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Inbreeding in three-spined sticklebacks (*Gasterosteus aculeatus* L.): effects on testis and sperm traits

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Mating between relatives often results in inbreeding depression, and is assumed to have a strong effect on fitness traits such as fertility and gonad/gamete quality. However, data concerning this topic are contradictory and particularly scarce in fishes. Three-spined sticklebacks (*Gasterosteus aculeatus* L.) show inbreeding depression in fertilization and hatching success, survival rates, body symmetry and behavioural traits. To date, any knowledge of the impact of inbreeding on males' gonads and gametes is lacking in this species. In the present study, testis and sperm traits were quantified in outbred and inbred males. Overall, these traits were not generally impaired by inbreeding, and this result was not changed by a second/third generation of brother–sister matings. However, testes brightness, a potential measure of oxidative stress, was negatively correlated with sperm number. Additionally, inbred males with higher body condition had significantly brighter testes, whereas their sperm number was significantly negatively correlated with sperm quality (as estimated by head volume). Such a trade-off did not appear in outbred males. The comparatively small impact of inbreeding on testis and sperm traits might be explained by the low number of inbred individuals that reached the reproductive phase. © 2012 The Linnean Society of London, *Biological Journal of the Linnean Society*, 2012, ••, ••–••.

ADDITIONAL KEYWORDS: fish – heterozygosity – reproduction – sexual selection – sperm competition – testicular melanization.

INTRODUCTION

Mating with close relatives leads to an increased level of homozygosity (Charlesworth & Charlesworth, 1987), resulting in a loss of fitness in many species (Crnokrak & Roff, 1999; Armbruster & Reed, 2005). This inbreeding depression might influence many different traits. For example, inbred individuals face a reduced resistance to parasites (Coltman *et al*., 1999) and are more vulnerable to environmental changes (Keller *et al*., 1994). Furthermore, inbreeding may lead to a higher degree of body deformations, resulting in a higher mortality rate (Waldman & McKinnon, 1993). However, sometimes mating with close relatives has no detrimental effects (Keane, Creel & Waser, 1996; Sheridan & Pomiankowski, 1997; Peer & Taborsky, 2005; Thünken *et al*., 2007) or even increases an individual's fitness, as an individual that mates with a relative will enable that relative to spread genes identical by descent (Kokko & Ots, 2006).

Traits closely related to fitness (such as fertility or survival) are known to be more strongly influenced by inbreeding depression than traits that are less closely related to fitness (Falconer, 1989; DeRose & Roff, 1999). This difference might be explained by larger directional dominance in fitness-related traits (DeRose & Roff, 1999) or by the fact that, on average, fitness-related traits are influenced by more loci (Houle, 1992; Keller & Waller, 2002). Thus, fertility as well as gonadal and gamete traits are expected to suffer strong consequences of inbreeding. However, studies investigating such a connection have yielded

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contradictory results. For example, a study on Shetland ponies (*Equus ferus caballus* L.) found decreasing fertility with increasing homozygosity (van Eldik *et al*., 2006), whereas a study on Austrian Noriker draught horses did not find such a negative effect (Aurich, Achmann & Aurich, 2003). Margulis & Walsh (2002) demonstrated that testes mass of Alabama beach mice *Peromyscus polionotus* (Wagner) decreased when inbreeding coefficients increased. Similarly, in wild rabbits *Oryctolagus cuniculus* (L.), the production of abnormal sperm was negatively correlated with heterozygosity (Gage *et al*., 2006). Furthermore, in some species, the negative impact of inbreeding on fertility is limited to only one sex, whereas the other sex remained unaffected (Brekke *et al*., 2010; Rioux-Paquette, Festa-Bianchet & Coltman, 2011). For example, in the butterfly *Bicyclus anynana* (Butler), approximately one-half of the male offspring of brother–sister matings was infertile, whereas female fertility was unaffected by inbreeding (Saccheri *et al*., 2005). Also, in a small Swiss human population, inbred women had fewer children, whereas the reproductive success of men was not affected (Postma, Martini & Martini, 2010).

Fluctuating asymmetry is known to be a reliable indicator of developmental and environmental stress (Leary & Allendorf, 1989; Sopinka *et al*., 2012). Also, genetic stress as a result of inbreeding could lead to fluctuating asymmetry. For example, the degree of fluctuating asymmetry was positively correlated with the inbreeding coefficient and negatively correlated with the proportion of normal sperm in the Cuvier's gazelle *Gazella cuvieri* (Ogilby) (Roldan *et al*., 1998). A comparative study in three more species of ungulates showed that fluctuating asymmetry is related to individuals semen quality (Gomendio, Cassinello & Roldan, 2000). However, fluctuating testis asymmetries have seldom been investigated. Furthermore, the impact of inbreeding on males' gonadal and sperm traits has been less well explored, especially in fish (but see Zajitschek & Brooks, 2010). Thus, the present study aimed to increase the knowledge of the effects of inbreeding on males' reproductive traits.

The three-spined stickleback (*Gasterosteus aculeatus* L.) is an ideal model species for studying the influence of inbreeding on testis and sperm traits for several reasons. First, anadromous sticklebacks frequently colonize new fresh water environments and establish new populations consisting of only a few founder individuals, hence representing a rather small gene pool (Raeymaekers *et al*., 2005; Ólafsdóttir, Snorrason & Ritchie, 2007; Wund *et al*., 2008). The risk of inbreeding and associated inbreeding depression in such newly-established populations should be high (Aeschlimann *et al*., 2003). Indeed, the three-spined stickleback show inbreeding depression in life history traits such as fertilization success, hatching rates, and mortality of both juveniles and adults (Frommen *et al*., 2008), as well as in behavioural traits (Frommen *et al*., 2007) and body symmetry (Mazzi *et al*., 2004). Inbreeding depression in this species can be caused by just one generation of brother–sister mating, and is further intensified by a second generation of incestuous mating (Frommen *et al*., 2008). However, several traits under sexual selection such as mating decisions (Frommen & Bakker, 2006; Mehlis, Bakker & Frommen, 2008; but see also Mazzi *et al*., 2004), aggressive interactions (Mehlis *et al*., 2009) or the expression of male breeding coloration (Frommen *et al*., 2008), appeared not to be affected by inbreeding.

Second, the stealing of fertilizations, so-called 'sneaking', is a common behavioural tactic in this species (Goldschmidt, Foster & Sevenster, 1992; Jamieson & Colgan, 1992; Rico, Kuhnlein & Fitzgerald, 1992; Largiadèr, Fries & Bakker, 2001; Vlieger & Candolin, 2009), which consequently results in sperm competition (Parker, 1990). Spermatogenesis in sticklebacks takes place in the months in which photoperiod is short (Borg, 1982). Thus, sperm are not produced during the breeding season, which takes place between April and August, resulting in a larger number of sperm stored in testes of virgin males (e.g. early spring) compared to those of multiply-mated males (e.g. late summer) (Zbinden, Largiadèr & Bakker, 2001). As the sperm quantity decreases during the course of the breeding season, sperm are allocated carefully (Zbinden *et al*., 2003; Zbinden, Largiadèr & Bakker, 2004). Furthermore, gonadal mass is related to reproductive success (Cubillos & Guderley, 2000), and the number of stored sperm is significantly positively correlated with the gonadosomatic index (Zbinden *et al*., 2001). Thus, a stickleback male that is unable to produce sufficient amounts of fertile sperm because of inbreeding depression will most probably suffer a reduced fertilization success.

Using the same breeding line as that described by Frommen *et al*. (2008), the present study investigated how inbreeding affects testis and sperm traits of the three-spined stickleback. We measured the potential impact of inbreeding on the gonadosomatic index, sperm number, and sperm morphology. Furthermore, we analyzed melanophore pigmentation of the testes, which might play a role in protection against ultraviolet (UV)-light and/or oxidative stress (Kaidbey *et al*., 1979; Plonka *et al*., 2009; Galván, Møller & Erritzøe, 2011). Finally, asymmetries of testis traits were considered as a measure of developmental and genetic stress (Leary & Allendorf, 1989; Mazzi, Largiadèr & Bakker, 2002). All measures were

compared between males that were outbred or inbred for one or several generations.

MATERIAL AND METHODS

Experimental subjects

Three-spined sticklebacks from an anadromous population were caught during their spring migration in April 2002, 2003, and 2004 on the island of Texel, the Netherlands. During this time, large numbers of sticklebacks migrate from the ocean into freshwater habitats (Kemper, 1995). Furthermore, individuals captured on Texel are representative of the whole population, and the gene pool is highly heterogeneous (Heckel *et al*., 2002).

Fish used in the present study were outbred and first-, second- or third-generation inbred descendants of these wild-caught parents. Outbred fish were the F_1 progeny of randomly crossed wild-caught fish. To achieve different outbred families (full-sib groups), males were allowed to spawn with one single randomly chosen female. Inbred fish were then produced by three subsequent generations of brother–sister matings. Thus, first-generation inbred fish were the F_2 progeny, second-generation inbred fish were the F_3 progeny, and third-generation inbred fish were the F_4 progeny of the wild-caught fish. To avoid pseudoreplication, inbred clutches were obtained by using only one parental pair for each family. Outbred clutches were produced in April and May 2004, whereas inbred clutches were produced in December 2004 and January 2005. To exclude paternal and tank effects, all clutches were removed from the males' tank and split into two groups 1 h after fertilization (Frommen *et al*., 2008). Fish were kept in full-sib groups of 10–20 individuals. The holding tanks measured 50 cm × 30 cm × 30 cm (length × width × height) and were placed in an air-conditioned room under standardized winter light-regime (8 : 16 h light/dark cycle at 17 ± 1 °C). To simulate the start of the breeding season, the light regime was changed to summer conditions in August 2006 (16 : 8 h light/dark cycle at 17 ± 1 °C). Males that showed initial signs of breeding coloration in the holding tanks were isolated in separate tanks (40.5 × 20.5 × 25 cm) for nest building (Mehlis *et al*., 2008). All males used were virgin because they had no possibility to build a nest in their holding tank and were not paired with any female in their nesting tank. In addition, only males that had built a nest were used, which ensured that they were reproductively active. On average, males needed one day (median and quartiles: 1, 1, and 2) to build their nest. They were allowed to care for the nest for up to 18 days (median and quartiles: 10, 8, and 18). Before they were quickly sacrificed, males' standard length (SL) and body mass (M) were measured, to calculate their body condition (BC) *sensu* Bolger & Connolly (1989):

$$BC = 100 \times M/SL^3 \quad (1)$$

In total, 125 males were used originating from 31 outbred families (a total of 85 males) and 15 inbred families (a total of 40 males). Of the inbred families seven families (18 males) were inbred for one generation, six families (18 males) were inbred for two generations and two families (four males) were inbred for three generations. Males from outbred and inbred families did not differ significantly with respect to the duration of nest building, in the time they cared for a nest, in SL, in M or in BC ($N_{outbred} = 31$, $N_{inbred} = 15$; unpaired Wilcoxon-test, all $W < 291$, all $P > 0.173$; see Supporting information, Table S1).

Measurement of testis traits

Before testes dissection (April 2008 and June 2009), all males had been stored in 70 % ethanol for at least 2 years, which might have led to dehydration and hence organ deformation. Thus, new body measurements of males were taken using the same protocol as before and the new measurements were used for all further analyses. Afterwards, both testes were removed and placed separately on a tissue to remove adhered liquids. Then they were weighed to the nearest milligram (OHAUS, Explorer, E11140) and stored separately in Eppendorf tubes containing 500 µl of 70 % ethanol. The gonadosomatic index (GSI) was calculated *sensu* de Vlaming, Grossman & Chapman (1982):

$$GSI = (TM\ M^{-1}) \times 100 \quad (2)$$

where TM is the sum of the mass of both testes ($TM_{left} + TM_{right}$).

To determine the intensity of melanophore pigmentation, a standardized image of each testis was taken with a digital camera (HV-C20AMP; Hitachi Denshi Ltd) mounted on a binocular (Leica S8AP0) using Diskus 4.6 (C.H. Hilgers Technical office). To ensure comparable light regimes, all specimens were illuminated using a cold light source (KL 1500 LCD Leica), which was always placed in the same position. Furthermore, the orientation of the testis was always the same with the outside upturned. (Fig. 1A, B, C). Pictures were analyzed in ADOBE PHOTOSHOP CS4 (Adobe Systems Inc.) using the CIE L*a*b* colour space, which is a method that is frequently used in fish colour evaluation (Adachi *et al*., 2005; Svensson *et al*., 2005). A white Munsell card (N10) visible on each picture served as white balance. To obtain the

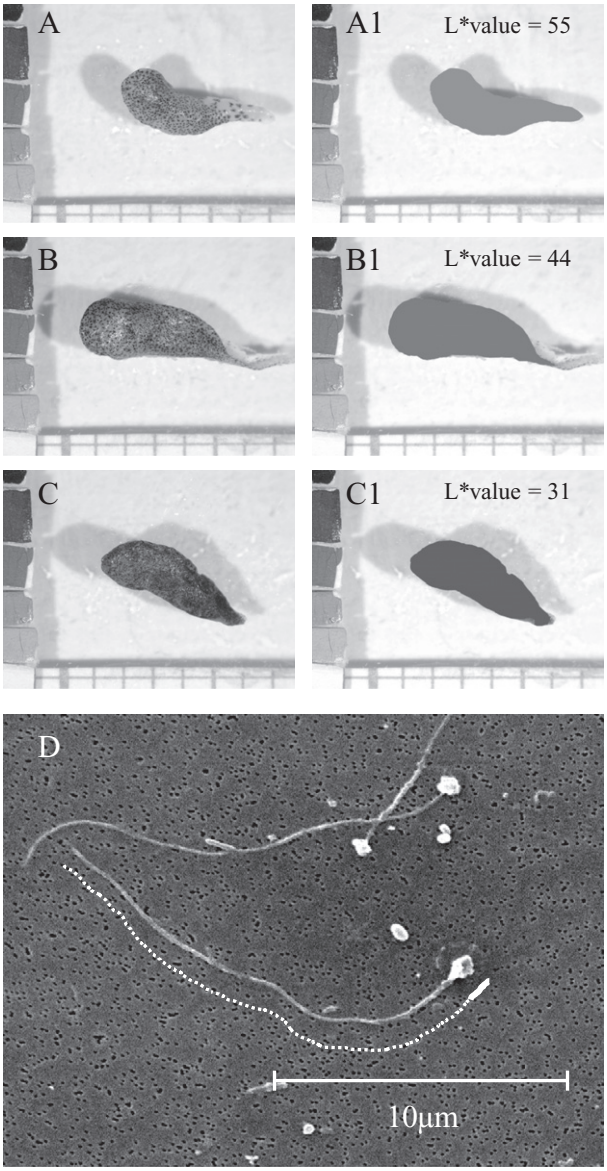

Figure 1. Testes and sperm of three-spined stickleback. A, B, C, variation in testis melanophore pigmentation and the corresponding mean brightness (L* value; A1–C1). Munsell chips are visible on the left side and a millimetre scale is provided at the bottom of each image. D, sperm morphology traits, the dotted line indicates flagellum length and the solid line indicates head length.

mean L* value [brightness; ranging from black (0) to white (100)] of each testis, testis shape was marked using the magnetic-lasso-tool. The testes of three-spined sticklebacks show a high variability in melanophore pigmentation (Fig. 1A, B, C) and the L* value (brightness) is a good indicator of melanophore density (Fig. 1A1, B1, C1). Brightness was determined using the entire area inside of the magnetic-lasso-tool for each male's testis (L^*_{left} and L^*_{right}) and averaged (L^*_{total}). Finally, asymmetries in testis mass (Eqn 3) and testis brightness (Eqn 4) were calculated:

$$\text{Testis mass asymmetry} = (TM_{heavy} - TM_{light})/TM_{heavy} \quad (3)$$

$$\text{Brightness asymmetry} = (L^*_{bright} - L^*_{dark})/L^*_{bright} \quad (4)$$

Measurements of sperm traits

The number of sperm stored in left and right testis are highly correlated in three-spined sticklebacks of the study population (Bakker *et al.*, 2006). Therefore, the measurement of sperm number was solely conducted on the left testis. To obtain sperm, each male's left testis was pestled in 200 µl of phosphate-buffered saline (Dulbecco & Vogt, 1954). Ten microlitres of this solution were diluted with 190 µl of tap water to reduce sperm density. Twelve microlitres of this dilution were filled into a Neubauer improved counting chamber (Labor Optik, 0.0025 mm^2, depth 0.1 mm). Sperm were counted in 64 cells, which were equally distributed over the chamber. Thus, the total number of sperm (S_T) was calculated using the equation:

$$S_T = S_A \times 4000 \times 200 \times 20 \quad (5)$$

where S_A is the mean sperm number of the 64 counted cell chambers.

Sperm morphology was determined by scanning electron microscopy (SEM; Fig. 1D) with a subsample of 23 males from different families [twelve outbred and eleven inbred males (four males first-, six males second- and one male third-generation inbred, which were pooled for analyses)]. The preparation of sperm for SEM was carried out *sensu* Mortimer (1994) and was adapted for stickleback sperm (see Supporting information, Appendix S1). Sperm variables were measured using 2500–4500 magnified SEM-images (digitized Leitz AMR 1000). They were digitized using DISS (Digital Image Scanning System, Point Electronic GmbH) and DIPS (Digital Image Processing System, version 2.5.2.1, Point Electronic GmbH). Although ethanol has been used as a preservative medium in a number of comparative studies concerning testes and sperm variables (Byrne, Roberts & Simmons, 2002; Kvarnemo & Simmons, 2004), sperm midpiece and head could not visually be distinguished as a result of the dehydrating effects of the alcohol and were thus measured together. However, because both inbred and outbred males had been stored in 70 % ethanol for the same time, data were comparable with each other. Head length (hl) and flagellum length (fl) were measured only on images that showed intact sperm with a well visible head and a complete tail (Fig. 1D). In total, 187 sperm were measured

(median and quartiles: 6, 4, 12.5 per male) using IMAGEJ (NIH). The measures used are known to be good proxies of reproductive success in several animal species (Murray, Kozlowska & Cutter, 2011), including fish (Boschetto, Gasparini & Pilastro, 2011). In addition, several studies revealed that sperm morphology is linked to sperm velocity (Fitzpatrick, Garcia-Gonzalez & Evans, 2010). Thus, as a proxy of sperm motility, the head length to flagellum length ratio (Eqn 6) (Humphries, Evans & Simmons, 2008) and the complete head volume (including the mid-piece volume; Anderson & Dixson, 2002) (Eqn 7) assuming the form of a sphere (Fig. 1D) were calculated:

$$\text{Head to flagellum ratio} = \text{hl}/\text{fl} \quad (6)$$

$$\text{Head volume} = \tfrac{1}{6} \times \pi \times (\text{hl})^3 \quad (7)$$

The head to flagellum ratio is a good measure of sperm motility in three-spined sticklebacks, and flagellum length, mid-piece volume and head volume were shown to predict fertilization success in this species (Marion Mehlis, Lukas K. Hilke, Theo C. M. Bakker unpubl. data and Theo C. M. Bakker, Michael Hollmann, Marion Mehlis, Marc Zbinden, unpubl. data).

Repeatability of testis and sperm traits

To calculate the repeatability (R) and its standard error (SE; after Becker, 1992) of the measurements of testis traits, one male was randomly chosen from each family (approximately 37 % of total sample size). From these males, TM_{right} and L^*_{right} were measured three times. Furthermore, from the subsample of 23 males, one randomly chosen sperm was measured twice from each male (approximately 12 % of total sample size). The formula used to calculate repeatability was:

$$R = ([(MS_a - MS_w)/N_0]/\{[(MS_a - MS_w)/N_0] + MS_w\}) \times 100 \quad (8)$$

where MS_a is the between sperm mean square, MS_w is the within sperm mean square, and N_0 equals three (testis traits) and two (sperm traits) respectively. Repeatability was high for the measurement of testis mass (89.71 ± 0.15 %), as well as for testis brightness (98.91 ± 0.02 %). The repeatability values were also high for the measurement of sperm traits. This was true for head length (94.24 ± 0.35 %), as well as for flagellum length (99.92 ± 0.01 %).

Statistical analysis

The R 2.9.1 statistical package was used for analyses. The mean values of the different families were used to avoid pseudoreplication. Thus, the sample size for analyses of testis traits was $N_{outbred} = 31$ and $N_{inbred} = 15$ ($N_{1st\ inbred} = 7$, $N_{2nd + 3rd\ inbred} = 8$) and, for analyses of sperm traits, $N_{outbred} = 12$ and $N_{inbred} = 11$. Second- and third-generation inbred families were pooled because of the low sample size ($N_{3rd\ inbred} = 2$). For statistical analyses, linear models ('lm') were conducted using the respective testis or sperm traits as dependent variables and the breeding regime (outbred versus inbred and outbred versus various levels of inbreeding (first- and second + third-generation inbred) as an explanatory variable (for a detailed list of relevant models, see the Supporting information, Table S2). Inbred males were approximately 8 months younger than outbred ones. Fish grow throughout their life and, in most cases, reproductive success is positively related with size (Reznick, Ghalambor & Nunney, 2002), which could also be shown for three-spined sticklebacks (Allen & Wootton, 1982; Kraak, Bakker & Mundwiler, 1999). Similarly, in the present study, age was significantly correlated with male SL (Spearman correlation: $N = 125$; $r_s = 0.206$, $P = 0.021$). Thus, as a surrogate for age, SL was included as an explanatory variable in all models and never removed to control for possible age influences. To check for differences in the relationship among testis and sperm investment between outbred and inbred males, additional linear models ('lm') were conducted, in which interactions between the breeding regime and different testis respectively sperm traits were included (for a detailed list of relevant models, see Supporting information, Table S2). For all models, tests of significance were based on likelihood-ratio tests (LRT). Because normally distributed dependent variables are a prerequisite for running linear models, sperm number was square-root transformed, GSI was inverse transformed, and head volume and asymmetries in testis mass and testis brightness were logarithmically transformed, to achieve normal distributions of the residuals of the best explaining model, according to Kolmogorov–Smirnov tests. All P-values are based on two-tailed tests.

RESULTS

Testis traits

Males showed considerable variation in GSI and testis brightness (L^*_{total}) (Fig. 1, Table 1), which did not differ significantly between outbred and inbred males ($N_{outbred} = 31$, $N_{inbred} = 15$; 'lm', both $\chi^2 < 0.082$, both $P > 0.776$; Table 1). Differences in GSI and testis brightness did not differ significantly between different levels of inbreeding ($N_{outbred} = 31$, $N_{1st\ inbred} = 7$, $N_{2nd + 3rd\ inbred} = 8$; 'lm', both $\chi^2 < 0.913$, both $P > 0.409$; Table 1). Asymmetries of testis mass or brightness were not significantly different between outbred and inbred males ($N_{outbred} = 31$, $N_{inbred} = 15$; 'lm', both $\chi^2 < 1.215$, both $P > 0.277$; Table 1). The level

Table 1. Descriptive statistics (median, first and third quartile) of testis and sperm traits used in the analyses

	Outbred			Inbred			First-generation inbred			Second + third-generation inbred		
	Median	First quartile	Third quartile	median	First quartile	Third quartile	median	First quartile	Third quartile	median	First quartile	Third quartile
Gonadosomatic index	0.52	0.44	0.71	0.48	0.43	0.60	0.52	0.41	0.75	0.46	0.43	0.55
Testis brightness (L^*_{total})	49.50	41.44	51.70	50.63	43.10	52.91	42.00	41.29	48.11	52.38	49.34	54.98
Testis mass asymmetry	0.210	0.167	0.337	0.239	0.139	0.265	0.227	0.138	0.253	0.255	0.195	0.282
Testis brightness asymmetry	0.092	0.055	0.124	0.098	0.074	0.156	0.118	0.075	0.156	0.096	0.074	0.157
Sperm number	12.6×10^6	8.9×10^6	14.8×10^6	11.9×10^6	8.9×10^6	16.5×10^6	16.3×10^6	12.5×10^6	17.0×10^6	9.9×10^6	7.8×10^6	12.1×10^6
Flagellum length (μm)	18.19	16.37	19.11	16.84	15.84	21.21	–	–	–	–	–	–
Head length (μm)	0.72	0.65	0.79	0.69	0.67	0.77	–	–	–	–	–	–
Head to flagellum ratio	0.042	0.038	0.045	0.039	0.035	0.044	–	–	–	–	–	–
Head volume (μm^3)	0.217	0.155	0.280	0.197	0.165	0.272	–	–	–	–	–	–

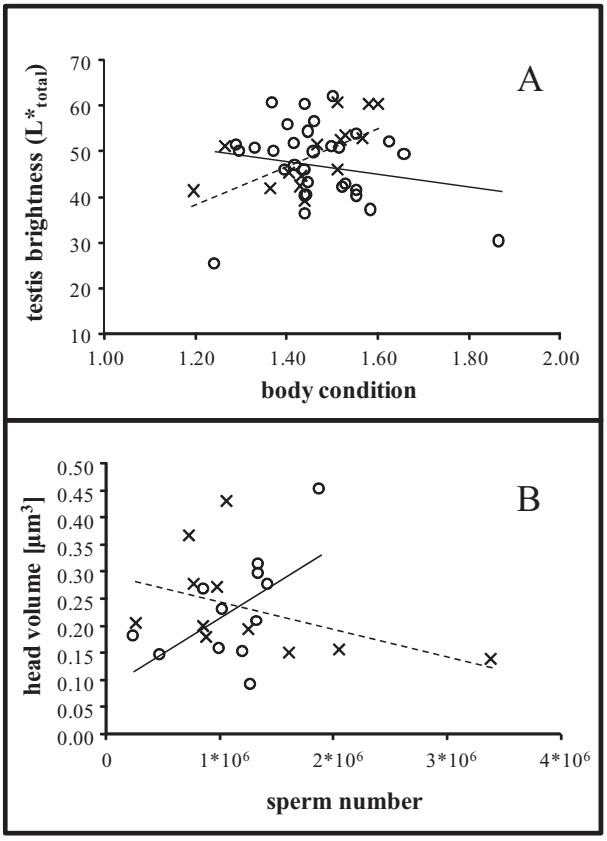

Figure 2. Significant interactions between outbred (circles; solid lines) and inbred (crosses; dotted lines) males. A, relationship between male body condition and testis brightness (L^*_{total}). B, relationship between sperm number and head volume (μm^3).

of inbreeding was not significantly related to asymmetries of testis mass or brightness ($N_{outbred} = 31$, $N_{1st\ inbred} = 7$, $N_{2nd + 3rd\ inbred} = 8$; 'lm', both $\chi^2 < 0.848$, both $P > 0.436$; Table 1).

Sperm traits

Sperm number did not differ significantly between outbred and inbred males ($N_{outbred} = 31$, $N_{inbred} = 15$; 'lm', $\chi^2 = 0.417$, $P = 0.522$; Fig. 2, Table 1). Analysing the different generations of inbreeding separately revealed no significant differences in number of sperm with increasing levels of inbreeding ($N_{outbred} = 31$, $N_{1st\ inbred} = 7$, $N_{2nd + 3rd\ inbred} = 8$; 'lm', $\chi^2 = 2.021$, $P = 0.145$; Table 1). Furthermore, outbred and inbred males did not differ significantly in sperm morphology traits, neither in head length, flagellum length, head to flagellum ratio, nor in head volume ($N_{outbred} = 12$, $N_{inbred} = 11$; 'lm', all $\chi^2 < 0.718$, all $P > 0.407$; Table 1).

Interactions between testis and sperm traits

The number of sperm was negatively correlated with testis brightness (L^*_{left}), which was true for

inbred (Spearman correlation: $N_{inbred} = 15$; $r_s = -0.654$, $P = 0.010$), as well as for outbred males (Spearman correlation: $N_{outbred} = 31$; $r_s = -0.371$, $P = 0.040$). This means that, in all males, brighter testes contained less sperm. Furthermore, inbred males in better BC had significantly brighter testes (L^*_{total}) (Pearson correlation: $N_{inbred} = 15$; $r_p = 0.640$ $P = 0.010$; Fig. 2A), whereas this relationship was not found in outbred males (Pearson correlation: $N_{outbred} = 31$; $r_p = -0.191$, $P = 0.304$; Fig. 2A), leading to a significant interaction between outbred and inbred males with respect to the relationship between testis brightness and BC ($N_{outbred} = 31$, $N_{inbred} = 15$; 'lm', $\chi^2 = 4.676$, $P = 0.037$). Furthermore, the relationship between sperm number and head volume differed significantly between outbred and inbred males ($N_{outbred} = 12$, $N_{inbred} = 11$; 'lm', $\chi^2 = 6.054$, $P = 0.024$). Inbred males with higher numbers of sperm had significantly smaller head volumes (Spearman correlation: $N_{inbred} = 11$; $r_s = -0.673$, $P = 0.028$; Fig. 2B), whereas the opposite was true for outbred males (Pearson correlation: $N_{outbred} = 12$; $r_p = 0.596$, $P = 0.041$; Fig. 2B).

DISCUSSION

Outbred and inbred males did not differ in GSI, testis coloration, degree of gonadal asymmetries, sperm number, and sperm morphology. As discussed by Keller & Waller (2002), it might be difficult to detect the effects of inbreeding when inbreeding depression is high. It is argued that, in such a case, homozygous individuals would suffer from lethal mutations early in development and might not reach the adult stage. This could lead to low estimates of the effects of inbreeding, as shown for example in the Scots pine (*Pinus sylvestris* L.) (Savolainen & Hedrick, 1996). The present study used the same breeding line as that employed in Frommen *et al*. (2008). Frommen *et al*. (2008) showed that sticklebacks are adversely affected by inbreeding depression at an early life stage because fertilization and hatching rates in inbred offspring were low and the mortality of juveniles was high. It is therefore possible that inbred fish, which had suffered strongly from inbreeding depression, died before they reached the reproductive phase and were not measured in the present study. Thus, inbred fish that do reach adulthood may not suffer as much from inbreeding depression and their sperm and gonadal traits may be of similar quality to those of outbred conspecifics. Furthermore, in the present study, both outbred and inbred males were laboratory-bred. Standardized near-optimal laboratory rearing conditions could have overruled the possible negative effects of inbreeding. In the wild, diseases and restricted food availability may have detrimental effects on males, so that they may not be able to show comparable fertility and hence fitness with respect to outbred males. For example, in male house mice (*Mus musculus* L.), reproductive success was more strongly influenced by inbreeding when mice were reared in large semi-natural enclosures (Meagher, Penn & Potts, 2000), implying that competition in the wild might increase the harmful effects of inbreeding.

A further reason for a lack of inbreeding depression in testis and sperm traits could be that the applied levels of inbreeding did not reduce heterozygosity sufficiently strongly to unmask deleterious recessive alleles. Indeed, several studies found that inbreeding depression was intensified by on-going incestuous matings (Richards, 2000; Crnokrak & Barrett, 2002; Dolgin *et al*., 2007). The results of Frommen *et al*. (2008) support this idea as well. One generation of inbreeding by brother–sister matings significantly lowered the fertilization success and hatching rate of eggs. This effect was intensified by a second generation of inbreeding (Frommen *et al*., 2008). In the present study, the increase of inbreeding depression by a second generation of incestuous mating was marginal if anything. GSI, testis brightness, and asymmetries, as well as sperm number and morphology, did not differ significantly between outbred, one generation inbred and second + third-generation inbred males.

In several vertebrates, testes are covered by melanophores that contain melanin, such as in fish (Louiz, Ben-Attia & Ben-Hassine, 2009), amphibians (Zieri, Taboga & De Oliveira, 2007), reptiles (Guillette, Weigel & Flater, 1983), birds (Galván *et al*., 2011), and mammals (Poole & Lawton, 2009). Melanophores contain dark pigments, which are assumed to protect the testes from deleterious UV light as a result of their strong light absorbance (Kaidbey *et al*., 1979; Plonka *et al*., 2009) or to protect male germ cells from oxidative stress (Galván *et al*., 2011). In birds, for example, testis melanization has evolved in species with high rates of accumulated mitochondrial mutations and is assumed to be an adaptive response related to the protective capacity of melanin against oxidative stress (Galván *et al*., 2011). In the present study, a higher number of melanophores was related to a higher number of sperm, both in outbred and inbred males. Thus, males that were of generally higher quality in terms of sperm production were also of higher quality in terms of sperm protection. However, testes melanization in inbred males was negatively correlated with BC, which means that males with higher BC had testes with fewer melanophores. Thus, inbred males might face a trade-off between investment in BC and testis melanization: the higher their investment in BC the smaller was the investment into melanophores. Because sperm

are known to be highly prone to suffer from oxidative stress (Aitken & Baker, 2006), inbred sticklebacks may not be able to keep a high BC and protect their sperm from oxidative stress at the same time. By contrast, such a trade-off was not found in outbred fish, and might thus be interpreted as a cryptic case of inbreeding depression. Additionally, inbred males (in contrast to outbred males) faced a trade-off between sperm quantity and sperm quality (as estimated by head volume). Hence, inbred males might either be able to invest into producing larger ejaculates, or in smaller ejaculates containing better sperm. Taken together, these results show that, for inbred males, it is physiologically taxing to produce large numbers of sperm of high quality, and protect them from potential damage caused by UV light and oxidative stress, at the same time as maintaining a high BC. In nature, sticklebacks face a high risk of sperm competition (Goldschmidt *et al*., 1992; Jamieson & Colgan, 1992; Largiadèr *et al*., 2001). Under these conditions, it is advantageous for an individual to possess both a large amount of sperm and high-quality sperm. Furthermore, stickleback males in good BC are more successful in attracting mates and withstanding competitors (Bakker & Milinski, 1993). According to the present results, inbred males should face problems in both of these terms.

Outbred and inbred males were of different age, with the inbred fish being, on average, 8 months younger than the outbred fish. Hence, an influence of age on the experimental results cannot be completely ruled out. For example, other studies found a negative effect of age on testis and sperm traits even in three-spined sticklebacks (M. Mehlis & T. C. M. Bakker, unpubl. data; de Fraipont, FitzGerald & Guderley, 1993). However, these studies compared stickleback males that had experienced one breeding season with older males that had experienced two breeding seasons. In the present study, both groups were in their first reproductive season when measurements were taken, and were exposed to the same duration of winter conditions before the light-regime was switched to resemble summer again. Borg (1982) showed that, in three-spined sticklebacks, spermatogenesis was only active during short photoperiods. The time span for sperm production was the same for both outbred and inbred males, indicating that stored sperm were of similar age in both groups. In addition, despite the age difference between outbred and inbred males, they did not differ significantly in body measurements, supporting the comparability of the two groups. Moreover, we added SL as an additional variable to our models, adjusting for nonsignificant differences in body size between the groups.

In conclusion, the present study shows that sperm and gonadal traits are not generally impaired by one or more generations of brother-sister matings. However, inbred males appeared to face rather cryptic inbreeding depression because they were unable to produce large numbers of sperm, adequately protected against potential damage caused by UV light or oxidative stress, and, at the same time, maintain a good BC. Finally, inbred fish were unable to both produce large amounts of sperm and maintain high-quality sperm. Such a trade-off did not manifest in outbred males, which might imply that inbred males were of lower quality and may have a fitness disadvantage, especially when confronted with sperm competition under natural conditions.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Table S1. Descriptive statistics (median, first, and third quartile) of male body measurements (before storage in 70% ethanol), including the values for the duration of nest building and the time that males cared for a nest (days).

Table S2. Table of linear models ('lm'). In all models, standard length was additionally included as an explanatory variable and never removed to control for possible age influences. 1. 'lm' to compare different testis and sperm traits between breeding regime [outbred versus inbred and outbred versus various levels of inbreeding (first and second + third-generation inbred)]; 2. 'lm' including interactions between different testis respectively sperm traits and breeding regime.

Appendix S1. Preparing of stickleback sperm for scanning electron microscopy, modified *sensu* Mortimer (1994).

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