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Developmental social experience of parents affects behaviour of offspring in zebrafish

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Interactions between conspecifics early in life have the potential to shape phenotypic differences between individuals. These changes in phenotype may subsequently be passed to future offspring, something that has been studied in live-bearing mammals where there is often an element of parental care. The present study considers the transgenerational effects of social environment in zebrafish, *Danio rerio*, an egg-laying animal that shows no parental care, thus removing any influence of parental interaction and allowing the effects of conspecific interaction to be clearly determined. Zebrafish (F₀) were reared from fertilization to reproduction in three different social treatments: isolation, groups of 30 or groups of 100. At 28 days post fertilization, individuals were tested for anxiety and activity and at 3 months old for aggression. These F₀ fish were raised to sexual maturity and bred within their treatment group. The F₁ generation were then raised in groups of 30, irrespective of parental social environment and were assessed for behaviour in the same way as their parents. Social isolation increased anxiety and decreased aggression in the F₀ fish compared to those raised in social groups of 100. F₀ fish raised in social groups of 30 showed an intermediate response. Differences in anxiety were not passed to the F₁ generation; however, offspring of socially

isolated fish were less aggressive than offspring of parents from social groups of 30 and 100.

The social environment that an individual experienced from fertilization to reproduction affected their own behaviour and the behaviour of their offspring.

Key words: activity, aggression, anxiety, behaviour, isolation, traits, transgenerational effects.

The early social environment experienced by an individual influences the development of behaviour as individuals in a social environment can learn from their conspecifics by observing them engaging in particular activities (Suboski & Templeton, 1989; Brown & Laland, 2001). Deprivation of social interaction early in development can, therefore, affect a variety of behaviours in a range of animals. For example, dairy calves housed individually immediately after birth were more reactive to environmental and social novelty than group-housed calves and calves housed with an older companion (Vieira, de Passille, & Weary, 2012). The majority of mammalian studies have considered the effects of social interactions after birth, although there is also evidence in rodents that interfetal communication can have a significant effect on behaviour later in life (vom Saal, 1989). In oviparous fish, which lay eggs into the external environment, exogenous cues such as the smell of predators or alarm cues from adults can alter developmental processes (Mirza, Chivers & Godin, 2001; Mourabit, Rundle, Spicer, & Sloman, 2010). For example, in rainbow trout, *Oncorhynchus mykiss*, raised in different social group sizes from fertilization, presence of conspecifics affected both physiology and behaviour. Trout raised in isolation had lower oxygen consumption rates and were less aggressive towards their own mirror image than individuals raised in social groups (Sloman & Baron, 2010).

51

52 The developmental environment experienced by an organism may alter not only its own
53 phenotype but also the behaviour of its offspring. Parental influence on offspring phenotype
54 (both maternal and paternal) can occur by genetic and epigenetic mechanisms. Maternal
55 effects are traditionally considered to be nongenomic, that is, they are not related to gene
56 sequence, although there is variation in the way 'maternal effects' are defined (Wolf & Wade,
57 2009). Both paternal and maternal effects have been documented in the literature, although
58 maternal effects have received the most attention and have been studied in a wide variety of
59 animals (mammals: Inhasz Kiss, Woodside, Felicio, Anselmo-Franci, & Damasceno, 2012;
60 birds: Guibert et al., 2011, Rubolini et al., 2005; reptiles: Robert, Vleck, & Bronikowski,
61 2009; Uller & Olsson, 2006; fish: Andersson, Silva, Steffensen, & Höglund, 2013; Eriksen et
62 al., 2011; Sloman, 2010). For example, a study on the pea aphid, *Acyrtosiphon pisum*,
63 showed that exposure of females to the alarm pheromone (E)- β -farnesene prior to
64 reproduction, a cue for predation risk, resulted in a change of feeding sites in offspring
65 (Keiser & Mondor, 2013). Postnatal communal rearing in Balb/c mice, *Mus musculus*,
66 induced transgenerational effects on emotional and reproductive behaviour of offspring
67 (Curley, Davidson, Bateson, & Champagne, 2009). Communally raised females that received
68 more postpartum maternal care exhibited lower anxiety, built higher quality nests and showed
69 more postpartum care as adults than standard reared females. This behaviour was further
70 carried through to F₂ mice that exhibited lower anxiety, larger litter size and increased
71 nursing, suggesting the effect of postnatal social environment on the behaviour of offspring
72 across generations (Curley et al., 2009).

73

74 Early in development the epigenome of an individual can be influenced by environmental and
75 nutritional factors, such as transfer of hormones or nutrient provision by mothers to eggs or

offspring (Nafee, Farrell, Carroll, Fryer, & Ismail, 2007). Parental conditions can alter the phenotype of offspring (Chen et al., 2013; Franzke & Reinhold, 2013; Krause & Naguib, 2014; Pittet, Le Bot, Houdelier, Richard-Yris, & Lumineau, 2014) and while some authors may conclude that these transgenerational effects occur via epigenetic mechanisms (Youngson & Whitelaw, 2008), others would argue that when considering environmentally-induced effects, an epigenetic basis can be inferred only if changes last over multiple generations (Grossniklaus, William, Ferguson-Smith, Pembrey, & Lindquist, 2013; see also Burggren, 2014). Mechanisms that allow transfer of information about the maternal environment to offspring are likely to be advantageous if they prepare the offspring for the environment they will be born into (Love, McGowan, & Sheriff, 2013; Sheriff & Love, 2013). Parental mechanisms that allow adjustments of offspring phenotype based on immediate parental environment may allow more flexibility than selection on genotypes (Crews, 2008; Wisenden, Sailer, Radenic, & Sutrisno, 2011).

A number of studies have looked at the effect of variations in the social environment during development. However, it is not known whether changes in phenotype induced by early social interactions can be passed across generations. Furthermore, previous studies have addressed transgenerational effects wherein the parents were exposed to the experimental manipulation only during a certain point of their own development and transferred to control conditions before reaching sexual maturity; any responses in offspring could thus be the result of differences in natal and adult environments (reviewed in Burton & Metcalfe, 2014). Therefore, the aim of the present study was, first, to examine the effects of different social environments maintained from fertilization to reproduction in F_0 zebrafish, *Danio rerio*, and, second, to investigate any transfer of behavioural effects to the subsequent F_1 generation.

Social environments were varied by number of individuals rather than stocking density; it

was hypothesized that zebrafish raised in different social environments would exhibit differences in behaviour later in life and these differences in behavioural phenotype may be transmitted to future generations.

Methods

Adult zebrafish (AB, TL mixed strains) from an existing stock at the University of the West of Scotland were held on a recirculating system (27 ± 1 °C; pH 7.1 ± 0.4 ; dissolved oxygen $90\pm5\%$; 14:10 h light:dark) and fed *Artemia* or Aquarian tropical flake twice daily. Fish were bred to produce embryos (F_0 generation) which were collected within 30 min of fertilization and placed into group sizes of 1 ($N=36$), 30 ($N=3$) and 100 ($N=3$). The different group sizes were held in 50, 500 and 5000 ml containers with 10, 30 and 1000 ml of system water, respectively, and placed in a water bath at 28.5 °C. The walls of the containers were opaque to block any visual cues and a 30% water change was carried out daily. From 5 days post fertilization (dpf) larvae were fed Liquifry and ZM 00 daily. One week after hatching, fry were transferred in their group sizes into flow-through tanks with opaque sides on the main recirculating system. Tanks sizes were 1, 3 and 12 litres containing 0.94, 2.95 and 11.5 litres of water, respectively. Fry were fed ZM 100 from 11 dpf and ZM 200 and *Artemia* from 15 dpf. From 30 dpf they were fed twice daily with flake food and *Artemia*. Variations in tank sizes between the different treatments ensured that treatments represented a change in number of individuals within the social group, not differences in stocking density. An overview of the experimental design is shown in Fig. 1.

Breeding of F_0 fish

When the F_0 fish were 3 months old, they were bred to produce the F_1 generation. To achieve this, F_0 fish raised in social isolation were combined within their treatment resulting in three replicate breeding tanks for each treatment. Thirty F_1 embryos from each replicate of each F_0 treatment (i.e. $N=3$) were then raised in 1.5-litre containers held at 28.5 °C. Thus, F_1 offspring from all F_0 treatments were held at the same density and the only difference between F_1 treatments was the social environment experienced by their parents. Two weeks after hatching, fry were transferred into 3-litre flow-through tanks on the main recirculating system. The feeding regime was the same as for the F_0 generation.

Behavioural testing of F_0 and F_1 fry

At 28 dpf F_0 ($N=27$) and F_1 ($N=18$) fry were tested for their behaviour in the light and dark box test and the novel tank diving test. At 3 months old, fish ($N=27$ F_0 ; $N=18$ F_1) were tested for their response to their own mirror image as zebrafish show similar levels of aggression towards a mirror image as towards an opponent (Ariyomo & Watt, 2013).

Light and Dark Box Test

The experimental tank (16 x 10 cm and 10.5 cm high) was divided vertically in half and covered externally on the sides and bottom by white or black paper. The top was left completely open. The half of the tank covered externally with a white background represented the light compartment and the half with a black background was the dark compartment. An opaque cylinder was placed in the centre of the tank creating a compartment (5 cm diameter) for acclimating the fish. The tank was filled with system water which was replaced at the end of every trial (Blaser & Rosemberg, 2012; Maximino et al., 2010a; Maximino, de Brito, Dias, Gouveia, & Morato, 2010b). Fish were individually placed

into the central compartment for 5 min, after which time the walls of the central compartment were removed, allowing the fish to freely explore the tank. Behaviour was recorded for 10 min with a video camera (Panasonic SD video camera, SDR-S50) placed above the arena. The tank was illuminated with a white daylight bulb (70 W) and the illumination kept constant between trials. The behavioural end points measured were the proportion of time the fish spent in the dark compartment (scototaxis), the time spent beside the walls (thigmotaxis) and the number of transitions between the light and dark compartment (activity) (Blaser & Rosemberg, 2012; Maximino et al., 2010a, b).

Novel Tank Diving Test

A trapezoidal tank, greater in horizontal cross-sectional area at the top than the bottom, (top: 16 x 10 cm; bottom: 15 x 9 cm; 10.5 cm deep) was visually divided horizontally on the outside, half way between the top water line (8 cm) and the bottom of the tank (Cachat et al., 2010; Egan et al., 2009; Levin, Bencan, & Cerutti, 2007). The tank was filled with system water which was replaced at the end of every trial. Individual fish were transferred to the experimental tank and behaviour was recorded for 6 min with a video camera. The behavioural parameters measured were latency to enter the upper compartment of the tank, time spent in the upper and lower compartments of the tank and number of entries into the upper and lower compartments (activity). A longer latency to reach the upper compartment and surface and reduced entries into the upper compartment are considered to be indicators of stress and anxiety (Egan et al., 2009; Levin et al., 2007).

Mirror Image Test

Individual fish were placed in a test tank (16 x 10 cm and 10.5 cm high) containing a covered mirror and left to acclimate for 20 h. Before commencement of behavioural recordings, the mirror was revealed for 1 min to allow the fish to get used to the disturbance caused by revealing the mirror. After a further 15 min, the mirror was revealed once again and behaviour was video recorded for 1 h. The behavioural parameters measured were latency to first attack the mirror, frequency of mirror biting and time spent within 5 cm of the mirror.

Ethical Note

All experiments adhered to the ASAB/ABS guidelines for the use of animals in research and the U.K. Home Office guidelines (licence: 70/8539). All fish were held in appropriate water quality (as documented above) and water quality was checked daily for all treatments. All experiments were mild and did not cause significant pain or lasting harm to the fish; a mirror image test was used to measure aggression rather than pairing of conspecifics which can lead to physical damage.

Data analysis

All the behavioural videos were observed blind, that is, the observer did not know which treatment groups they came from. Data analysis was carried out using the statistical software SPSS Statistics 20 (IBN, Armon, NY, U.S.A.). Data were tested for normality using the Kolmogorov–Smirnov test and found to be normally distributed. Analysis of variance was used to compare behaviour between the three treatment groups in the F_0 and F_1 generations, respectively. Post hoc testing with a least significant difference test was performed on the behavioural parameters that showed significant results ($P < 0.05$) and is presented in the figures. For the F_1 generation, tank replicate was included as a random effect in the model.

Results

Light and Dark Box Test

For the F_0 generation, fish raised in social groups of 100 displayed significantly less scototaxis ($F_{2, 66} = 4.891$, $P=0.010$; Fig. 2a), spending less time in the dark compartment, than those reared in groups of 30 or in social isolation. Fish raised in isolation displayed significantly more activity ($F_{2, 66} = 4.783$, $P=0.011$; Fig. 2b) than those in social groups of 100. Time spent beside the walls (thigmotaxis) was not significantly different between treatments ($F_{2, 66} = 0.310$, $P=0.735$). Neither was there any significant difference in thigmotaxis between treatments when data were analysed separately for the light and dark compartments (light: $F_{2, 66} = 0.602$, $P=0.179$; dark: $F_{2, 66} = 2.129$, $P=0.076$). In the F_1 generation, there were no significant effects of treatment on scototaxis ($F_{2, 51} = 0.183$, $P=0.840$), activity ($F_{2, 51} = 1.528$, $P=0.321$) or thigmotaxis ($F_{2, 51} = 0.399$, $P=0.695$).

Novel Tank Diving Test

In the F_0 generation, there was a reduced latency to reach the upper compartment ($F_{2, 64} = 3.563$, $P=0.034$; Fig. 3a) in the fish raised in groups of 100 compared to those from isolation. However, time spent in the upper or lower compartment was not significantly different ($F_{2, 64} = 0.949$, $P=0.392$) between treatments. Fish raised in isolation displayed significantly more activity ($F_{2, 64} = 10.597$, $P<0.001$; Fig. 3b) than those raised in social groups. For the F_1 generation, there was no significant effect of treatment on latency to reach the upper compartment ($F_{2, 51} = 0.674$, $P=0.559$), time spent in the upper compartment ($F_{2, 51} = 0.175$, $P=0.845$) or activity ($F_{2, 51} = 1.990$, $P=0.251$).

Mirror Image Test

In the F_0 generation, fish raised in social isolation displayed significantly fewer attacks towards the mirror than those raised in social groups of 100 and 30 ($F_{2, 73} = 3.626$, $P=0.032$; Fig. 4a). There was no difference in latency to attack the mirror ($F_{2, 73} = 1.699$, $P=0.190$) or the time spent beside the mirror ($F_{2, 73} = 1.986$, $P=0.145$). In the F_1 generation the latency of fish to attack their own mirror image was not significantly different between F_1 fish whose parents were raised in different social environments ($F_{2, 51} = 0.425$, $P=0.680$). However, F_1 fish from parents raised in social isolation displayed significantly fewer attacks ($F_{2, 51} = 142.039$, $P=0.007$; Fig. 4b) and spent less time beside the mirror ($F_{2, 51} = 322.775$, $P<0.001$; Fig. 4c) than those from parents raised in social groups.

Discussion

The present study has shown that the social environment experienced by zebrafish from fertilization to reproduction affects anxiety and aggression later in life and that differences in aggression are passed on to offspring regardless of their rearing environment. To our knowledge, this is the first study in fish to have considered whether behavioural changes in the F_0 generation induced by their social environment can be passed on to their offspring. In fish, social isolation has been shown to alter behaviour (Ichihashi, Ichikawa, & Matsushima, 2004; Gomez-Laplaza & Morgan, 2000; Zellner, Padnos, Hunter, MacPhail, & Padilla, 2011); however, most of the previous studies in fish have manipulated the social environment after hatching, whereas in the present study zebrafish embryos were separated immediately after fertilization.

244 In the present study, in the F₀ generation, individuals raised in either social isolation or in
245 groups of 30 were more scototactic (spent more time in the dark) than those raised in groups
246 of 100. Scototaxis as a measure of anxiety in zebrafish has been validated in a number of
247 previous studies (Blaser & Rosemberg, 2012; Maximino et al., 2010a,b), anxious fish being
248 more scototactic (Maximino et al., 2012; Maximino et al., 2010a). It has been suggested that
249 scototaxis is a form of defence where camouflaging with the substratum can help avoid
250 predation (Maximino et al., 2010b). Thigmotaxis (wall hugging) is another indicator of
251 anxiety. This phenomenon has been found in mice and it is proposed to assist in search for
252 shelter and protection (Treit & Fundytus, 1988). Anxious fish exhibit higher thigmotaxis
253 (Blaser & Gerlai, 2006; Champagne, Hoefnagels, de Kloet & Richardson, 2010; Maximino et
254 al., 2010a) but in the present study F₀ fish showed no difference in thigmotaxis. As previous
255 studies have shown that more thigmotaxis can occur in the dark (Blaser, Chadwick, &
256 McGinnis, 2010), the thigmotaxis data were further analysed for the dark and light
257 compartments separately but no significant effects were found. Therefore, one anxiety
258 measure (scototaxis) indicated that the fish raised in isolation and small social groups were
259 more anxious, whereas the second measure of anxiety (thigmotaxis) found no effects. These
260 results are in line with studies by Blaser et al. (2010) who also found thigmotaxis behaviours
261 difficult to interpret. They suggested that 'thigmotaxis' includes a greater variety of
262 behaviours than simply 'proximity to the walls' and therefore may require additional
263 behavioural measurements to relate to anxiety. The effects on anxiety levels indicated by
264 differences in scototaxis in the light and dark box test, however, were further substantiated by
265 the results of the novel tank diving test where anxious fish take longer to reach the upper part
266 of the tank (Egan et al., 2009; Gerlai, 2003; Levin et al., 2007). Fish raised in isolation were
267 more anxious than those raised in groups of 100 as they showed a longer latency to reach the
268 upper compartment, with fish raised in groups of 30 showing an intermediate response.

270 In both the light and dark box test and the novel tank diving test, fish raised in social isolation
271 were more active than those raised in social groups of 100. In the light and dark box test, fish
272 raised in social groups of 30 were more active than those raised in groups of 100, whereas in
273 the novel tank diving test fish raised in groups of 30 showed a more intermediate response.
274 Test-specific differences in the behaviour of zebrafish have been reported in previous studies
275 (Blaser & Gerlai, 2006); however, the overall pattern in both tests shows an increased level of
276 activity with decreasing social experience. Activity has previously been suggested as a
277 measure of anxiety (Blaser et al., 2010; Maximino et al., 2010a; Peng et al., 2016); therefore,
278 the increased activity seen in the fish raised in social isolation supports the higher anxiety
279 suggested by scototaxis and latency to reach the upper compartment.

280

281 None of the behavioural parameters measured in either the light and dark box test or the
282 novel tank diving test were significantly different in the F_1 generation where offspring of F_0
283 fish reared in social isolation and social groups of 30 and 100 were all reared in groups of 30.
284 This would suggest that although the F_0 socially isolated fish were more anxious, these
285 effects were not passed on to their offspring. The fact that differences in anxiety were not
286 transferred to offspring may be because anxiety can be a disadvantageous trait to pass on as it
287 can lead to increased predation (McGhee & Bell, 2014). In the present study, the genetic pool
288 was assumed to be equally distributed between treatments at the start, as we were specifically
289 interested in nongenomic effects of parental social environment. As both mothers and fathers
290 were exposed to the same social environments, we cannot separate maternal from paternal
291 effects. There is evidence that some behavioural traits such as boldness and aggression have a
292 heritable component (Brown, Burgess, & Braithwaite, 2007; Ariyomo, Carter, & Watt, 2013),

and further studies on the heritability of anxiety, and potential interactions with parental effects, are warranted.

At approximately 100 dpf, F₀ zebrafish raised in different social environments displayed differences in their response to their own mirror image. The mirror image test was used to measure aggression as it represents a measure of how individuals would interact with conspecifics (Moretz, Martins, & Robison, 2007). Fish are unable to recognize their own image and respond to the mirror image as if it were an intruder (Rowland, 1999). Aggression is exhibited by both sexes in zebrafish (Moretz et al., 2007) and is important in reproductive success and establishment of dominance hierarchies (Paull, Filby, Giddins, Coe, Hamilton, & Tyler., 2010). Although the mirror image test is generally considered to be good for measuring some behavioural parameters over others (Balzarini, Taborsky, Wanner, Kock, Felizia, & Frommen, 2014; Elwood, Stoilova, McDonnell, Earley, & Arnott, 2014), Ariyomo and Watt (2013) found that there were no differences in the rate of aggression of zebrafish towards a mirror image and an opponent, thus demonstrating the validity of the mirror image test as an indicator of aggression in zebrafish. Overall, fish raised in social groups of 30 and 100 were more aggressive than those held in social isolation. The results of the present study are similar to results obtained by Sloman and Baron (2010) who found that rainbow trout raised in social isolation performed half as many bites as those raised in social groups. In both their study and ours, fish were raised in their respective social conditions from fertilization until they were used in the mirror image test, so those raised in social isolation had never encountered another fish. In the present study, there was no difference between treatments in latency to attack the mirror or the time spent beside the mirror, suggesting an innate fighting ability of fish raised in social isolation.

It is possible that the absence of conspecifics during the very early stages of development might have led to adults with reduced aggressive behaviour since they were not receiving cues about the presence of conspecific competition. Sloman and Baron (2010) suggested that the higher prehatching metabolic rate of embryos raised in social groups indicated their ability to detect cues from conspecifics in their environment during development and through their higher metabolic rate have an increased competitive ability at first feeding. Similarly, it is likely that in the present study embryos raised in social groups could detect cues from conspecifics during development which increased aggression, reinforced by social interaction after hatching. In social groups individuals learn from their conspecifics (Suboski & Templeton, 1989) whereas fish raised in social isolation had no opportunity to learn. The present study confirms that fish raised in social groups are more aggressive and here we found that this behaviour is passed to their offspring. This is likely to be advantageous for individuals born into a competitive environment. In contrast, social isolation appears to reduce aggression, which persists in the next generation even when offspring are raised in social groups. In this scenario, where there is a mismatch between parent and offspring environment, reduced aggression levels may be disadvantageous. To examine this further, it would be interesting to know how long social isolation needs to persist in the F_0 generation for it to be transferred to the next generation. For example, if an embryo is separated from its conspecifics from fertilization until hatching only, does it recover normal levels of aggression? Also, it would be interesting to know whether the higher aggression seen in the F_1 generation whose parents were raised in social groups persists if the F_1 generation are all reared in social isolation. Aggression levels were much higher in the F_1 than in the F_0 generation (Fig. 4). At present, we have no clear explanation for this difference and future studies are required to see if this is maintained across further generations.

The advantages of a mother transferring information about her environment to her offspring, mostly associated with maternal transfer of stress hormones, have been reviewed previously (Love et al., 2013; Sheriff & Love, 2013). For example, female sticklebacks, *Gasterosteus aculeatus*, can transfer information about the level of predation in their environment to their offspring, and it has been suggested that this occurs via cortisol transfer (Giesing, Suski, Warner, & Bell, 2011). Eaton, Edmonds, Henry, Snellgrove, and Sloman (2015) speculated that the lack of associative learning by guppy offspring of mildly stressed mothers may be advantageous. If offspring are to be born into an unpredictable environment then a lack of associative learning, or ability to forget, may be adaptive (Warburton, 2003). The mechanisms by which differences in aggression levels are transferred between generations in the present study remain unknown but it is possible that different social environments resulted in different levels of cortisol or nutrients being deposited in eggs during oogenesis.

In conclusion, offspring of fish reared in social isolation were less aggressive than the offspring of group-reared fish. However, there were no significant differences in behavioural parameters related to anxiety and activity in the F₁ generation. These results suggest that zebrafish can transfer information about their environment to offspring, leading to nongenomic transgenerational transmission of behavioural phenotypes. The mechanisms underlying these differences are not clear and future studies should look both at whether these effects persist across multiple generations and for evidence of epigenetic mechanisms.

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367

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Figure Legends

Figure 1. Schematic representation of experimental design. Grey boxes represent experimental units, text within black boxes indicates behavioural measurements and arrows represent progression of each treatment through time. Not to scale.

Figure 2. Behaviour of F_0 fish in the light and dark box test. (a) Time spent in the dark compartment and (b) activity, i.e. number of transitions between compartments for F_0 fish in the light and dark box test. Boxes represent the 25th and 75th percentiles and the line in between represents the median. Error bars are 95% confidence intervals. Sample sizes are given within the bars. Letters denote significant differences ($P < 0.05$), where bars sharing a letter are not significantly different.

Figure 3. Behaviour of F_0 fish in the novel tank diving test. (a) Latency to reach the upper compartment and (b) activity, i.e. number of transitions for F_0 fish in the novel tank diving test. Boxes represent the 25th and 75th percentiles and the line in between represents the median. Error bars are 95% confidence intervals. Sample sizes are given within the bars. Letters denote significant differences ($P < 0.05$), where bars sharing a letter are not significantly different.

Figure 4. Behaviour of F_0 and F_1 fish in the mirror image test. (a) Number of attacks/min in the mirror image test for F_0 fish, (b) number of attacks/min by offspring (F_1) and (c) time spent beside the mirror by offspring (F_1). Boxes represent the 25th and 75th percentiles and the line in between represents the median. Error bars are 95% confidence intervals. Sample sizes are given within the bars. Letters denote significant differences ($P < 0.05$), where bars sharing a letter are not significantly different.

Figure 1

Figure 2

Figure 3

Figure 4