Inbreeding depression affects fertilization success and survival but not breeding coloration in threespine sticklebacks

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Summary

Inbreeding depression is a well-studied phenomenon which has been demonstrated in many animal and plant species. In fishes, most studies focus on species of commercial interest. Sticklebacks often colonize new habitats by starting with a small founder population which, thus, suffers a high risk of inbreeding. However, little is known about the degree of inbreeding depression of sticklebacks' life-history traits like fertilization success or hatching and survival rate. Furthermore, there is a general lack of knowledge about the impact of inbreeding on sexually selected traits like males' breeding coloration. In our study, one generation of inbreeding by brother–sister mating of wild-caught, anadromous sticklebacks significantly lowered the fertilization and hatching success of eggs. This effect was intensified by a second generation of inbreeding. Furthermore, fewer inbred individuals reached and survived the reproductive phase than outbred ones. However, surviving inbred and outbred males did not differ significantly in the intensity of red throat or blue eye coloration. Our data indicate that even one generation of inbreeding leads to a loss of fitness in threespine sticklebacks.

Keywords: hatching rate, *Gasterosteus aculeatus*, inbreeding depression, population size, sexual selection, breeding coloration, fish, heterozygosity.

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Introduction

Inbreeding depression, described as a loss of fitness through an increase of homozygosity (Wright, 1921; Charlesworth & Charlesworth, 1987), is a well-known phenomenon, already discussed by Darwin (1868). The two most plausible mechanisms of inbreeding depression are that an increased level of homozygosity unmasks deleterious, recessive alleles, and a general loss of heterosis (Charlesworth & Charlesworth, 1987). Inbreeding depression has been demonstrated in many groups of animals and plants (e.g., Crnokrak & Roff, 1999; Armbruster & Reed, 2005). In birds and mammals, for example, inbreeding depression affects body mass at birth, survival and reproductive success as well as resistance to disease, predation and liability to environmental stress (see Keller & Waller, 2002 for an overview). Thus, inbreeding depression is a problem receiving growing attention in conservation biology (Vrijenhoek, 1998) and animal breeding (Kristensen & Sørensen, 2005).

Inbreeding depression is also described for an array of fish species (see Waldman & McKinnon, 1993 for a review). Inbred rainbow trout, *Oncorhynchus mykiss* had more body deformations and a reduced fry survivorship (reviewed in Waldman & McKinnon, 1993), while the specific growth rate of inbred coho salmon (*Oncorhynchus kisutch*) was reduced (Gallardo & Neira, 2005). Inbreeding in zebrafish (*Danio rerio*) led to a reduced fertilization success, but not to a reduction in hatching rate (Mrakovčić & Haley, 1979). Furthermore, inbred zebrafish had a reduced survival, a lower growth rate and a higher number of fry suffering body deformations (Mrakovčić & Haley, 1979). Inbreeding in guppies (*Poecilia reticulata*) reduced males' sexual activity in several populations (Farr & Peters, 1984; Sheridan & Pomiankowski, 1997; van Oosterhout et al., 2003; Mariette et al., 2006). In contrast, an influence on sexually selected male coloration patterns was evidenced in some (Sheridan & Pomiankowski, 1997; van Oosterhout et al., 2003) but not in other populations (Sheridan & Pomiankowski, 1997; Mariette et al., 2006).

The threespine stickleback (*Gasterosteus aculeatus*) is a widely distributed species, which often migrates between freshwater habitats inhabited in summer and the sea inhabited in winter (Wootton, 1984). During the reproductive phase in spring and early summer male sticklebacks establish territories in shallow parts of their habitats where they build small nests using

plant material (Wootton, 1976). Males court females by presenting their red throat and blue eye coloration in a so called zig-zag dance (van Iersel, 1953). Females prefer males with a more intensely red-colored throat region (see Bakker & Milinski, 1993, for a review) and bluer eyes (Rowland, 1994; Bakker & Rowland, 1995; Bakker et al., 2006b). After spawning, males guard the eggs until hatching and the fry for a limited period after hatching. In most populations, males die after one breeding season. Fry start their reproductive phase the following year; thus, the ability to survive the winter period is a crucial fitness component.

As a colonizing species (Bell & Foster, 1994; von Hippel & Weigner, 2004), sticklebacks often establish new populations with a rather small gene pool (e.g., Raeymaekers et al., 2005). The risk of inbreeding in such newly established, small populations is supposed to be high (Heckel et al., 2002; Aeschlimann et al., 2003). Sticklebacks are able to minimize the risk of inbreeding by actively avoiding kin as mating partner (Frommen & Bakker, 2006). Furthermore, sneaked fertilizations are common in this species (Largiadèr et al., 2001; Zbinden et al., 2003), and females produce an egg-coating mucous that prolongs sperm life (Bakker et al., 2006a), provoking sperm competition. This may further increase the heterozygosity of the offspring (e.g., Tregenza & Wedell, 2002).

In contrast to the knowledge of inbreeding avoidance mechanisms, little is known about the impact of inbreeding on the biology of sticklebacks. Mazzi et al. (2002) showed that inbred stickleback males had greater body asymmetries that made them less attractive as mating partners (Mazzi et al., 2003). In experiments concerning shoaling decisions, inbred as well as outbred individuals preferred to shoal with familiar kin (Frommen & Bakker, 2004; Frommen et al., 2007). While outbred fish also preferred unfamiliar kin over non-kin, this preference was lost in inbred fish (Frommen et al., 2007), indicating an effect of inbreeding on cognitive abilities. On the other hand, inbreeding seemed not to impair female mate-choice decisions (Mazzi et al., 2004; Frommen & Bakker, 2006). However, nothing is known about the impact of inbreeding on life-history traits like fertilization success or survivorship to maturity. Furthermore, there is a lack of knowledge of the impact of inbreeding on sexually selected traits like males' breeding coloration.

Thus, our study aims to answer three questions: First, does inbreeding influence the fertilization and hatching success of stickleback eggs? Second, do relatively more outbred than inbred sticklebacks reach and survive the

reproductive phase? Third, is there an impact of inbreeding on male nuptial coloration?

Methods

All sticklebacks used in this study were descendants of fish caught in the time period 1998–2004 during their spring migration from a large, genetically heterogeneous (Heckel et al., 2002), anadromous population on the island of Texel, The Netherlands (Kemper, 1995). In the laboratory, males were allowed to spawn with a single female in order to produce kin groups. Inbred groups were produced by brother–sister matings.

Fertilization success and hatching rate

The sticklebacks used in the experiments were tested in 1999 and 2004. Therefore, they are referred to as 1999 and 2004 fish, respectively. Clutches used in the 1999 experiment were descendants of parents raised in the lab, which were the offspring of haphazardly mated wild caught fish. Thus, all clutches were the F2 of wild caught fish. Twenty-five clutches were outbred by crossing unrelated individuals, 28 were inbred for one generation by brother–sister matings (see Mazzi et al., 2002, for details). Each clutch was produced using parents of a different family to avoid pseudoreplication. Clutches used in the 2004 experiment were outbred ($N = 36$) and one or two-generations inbred ($N = 20$ and 14, respectively) descendents of wild-caught fish. Outbred fish were the F1 of haphazardly crossed wild caught fish. Inbred fish were produced by one or two generations of brother–sister matings. Thus, one-generation inbred fish were the F2 and two-generations inbred fish the F3 of wild caught fish. Again, each family provided only one clutch to avoid pseudoreplication. To exclude paternal effects, all clutches were removed from the males' tank 1 h after fertilization. At this time fertilization should be finished (Bakker et al., 2006a) and the chorion has been hardened (Wootton, 1976). Eggs were placed in a tank filled with 1 l of water from a well (1999) or tap-water (2004). Water was aerated by an airstone and changed twice a day in 1999 and once a day in 2004. Eggs were kept under summer conditions, that is, 16 h of light and a water temperature of 16 ± 1°C. After 24 h, all eggs were counted; unfertilized eggs, which could

easily be spotted because of their aberrant coloration and the lack of a developing embryo (Swarup, 1958), were counted and removed. Fertilized eggs were kept under the described conditions until hatching at day 8. They were daily controlled for dead embryos, which could be spotted by the lack of development. These dead eggs were removed to avoid fungal infestations. At the day of hatching, all surviving fry were counted and hatching rates for the fertilized eggs were calculated.

Survival

Outbred ($N = 19$) and one-generation inbred ($N = 18$) groups of kin were obtained in the year 2003 as described above for the 2004 fish. Prior to hatching, clutches were divided into two subgroups to reduce possible tank effects. At an age of three weeks, group sizes were reduced to 25 individuals per tank. Between weeks 4 and 18, group size was again reduced to 15 individuals. The age at which reduction was done, did not differ significantly between inbred and outbred groups (Mann–Whitney U-test, $N_{\text{out}} = 19$, $N_{\text{in}} = 18$, $Z = -0.585$, $p = 0.578$). Furthermore, age at group-size reduction did not significantly influence the survival till sub-adulthood and adulthood of outbred (Spearman rank correlation, $N = 19$, $r_S = 0.067$ and 0.234, $p = 0.786$ and 0.334, respectively) or inbred fish ($N = 18$, $r_S = 0.121$ and 0.115, $p = 0.632$ and 0.65, respectively). Thus, we raised 30 individuals reared in two tanks for each kin group. After the second reduction of group size, all fish were kept in an air-conditioned room under standardized winter light-regime (day length 8L:16D, temperature 16 ± 1°C) for 12 months. Afterwards the light regime was changed to summer conditions (day length 16L:8D, temperature 16 ± 1°C) for five months before winter conditions were re-established. Tanks contained 50 l of tap water, of which a third was replenished once a week. They were separated from each other by grey opaque partitions. Water in the tanks was cleaned and aerated through an internal filter. The fish were fed daily on frozen *Chironomus* larvae. At an age of 11 months, just before the start of the breeding season, all fish were counted. The second count was conducted at an age of 23 months.

Coloration

The sticklebacks used in coloration trials were tested in 2001 and 2004. We, therefore, refer to them as 2001 and 2004 fish, respectively. Individuals used

in the 2001 measurements were the F2 generation of fish caught in the wild during their 1999 spring migration. Fish were reared and kept under conditions described in Mazzi et al. (2002). Males that showed signs of nuptial coloration were isolated in tanks filled with 10 l of water from a well. Nest building was stimulated by daily presentation of a gravid female. Males that finished a nest, which could be recognized by intensified courtship behaviour (Wootton, 1976), were presented a gravid female for 30 min. Subsequently, slides were taken using the photo-box described in Bakker & Mundwiler (1994) which was a transportable version of the set-up of Frischknecht (1993). Of each fish, one slide was taken from the anterior half of the ventral and two from a randomly chosen lateral side of the male. From these slides the coloration was measured at ten standardized points of the throat and at six points of the eye (Figure 1) using a densitometer (X-Rite 310 Photographic Densitometer). The RGB-values were corrected for illumination differences between pictures by subtracting them from the optical density of R, G, or B of a white Munsell card (N 9.5) visible on each picture (Bakker & Mundwiler, 1994). The corrected red or blue index for throat and eye were

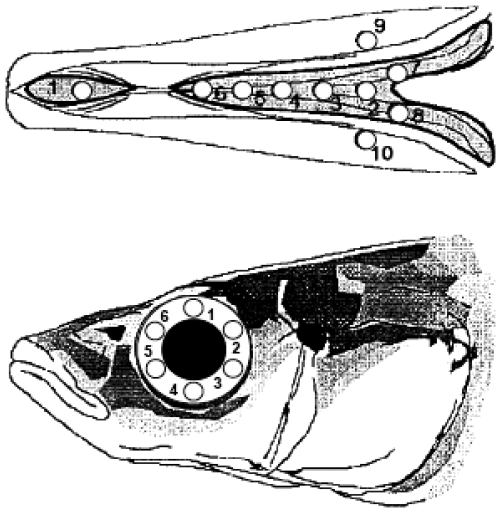

Figure 1. Points at the male's throat (upper diagram) and iris of the eye (lower diagram) for which breeding coloration was quantified (modified from Mundwiler, 1993). The optical densities of red, green and blue were measured at ten standardized points in the throat region and at six standardized points in the iris of the eye. From these measurements the maximum red and blue index for each male was estimated.

calculated following Bakker & Mundwiler (1994):

$$\text{red-jaw color intensity} = 1 - R_{\text{cor}}/(R_{\text{cor}} + G_{\text{cor}} + B_{\text{cor}})$$
$$\text{blue-eye color intensity} = 1 - B_{\text{cor}}/(R_{\text{cor}} + G_{\text{cor}} + B_{\text{cor}})$$

The range of the color intensities lay between zero and one, with a high value standing for a high intensity of red or blue. In the analyses, the highest index for the throat and the eye region was used. Coloration of 18 outbred and 17 inbred groups were tested. Each group provided three to five males. Mean values per family were calculated to avoid pseudo-replication.

For the 2004 measurements, fish from the above-described survival trials were used. During the period of summer conditions 34 outbred and 33 inbred, haphazardly chosen males that showed nuptial coloration were isolated into glass tanks containing 10 l of aerated tap water. To avoid visual contact between neighboring males, tanks were separated from each other by grey plastic sheets. They were equipped with a Petri dish filled with sand and 2 g of java moss (*Vesicularia dubyana*) for nest building. Nest building was stimulated by presenting a gravid female in a clear plastic box for 30 min each day. Males that finished a nest were presented a gravid female for 30 min. Immediately afterwards, males were carefully caught and placed into a transparent plastic box (6 cm $\times$ 7 cm $\times$ 7 cm) containing water (Bakker & Mundwiler, 1994). This box had two high-quality glass windows (Hoya HMC ultraviolet filter, 6 cm $\times$ 1.87 cm) through which the male could be photographed from two sides. The fish was gently fixed in position against the two windows using a wet, soft, black sponge. Two digital pictures were taken from each fish, one from the anterior half of the ventral and one from a randomly chosen lateral side of the male. Pictures were taken using a Canon Powershot G2 digital camera under standardized lighting conditions using two Metz Mecablitz 32 CT-3i flashes, each mounted on a tripod. The coloration of the males' throat and eye was measured at the same points as in the 2001 individuals (Figure 1). The RGB-values were measured using Photoshop 5.0 and were corrected for illumination differences as described above. Again, the maximum red and blue index was estimated following Bakker & Mundwiler (1994). Some of the 34 outbred or 33 inbred fish were brothers. In these cases the mean red and blue index per family was used in the analysis to avoid pseudoreplication. Thus, we obtained data for 19 outbred and 20 inbred families.

Statistical analysis

Because most data were not normally distributed according to Kolmogorov–Smirnov tests, non-parametric statistics were applied. Given test probabilities are two-tailed throughout. Analyses were performed using SPSS 12.0.

Results

In 1999, the sizes of outbred (median, quartiles: 101.0, 84.0, 126.0) and inbred (94.0, 85.0, 111.5) clutches did not differ significantly (Mann–Whitney U-test, $N_{out} = 25$, $N_{in} = 28$, $Z = -0.579$, $p = 0.562$), and clutch size was not significantly correlated with fertilization success (Spearman correlation, $N = 25$ and 28, $r_S = 0.309$ and -0.199, $p = 0.133$ and 0.310, respectively). Outbred clutches had a significantly higher fertilization rate than inbred ones (Mann–Whitney U-test, $N_{out} = 25$, $N_{in} = 28$, $Z = -3.279$, $p = 0.001$, Figure 2). In 2004 outbred (98.0, 95.5, 100.25) and one (97.5, 81.75, 104.0) or two (99.5, 84.0, 112.25) generations inbred clutches also did not differ significantly in clutch size (Kruskal–Wallis test, $\chi^2 = 0.532$, $p = 0.766$), but there were significant correlations between clutch size and fertilization success (Spearman correlations, $N = 36$, 20 and 14, $r_S = -0.514$, -0.422 and -0.540, $p = 0.001$, 0.064, 0.046, respectively). Furthermore, breeding regime had a significant effect on fertilization rate (Kruskal–Wallis test, $\chi^2 = 13.447$, $p = 0.001$, Figure 2). Clutches inbred for one and two generations had a lower fertilization rate than outbred ones, in the former this result only approached significance (Mann–Whitney U-test, $N_{out} = 36$, $N_{one} = 20$, $Z = -1.72$, $p = 0.085$, $N_{out} = 36$, $N_{two} = 14$, $Z = -3.63$, $p < 0.001$, respectively, Figure 2). Furthermore, one-generation inbred clutches tended to have a higher fertilization rate than two-generations inbred clutches ($N_{one} = 20$, $N_{two} = 14$, $Z = -1.864$, $p = 0.066$, Figure 2).

Outbred clutches in 1999 had a higher hatching rate than inbred ones. However, this difference was not statistically significant (Mann–Whitney U-test, $N_{out} = 25$, $N_{in} = 28$, $Z = -1.375$, $p = 0.169$, Figure 3). In 2004, outbred and inbred clutches showed significant differences in their hatching rate (Kruskal–Wallis test, $\chi^2 = 17.063$, $p < 0.001$, Figure 3). Outbred clutches had a significantly higher hatching success than one- ($N_{out} = 36$, $N_{one} = 20$, $Z = -2.327$, $p = 0.02$, Figure 3) as well as two-generations

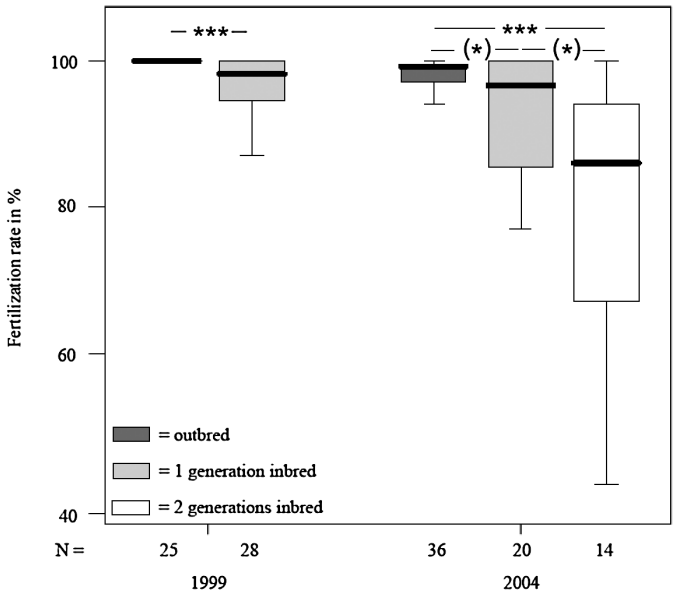

Figure 2. Fertilization rates of the 1999 and 2004 clutches. Plotted are medians, quartiles and ranges. In 1999, outbred clutches had a significantly higher fertilization rate than inbred ones. In 2004 outbred clutches had a significantly higher fertilization rate than two-generations inbred clutches. Furthermore there were differences between outbred and one-generation inbred, as well as one-generation and two-generation inbred clutches, which approached significance. $^{***}p < 0.001$, $(*)p < 0.1$.

inbred clutches ($N_{\text{out}} = 36$, $N_{\text{two}} = 14$, $Z = -4.056$, $p < 0.001$, Figure 3). One- and two-generations inbred clutches differed in their hatching success, but this result failed to reach statistical significance ($N_{\text{one}} = 20$, $N_{\text{two}} = 14$, $Z = -1.402$, $p = 0.169$, Figure 3).

Both subadult and adult fish showed different survival rates when they were inbred or outbred (Figure 4). In both cases, these difference approached significance (Mann–Whitney U-test, $N_{\text{out}} = 19$, $N_{\text{in}} = 18$, $Z_{\text{subadult}} = -1.863$, $Z_{\text{adult}} = -1.858$, both $p = 0.066$).

Coloration of 2001 outbred and inbred males did not differ significantly. Maximum values of red throat and blue eye coloration did not show any differences between outbred and inbred fish (Mann–Whitney U-test, $N_{\text{out}} = 18$, $N_{\text{in}} = 17$, $Z = -0.25$ and -0.951, $p = 0.807$ and 0.351, respectively, Figure 5). Similar results were obtained for the 2004 males. Red throat coloration as well as blue eye coloration of outbred and inbred fish did not differ

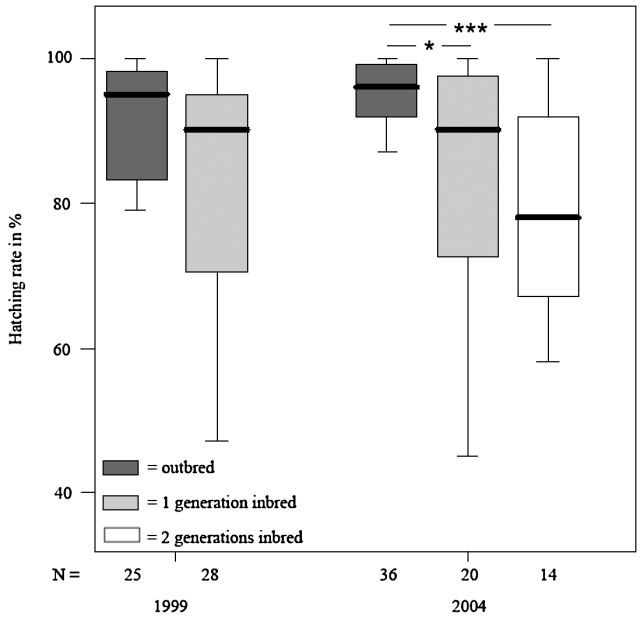

Figure 3. Hatching rates of the 1999 and 2004 clutches. Plotted are medians, quartiles and ranges. Inbred clutches in the 1999 trials had a lower hatching rate than outbred ones. However, this result was not statistically significant. In 2004, clutches which had been inbred for one or two generations had a significantly lower hatching rate than clutches which had been outbred. *** $p < 0.001$, * $p < 0.05$.

significantly (Mann–Whitney U-test, $N_{\text{out}} = 19$, $N_{\text{in}} = 20$, $Z = -0.751$ and -0.099, $p = 0.453$ and 0.921, respectively, Figure 5).

Discussion

Sticklebacks have been a model species in the fields of evolutionary ecology and ethology for over 50 years (reviewed in Bakker & Sevenster, 1995; Peichel & Boughman, 2003). They are a typical colonizing species (Bell & Foster, 1994; von Hippel & Weigner, 2004), rapidly inhabiting newly established habitats (see McKinnon & Rundle, 2002, for a summary). Freshwater sticklebacks are often shown to have originated from anadromous ancestors (McPhail, 1994). Reduced genetic variation within recently founded freshwater populations relative to anadromous source populations has been observed several times (e.g., Raeymaekers et al., 2005, and citations therein).

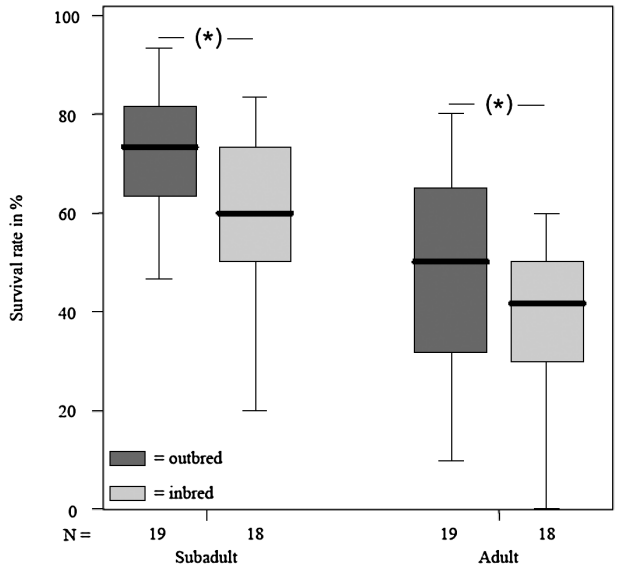

Figure 4. Survival rates of outbred and inbred fish until sub-adulthood and adulthood. Plotted are medians, quartiles and ranges. In both age classes, outbred fish had a higher survival rate than inbred ones, although these results only approached significance. $^{*}p < 0.1$.

Ólafsdóttir et al. (2007) for example found a reduction of genetic variability in recently established Icelandic stickleback populations, although none of the populations showed signs of significant bottleneck effects. As a consequence of the small population size the risk of inbreeding in such newly founded populations should be higher, favouring the evolution of inbreeding avoidance mechanisms. While recent studies demonstrate different ways to reduce the risk of inbreeding (Largiadèr et al., 2001; Bakker et al., 2006a; Frommen & Bakker, 2006), little is known about the strength of inbreeding depression in sticklebacks. This study shows that only one generation of brother–sister mating leads to a loss of fitness due to a reduced fertilization and hatching rate. Furthermore, inbred individuals had a lower probability to reach and survive the reproductive phase than outbred ones, although this effect only approached significance. An influence of inbreeding on males' breeding coloration could not be found.

The finding that one generation of inbreeding leads to only a moderately reduced adult survival as well as no reduction of males' breeding coloration might have several explanations. Sticklebacks used to establish the inbred lines were wild caught fish from a large and genetically heterogeneous popu-

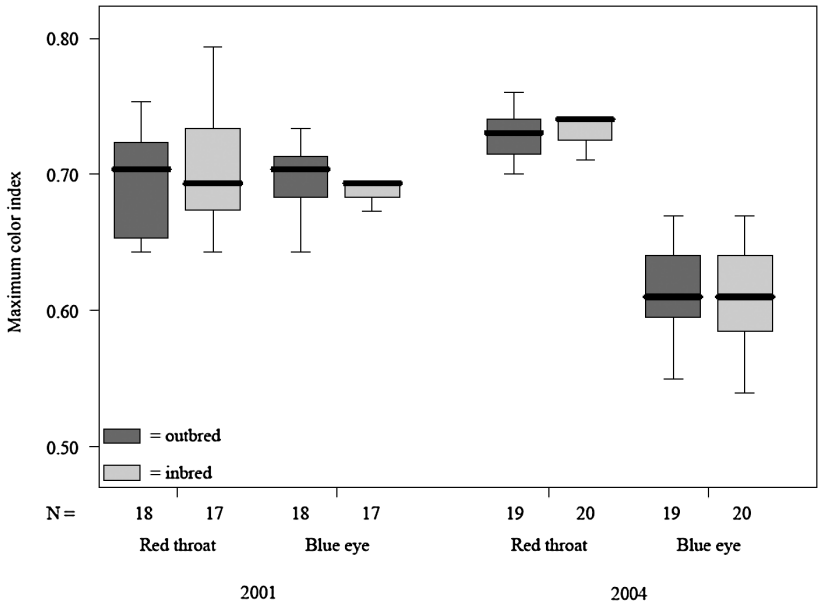

Figure 5. Maximum index for the intensity of red throat and blue eye breeding colors. See the text for explanation. Plotted are medians, quartiles and ranges. Neither in 2001 nor in 2004 did inbred and outbred males differ significantly in their red throat and blue eye coloration.

lation (Heckel et al., 2002). Thus, it might be possible that one generation of inbreeding does not reduce genetic heterozygosity enough to unmask deleterious recessive alleles. The results of our 2004 trials on fertilization success and hatching rate support this assumption. Although not statistically significant, eggs produced by two subsequent generations of brother–sister matings had a lower fertilization success and hatching rate than one-generation inbred clutches, suggesting that inbreeding depression increased with increasing inbreeding coefficient. This indicates that the negative genetic load has not been purged (Crnokrak & Barrett, 2002) after only one generation of inbreeding. The finding that coloration is not affected by one generation of inbreeding is in concordance with studies on guppies (van Oosterhout et al., 2003). Here, male color traits only revealed inbreeding depression after several generations of brother–sister matings. In sticklebacks, one generation of inbreeding may also be too weak to cause detectable reduction of breeding coloration.

Alternatively, inbreeding may affect coloration only under harsh environmental conditions, for example when food supply is limited. In our study,

males were fed daily frozen *Chironomus* larvae and showed a good body condition (Frommen et al., 2007). Therefore, it is possible that males of lower genetic quality, which would have shown a reduced breeding coloration under food-deprived conditions, were able to express a breeding coloration similar to that of high-quality males under ideal laboratory conditions. Furthermore, all fish experienced the same diet in the lab, which might have led to a reduction of color variation. Indeed, inbred and outbred lab-raised males remained paler than wild caught males from the same population (J.G.F. and T.C.M.B., pers. observation). Another possible explanation for the lack of inbreeding effects on breeding coloration is that individuals that suffered more from inbreeding did not survive until adulthood, so that surviving inbred males were of similar genetic quality as outbred ones.

Reduced fertilization success, hatching rate and survival until adulthood as a result of inbreeding in sticklebacks are consistent findings with several other studies on inbreeding in fishes. In different salmonid species, a reduction of inbred egg and fry survivorship as well as a lowered body mass, reduced growth rate, and survivorship to various ages were demonstrated (reviewed in Waldman & McKinnon, 1993). However, studies on fish which are not of commercial interest are scarce thus far. In zebrafish, inbred eggs showed a reduced fertilization rate and a reduction in the probability to reach an age of 30 days (Mrakovčić & Haley, 1979). Furthermore, inbred progeny had a higher amount of body deformations. Inbred convict cichlids, *Archocentrus nigrofasciatus* showed a reduced survivorship to an age of five months as well as a higher frequency of body deformations (Winemiller & Taylor, 1982). In contrast, one generation of brother–sister mating had no effect on juvenile survival or growth rate of the cichlid *Pelvicachromis taeniatus*, a species in which both sexes even prefer incestuous matings (Thünken et al., 2007).

In 2004, there were significant negative correlations between clutch size and fertilization success. Threespine sticklebacks are known to be sperm-limited, because they do not produce sperm during the breeding season (Borg, 1982). However, effects of sperm depletion on fertilization success are thus far only documented for males which sired high numbers of clutches (Zbinden et al., 2001), making shortage of sperm an unlikely explanation for these results. Clutches in the 2004 experiments were removed from the nest 1 h after spawning. As stickleback eggs show a very slow fertilization (Bakker et al., 2006a) it might, thus, be possible that in larger clutches not all eggs

were fertilized when they were removed from the nest. However, as clutch size did not differ significantly between the different treatments, differences in egg numbers cannot explain the decrease of fertilization success in inbred clutches.

In conclusion, this study revealed that only one generation of inbreeding by brother–sister matings leads to a loss of fitness in sticklebacks. This is due to a reduced fertilization rate, as well as a lowered survivorship of inbred eggs and fish. An influence of inbreeding on males' breeding coloration could not be assessed after one generation of inbreeding.

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