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Antipredator defences of young are independently determined by genetic inheritance, maternal effects and own early experience in mouthbrooding cichlids

Ariane Stratmann[1,2] and Barbara Taborsky*[,1]

[1]*Behavioural Ecology, Institute of Ecology and Evolution, University of Bern, Wohlenstrasse 50A, CH-3032 Hinterkappelen, Switzerland; and* [2]*Division of Animal Welfare, Veterinary Public Health Institute, University of Bern, Burgerweg 22, CH-3052 Zollikofen, Switzerland*

Summary

1. Predation is a prime force of natural selection. Vulnerability to predation is typically highest early in life, hence effective antipredator defences should work already shortly after birth. Such early defences may be innate, transmitted through non-genetic parental effects or acquired by own early experience.

2. To understand potential joint effects of these sources of antipredator defences on phenotypic expression, they should be manipulated within the same experiment. We investigated innate, parental and individual experience effects within a single experiment. Females of the African cichlid *Simochromis pleurospilus* were exposed to the offspring predator *Ctenochromis horei* or a benign species until spawning. Eggs and larvae were hand-reared, and larvae were then exposed to odour cues signalling the presence or absence of predators in a split-brood design.

3. Shortly after independence of maternal care, *S. pleurospilus* undergo a habitat shift from a deeper, adult habitat to a shallow juvenile habitat, a phase where young are thought to be particularly exposed to predation risk. Thus, maternal effects induced by offspring predators present in the adult habitat should take effect mainly shortly after independence, whereas own experience and innate antipredator responses should shape behaviour and life history of *S. pleurospilus* during the later juvenile period.

4. We found that the manipulated environmental components independently affected different offspring traits. (i) Offspring of predator-exposed mothers grew faster during the first month of life and were thus larger at termination of maternal care, when the young migrate from the adult to the juvenile habitat. (ii) The offspring's own experience shortly after hatching exerted lasting effects on predator avoidance behaviour. (iii) Finally, our results suggest that *S. pleurospilus* possess a genetically inherited ability to distinguish dangerous from benign species.

5. In *S. pleurospilus,* maternal effects were limited to a short but critical time window, when young undergo a niche shift. Instead, own environmental sampling of predation risk combined with an innate predisposition to correctly identify predators appears to prepare the young best for the environment, in which they grow up as juveniles.

Key-words: cichlids, developmental plasticity, growth, innate predator defences, maternal effects, predator recognition

Introduction

Predation is one of the most important selective forces in nature. Evolving efficient antipredator strategies is thus a pivotal component of Darwinian fitness (e.g. Lima & Dill 1990). In many species, the vulnerability to predation is particularly high at very early life stages, when young can become an easy treat for predators because of their small size, limited body strength or constrained escape potential (e.g. Gosselin & Qian 1997; Sogard 1997). Therefore, it is not surprising that young often possess efficient antipredator defences already at or shortly after birth, as shown in a

*Correspondence author: E-mail: barbara.taborsky@iee.unibe.ch

number of vertebrates (e.g. Laurila, Kujasalo & Ranta 1997; Veen *et al*. 2000; Göth 2001; Vilhunen & Hirvonen 2003; Fendt 2006; Hawkins, Magurran & Armstrong 2007) and invertebrates (e.g. Abjörnsson, Hansson & Brönmark 2004; Storm & Lima 2010).

Causal mechanisms that have been proposed to be responsible for the expression of early antipredator responses include genetic predisposition for predator recognition (Magurran 1990; Abjörnsson, Hansson & Brönmark 2004; Scheurer *et al*. 2007), non-genetic maternal effects (Dzialowski *et al*. 2003; Sheriff, Krebs & Boonstra 2010; Coslovsky & Richner 2011; Giesing *et al*. 2011; McGhee *et al*. 2012; Segers & Taborsky 2012), learning before (Mathis *et al*. 2008; Colombelli-Negrel *et al*. 2012) or shortly after birth (Brown, Ferrari & Chivers 2011) and the use of cues from the diet of predators (rev. in Ferrari *et al*. 2007). Studies comparing the relative influence of non-genetic maternal effects and own early predator experience on the phenotype of young animals showed that maternal effects can act in isolation (Dzialowski *et al*. 2003) or interactively with own experience (Tollrian 1995; Kaplan & Phillips 2006). To obtain a sound understanding of the action of the sources of antipredator responses, these sources should be studied jointly. Here, we present a factorial experiment allowing us to identify separate or joint effects of three main sources of phenotypic variation, namely of innate, non-genetically inherited and individually acquired early life antipredator defences and to test for short- and long-term consequences of these effects on behaviour and life-history traits. If parents can reliably predict the conditions of their offspring's environment, they may adjust offspring phenotypes via anticipatory parental effects potentially enabling their offspring to better cope with these conditions (e.g. Uller 2008). In contrast, if parents cannot sample the offspring environment, or if conditions are strongly fluctuating, anticipatory parental effects may be of little use to offspring, in which case offspring may do better to solely rely on innate information and own experience.

We chose the African mouthbrooding cichlid fish *Simochromis pleurospilus* as model species (Fig. 1), as it is one of the few species in which the parents' possibilities to predict the offspring environment in the wild (Kotrschal *et al*. 2012) and to adjust offspring traits to the environment (Taborsky 2006a,b) have been explicitly studied. These earlier studies addressed the resource availability for young. In this study, we focused on environmental cues elicited by offspring predators. Adult and juvenile *S. pleurospilus* occupy different, slightly overlapping niches along the depth gradient of Lake Tanganyika with juveniles inhabiting shallower depth than adults. When young become independent of maternal care at an age of 4 weeks, they start to move from the adult to the juvenile depth range. While we cannot entirely exclude that some brooding mothers actively deliver their offspring to the juvenile habitat, field observations of the distribution of brooding females and the sizes of young held in the mouths suggest that juveniles are released in the adult habitat and then migrate to the shallow zone on their own (A. Kotrschal pers. obs; B. Taborsky, pers. obs). The main offspring predators of *S. pleurospilus* are other cichlids fish, which either feed on small invertebrates and on cichlid fry opportunistically, or which represent specialized piscivores, such as *Ctenochromis horei*, the predator used in this study. The distribution of *C. horei* is predominantly bound to the existence of underwater vegetation, which occurs patchily in the lake at various depths (Ochi 1993). Therefore, large, dangerous *C. horei* can occur in the juvenile or in the adult niches of *S. pleurospilus,* or both (Ochi 1993, Sefc *et al*. 2009). Thus, we should expect that maternal effects induced by offspring predators take effect mainly shortly after the release of young, that is, while the young still travel to their juvenile habitats, whereas both innate and acquired antipredator responses should shape the behaviour and life history of *S. pleurospilus* during the entire juvenile period or at least until they reach a size above the gape size of their main predators.

We performed a factorial experiment with three levels of manipulations. (i) To test for predator-induced non-genetic maternal effects, we exposed mothers either to an environment with perceived offspring predation threat or to a control environment during the egg formation phase until spawning. (ii) To test for innate antipredator responses, we hand-reared eggs individually under highly controlled conditions, preventing any environmental maternal effects or

Fig. 1. Female Lake Tanganyika cichlid *Simochromis pleurospilus* recalling her young to the mouth (Photo by Christoph Grüter).

own experience to take effect after egg laying. (iii) To test for acquired antipredator responses, we either exposed predator-naïve offspring to a chemical predator experience shortly after birth, or to control cues. This set-up allowed us to test jointly for innate, maternally mediated or acquired effects on offspring growth and long-term antipredator behaviour in a single experiment.

Materials and methods

STUDY SPECIES

Simochromis pleurospilus (subfamily Tropheini) is a mouthbrooding cichlid endemic to Lake Tanganyika, East Africa. It lives along the rocky shores of the lake down to a depth of 12 m (Taborsky 2006a; Kotrschal *et al*. 2012), where it feeds on epilithic algae. *S. pleurospilus* reproduce year-round. Males defend small breeding territories against conspecific and heterospecific food competitors (Kotrschal & Taborsky 2010). Females visit these breeding sites for mating and leave directly after spawning with the clutch held in their buccal cavity (Taborsky 2006b; Kotrschal & Taborsky 2010). Females brood the clutches in their buccal cavities continuously for 2 weeks during which the young use up most of their yolk reserves. During the following 2 weeks, the mothers release the offspring temporarily allowing the young and herself to feed, but she takes them back in case of danger or disturbance (Segers, Gerber & Taborsky 2011). Four weeks after spawning, the young are independent.

ANIMAL HUSBANDRY

The study was conducted at the University of Bern, Switzerland, under license 21/08 of the Veterinary Office of the Kanton Bern. The experimental animals were second- and third-generation offspring from fish originating from Nkumbula Island, Lake Tanganyika, Zambia. Parental fish were kept in four 400-L tanks until spawning. Offspring were reared individually in 20-L tanks. All fish were kept under standard housing conditions described in Segers & Taborsky (2011). All adult cichlids used in this study were fed twice per day on 6 days per week with commercial tropical fish flake food (4 days) or a mixture of frozen zooplankton (2 days).

FEMALE TREATMENT

To induce potential non-genetic maternal effects via perceived offspring predation risk on offspring phenotypes, we exposed adult *S. pleurospilus* females to an offspring predator ('predator treatment') or a non-predatory fish ('control treatment') during the phase when they form the egg for their next clutch. As stimulus offspring predator, we chose adult *Ctenochromis horei*, which are a dangerous predator of small juvenile *S. pleurospilus*, but pose no threat to adult females as they are of similar body size. This approach contrasts previous work, where the perceived predation risk for adult females rather than for offspring was manipulated (e.g. McCormick 1998, 2006; Coslovsky & Richner 2011; Giesing *et al*. 2011; McGhee *et al*. 2012). We did so, because where we were explicitly interested in whether mothers adjust offspring phenotype to the expected offspring environment ('anticipatory maternal effects'; sensu Uller 2008). In the control treatment, we exposed adult females to individuals of a non-predatory algae eater (*Ophtalmotilapia ventralis*) of a similar body size to the adult *S. pleurospilus* females. Both the predatory and the control stimulus species are endemic to Lake Tanganyika and occur sympatrically with *S. pleurospilus*.

The experimental clutches were produced in four 400-L tanks, two assigned to the predator treatment and two assigned to the control treatment, inhabited by groups of six to nine *S. pleurospilus* females, one male and one heterospecific cichlid (a *C. horei* or an *O. ventralis*). Adult *S. pleurospilus* were captured from their home tanks and were randomly assigned to the four breeding tanks. At introduction in the experimental tanks and also directly after spawning, we measured the females' total lengths (TL; tip of mouth to end of caudal fin) on a measuring board with 1-mm grid, estimating their length to the nearest of 0·5 mm, and we weighed them to the nearest of 0·01 g on an electronic balance.

The tanks were checked daily for females with clutches in their buccal cavity. As soon as a female had spawned, the eggs were collected by slightly pressing her cheeks. Then, the female was placed in a 20-L tank for recovery for 50 days. After the 50 days, she was transferred to a 400-L tank of the opposite treatment to produce a second clutch. To maintain stable densities in the breeding tanks, the removed female was replaced by a new female. In total, we obtained eight clutches from the control treatment and ten clutches from the predator treatment. Only three females produced one clutch each in both treatments, and only in one case both clutches of a female hatched; all other females contributed just one clutch to this experiment.

We exchanged the males several times during the experiment (3 males produced offspring in the predator treatment and 3 males did so in the control treatment). Our treatment aimed at inducing environmental *maternal* effects, and maternal effects are indeed more likely to occur than paternal effects in this species, which has a very high maternal reproductive investment (females produce very large, energy rich eggs and exhibiting a long female-only care period), but only a small paternal investment. We are aware, however, that our experimental design does not allow the exclusion of the possibility of environmental paternal effects.

REARING OF EXPERIMENTAL BROODS

Each single egg of the 18 clutches was individually reared in a 250-mL Erlenmeyer flask filled with clean tap water that was mounted in a self-constructed egg tumbler described in Segers & Taborsky (2011). Each flask was individually oxygenated by an air flow. Eggs take 5 days to hatch (Segers & Taborsky 2011). At experimental day 8 (see experimental timeline, Table S1; 'experimental days' correspond to days after hatching), the larvae were moved individually to net cages (16·5 × 12 × 13·5 cm) placed in individual 20-L tanks. From day 18, when yolk sacs were absorbed, juveniles were released in the 20-L tanks and fed a near *ad libitum* ration (12% of their body mass) of fine-grained Tetramin Baby® flake food 6 days per week with adjustment to increasing body mass every 2 weeks (see Taborsky 2006b).

LENGTH, MASS AND GROWTH

Eggs were placed individually on a moistened cotton pad and weighed to the nearest of 0·1 mg on an electronic balance. We obtained egg weights from 17 of the 18 experimental clutches. Offspring lengths and weights were taken every 4 weeks from day 28 until day 168. Body condition was calculated using Fulton's index F as $F = \text{mass [g]}/\text{TL[cm]}^3 \times 100$. Length growth of individual larvae was modelled as larval length controlled for individual egg mass. Larval mass growth was directly calculated as the differences between individual larval mass at day 28 and individual egg mass. For juveniles, we calculated the specific length growth rate for each 4-weekly measuring interval as (Ln TL_2-Ln TL_1)/28 d × 100, where TL_1 and TL_2 are the two successive measurements. Specific growth rates give the percentage of daily growth.

OPERCULAR BEAT RATES AND OFFSPRING TREATMENT

Predator-naïve larvae of both maternal treatments were exposed to a chemical predator cue (*C. horei*) or to a control cue (tap water) in a split-brood design. We used olfactory cues to elicit responses in larvae, as in fish olfaction is an essential source of information about predators (Ferrari, Wisenden & Chivers 2010). The heterospecific cues were produced by confining an adult *C. horei,* an *O. ventralis* or several snails for 1 h in 700 mL water taken from their respective holding tanks. Afterwards, the 700-mL sample was filled in 2-mL Eppendorf tubes and kept at −20°C until use.

We tested for the strength of responses to the chemical cues by recoding the opercular beat rates (OBR) of larvae. Under standardized conditions, ventilation frequency is known as a sensitive measure of response to disturbance (Brown, Gardner & Braithwaite 2005). Repeated exposures were carried out at days 8, 13 and 18. Two fish per brood each were haphazardly assigned to the predator cue and to the control cue, except in five broods where only three individuals survived until day 8 (individuals in total: predator cue = 36; control cue = 33). To measure opercular beat rates (OBR), a larva was placed in a small glass tube (1·2 cm diameter, 4 cm length), filled with water from its holding container. The tube was placed upright under a binocular microscope connected to a video camera. The tube holding the fish and containers holding the water cues were kept at a constant temperature of 28°C by a thermostat-controlled water bath. After 5 min of acclimatization, OBR was video-recorded for 40 s ('baseline 1'; see Supporting Information, Appendix S1, Fig. S1). Then, the fish holding water was removed with a pipette such that the larva was still covered with water, and the water was quickly replaced by the respective treatment cue water (control cue, i.e. tap water, or heterospecific cue), followed by another 40-s video-recording of OBR ('treatment'). Finally, the treatment water was exchanged against tap water ('baseline 2') followed by a third 40-s video-recording. 'Baseline 2' was mainly carried out to detect a potential fear response to the control cue (tap water) in the 'treatment' recording.

To test whether OBR changes after exposure to *C. horei* odour represent a specific antipredator response or a general neophobic fear response towards other fish (Hirvonen *et al.* 2000), we exposed additional larvae obtained from those broods with sufficient surviving young ($N = 5$ broods) to the odours of the herbivorous cichlid *O. ventralis*. To test further whether larvae are able to discriminate between fish and non-fish odour, we exposed additional larvae of these five large broods to the odour of an aquatic snail of the family Thiaridae, a gastropod family occurring in Lake Tanganyika. *C. horei* and *O. ventralis* used to produce the odour cues were fed on identical standard diets (see above), thereby controlling for potential clues larvae may obtain about the danger exerted by a stimulus species only based on the species' diet (rev. in Ferrari *et al.* 2007).

For data analysis, we compared the absolute values of beat rates between treatments, and we compared the differences of OBR between treatment and baseline 1 ('difference 1') and between treatment and baseline 2 ('difference 2'), respectively.

LONG-TERM EFFECTS ON BEHAVIOUR

We tested for long-term effects of our treatments on behaviour both in generally threat-related contexts (novel object, unspecific startle stimulus) and in a predation context (presentation of predator). We conducted (i) a novel object test of general explorative or neophobic tendencies in a non-predatory context; (ii) a startle response test recording recovery from a fear response induced by non-predator stimulus; and (iii) a visual and olfactory exposure to the offspring predator *C. horei.*

The novel object test was performed at day 94 in the home tank of a focal fish. We removed the filter temporarily and placed a shelter (flowerpot half) in a distance of 15 cm to both front corners of the tank. After letting a test fish acclimatize to this set-up for 15 min and verifying that it stayed in the shelter, a novel object, randomly chosen from either a blue-red rubber eraser or a blue or a red clothes peg, was gently placed in one of the corners. We recorded the latency until first emergence from the shelter and the closest distance the fish approached the novel object. Observation time was 10 min.

The startle response test was carried out twice, at days 33 and 84, to capture possible developmental changes of risk-related behaviour as have been previously observed in this species (Segers & Taborsky 2011). In this test, we measured the time until fish resume feeding after being startled by a short but strong threat stimulus. The home tank of a focal fish was divided by a partition into two equally sized compartments; the test was performed in the frontal compartment. A shelter (a short PVC tube) was placed at the right screen of the compartment. Near the centre of the frontal screen, standard flake food diluted with water was supplied with a pipette directly to the sandy bottom creating a food patch of approximately 0·5 cm diameter. Immediately after the fish started feeding, a glass marble of 2 cm diameter was dropped next to the patch, and the response of the focal fish was video-recorded. From the videos, we recorded the behavioural responses (fleeing, freezing or no response; none of the fish entered the shelter), and we analysed the time until fish resumed feeding after being startled.

The presentation of *C. horei* was conducted at day 140. We presented visual and chemical cues of an adult male *C. horei* (11·5 cm TL) to *S. pleurospilus* juveniles. For each trial, a focal juvenile was transferred to a 20-L tank placed directly to the left or right of another 20-L tank containing the *C. horei*. A shelter (flowerpot half) was provided at the furthest possible distance to the predator's tank. The testing order of siblings tested the same day, and the testing position (left or right of predator) was balanced with regard to maternal and offspring treatments. Before the test, the tanks were visually separated by an opaque divider. After 5 min of acclimatization of the focal fish, 15 ml of *C. horei* holding tank water was added to the focal's tank, the opaque divider was removed, and the behaviours of focal fish and predator were observed for 10 min. Afterwards, the opaque divider was put in place again and the test fish was transferred back to its home tank.

Every 30 s during the 10-min recording ($n = 20$ observations per trial and fish), we noted the position of focal fish and predator and whether they were active (i.e. moving around). To record the positions, we divided the volume of each 20-L tank in 18 virtual, three-dimensional sections of 13·3 × 8·3 × 8·3 cm by applying marks at the tank screens. We simultaneously determined the two sections where the predator and the focal fish were located and calculated the distances between the mid-points of these sections by applying Pythagoras' law. For statistical analysis, we used the mean distances of the 20 observations, and the percentages of observations the focal was active. The activity of the predator was included as covariate.

Furthermore, we recorded any actions of the focal fish towards the predator on an all-occurrence basis, as these actions were rare. We ranked them from most defensive to most offensive: fleeing (rank 1), freezing (rank 2), inspection of predator (rank 3) and aggression (rank 4). For each fish, we calculated the weighted mean of ranks by multiplying the frequency of an action by its rank, summing these products for the four actions and dividing by the total number of actions in 10 min. Additionally, we counted the total occurrence of all above-mentioned actions without distinguishing the type of the response. This gives only a coarse measure of responsiveness, but it allows the inclusion of fish which showed zero responses.

STATISTICAL ANALYSIS

When the data structure fulfilled the conditions for parametric testing, we analysed our data by linear mixed-effects models (LME) with identity link functions and by AN(C)OVAS; otherwise, we used nonparametric tests. Some variables were log-transformed to allow for parametric analyses (transformed variables are indicated in the respective result tables). All fixed factors and covariates included in the models are explicitly listed in the main text and result tables. In the initial models, we also fitted all interactions between the fixed factors. To simplify our models, we used stepwise backward elimination of non-significant interaction terms of the fixed factors (Bolker *et al.* 2009), but the fixed factors were always kept in the models even if non-significant. We determined the variance components of all potentially relevant random factors for each of these models (percentages of variance are given in the Appendix S1, Tables S2, S3 and S4), namely breeding tank, male identity, female identity, clutch identity and for the repeated measures in the OBR analysis, also individual identity. None of these random factors accounted for a significant amount of variance in the random term, except for clutch identity in some of the models on offspring size and growth (see Table S3). To avoid overparametrization of our models, for the final models presented in Tables 1, 2 and 3, we kept only clutch identity in the random term and, for the repeated OBR analyses, we also kept individual identity in the random term to account for the repeated measures. All analyses were carried out using SPSS 17, SPSS Inc., Chicago, IL, USA.

Results

PREDATOR RESPONSES OF PREDATOR-NAÏVE LARVAE

Larvae receiving the offspring predator cue had strongly reduced OBRs compared with larvae receiving the control cue (Fig. 2a, Table 1a). OBRs before the presentation (baseline 1) did not differ between larvae assigned to predator and control treatment (Figure 2a, Table 1a), whereas OBRs after the presentation (baseline 2) were still slightly lower after the predator cue than after the control cue (Table 1a, Factor 'O'). The reduction in OBR in response to the predator cue was particularly strong on the first experimental day, whereupon the effect decreased gradually (Fig. 2b, Table 1a). In addition, OBRs during both baselines and the olfactory treatment decreased over the experimental days 8, 13 and 18 (Table 1a, Factor 'Day'). The female treatment did not influence OBR significantly (Table 1a).

In the five broods tested with cues of three different species and the control, the OBR responses differed significantly between the four presented cues both when analysing the differences to the first ('difference 1'; Fig. 2c) and to the second baseline ('difference 2'). Again, the responses were strongest in naïve fish (day 8) and declined afterwards (significant effects of 'Day'; Table 1b). Pairwise analyses of the initial responses of naïve fish (i.e. 'Difference 1' at day 8; Table 1c) revealed that OBRs were significantly more reduced in response to the cues of the offspring predator and, unexpectedly, also towards the snail odour compared with tap water or the herbivorous cichlid. The responses to tap water and the herbivore did not differ. Interestingly, naïve larvae reduced OBR even more strongly when exposed to the snail cue than to the predator cue. This difference vanished, however, when taking all three exposure days into account, whereas all other differences remained significant in an analysis including all days (results not shown).

Table 1. Treatment effects on opercular beat rates

Dependent variable	Fixed factors	d.f.	*F*	*P*
(a) Predator vs. control treatment				
Baseline 1	F	16·08	0·07	0·79
	O	189·13	1·88	0·17
	Day	186·22	47·65	**< 0·001**
Baseline 2	F	31·60	0·04	0·84
	O	50·63	4·99	**0·030**
	Day	136	47·25	**< 0·001**
Treatment	F	15·85	0·15	0·71
	O	50·55	71·48	**< 0·001**
	Day	134	26·51	**< 0·001**
	O × Day	134	7·62	**0·001**
Difference 1	F	15·01	0·08	0·78
	O	50·94	155·05	**< 0·001**
	Day	134	4·37	**0·014**
	O × Day	134	4·73	**0·010**
Difference 2	F	14·12	0·67	0·43
	O	50·72	45·76	**< 0·001**
	Day	134	5·53	**0·005**
	O × Day	134	9·59	**< 0·001**
(b) Treatment with four odour cues				
Difference 1	O	36	24·25	**< 0·001**
	Day	72	14·64	**< 0·001**
	O × Day	72	2·31	**0·043**
Difference 2	O	32·66	7·67	**0·001**
	Day	72	23·39	**< 0·001**
	O × Day	72	3·18	**0·008**
(c) Multiple comparisons (day 8)				
	Herbivore	Predator	Snail	
Tap water	0·16	**< 0·001**	**< 0·001**	
Herbivore		**0·023**	**0·004**	
Predator			**0·027**	

'F' refers to female treatment, 'O' refers to offspring treatment; 'Day' refers to the experimental day 8, 13 or 18 after hatching; 'Difference 1' and 'Difference 2' refer to the differences between 'Treatment' and 'Baseline 1' and between 'Treatment' and 'Baseline 2', respectively; significant *P*-values are marked in bold; in *(c)* all bold *P*-values are significant after accounting for false discovery rate using the Benjamini–Hochberg method (Verhoeven *et al.* 2005).

BODY SIZE, CLUTCH TRAITS AND GROWTH

Offspring of mothers that had been exposed to an offspring predator during clutch production were larger (Fig. 3a) and heavier 28 days after hatching than offspring from females exposed to a herbivorous control fish (Table 2), whereas their condition factor did not differ (Table 2). The treatment effect on offspring size cannot be explained by variation in female total length, as female size and mass after spawning did not differ significantly (there was even a weak tendency of females being shorter in the predator treatment: total length: $F_{1,15} = 3{\cdot}23$, $P = 0{\cdot}090$, body mass: $F_{1,15} = 1{\cdot}89$, $P = 0{\cdot}19$; ANOVAS). The treatment

Table 2. Treatment effects on offspring body size and growth; 'F' refers to female treatment, 'O' refers to offspring treatment, TL is total length; significant *P*-values are marked in bold

Dependent variable	Fixed factors	d.f.	*F*	*P*
TL at day 28	F	16·31	6·07	**0·025**
	O	43·73	1·02	0·19
Weight at day 28	F	15·88	8·47	**0·010**
	O	45·68	0·042	0·84
Condition factor *K*	F	16·13	0·44	0·52
	O	47·63	0·25	0·62
TL at day 28*	F	15·34	6·97	**0·018**
	O	42·62	1·91	0·17
	Egg mass	54·93	0·054	0·82
Juvenile specific growth rate	F	262	14·73	**< 0·001**
	O	262	0·33	0·57
	Age	262	136·98	**< 0·001**
	F × age	262	9·70	**0·002**
Final TL	F	13·55	0·68	0·42
	O	35·35	0·17	0·68

*This model fits larval length at day 28 corrected for the individual egg size of this larvae; as larger larvae hatch from larger eggs (Segers & Taborsky 2011), this analysis actually models larval growth.

Table 3. Results of Mann–Whitney *U*-tests ('Distance to object'), chi-squared tests ('Response types') and LME models (all other results) of the experimental tests for long-term effects of female and offspring treatments on behaviour; 'F': female treatment; 'O': offspring treatment; 'Predator': Predator activity; significant *P*-values are marked in bold

Dependent variable	Fixed factors	d.f.	*F*	*U*	χ^2	*P*
Novel object test						
Latency to 1st emergence*	F	13·51	0·55			0·47
	O	45·44	2·08			0·16
Distance to object	F			389		0·81
	O			401		0·97
Startle response tests						
Time to feed (day 33)*	F	15·25	0·35			0·56
	O	46·07	0·05			0·82
Time to feed (day 84)	F	22·93	0·48			0·50
	O	42·99	0·62			0·43
Response type (day 33, $n = 65$)	F				0·90	0·64
	O				0·34	0·84
Response type (day 84, $n = 50$)	F				3·44	0·18
	O				0·62	0·73
Response to 'familiar' predator *C. horei*						
Mean distance (cm)	F	10·26	0·43			0·52
	O	32·33	13·72			**0·001**
	Predator	40·84	3·47			0·070
Activity	F	9·37	1·05			0·33
	O	32·66	0·33			0·57
	Predator	43·25	0·57			0·45
Type of behaviour	F	6·97	0·43			0·53
	O	12·31	0·046			0·83
	Predator	14·94	0·88			0·36
Frequency of any behaviour*	F	9·48	0·45			0·52
	O	30·69	0·011			0·92
	Predator	40·84	0·13			0·72

*Variables Log_{10}-transformed.

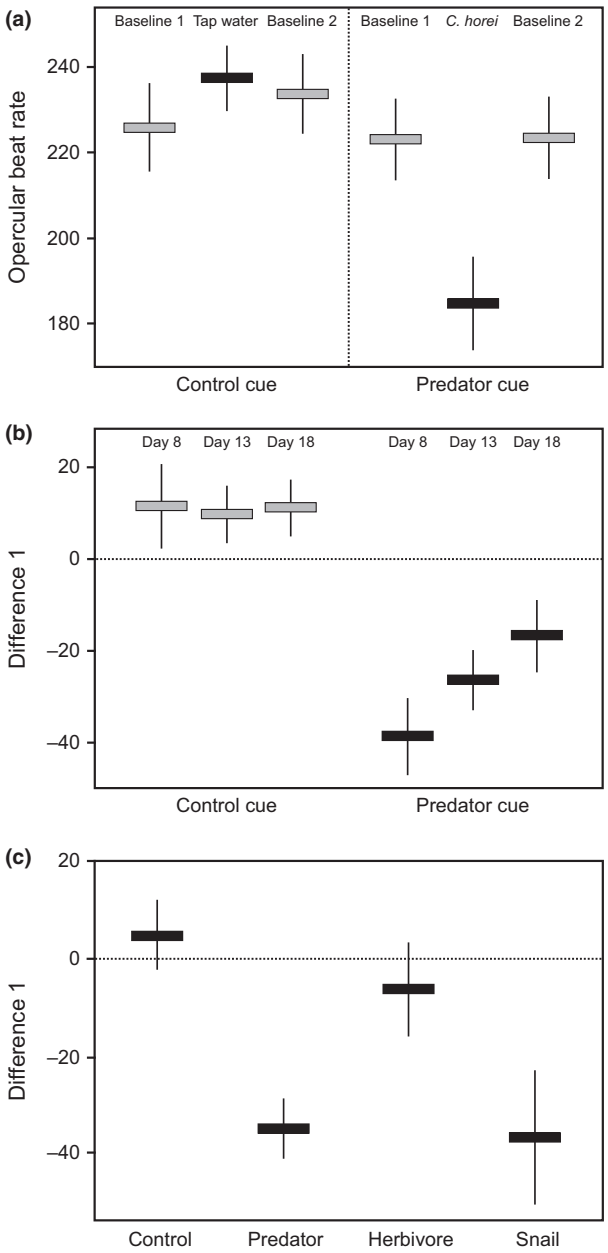

Fig. 2. Opercular beat rates (OBR; beats per 40 s) of larvae during the exposure to different odour cues, shown for the combined predator and control female treatments, as the female treatments did not affect OBR (see Table 1); means (±SE). (a) OBR of predator-naïve larvae (i.e. at first exposure, day 8) during exposure to baseline and treatment cues; (b) difference in OBRs between treatment cue and baseline 1 ('difference 1') on all three exposure days; (c) OBRs towards the control cue (tap water), offspring predator (*C. horei*), herbivore (*O. ventralis*) and a Thiarid snail, averaged over all three observation days.

effect can also not be explained by differences in clutch size ($F_{1,14} = 0{\cdot}24$, $P = 0{\cdot}63$; female TL included as covariate: $F_{1,14} = 5{\cdot}24$, $P = 0{\cdot}038$ as larger females lay more eggs; ANCOVA) or egg mass between treatments (Female treatment: $F_{1,14} = 0{\cdot}073$, $P = 0{\cdot}79$; clutch size included as covariate: $F_{1,14} = 56{\cdot}74$, $P = 0{\cdot}021$ as there is an egg size/number trade-off; ANCOVA). Instead, young produced by

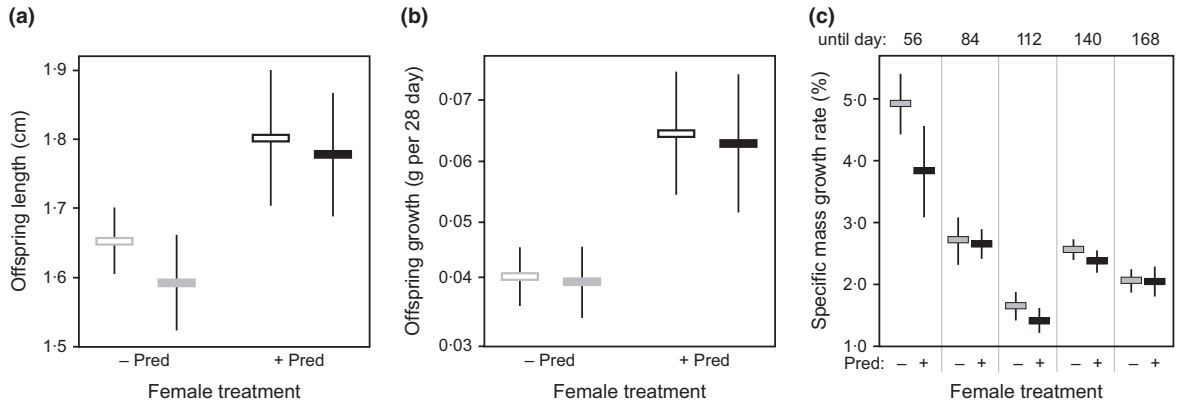

Fig. 3. Size and growth rates of juveniles; means (±SE). (a) Differences in total length at first size measurement (day 28); (b) Individual larval mass increase (weight at day 28 minus egg weight); (c) specific growth rate of juveniles; data of the two offspring treatments are combined, as offspring treatment did not affect growth. Open bars in (a) and (b): offspring control treatment, closed bars: offspring predator treatment; grey bars in (c): female control treatment; black bars: female predator treatment.

predator-exposed mothers grew faster during their first 4 weeks of life (Table 2; raw mass-based growth rates are shown in Fig. 3b). This effect was reversed during the following 4 weeks (weeks 5–8), when offspring of mothers exposed to the control treatment had higher growth rates (Table 2, Fig. 3c). After week 8, until an age of 24 weeks, female treatment no longer influenced growth. At the day of final measurement, the TL of fish did not differ anymore (Table 2). Larval growth, juvenile growth and size at final measurement were not influenced by the offspring treatment (Table 2).

LONG-TERM EFFECTS ON BEHAVIOUR

Neither female nor offspring treatment influenced (i) the latency until fish first emerged from their shelters after insertion of a novel object in their tank or (ii) the closest distance to which juveniles approached a novel object (Table 3). Also in the startle response test, female and offspring treatment did not influence (i) the distributions of the three occurring types of behavioural responses (fleeing, freezing or no response) towards the startle stimulus or (ii) the time until feeding was resumed after being startled (Table 3).

Long-term effects on behaviour were detected, however, during the presentation of the predator *C. horei*. Focal fish that had received the chemical predator cue during the offspring treatment kept a greater distance from the predator than young that had experienced the control cue, irrespective of female treatment (Table 3, Fig. 4). The activity, the type of behavioural responses and the frequency of responses were not affected by offspring or female treatment (Table 3).

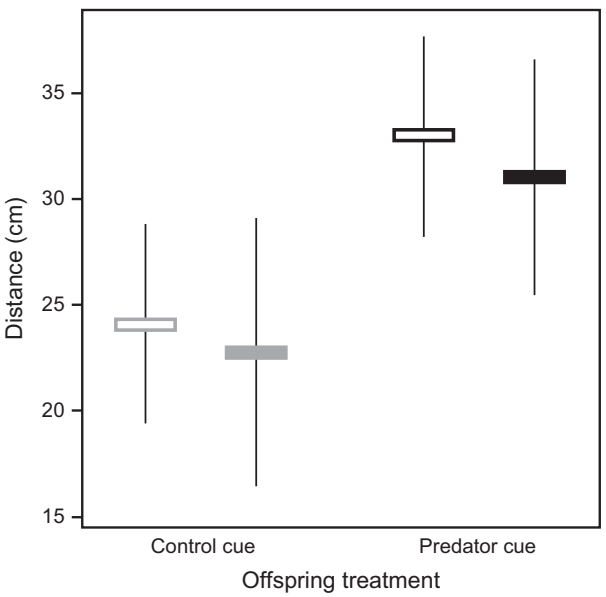

Fig. 4. Mean distance (cm) (±SE) between focal fish and an offspring predator *C. horei* during predator presentation trials. Open bars: offspring control treatment, closed bars: offspring predator treatment.

Discussion

Our results suggest that *S. pleurospilus* young possess an innate ability to recognize predators and to distinguish these from cichlids that pose no risk to them, based on chemical information obtained from odour cues. This ability was not modulated by the perceived threat of offspring predation experienced by mothers. Instead, environment-induced maternal effects influenced offspring growth; offspring of predator-exposed females grew faster during their first month of life. Early experience, but not maternal effects, exerted a long-term effect on the distance kept towards predators, but not on specific antipredator behaviours.

Predator-naïve *S. pleurospilus* larvae responded strongly to the odour of an offspring predator, but neither to a control cue nor to the cue of a benign herbivorous cichlid. This shows that *S. pleurospilus* have a sophisticated ability

to distinguish different odour cues already at birth. The maternal exposure to an offspring predator or to a benign species during egg production did not affect the predator recognition abilities of larvae as judged from the intensity of their ventilation response. Moreover, due to hand-rearing, none of our tested fish had the possibility to experience heterospecific odour cues prior to the first behavioural test, and they also could not obtain clues about the potential danger of the stimulus species by the latters' diet as all fish were fed the same type of food. It is furthermore highly unlikely that genetic maternal or paternal effects induced predator recognition, because the maternal and paternal identity did not explain a significant amount of variance in the models testing for responses to odour cues (the variance explained by the identity of mother or father never exceeded 2% in the models testing for OBR responses; Table S2). Together, these results suggest that *S. pleurospilus* possess a genetically inherited predisposition to distinguish predatory from non-predatory species. Previous reports of innate predator recognition (e.g. invertebrates: Abjörnsson, Hansson & Brönmark 2004; fish: Magurran 1990; Hawkins, Magurran & Armstrong 2007; Scheurer *et al*. 2007; amphibians: Laurila, Kujasalo & Ranta 1997; birds: Veen *et al*. 2000; Bize, Diaz & Lindström 2012) could not exclude (i) that predator recognition was induced by environmentally mediated maternal effects because they tested offspring of wild-born, unmanipulated mothers or (ii) that predators were recognized by odour cues of their piscivorous or carnivorous diet (e.g. Vilhunen & Hirvonen 2003, Fendt 2006; see Ferrari *et al*. 2007 for discussion of diet effects). Thus, to the best of our knowledge, here we report the first unconfounded evidence for a genetically inherited ability in animals to distinguish a dangerous from a benign species.

Unexpectedly, the odour of an aquatic snail elicited the strongest response in larval *S. pleurospilus*. There are three alternative explanations for this result. (i) The larvae might have recognized the snail odour and responded strongly, because snails may pose a real threat to larvae; in our laboratory, F. Segers (pers. comm.) found a snail that ate a yolk-sac larva while staying within the mouth of a brooding *S. pleurospilus* female. (ii) Alternatively, snail odour might represent a novel, unrecognized stimulus to the larvae eliciting a strong neophobic response. Strong neophobic responses can be adaptive, because they can help to survive first encounters with unknown potential dangers before an individual had the opportunity to collect information about a novel stimulus (Hirvonen *et al*. 2000). (iii) Finally, snails might emit odours that are chemically similar to odours of natural predators of *S. pleurospilus* young.

Typically, fish increase their ventilation rate in face of danger (e.g. Brown, Gardner & Braithwaite 2005; Hawkins, Magurran & Armstrong 2007). In contrast, *S. pleurospilus* young reduced their OBR, a response which has only recently been reported for the first time in a fish species (Kempster, Hart & Collin 2013). Reducing the ventilation rate might be part of a freezing response in the face of danger which is well known from young mammals, where it is accompanied by bradycardia (Smith & Woodruff 1980; Espmark & Langvatn 1985). Freezing rather than preparing for flight may be the most appropriate antipredator response by *S. pleurospilus* larvae, which still have huge yolk sacs particularly during the first 2 weeks of life preventing an effective escape. If by accident a mouthbrooding female drops a larva, freezing and reducing ventilation is likely to induce visual and chemical crypsis. Furthermore, mouthbrooding mothers might also benefit from a reduced larval OBR and thus reduced oxygen expenditure of offspring in the mouth when the female is threatened by a predator.

At first exposure, predator-naïve *S. pleurospilus* larvae showed the highest baseline OBRs and the strongest responses towards predator odour, and baselines and responses then gradually declined with age. This decline is most likely an effect of increasing body size and a corresponding decrease in metabolic rate (Jones 1971) rather than a habituation response, as in the latter case, baseline OBRs should not change with age as well.

Offspring from predator-exposed mothers grew faster during the first 4 weeks after hatching and consequently were larger and heavier at day 28 compared with offspring from control mothers. In small fish, mortality is strongly negatively size-dependent, as the most important predators are other fish species, which are gape-size limited (Sogard 1997). Faster growth will allow the young to outgrow the time window of highest juvenile mortality more quickly (Sogard 1997; Segers & Taborsky 2011). Moreover, even slightly larger body sizes allowed for higher burst speeds of juvenile mouthbrooders (Schürch & Taborsky 2005; Segers & Taborsky 2011), which should enable them to better escape predation (e.g. Husak 2006). Interestingly, offspring of predator-exposed females reached their larger size at about the age when they would become independent of maternal care in nature and start migrating from the deeper adult habitats to the shallow juvenile habitat (Kotrschal *et al*. 2012). Thus, our results suggest that the maternally mediated growth boost detected in our experiment prepares those offspring born into a predator-dense adult surrounding to better cope with predator attacks during their highly dangerous migration to the juvenile habitat. Moreover, it may reflect a general tendency of maternal effects to vanish quickly with time (Lindholm, Hunt & Brooks 2006). Soon after this initial growth boost, juveniles of predator-exposed mothers grew more slowly than offspring of control mothers, so that already at an age of 3 months, offspring sizes did not differ anymore between the two maternal treatments. This decrease in growth rates after a growth boost might help to buffer potential negative effects resulting from possible costs incurred during the phase of fast growth (Metcalfe & Monaghan 2001).

In contrast to previous results in fish (Giesing *et al*. 2011; Segers, Berishvili & Taborsky 2012), egg size was not affected by the maternal treatment. Currently, we can only

speculate about the possible mechanisms underlying the maternal effect on growth. In *S. pleurospilus*, larval growth depends on the post-hatching expression of the gene coding for growth hormone receptor (GHR) (Segers, Berishvili & Taborsky 2012), and it is possible that females can influence the larval expression of this gene.

In the long term, juvenile traits were only influenced by own experience. Juveniles that had experienced *C. horei* odour 5 months ago kept a greater distance from this predator than did control fish, indicating that early predator experience induces an adjustment of risk-taking behaviour. In contrast, none of the specific behaviours we recorded at this age were affected by early predator exposure. This overall rather weak long-term effect may reflect the fact that at this advanced age and body size, *C. horei* does not pose a severe life-threatening risk to *S. pleurospilus* anymore. The absence of treatment effects on the behaviours measured during the novel object and the startle response tests suggests that the early odour exposure induced behavioural changes specific to a predation context, rather than altering the general fearfulness of fish. This adds to previous findings showing that behavioural syndromes present in a predation context do not necessarily match behavioural syndromes in non-predatory contexts (Coleman & Wilson 1998; Dingemanse *et al.* 2007; Adriaenssens & Johnsson 2009).

In summary, maternal effects were effective for a short, critical time window after birth, and they might have a specific, but important effect on offspring survival by increasing body size during offspring migration. Own predator odour experience modulated the behavioural response of fish in the long-run, in particular the 'wariness' of juveniles towards large predatory individuals. Own environmental sampling of predation risk combined with a genetically inherited predisposition to correctly identify predatory species is likely to reveal the best possible prediction of environmental risk for juveniles in *S. pleurospilus*, a species in which adults and juveniles occupy different ecological niches. To understand the general principles of separate and joint action of parental and individual environmental effects, we would like to encourage further factorial experiments in species where the environmental predictability is known both from the perspective of adults and of offspring.

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Supporting Information

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

Appendix S1. Additional methodological and statistical information.