

Olfactory self-recognition in a cichlid fish

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Abstract Animal self-cognizance might be of importance in different contexts like territoriality, self-referent mate-choice or kin recognition. We investigated whether the cichlid fish *Pelvicachromis taeniatus* is able to recognize own olfactory cues. *P. taeniatus* is a cave breeding fish with pronounced brood care and social behavior. In the experiments we gave male cave owners the choice between two caves in which we introduced scented water. In a first experiment males preferred caves with their own odor over caves with the odor of an unfamiliar, unrelated male. To examine whether self-recognition is based rather on individual or on family cues we conducted two further experiments in which males could choose between their own odor and the odor of a familiar brother and between the odor of a familiar brother and an unfamiliar, unrelated male, respectively. Males preferred their own odor over that of a familiar brother suggesting individual self-referencing. Interestingly, males (at least outbred ones) preferred the odor of an unfamiliar, unrelated male over that of a familiar brother, maybe to avoid competition with kin. We discuss the results in the context of animal self-cognizance. All experiments were conducted with in- and outbred fish. Inbreeding did not negatively affect self-recognition.

Keywords *Pelvicachromis taeniatus* · Self-reference · Phenotype matching · Chemical communication · Cichlidae · Kin discrimination · Kin selection · Competition · Individual recognition · Odor · Inclusive fitness

Introduction

The ability to distinguish own from foreign in the broadest sense is of fundamental importance in many species (Tsutsui 2004). For instance, mechanisms to recognize individuals of one's own species (species recognition, for a recent review see Ritchie 2007) or, within species, to recognize individuals of one's own family (kin recognition, e.g., Hepper 1991; Tang-Martinez 2001; Mateo 2004) have evolved in many taxa and are extensively explored. However, cognizance of oneself and its dependence on social environment and life history of species is less well investigated (Bekoff and Sherman 2004). Own cues, i.e., self-reference, might be used in order to recognize one's own territory (e.g., Bonadonna and Nevitt 2004), to recognize kin (i.e., kin recognition based on self-referent phenotype-matching, e.g., Mateo and Johnston 2000; Hauber and Sherman 2001; Hain and Neff 2006; Schielzeth et al. 2008) or to find a compatible mating partner (self-referent mate-choice, for reviews see Milinski 2006; Pieltney and Oliver 2006).

The aim of this study was to investigate whether males of the cichlid fish *Pelvicachromis taeniatus* are able to recognize own cues and whether recognition is based on self-reference. Cichlids in general show a wide range of social interactions in numerous contexts like mating, brood care, territorial behavior and competition (e.g., Taborsky 2001; Stiver et al. 2005; Grosenick et al. 2007)

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and should have evolved cognitive abilities accordingly. *P. taeniatus* is a small, socially monogamous cave breeder with biparental brood care. Reproductively active males occupy appropriate caves and defend them against rivals. After mutual mate-choice and spawning, initially mainly the female cares for the eggs while the male defends the territory against intruders. The fry swims freely after about 1 week and is then guarded by both parents. Diverse intra-specific social interactions during brood care and in other contexts clearly acquire pronounced cognitive abilities. Previous experiments, for instance, showed that males and females were able to recognize familiar and unfamiliar kin in a mate-choice and parental care context when both visual and olfactory cues were available (Thünken et al. 2007a, 2007b). Here, we focus on olfactory cues because in cichlids scents have been shown to play a role in species recognition (e.g., Plenderleith et al. 2005), parent-offspring recognition (for review see Nelissen 1991), mate recognition (Reebs 1994) and in aggressive interactions (Barata et al. 2007). In *P. taeniatus* odors might contribute to detect the own breeding cave.

In the experiments, test males (which were habituated to a cave before) were given the choice between two caves supplied with different scented waters. Males' ability to distinguish different odors was thus tested in a natural context fulfilling the requirements of a functional test (*sensu* Thom and Hurst 2004). In order to test whether males generally discriminate own from foreign odor cues test males were given a choice between self-odor and the odor of an unrelated, unfamiliar male in our first experiment. Potential discrimination in this experiment could be based either on the recognition of individual self-cues or of common family self-cues. In order to investigate, which cues might be involved, we conducted two further experiments. First, we tested whether males are able to discriminate between self-odor and the odor of a familiar brother, which would indicate individual self-referential recognition. In a further experiment, males' response to familiar family cues was tested by giving the choice between the odor of a familiar brother and the odor of an unrelated, unfamiliar male.

In the experiments we used inbred as well as outbred males. In previous mate-choice experiments, both sexes of *P. taeniatus* preferred close kin as mating partners (Thünken et al. 2007a, 2007b). Active inbreeding in this species might be adaptive because related breeding pairs were better parents and inbreeding individuals might increase their inclusive fitness (Thünken et al. 2007a). Furthermore, we found no evidence for inbreeding depression in terms of survival and growth rate of the young (Thünken et al. 2007a).

Materials and methods

Experimental animals

Experimental fish were the F2 generation of wild-caught fish, which had been collected in 2003 from the Moliwe River near Limbe in Cameroon (04°04'N/09°16'E). Breeding was performed in spring 2006. Fish used in the experiments were separated from the parents 4 weeks after hatching and raised in mixed-sex, full-sib groups of 30 individuals. Holding tanks (50 cm × 30 cm × 30 cm) were separated by opaque plastic sheets to avoid visual contact of adjacent families. The water temperature was kept at $25 \pm 1^\circ\text{C}$. A 12 h: 12 h light–dark cycle simulated natural light conditions. Fry was fed daily with living *Artemia* nauplii and adult fish with frozen *Chironomus* larvae and *Artemia*.

All test fish were reproductively active males and showed courtship coloration. Prior to the experiments, individual males were kept solitary in isolation tanks (30 cm × 20 cm × 20 cm) equipped with an air stone and a standard breeding cave (volume of 215 ml). All males occupied their caves. Fish were fed with red mosquito larvae (*Chironomus*). Leftovers and excrements were removed daily immediately after feeding. Shortly before the experiments, using a small 1 l tank, a sufficient quantity of water was taken from these isolation tanks, which served as stimulus water in the experiments.

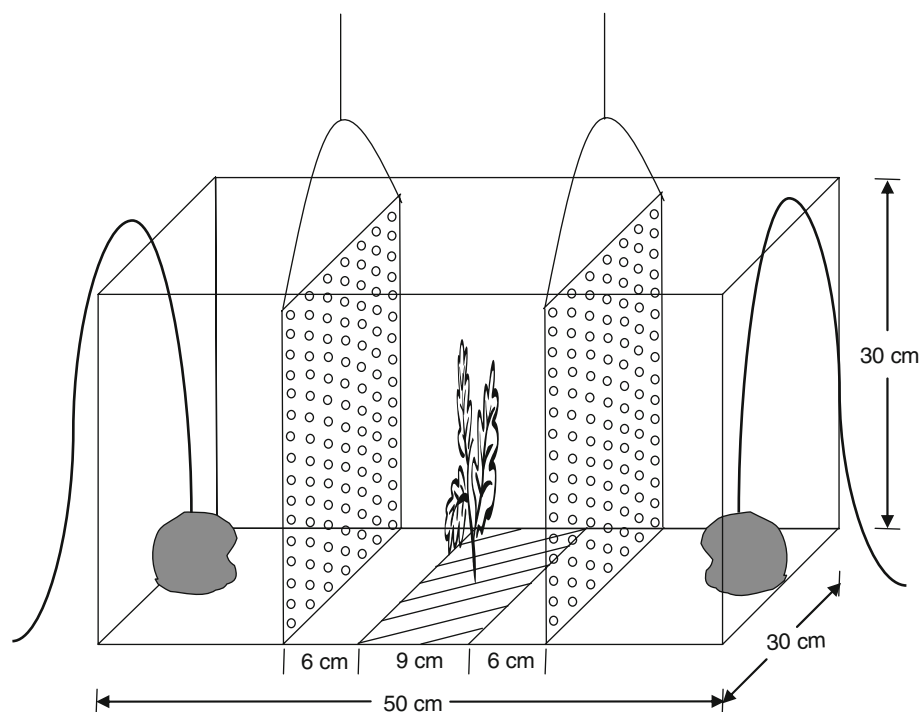
Experiments

In three dichotomous choice experiments, we presented different odor combinations to the test fish: (A) “self-odor”, i.e., odor of the test fish itself versus “foreign odor”, i.e., odor of an unfamiliar unrelated male, (B) “self-odor” versus “kin odor”, i.e., odor of a familiar brother and (C) “kin odor” versus “foreign odor”. All experiments were conducted in the same test aquarium. Because the experimental procedure differed slightly between experiments, we describe the general setup at first and thereafter the single experiments in detail.

General experimental setup

The test aquarium (50 cm × 30 cm × 30 cm) was divided into three compartments (Fig. 1). The center compartment for the test fish contained a plastic plant as shelter. The two outer compartments contained a standard cave each. A small flexible tube was inserted into the top of each cave to introduce the stimulus water. The scents could spread through the entrance of the caves into the test tank. In the beginning of the experiment, the central compartment was separated by two transparent, perforated plastic sheets that

Fig. 1 Test tank with the caves in which scented water of two stimulus males was added. The shaded area in the central compartment marks the neutral zone. In experiment A we measured the time the test fish spent in the areas left and right of the neutral zone (“choice areas”, 20.5 cm broad) over 30 min after lifting the dividers. In the experiments B and C we measured the time the test fish spent in the choice zones (6 cm) of the central compartment over 30 min before lifting the dividers



allowed the test fish to perceive the two standard caves with the respective odors in the outer compartments. To avoid side effects, a particular stimulus was presented alternately on the left and right side. The test tank was surrounded with black painted styrofoam and a black curtain to reduce disturbance from outside.

To simulate a competitive situation directly before a trial, we placed an adult male (unfamiliar and unrelated to the test male) in a 1-l tank in front of the test males' isolation tank for 5 min. Males usually responded aggressively against the rival (the self-scented water was taken before rival presentation because stress might influence the scent). After rival presentation, the focal male was gently taken out of the isolation tank and placed in the center of the test arena. The behavior of the test males was recorded by a webcam (Video Blaster webcam 3USB [WDM]). Thirty minutes of the digital film recordings were subsequently analyzed by a person who was naïve concerning the presentation side of the odors. We measured the time the test fish spent next to both odors (for details see below). After each trial the test aquarium and the tanks containing the stimulus water were thoroughly cleaned and the flexible tubes were rinsed thoroughly for several minutes with tap water.

Experiment A (“self-odor” vs. “foreign odor”)

Experiment A was conducted in December 2006. Altogether 17 test males (inbred: 8, outbred: 9) from twelve families (inbred: 6, outbred: 6) were used. Thus, five

families provided two males each. Individual isolation before the tests lasted 3–7 days. Stimulus water for the experiments was taken from the isolation tanks: 1 l from the tank of the subsequent test male itself (“self-odor”) and 1 l from the isolation tank of an (with respect to the test male) unrelated, unfamiliar male (“foreign odor”). Males served as donor for self-odor as well as for foreign odor. The two containers with stimulus water were placed above the test aquarium. Flexible tubes of 5 mm diameter led from each container to the respective cave. Water flow followed the principle of communicating vessels. Each tube was provided with two small stop-cocks. One started the water flow; the other regulated the flow rate (12 ml/min).

The experiment started by placing the test fish into the central compartment. The introduction of the scent was started after half an hour of acclimatization. Another 15 min later, the plastic sheets were removed so that the fish were able to swim freely in the whole aquarium. From this point on, we measured for 30 min how much time the fish spent within the choice areas left and right of the neutral zone (see Fig. 1 for details). When a test fish entered one of the caves the test was stopped (“final cave choice”). Two test males entered the cave before the end of the 30 min observation period. Thus the *absolute* times at the stimuli of these two trials were not comparable to the other 15 trials. Therefore, we analyzed the relative times (time at the respective odor in relation to the sum of the times at both odors) spent at both stimulus sides ($N = 17$). The results were similar to those of the absolute times (see “Results”). However, for the sake of clarity and comparability with the

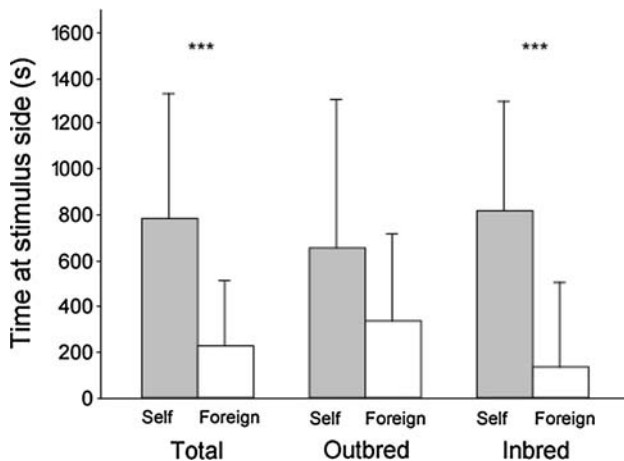


Fig. 2 Mean time (seconds \pm SD) males spent within 30 min at the side with self-odor (gray bars) and at the side with odor of a foreign, i.e., an unfamiliar, unrelated male (open bars). Shown is the time that all males and outbred and inbred males separately, spent in the choice area (for definition see Fig. 1). Asterisks denote significantly different responses to the two odors ($***P < 0.001$)

other experiments, we present the absolute times of the reduced data set ($N = 15$) in Fig. 2.

After the 30 min of recording, we observed whether a male chose a cave for another 15 min. This happened five times.

Experiment B (“self-odor” vs. “kin odor”) and C (“kin odor” vs. “foreign odor”)

Trials of these two experiments were conducted in alternating order in summer 2007 with 40 males of 15 families (20 males from 8 inbred and from 7 outbred families, respectively). Six inbred families provided two males and two inbred families provided four males each. Similarly, four outbred families provided two males and three further families provided four males each. Every male was used in both experiments and served as test fish and as scent donor in both experiments. Half of the males started with experiment B, the other with experiment C. The experimental procedure was similar to that of experiment A, but differed in a few aspects: Individual male isolation before the experiment was increased to at least 2 weeks in order to increase odor concentration. Additionally, brightly colored females were regularly presented to the males in a small tank in order to increase and match males’ sexual activity. Once a week 1 l of isolation tank water was replaced with fresh tap water. Unlike experiment A, stimulus water was supplied by a peristaltic pump (Ismatec MS-CA4/640) during the experiments, which allowed a continuous flow rate of 4 ml/min.

After 30 min of acclimatization of the test fish to the aquarium, the scented water was continuously added until the end of the test. From this point onwards, the time the

test male spent in a 6 cm choice zone (see Fig. 1 for details) was measured for 30 min before lifting the dividers. This modification, compared to experiment A, was made because two males had entered one of the caves before expiration of the 30 min in that experiment. After lifting the plastic sheets it was noted which cave the male entered (“final cave choice”). The trials were stopped when one of the males had not entered one of the caves within 4 h. Test males which did not move at all during the trials were removed from the analyses. Therefore, sample size of experiment B and C was reduced to 37 and 38, respectively.

Statistical analyses

Parametric statistics were used when data did not significantly deviate from normal distribution according to Kolmogorov–Smirnov test with Lilliefors correction. In experiment A absolute and relative times were transformed (square root and arc sine of the square root, respectively) in order to reach normality. For analysis, linear mixed effect models were conducted using the “lme” function in the “nlme” library of the R 2.5.1 statistical package. The absolute time the test male spent on each stimulus side was entered as dependent variable. Fixed factors were “odor” and “breeding regime” (inbred or outbred). Furthermore, the interaction between “odor \times breeding regime” was included in order to test whether in- and outbred males differed in discrimination behavior. Non-significant factors and interactions were removed from the analysis (Engqvist 2005). Tests of significance were based on likelihood-ratio tests (“LRT”) that follow a χ^2 -distribution. Hence, degrees of freedom always differed by one. To avoid pseudo-replication all test fish were only used once in each experiment. Since families were multiply used, family origin of the test males as well as of the stimulus males was entered as random factor into the model. Family origin of the test fish themselves as well as of the stimulus fish had no significant influence on choice behavior (LRT, all $\chi^2 < 1.190$, all $P > 0.18$) and was thus excluded from further analyses. Test probabilities are two-tailed throughout.

Results

Experiment A (“self-odor” vs. “foreign odor”)

At large (in- and outbred fish pooled), males spent significantly more time near the self-odor than near the odor of a foreign male ($N = 15$, $\chi^2 = 10.858$, $P = 0.001$, Fig. 2). Inbred and outbred males did not differ significantly in their behavior ($N = 15$, $\chi^2 = 1.498$, $P = 0.221$). However, when analyzed separately, discrimination was—probably due to the small sample size—only in the case of inbred males

Table 1 Results of the detailed analysis of experiment B (self vs. kin)

Factors	Time interval											
	1st 5 min		2nd 5 min		3rd 5 min		4th 5 min		5th 5 min		6th 5 min	
	χ^2	<i>P</i>	χ^2	<i>P</i>	χ^2	<i>P</i>	χ^2	<i>P</i>	χ^2	<i>P</i>	χ^2	<i>P</i>
Odor × breeding regime	5.207	0.022	1.170	0.279	0.029	0.862	0.171	0.678	0.233	0.629	0.237	0.626
Odor	0.008	0.927	0.761	0.382	0.031	0.860	0.020	0.886	0.102	0.749	15.141	<0.001

In this analysis the total observation duration of 30 min was sub-divided into six 5-min intervals. Results that remained statistically significant after Bonferroni correction are printed in bold. The results of the interaction term “odor × breeding regime” indicate the impact of inbreeding on male discrimination. The results of the term “odor” indicate whether males discriminated between the self- and kin odor

statistically significant ($N = 8$, $\chi^2 = 13.999$, $P = 0.001$, Fig. 2), but not in the case of outbred males ($N = 7$, $\chi^2 = 1.673$, $P = 0.196$, Fig. 2). Analyzing the relative choice time (see “Materials and methods”) showed a similar pattern (total: $N = 17$, $\chi^2 = 18.831$, $P < 0.001$; inbred males: $N = 8$, $\chi^2 = 23.967$, $P < 0.001$; outbred males: $N = 9$, $\chi^2 = 3.680$, $P = 0.055$). Regarding “final cave choice” males at large showed no significant discrimination (self/foreign: 5/2, binomial test, $P = 0.453$). Inbred and outbred males did not differ significantly in “final cave choice” (self/foreign: 4/0 vs. 1/2, Fisher’s exact test, $P = 0.143$).

Experiment B (“self-odor” vs. “kin odor”)

At large (in- and outbred males pooled), males did not significantly differentiate between self-odor and kin odor over the total 30 min (average time in seconds \pm SD test males spent at self-odor: 750.5 ± 406.7 and kin odor: 684.1 ± 364.4 ; $N = 38$, $\chi^2 = 0.902$, $P = 0.335$). Also, there was no significant effect of inbreeding on discrimination ($\chi^2 = 1.082$, $P = 0.298$). Additionally, we conducted a more detailed analysis by dividing the 30 min in six 5-min intervals (see also Mehlis et al. 2008). This analysis revealed that males at large spent significantly more time near the self-odor than near the kin odor in the last 5 min interval ($\chi^2 = 15.141$, $P < 0.001$, Table 1, Fig. 3). This result remained significant after Bonferroni correction for multiple testing ($\alpha = 0.008$). In- and outbred males did not significantly differ from each other in this interval ($\chi^2 = 0.237$, $P = 0.626$). Inbred males spent on average (time in seconds \pm SD) 161.3 ± 95.4 near the self-odor and 81.6 ± 65.7 near the kin odor ($\chi^2 = 8.858$, $P = 0.003$); outbred males spent 170.3 ± 77.2 near self-odor and 83.9 ± 74.8 near kin odor ($\chi^2 = 6.328$, $P = 0.012$).

In the “final cave choice”, in- and outbred fish differed significantly (Fisher’s exact test, $P = 0.0498$). Inbred males significantly discriminated the cave with the self-odor from the cave with kin odor (self/kin: 8/1, binomial test, $P = 0.039$) whereas outbred males did not (self/kin: 3/6, binomial test, $P = 0.508$). Pooling the data of the experiments A and B showed a more pronounced difference

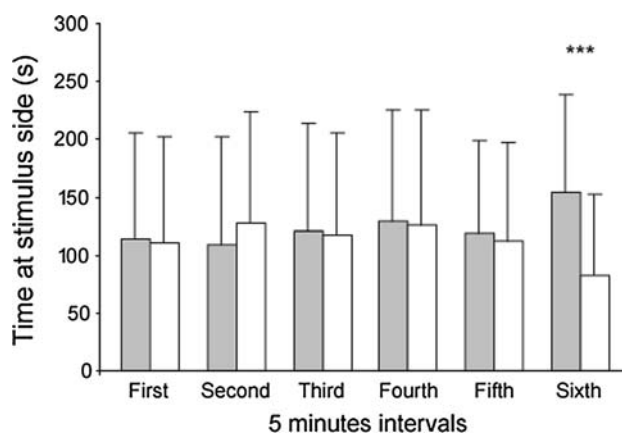


Fig. 3 Mean time (seconds \pm SD) males spent at the side with self-odor (gray bars) and at the side with kin odor, i.e., with odor of a familiar brother (open bars). For the detailed analysis, the recording time of 30 min was subdivided into 5 min intervals. Asterisks denote significantly different responses to the two odors (** $P < 0.001$)

between in- and outbred males concerning the discrimination of caves with self- and foreign odors (self/foreign including kin: inbred: 12/1 vs. outbred: 4/7, Fisher’s exact test, $P = 0.008$).

Experiment C (“kin odor” vs. “foreign odor”)

At large (in- and outbred males pooled), males spent more time near the odor of a foreign male than near kin odor ($N = 37$, $\chi^2 = 3.343$, $P = 0.068$, Fig. 4). The result was statistically significant only for outbred males ($N = 20$; $\chi^2 = 5.168$, $P = 0.023$, Fig. 4), but not for inbred males ($N = 17$, $\chi^2 = 0.143$, $P = 0.705$, Fig. 4). The difference between both groups, however, was not significant ($\chi^2 = 1.376$, $P = 0.241$). Regarding “final cave choice” males at large showed no significant discrimination between the two odors (kin/foreign: 11/9, binomial test, $P = 0.824$). There was also no significant difference between in- and outbred individuals (kin/foreign: 4/5 vs. 7/4; Fisher’s exact test, $P = 0.653$).

In order to test whether males responded differentially to the two related odors “self” and “kin”, we compared the

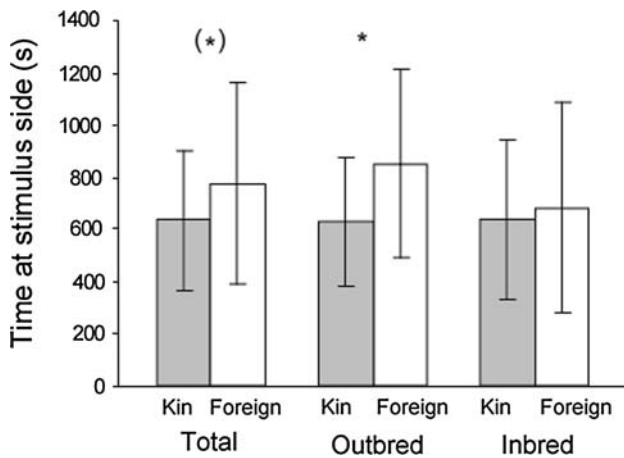


Fig. 4 Mean time (seconds \pm SD) males spent within 30 min at the side with kin odor (gray bars) and at the side with odor of a foreign, i.e., an unfamiliar, unrelated male (open bars). Shown is the time that all males and outbred and inbred males separately, spent in the choice zones (for definition see Fig. 1). Asterisks denote significantly different responses to the two odors (* $P < 0.05$, (*) $P < 0.1$)

results of experiment A and C in which males had a choice between a related odor (self or kin) and a foreign odor. There was a significant difference in the preference for the related odor between experiments (most time spent at the related odor vs. foreign odor: 11/3 in experiment A and 16/21 in experiment C, G-Test, $G = 5.361$, $P = 0.021$).

Discussion

The aim of this study was to investigate whether male *P. taeniatus* are able to recognize own cues and whether self-recognition is rather based on individual or on family cues. We examined this question via olfactory cues because they are known to play an important role in social cognition in many fish species (Ladich et al. 2006).

In experiment A males clearly preferred their own odor, i.e., they spent more time in front of the cave with the own odor than in front of the cave with the odor of an unfamiliar, unrelated male suggesting self-recognition based on olfactory cues. To our knowledge, there are only a few studies investigating olfactory self-recognition in animals. Waldman and Bishop (2004) showed that the frog *Leiopelma hamiltoni* preferred habitats with the own odor over habitats with the odor of conspecifics. Wild house mice (Hurst et al. 2001) as well as Antarctic prions also discriminated their own scent from that of conspecifics (Bonadonna and Nevitt 2004). In humans, females were able to recognize the own body odor whereas males were not (Platek et al. 2001).

The question whether self-recognition is based on individual self-cues (individual self-reference) or rather on family cues which might be derived from oneself (self-ref-

erence based on family cues) or from sibs was usually not in the focus of investigation. One exception was revealed by the study of Hurst et al. (2001) in which mice discriminated the own scent against the scent of a sib, indicating individual self-referential recognition. True individual recognition has rarely been demonstrated because class specific recognition is often difficult to exclude (Mateo 2006; Tibbetts and Dale 2007). However, indirect evidence for individual self-perception has been provided by studies in three-spined sticklebacks (Aeschlimann et al. 2003), Atlantic salmon (Rajakaruna et al. 2006) and mice (Sherborne et al. 2007) revealing self-referential mate-choice based on variation in MHC or MUP's, respectively. In experiment B of the present study, males again were able to discriminate and preferred their own scent over that of a familiar brother, although the two scents seemed to be difficult to distinguish for the males. The result suggests *individual* self-reference because if solely general family cues were used, the own odor and the odor of the related male should have been regarded as equal and no discrimination would have occurred. Differences between the odors of brothers probably actually reflected individual genetic differences because brothers were kept under standardized laboratory conditions and were of the same age. Hence, environmental or developmental differences, which might also contribute to variation in scents, were minimized.

In experiment C, at least outbred males discriminated between the odors of familiar brothers and those of unfamiliar, unrelated males. Interestingly, in this experiment males preferred to associate with the unfamiliar and unrelated odor. Thus, the preference for the own odor in experiments A and B probably cannot simply be explained by a general preference for the most related/familiar odor or a general avoidance of unfamiliar odors. The results suggest that *P. taeniatus* regarded self-scent as qualitatively different from family scents, maybe indicating that the self-odor was recognized as one's own or "mine". Assuming that males actually recognized the cave with the self-scent as own and the cave with non-self-odor as one of a brother or a foreign male, the behavior of the test male makes sense. To avoid competition, it is clearly adaptive to choose the own cave instead of that occupied by a foreign male (irrespective whether that is a brother or non-kin). In case of competition with either a brother or non-kin, kin selection should favor a behavior that evades kin competition (see West et al. 2002; Frommen et al. 2007a).

Individual cues (experiment B) as well as family cues (experiment C) in isolation induced a rather weak response suggesting that the combination of individual and family self-cues has contributed to self-recognition shown in experiment A. The results of experiment C confirm previous results that *P. taeniatus* are capable of kin recognition (Thünken et al. 2007a, 2007b), although further

experiments are necessary to disentangle the effects of familiarity and relatedness on odor preferences. What could be the genetic basis of olfactory recognition? The MHC is a potential candidate causing the observed individual self-recognition because it influences individual odors and is extremely variable (e.g., in cichlids: Klein et al. 1993). However, other molecules may also be involved. For example, in mice major urinary proteins (MUPs) appeared to be important as well (Cheetham et al. 2007; Sherborne et al. 2007). The MHC has been shown to play a role in mate-choice (for a review see Milinski 2006). The MHC might also play a role in kin recognition (although it is variable even among kin, for discussion see Penn and Potts 1998).

The effect of inbreeding on the discrimination behavior of the males seems to be weak which corresponds with findings in sticklebacks (Frommen and Bakker 2006; Frommen et al. 2007b; Mehliis et al. 2008, but see Frommen et al. 2007b). In our experiments, inbreeding had a statistically significant effect only on self-recognition concerning “final cave choice”. During “final cave choice”, males were probably exposed only to the odor which emitted directly out of the respective cave. Inbred fish significantly preferred to enter the cave with the own scent whereas outbred fish did not. Without possibility of comparison it might be easier for inbred males relative to outbred males to distinguish the self-scent from the non-self-odor maybe because inbreeding leads to more pronounced individual odors or because of better cognitive abilities of inbred individuals. However, more research is needed to estimate the impact of inbreeding on signalers and receivers.

Conclusion

Here we provide evidence that male *P. taeniatus* are capable of olfactory self-recognition. The recognition of own olfactory cues might be important in locating the own breeding cave and in intra-sexual competition (Barata et al. 2007). Self-recognition seemed to be based on self-reference. Individual as well as family self-cues are probably used.

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