg size as egg yolk diameter. Yolk size can be a better indicator of the egg quality than egg size (Kamler 2005) because perivitelline space is not contributing substantially to the egg quality (Alderdice 1988). The eggs were photographed and the yolk diameter was measured from the photographs under a profile projector (Quick Scope; AT112-220 F; Mitutoyo; www.mitutoyo.co.jp) with an accuracy of 0.1 μm . Eggs for size measurements were selected from the first clutches spawned and these eggs were only used for size measurements, not for the subsequent analyses.

Post-fertilization egg survival was estimated from the first clutch females spawned. Egg quality can decrease in the course of spawning duration (Paull et al. 2008; Uusi-Heikkilä et al. 2010) and therefore eggs only from the first, and presumably highest quality, clutch were collected. From each female's first clutch, we transferred 15–48 fertilized eggs (depending on the total amount of fertilized eggs produced per couple) into a 24-well Multiwell Plates (BD Falcon; nontreated polystyrene; Jacob et al. 2007) so that one egg per well was incubated in 2 ml of tap water. Consequently, eggs were not

influenced by other eggs or their contaminants and could be treated as independent data points in the statistical analyses. After adding eggs to the plates we transferred the plates into a rearing incubator (Tintometer GmbH, 44287 Dortmund, Germany, www.tintometer.de) at 27°C. Water in the wells was not changed during the incubation (Jacob et al. 2007). The cell well plates were controlled during the next 48 h for the egg mortalities, which were estimated by counting the number of dead eggs from each plate.

Larval traits

Larval traits for the different sized parents were assessed as larval age-at-hatch, larval length-at-hatch, and larval yolk-sac volume. Larval traits, similarly as egg traits, were assessed from the first clutches spawned. Larvae were hatching in the cell well plates and the number of larvae hatched was recorded each day during 7 days. The standard length of each newly hatched larva was measured under the dissection scope. Larvae of age 4 dpf were used to compare the larval length-at-hatch between the different sized couples. Measures based on larval length, however, may not be a reliable indicator of the quality of the larva (Kamler 2008). Therefore, we photographed individual larva to measure larval yolk-sac volume as an indicator of larval energy resources (Kamler 2008). We used the digitizing software Image Tool for Windows (version 3.0) to measure the height and width of the yolk-sac from the photographs. The yolk-sac volume was estimated using the following formula (Chambers et al. 1989):

$$V = \pi/6LH^2,$$

where L represents the length (horizontal measurement; mm) and H the height (vertical measurement; mm) of the yolk-sac.

Statistical analyses

Due to non-normally distributed and heteroscedastic data, we used generalized linear models (GLM; Crawley 2007) to disentangle maternal and paternal-size effects on reproductive output and early life-history traits. In all the analyses, female and male size categories and their interactions were treated as fixed effects. Due to the fact that zebrafish establish

potentially body-size based dominance hierarchies (Pritchard 2001; Paull et al. 2010) and the effect of these hierarchies on zebrafish mating success and reproductive output is largely unknown, we additionally tested the effect of the relative size difference between female and male on all the reproductive traits. The individual couple was set as a random variable to account for the fact that other parental traits than body size could contribute to the differences in reproductive success among couples (Spence and Smith 2006; Hutter et al. 2010). Spawning day was also treated as a random variable when estimating the effects of parental body size on variables measured over the whole experimental period (i.e., spawning probability, clutch size and fertilization probability). The amounts of variance associated to the random variables were estimated through variance components. Couples which did not produce any eggs during the four days spawning period were excluded from the clutch-size analysis. Count data, such as clutch size and age-at-hatch, were modeled using Poisson regression. All probability data (i.e., spawning probability, egg fertilization probability, egg survival probability and hatching probability), were modeled using binomial regressions. In the analyses of larval age-at-hatch, larval length-at-hatch and larval yolk-sac volume, egg size could not be treated as a covariate because the eggs measured were not the same eggs from which the larvae hatched. Using an average value of egg size per couple as a covariate in these analyses was not feasible due to the low number of observations per couple for which both egg and larval traits were measured. Instead, we did a simple correlation analysis (Pearson's correlation) between the average egg size and the average larval age-at-hatch, length-at-hatch and yolk-sac volume. If data was over-dispersed, the quasi-Poisson or quasi-binomial distributions were used to account for the overdispersion. To estimate differences among size categories we first fitted the full model and then used the stepwise model reduction that in our case referred to aggregating size categories, which had most similar response variable values with each other.

To summarize the effects of individual traits on overall reproductive success we used spawning probability, clutch size, egg fertilization probability, egg survival probability and larval hatching probability as components to estimate an integrative measure of reproductive fitness (e.g., Mousseau and Roff 1987; Danzmann et al. 1989). The components (i.e., the

coefficients for each size combination predicted by the model) were multiplied to obtain the expected number of hatched larvae, i.e., our fitness measure was obtained by multiplying the model-based probabilities that an egg survives and hatches (as a product of spawning probability, egg fertilization probability and hatching probability) further multiplied with the predicted number of eggs for each size category of either males or females. This measure describes the effective offspring production as predicted by the statistical models and is not to be confused with lifetime fitness. The final fitness values are given as relative values where the values of different size combinations are standardized by the average value for the small female : small male size combination. In other words, this chosen reference value is used as a value of 1 and all other values are relative to this reference. Our final integrative measure of reproductive success described the expected number of hatching larvae, which was considered a proxy of fitness, as a function of female and male size, expressed relative to the small female : small male reproductive fitness.

All data were considered statistically significant at $P < 0.05$. All statistical analyses were performed with R 2.11.1 with packages MASS and lme4 (R Development Core Team 2009). Data are presented as mean values with standard errors (SE).

Results

Reproductive output

The spawning probability was not affected by female body size whereas male body size had a significant effect on the female's probability to spawn (Table 1). Females from all size categories had a significantly lower probability to spawn with very large males (0.17 ± 0.03) compared to mating with large (0.50 ± 0.04), medium-sized (0.38 ± 0.04) or small males (0.42 ± 0.04 ; Table 1). The interaction between female and male size and the relative size of males and females did not affect the probability to spawn (Table 1). Spawning day captured a relatively small amount of variance (5.5%) not explained by the parental body size, while the individual couple was responsible for relatively large amount of variance (72.9%) in terms of spawning probability.

Table 1 The effects of female, male, female \times male body size and relative size difference between female and male on zebrafish reproductive traits. Estimated values are given for the significant covariates, which are indicated in bold

Trait	Variable	Parameter values (SE)	χ^2 -value ^a (df)	<i>P</i> -value ^b
Spawning probability	Female		0.648 (10,7)	0.886
	Male			
	Small (Intercept)	-0.608 (0.110)	20.13 (5,4)	0.000
	Medium	-0.198 (0.131)		
	Large	0.631 (0.129)		
	Very large	-2.013 (0.140)		
	Female \times Male		5.008 (19,10)	0.834
Clutch size	Size difference		2.276 (12,10)	0.321
	Female		20.07 (9,4)	0.001
	Small (Intercept)	3.349 (0.299)		
	Medium	0.260 (0.254)		
	Large	0.501 (0.253)		
	Very large	1.007 (0.262)		
	Male		7.626 (6,5)	0.007
	Small (Intercept)	3.349 (0.299)		
	Medium	-0.029 (0.251)		
	Large	0.156 (0.236)		
Fertilization probability	Very large	-0.574 (0.281)		
	Female \times Male		7.937 (19,10)	0.541
	Size difference		0.386 (11,10)	0.535
	Female		0.714 (7,4)	0.870
	Male		2.188 (10,7)	0.534
Egg size	Female \times Male		14.21 (19,10)	0.115
	Size difference		5.172 (11,9)	0.075
	Female		2.856 (9,6)	0.414
	Male		6.246 (4,3)	0.012
Egg survival probability	Small (Intercept)	0.520 (0.004)		
	Medium	-0.010 (0.006)		
	Large	0.003 (0.005)		
	Very large	-0.006 (0.007)		
	Female \times Male		4.063 (18,9)	0.907
	Size difference		0.562 (11,9)	0.755
	Female \times Male		20.73 (17,8)	0.014
	Female			
	Small (Intercept)	2.227 (0.685)		
	Medium	1.714 (1.002)		
Large	-0.805 (0.943)			
Very large	1.378 (1.046)			
Egg survival probability	Male			
	Small (Intercept)	2.227 (0.685)		
	Medium	2.785 (1.559)		
	Large	-0.066 (0.897)		
	Very large	2.145 (1.138)		

Table 1 (continued)

Trait	Variable	Parameter values (SE)	χ^2 -value ^a (df)	<i>P</i> -value ^b
Hatching probability	Size difference		1.599 (11,9)	0.450
	Female		3.794 (8,5)	0.285
	Male		4.136 (3,2)	0.042
	Small (Intercept)	0.162 (0.449)		
	Medium	0.657 (0.630)		
	Large	0.598 (0.580)		
	Very large	1.631 (0.687)		
Larval age-at-hatch	Female × Male		5.999 (17,8)	0.740
	Size difference		1.193 (10,8)	0.551
	Female		2.049 (9,6)	0.562
	Male		4.594 (5,4)	0.032
	Small (Intercept)	1.599 (0.047)		
	Medium	−0.040 (0.062)		
	Large	−0.112 (0.057)		
Larval length-at-hatch	Very large	−0.153 (0.065)		
	Female × Male		11.13 (18,9)	0.267
	Size difference		2.562 (11,9)	0.278
	Female		0.150 (9,6)	0.985
	Male		7.541 (5,4)	0.006
	Small (Intercept)	3.252 (0.039)		
	Medium	0.025 (0.058)		
Larval yolk-sac volume	Large	0.123 (0.048)		
	Very large	0.071 (0.052)		
	Female × Male		14.49 (18,9)	0.106
	Size-difference		2.058 (11,9)	0.357
	Female		4.594 (5,4)	0.032
	Small (Intercept)	0.013 (0.001)		
	Medium	0.003 (0.002)		
Larval yolk-sac volume	Large	0.003 (0.002)		
	Very large	0.008 (0.002)		
	Male		2.049 (9,6)	0.562
	Female × Male		11.13 (18,9)	0.267
	Size difference		0.391 (11,9)	0.822

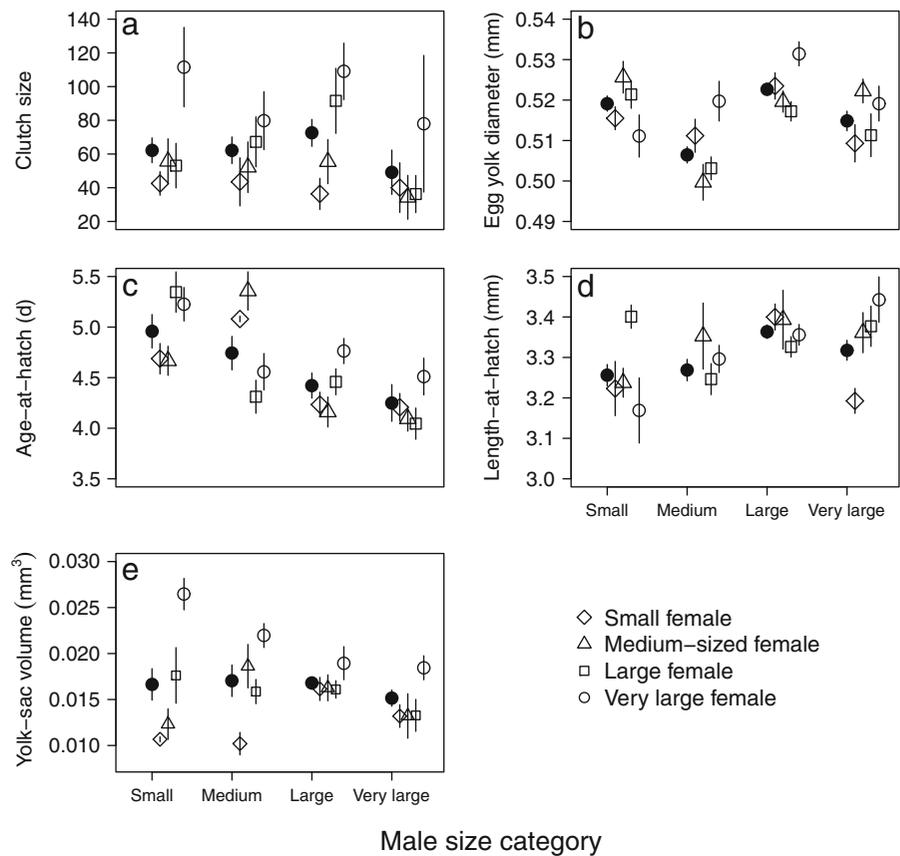
^a χ^2 -value from the deletion of the variable from the full model

^b*P*-values derived from the χ^2 -statistics

The number of eggs produced by zebrafish correlated with female's body size (Fig. 1a, Table 1). Very large females released significantly more eggs compared to large, medium-sized and small females (Table 1). Furthermore, females released on average significantly smaller clutches (number of eggs produced over four spawning days)

when mated with very large males (49.2 ± 13.1 eggs over four spawning days) compared to matings with large (72.5 ± 7.98), medium-sized (62.2 ± 7.88) or small (62.2 ± 7.33) males (Fig. 1a, Table 1). Neither the interaction nor the size difference between female and male body size did affect the number of eggs produced (Table 1). In terms of clutch size, 36.9% of

Fig. 1 **a** average clutch size (number of eggs over four spawning days), **b** average egg size, **c** average larval age-at-hatch, **d** average larval length-at-hatch and **e** average yolk-sac volume produced by each male size category across all female size categories (filled circles). The average value of crosses between each male and female size category is indicated by the open symbols. Error bars indicate standard errors



the variance not captured by parental body size was associated to the individual couples and 7.8% to the spawning day.

The probability of egg fertilization was not influenced by the parental body size or the female × male interaction (Table 1). After controlling for the effect of body size, only 1.2% of the variance was associated to the spawning days, whereas 51.8% of the variance was associated to the individual couples.

Egg traits

We found no difference in egg size (measured as egg yolk diameter) among female size categories and no significant female × male interaction (Table 1). However, females released significantly smaller eggs when crossed with medium-sized males (0.507 ± 0.002 mm) compared to the eggs released when mated with small (0.519 ± 0.002 mm), large (0.523 ± 0.001 mm) or very large males (0.515 ± 0.002 mm; Fig. 1b, Table 1). A large proportion of the variance

not explained by the parental body size (51.3%) was associated to the individual couples.

In terms of egg survival probability there was a significant interaction between female and male body size, but the pattern was not straightforward (Table 1). Certain combinations, for instance small and large females mated with small males, large males mated with either small or medium-sized females and very large males mated with very large females exhibited lower egg survival probabilities (<90%) compared to other size combinations where egg survival probabilities exceeded 90% (Table 2). 59.8% of the variance in egg survival probability was explained by characteristics other than body size of the individual couple.

Hatching probability was unaffected by female body size, but was affected by male body size. The average hatching probability of embryos fertilized by very large males (0.70 ± 0.02) was significantly higher than embryos fertilized by large (0.61 ± 0.02), medium-sized (0.60 ± 0.02) or small males (0.49 ± 0.02 ; Table 1). There was no interaction effect between male and female size.

Table 2 The average egg survival probabilities and their standard errors for different female and male size combinations in zebrafish

	Small male	Medium-sized male	Large male	Very large male
Small female	0.827 (± 0.03) (<i>N</i> =150)	0.989 (± 0.01) (<i>N</i> =94)	0.889 (± 0.03) (<i>N</i> =162)	0.979 (± 0.01) (<i>N</i> =146)
Medium-sized female	0.952 (± 0.02) (<i>N</i> =188)	0.945 (± 0.02) (<i>N</i> =201)	0.880 (± 0.02) (<i>N</i> =251)	0.990 (± 0.01) (<i>N</i> =105)
Large female	0.760 (± 0.04) (<i>N</i> =104)	0.956 (± 0.02) (<i>N</i> =189)	0.955 (± 0.01) (<i>N</i> =294)	0.963 (± 0.03) (<i>N</i> =54)
Very large female	0.944 (± 0.02) (<i>N</i> =124)	0.919 (± 0.02) (<i>N</i> =136)	0.946 (± 0.01) (<i>N</i> =350)	0.855 (± 0.03) (<i>N</i> =173)

N refers to the number of individual eggs used in the egg survival probability estimation

62.1% of the variance in hatching probability was associated to the individual couples.

Larval traits

Regardless of female body size, offspring sired by very large (4.25 ± 0.08 d) and large males (4.42 ± 0.06 d) hatched significantly earlier than offspring of medium-sized (4.74 ± 0.01 d) and small males (4.96 ± 0.01 d; Fig. 1c, Table 1). No variance in hatching time was associated to the individual couples. There was no correlation between the average egg size and the average larval age-at-hatch ($df=31$, $r=-0.127$, $P=0.483$).

Female body size was not a significant variable in determining larval length-at-hatch (Table 1). Instead, larvae which hatched from eggs fertilized by very large (3.32 ± 0.02 mm) and large males (3.36 ± 0.01 mm) exhibited greater standard length than larvae which hatched from eggs fertilized by medium-sized (3.27 ± 0.03 mm) and small males (3.26 ± 0.03 ; Fig. 1d). When aggregating size categories, very large and large males sired significantly larger offspring compared to offspring of medium-sized and small males (Table 1). In the larval length analysis, 21.5% of the variance, not explained by parental body size, was associated to the individual couples. The average larval length-at-hatch did not correlate with the average egg size ($df=31$, $r=0.180$, $P=0.316$).

Female body size, but not male body size, had a significant effect on larval yolk-sac volume. Very large females produced larvae with significantly greater yolk-sac volume (0.021 ± 0.001 mm³) relative to large (0.016 ± 0.001 mm³), medium-sized (0.015 ± 0.001 mm³) and small females (0.013 ± 0.001 mm³; Fig. 1e, Table 1). 25.1% of the variance in yolk-sac volume was associated to the individual couples. The

average yolk-sac volume did not correlate with the average egg size ($df=25$, $r=-0.031$, $P=0.880$).

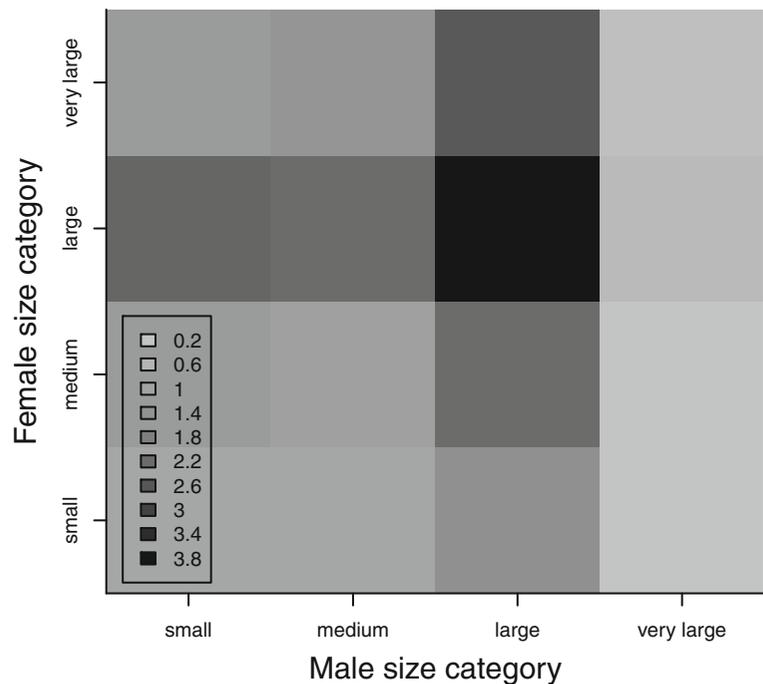
Integrative measure of reproductive fitness

The integrative measure of reproductive success (i.e., reproductive fitness) varied with both female and male body size from 0.2 to 4.0 relative to the reproductive value exhibited by small female: small male crossings chosen as the reference category (Fig. 2). Large males, independent of the crossing, had the highest predicted fitness values, which was almost three times higher compared to the reference value of a small female: small male size combination. Particularly the combination of large female and large male yielded the highest reproductive fitness value (4.0), which was four times higher than the reference value. Interestingly, the average absolute reproductive fitness value of very large females was somewhat lower (0.71) compared to the value of large females (1.13), but it was still larger than the average absolute fitness value of medium (0.61) and small females (0.46) across all male sizes. The very large males exhibited the lowest fitness values compared to all other male sizes.

Discussion

Our study is the first to disentangle maternal and paternal-size effects on reproductive success in zebrafish. As expected, female size contributed to reproductive output and larval quality, and more unexpectedly male size contributed to a wide variety of reproductive parameters involving spawning frequency, clutch size, egg size, embryo development rate and larval size-at-hatch. The integrated reproductive fitness measure showed that

Fig. 2 The estimated integrated reproductive fitness (i.e., the expected number of hatched larvae) for different female and male size combinations in zebrafish. Lighter colour corresponds to lower estimated fitness value. The values are expressed as relative to the reference size category of small female : small male=1. Colors in the figure change smoothly and the example colors in the legend correspond to the relative fitness values



large, but not very large, fish exhibited the highest reproductive success among both males and females, and while large females were reproductively superior to medium and small females, the very large males were the least reproductively fit of all male sizes (Fig. 2). Our results altogether showed that male body size contributes substantially to variation in several early life-history traits in zebrafish, and, therefore, size-dependent paternal effects might be more important for reproductive success in this species than previously believed.

The positive relationship between female body size and fecundity has been shown in several fish species (Wootton 1998) and was also evident in our study, similar to earlier reports in zebrafish (Spence and Smith 2006; Uusi-Heikkilä et al. 2010). In addition to egg number, egg size often correlates positively with female body size across a range of fish species (e.g., Green 2008; Marshall et al. 2008). This is in contrast with our results, which revealed that zebrafish egg size varied independently of female body size. However, it has been previously indicated that egg size measured as egg diameter may not be a biologically relevant parameter for determining zebrafish reproductive success (Uusi-Heikkilä et al. 2010) and this may explain the lack of correlation between egg size and female body size in the present study. The assumption is further supported by the lack of

correlation between egg size and a range of larval traits (e.g., length-at-hatch) in our study. In fact, in fish egg quality might be better reflected in embryo developmental rates or larval parameters than in egg size (Kamler 2005, 2008). In our study, very large females did not produce larger eggs, but they produced higher quality larvae, in terms of yolk-sac volume, compared to large, medium-sized or small females. This is consistent with the previous findings of greater egg and larval qualities produced by larger females of many fish species (Marteinsdottir and Steinarsson 1998; Kennedy et al. 2007), as it is known that larvae with larger yolk-sacs may show increased survival in the wild by being more resistant to starvation under food-limited conditions (Miller et al. 1988; Kjørsvik et al. 1990; Marshall et al. 2010). Yet, despite the greater egg numbers and larger larval qualities exhibited by the very large females in our study, they showed a consistently lower integrated reproductive fitness value compared to large females, while still maintaining higher relative fitness compared to medium and small females. This apparently inconsistent finding can be explained by the slightly lower model-predicted spawning probabilities, egg fertilization probabilities and hatching probabilities by eggs produced by very large females compared to large females. The multiplied effects, although

individually not statistically significant (Table 1), surmounted the significantly higher egg number produced by the very large females, resulting in slightly lower reproductive fitness values for very large females. Given the lack of trait-dependent significant differences for female size for many traits such as spawning and fertilization probabilities, one should cautiously interpret this finding and not prematurely discard the reproductive value of very large females.

We identified a range of pronounced paternal-size effects on several reproductive traits such as spawning probability, egg size, clutch size, embryo developmental rate (i.e., hatching time), larval hatching probability, and egg survival probability in zebrafish. Early hatching larvae have higher muscular activity during the embryogenesis compared to late-hatching larvae (Kimmel et al. 1995). Thus, the higher hatching probability and early hatching time of the larvae produced by the large and very large males could be an indicator of faster developmental rate and better larval condition. Previous studies have demonstrated maternal-size effects on embryo developmental rate (Marteinsdottir and Steinarsson 1998; Kennedy et al. 2007), but the evidence for paternal contributions to offspring development is limited (Saillant et al. 2001; Bang et al. 2006). In our study, larvae sired by very large and large males also hatched significantly larger, in terms of standard length, compared to larvae sired by medium-sized and small males. Larger body size at hatch may increase larval fitness in the wild due to the greater mouth gape and higher swimming activity, which allows the larva to predate more efficiently and use wider variety of prey (Miller et al. 1988).

Considering that the contribution of sperm to offspring development is mostly genetic, hypothesizing on the nature of direct, non-genetic paternal effects on early-life history traits is challenging. It has been shown that the amount and quality of sperm varies among males and this variation can be size-dependent (Howard et al. 1998). In zebrafish, however, sperm quality, quantity and motility have been shown to vary with fish age and swimming activity level (Kemadjou Njiwa et al. 2004) but not with body size (Skinner 2004). This is partly supported by our results, which showed no differences in egg fertilization probability explained by male body size. Therefore, we are not convinced that size-dependent sperm quality is a

sufficient explanation for the pronounced size-dependent paternal effects we identified.

Male size may also affect reproductive success in a less obvious indirect way through female mate choice and differential allocation patterns (Skinner and Watt 2007b). Differential allocation patterns are particularly likely when the experimental fish are allowed to spawn freely, as in our experiment, and eggs are not striped and fertilized artificially. Females benefit from the allocation of reproductive resources to better quality mates as these partners may provide better genes or more resources to the offspring (Andersson 1994). For example, in zebrafish territorial males are known to be larger (Spence and Smith 2005) and females allocating reproductive resources towards larger, territorial males may benefit from better oviposition sites. Zebrafish females have indeed been shown to prefer (Pyron 2003, but see Hutter et al. 2010) large males, and they have also been found to differentially allocate eggs towards larger males in a second spawning when mated in short sequences with either a large or a small male (Skinner and Watt 2007b). In our study, female zebrafish thus might have allocated higher quality eggs to larger males because the more territorial (i.e., larger) males may exhibit higher reproductive success, as is empirically shown to be true under low density conditions (Spence and Smith 2005; Spence et al. 2006). Such female preferences for large male body size would be revealed as a significant male-size effect in our statistical analysis. In earlier studies the higher reproductive success by larger males has not been consistently evident (Spence and Smith 2005, 2006; Spence et al. 2006). However, differences in study findings on the importance of male size for reproductive success should be viewed in terms of the male size gradient used in the experiments. For example, Spence and Smith (2006) did not find male size to be related to reproductive success in zebrafish while using males ranging between 33.8 and 37.4 mm. In our study, we used males ranging from 24 to 31 mm. Potentially, the larger size gradient of males in our study facilitated the emergence of clear paternal-size effects on reproductive fitness, which may have involved both direct (e.g., genetic quality, sperm quality) and indirect (differential allocation by females) male-size effects.

The relationship between male size and reproductive fitness in our zebrafish study was nonlinear. In

fact, we found that the very large males exhibited consistently lower reproductive fitness compared to large males (Fig. 2). Unlike among females, the very large males exhibited the lowest integrative reproductive fitness value of all male sizes. Interestingly, very large males sired high quality offspring once spawning occurred, but they had substantially lower spawning probabilities and they received significantly smaller clutches compared to the other-sized males. Because this effect was not caused by the relative size difference between females and males, it appears that the advantages of very large body size are traded off against unknown fitness costs of being too large. We can only speculate about the likely mechanisms, but mating-related physiological or behavioral factors (e.g., courtship behavior and sexual harassment; Partridge and Fowler 1990) may play a paramount role. In *Drosophila melanogaster* male body size have been shown to enhance male's mating success but simultaneously to have a detrimental effect on female's fitness leading to a lower egg number received by the large male (Fowler and Partridge 1989; Pitnick and García-González 2002). So far similar mechanisms of sexual conflict have not been demonstrated in fish, however it is possible that our experimental design facilitated continuous sexual harassment of females by very large males, which may have induced substantial stress on females resulting in reduced matings (Morgan et al. 1999; Small 2004). Thus, the persistent and partly aggressive spawning behavior of very large males, which could lead to a high mating success first, may not be advantageous in repeated spawnings and may introduce fitness costs for both females and males. Investigating the potential costs of mating with very large males in zebrafish constitutes an important avenue for further studies on size-dependent sexual conflict in this species.

Our experimental study controlled for density and sex ratio, which both can affect zebrafish reproductive success (Spence and Smith 2005; Spence et al. 2006) and additionally allowed the fish spawn naturally instead of using artificial fertilization. Zebrafish has been suggested to spawn in groups (Spence et al. 2008) but a recent behavioural study showed that wild zebrafish spawn in pairs rather than in groups (Hutter et al. 2010). Therefore, we believe that our experiment allowed us to determine reliably the effect of body size on reproductive success in zebrafish despite the unnatural spawning conditions the fish were

exposed to. Our experimental design allowed us to unravel some additional aspects related to zebrafish reproduction. For example, stocking two fish in a spawning box helped us to reveal the relatively high amount of variation in reproductive output and early life-history traits that was associated to individual couples independent of male or female body size (*sensu* Paull et al. 2008). This additional variation in reproductive success could be related to hormonal factors (van den Hurk and Lambert 1983; van den Hurk et al. 1987), genetic incompatibility (Gerlach and Lysiak 2006), or dominance hierarchies (Pritchard 2001). Our study thus suggests that one should expect a strong effect of body size on reproductive performance but additional factors, potentially related to mate choice, are paramount in explaining reproductive success in zebrafish. The great individual variance in reproductive output has implications for experimental design of studies that investigate reproductive success in zebrafish or use reproductive parameters in ecotoxicological studies as large sample sizes are needed to account for the large variability in individual reproductive performance (Paull et al. 2008).

To conclude, our study is the first to unambiguously identify the maternal and paternal-size dependent effects on zebrafish reproductive success. We revealed an insofar overlooked importance of male body size for reproduction in this species and our findings also suggest the importance of body size-dependent sexual conflict and female differential allocation as potential mechanism explaining the pronounced male-size effects. Implicating beyond our laboratory approach and assuming that similar effects exist in other fish species, our findings of paternal-size effects in addition to maternal-size effects are worth being considered when deriving harvest regulations designed to protect exploited stocks. In particular, our results suggest that ignoring the importance of male body size for recruitment of fish might constitute a shortcoming when assessing the impact of size-selective fishing and skewed sex ratio on recruitment dynamics (Langangen et al. 2011).

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