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# Kin recognition by phenotype matching is family- rather than self-referential in juvenile cichlid fish

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The ability to differentiate between kin and nonkin is of importance in nepotistic as well as in mate choice contexts. Phenotype matching is a significant kin recognition mechanism, which is widespread in animals. However, the underlying proximate mechanisms are still poorly understood. Phenotype matching can be based on either self-reference or familial imprinting. We investigated phenotype matching in juvenile *Pelvicachromis taeniatus* based on chemical cues. *Pelvicachromis taeniatus* is a socially monogamous cichlid fish with biparental brood care. Previous studies indicate that the adults use phenotype matching to recognize kin. Juvenile fish were reared under three different conditions to manipulate recognition templates: (1) reared with kin, (2) reared in isolation or (3) reared with foster siblings. *Pelvicachromis pulcher* families served as foster families. In the experiments, test fish had to choose between olfactory cues obtained from two stimulus shoals differing in relatedness to the test fish. Test fish reared with kin discriminated unfamiliar kin from unfamiliar nonkin indicating that juvenile *P. taeniatus* also use phenotype matching to recognize kin. Focal fish reared in isolation or with foster siblings did not significantly discriminate unfamiliar kin from unfamiliar nonkin suggesting that juveniles did not imprint on their own phenotypic traits. However, individuals reared with foster siblings preferred unfamiliar olfactory stimuli of the foster species over those of their own indicating they used rearing partners as reference. Thus, phenotype matching is probably based on familial imprinting rather than self-reference in juvenile *P. taeniatus*.

Kin recognition, that is, the cognitive ability to distinguish between kin and nonkin, as well as kin discrimination, that is, the differential treatment of kin and nonkin, have been studied in various taxa (mammals: Mateo 2003; birds: McGregor 1989; reviewed in Nakagawa & Waas 2004; anuran amphibians: Blaustein & Waldman 1992). Kin discrimination is essential for kin selection theory (Hamilton 1964), which predicts indirect fitness benefits for helping or cooperating with close kin. Phenotype matching is an important kin recognition mechanism (Holmes & Sherman 1982; Waldman 1987; Mateo 2004; also referred to as 'indirect familiarity', Porter 1988) because it enables an individual to recognize unfamiliar kin. In phenotype matching an individual learns phenotypic cues from itself and/or relatives it was reared with and forms a kin template. Later on, phenotypic cues of conspecifics are compared with this kin template and classified either as kin or nonkin. If an individual includes only cues from itself in that recognition template, the mechanism is referred to as self-referent phenotype matching (Holmes & Sherman 1982).

A kin template may include visual, olfactory and acoustical learned traits of relatives or the individual itself (Brown & Brown 1996; Nakagawa & Waas 2004; Sharp et al. 2005; Mehlis et al. 2008; Kaminski et al. 2009). Self-reference is advantageous in species in which multiple mating occurs and siblings differ in relatedness in one litter/clutch. Imprinting on nestmates, for example, would result in integrating phenotypic traits of half-siblings into kin recognition templates and lead to an inaccurate template. Kin recognition by phenotype matching has been demonstrated in various taxa, for example mammals (Holmes 1986a, b; Sun & Müller-Schwarze 1997; Wahaj et al. 2004), amphibians (Blaustein & Waldman 1992) and fishes (Gerlach & Lysiak 2006). However, the reference on which the kin template is based often remains unclear. Self-referent kin recognition has been suggested for instance in rodents (Mateo & Johnston 2000), birds (Schielzeth et al. 2008) and fishes (Hain & Neff 2006) but definitive evidence is still scare because any contact with kin has to be excluded during development (Hauber & Sherman 2001; Hare et al. 2003). Demonstration of self-reference is suggested if an individual reared in isolation or cross-fostered is able to discriminate unfamiliar kin (Mateo 2004, 2010; Mateo & Holmes 2004). In contrast to self-reference, imprinting on nestmates, also called

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familial imprinting, is indicated when unfamiliar foster siblings are recognized (mammals: Mateo 2003; birds: Nakagawa & Waas 2004; fishes: Olsen & Winberg 1996).

In this study, we aimed to identify the mechanism underlying kin recognition by phenotype matching in juveniles of the cichlid fish *Pelvicachromis taeniatus*, which is a small, socially monogamous cave breeder from West Africa. After hatching, the fry are herded by both parents for several weeks (Thünken et al. 2010). Juvenile *P. taeniatus* live in shoals after their parents have left them, which is common in juvenile cichlids (Lamboj 2006). In previous experiments, adult *P. taeniatus* discriminated unfamiliar kin from unfamiliar nonkin in a mate choice context when both visual and chemical cues were available, suggesting phenotype matching as the kin recognition mechanism (Thünken et al. 2007b). The results of subsequent experiments suggest that, as in other animal species, olfactory cues play an important role in this process (Thünken et al. 2009, 2011). To investigate how the kin template for phenotype matching is formed in juvenile *P. taeniatus*, that is, whether it relies on self-reference or whether the juveniles imprint on their rearing partner, we raised juveniles with kin, in isolation and with foster siblings. In a series of experiments juvenile *P. taeniatus* were then given the choice between differently scented water in a fluviarium (Y-maze).

## METHODS

### Experimental Fish

The experimental fish were bred between February and April 2010 in the laboratory at the Institute for Evolutionary Biology and Ecology in Bonn, Germany under standardized conditions. To create different families, each of 16 size-assorted breeding pairs of *P. taeniatus* was introduced into a breeding tank (45 × 40 cm and 30 cm high), which was equipped with a standard breeding cave, an aquarium heater and a filter (model: Hobby gully filter). The bottom was covered with 500 ml of autoclaved sand and java moss, *Taxiphyllum barbieri* (2.5 g) to provide shelter. The water temperature was kept at 25 ± 1 °C and the experimental subjects were held under a light:dark regime of 12:12 h. They were fed daily with a mixture of defrosted *Chironomus* larvae and *Artemia*. Until the breeding pairs spawned, approximately 30% of the tank water was changed weekly to enhance the spawning probability of fish; after they had spawned the same amount of water was changed once a month. Additionally, breeding pairs of *Pelvicachromis pulcher* were established under the same breeding conditions for the cross-fostering experimental design (see below). Breeding caves were checked for eggs daily. The eggs were then transferred to the different rearing conditions. The day the eggs hatched and the first day of free swimming was noted for every family. Free-swimming fry were fed once a day with living *Artemia* nauplii in the morning hours before the experiments started. Experimental fish were not tested before their 14th day of free swimming.

### Rearing Conditions

(1) Reared with kin. We placed 10–20 eggs of one family together in a plastic tank (16 × 9 cm and 10 cm) filled with 850 ml tap water and equipped with an airstone for oxygen supply. Tanks were surrounded by grey plastic sheets on each side to prevent visual contact with neighbouring individuals. Approximately two-thirds of the water in each tank was changed daily and refilled with aged tap water. Water temperature was 22 ± 1 °C. The tanks were checked daily for unfertilized eggs and dead individuals, which were removed. Two sibling groups of each family were established. If a female's clutch size was sufficiently large (at least 60 eggs), a third sibling group was left with their parents. Since sibling groups were reared separately from each other, this design allowed us to test kin recognition independent from direct familiarity (prior association).

(2) Reared in isolation. One egg was placed alone in a plastic tank (16 × 9 cm and 10 cm high) and raised under the same conditions as described above. No visual or olfactory contact with any other individual except itself was possible. Since only the individual's own cues were available for imprinting, this experimental design allowed to test whether kin recognition is self-referent in this species.

(3) Reared with foster siblings. Since interspecific as well as intraspecific brood adoption is common in cichlids (e.g. Greenberg 1963; Wisenden & Keenleyside 1994; Fraser 1996; Ochi & Yanagisawa 2005), cross-fostering provides an elegant opportunity to determine on which reference phenotype matching is based. On the one hand, it excludes or minimizes experience with kin but maintains a normal social environment for the growing individual. On the other hand, it allows an individual to imprint on nonkin. We used *P. pulcher* as the foster species because they show a similar shoaling behaviour but have a slightly different body coloration and morphology compared to juvenile *P. taeniatus* enabling us to identify *P. taeniatus* in a *P. pulcher* group. Cross-fostering was conducted by either rearing one *P. taeniatus* from the egg stage in a group of 10 *P. pulcher* (age difference ± 2 days) kept in plastic tanks or by introducing wrigglers (larval state after hatching and before free swimming) of *P. taeniatus* into the brood of a *P. pulcher* breeding pair. To set the wrigglers directly into the breeding cave of the foster family, the breeding pair was carefully netted and kept in one corner of the tank. The *P. taeniatus* wrigglers were then sucked individually into a plastic tube (diameter = 4.5 mm) and subsequently released into the breeding cave. Breeding pairs were set free after all foreign fry were transferred. Cross-fostering was chosen to demonstrate self-reference alongside rearing fish in isolation to control for possible social deficits. Furthermore, this design allowed us to test which references were used to set up kin recognition templates in this species since familial imprinting can be demonstrated by species preferences of cross-fostered individuals.

Four different experiments were conducted. Fish reared with kin were given the choice between the odour from unfamiliar kin and unfamiliar nonkin to test whether they use phenotype matching to discriminate kin (experiment 1). Fish reared in isolation or with foster siblings, respectively, were also given the choice between unfamiliar kin and unfamiliar nonkin to determine whether phenotype matching is self-referent (experiments 2 and 3). In a fourth experiment fish reared with foster siblings were given the choice between odour of unfamiliar heterospecifics (i.e. the foster species *P. pulcher*) and unfamiliar unrelated conspecifics to examine whether the focal individual imprinted on the odour of the foster species. The sides on which the odour was introduced were switched during each experiment. Therefore, each experiment consisted of two trials (1 and 2). Kin recognition experiments 1, 2 and 3 were of a paired design to control for potential differences in general attractiveness in stimulus odours. One paired experiment consisted of two single experiments with the same pair of stimulus shoals but a different test fish between successive experiments. Hence, the focal fish in the first experiment and the focal fish in the second experiment were related to different stimulus shoals. The experiment to determine species preferences in cross-fostered fish had an unpaired design. All experiments were conducted between March and May 2010.

### Experimental Set-up

Experiments were conducted in a dichotomous choice Y-maze (Fig. 1). The Y-maze was made of white PVC tubes with an internal