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# The ontogeny of kin recognition in tadpoles of the toad *Bufo melanostictus* (Anura; Bufonidae)

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The ontogeny of kin recognition and influence of social environment on the development of kin recognition behaviour was experimentally investigated in tadpoles of *Bufo melanostictus* that lived in aggregations and showed low larval dispersion. Embryos and tadpoles of the toad were reared as (i) kin only, (ii) with kin and non-kin (separated by a mesh screen), and (iii) in isolation. They were tested for the ability to discriminate between (i) familiar siblings and unfamiliar non-siblings, (ii) familiar siblings and familiar non-siblings and, (iii) unfamiliar siblings and unfamiliar non-siblings. All tadpoles were fed on boiled spinach before conducting trials. Preference of test tadpoles to associate near the end compartments whether empty or containing members of specific stimulus groups was assessed using a rectangular choice tank. When tested in tanks with empty end compartments, the test tadpoles showed random distribution and thus no bias for the apparatus or the procedure. In the presence of kin/non-kin in the end compartments a significantly greater number of test tadpoles spent the majority of the time near familiar or unfamiliar kin rather than near familiar or unfamiliar non-kin. Kin discrimination ability persisted throughout larval development. Familiarity with siblings is not required for discriminating kin from non-kin, and kin discrimination ability is not modified following exposure to non-kin. Also, involvement of dietary cues is unlikely to be the prime mechanism of kin recognition in *B. melanostictus* unlike in some other anurans.

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## 1. Introduction

An extensive body of information generated over the past two decades shows that kin recognition is a widespread phenomenon in organisms as diverse as social insects, fishes, amphibians, birds, mammals and even plants (Fletcher and Michener 1987; Pfennig and Sherman 1995). However, the functions of kin recognition are not well understood in most cases (Blaustein *et al* 1991). Anuran embryos and tadpoles serve as useful model organisms for the study of the significance and mechanisms of kin recognition (Blaustein 1988; Waldman 1991; Blaustein and Waldman 1992). The tadpoles of American anurans *Bufo americanus* (Waldman and Adler 1979; Waldman 1981, 1982), *Rana cascadae* (O' Hara and Blaustein 1981; Blaustein and O' Hara 1982a, b), *Bufo boreas* (O' Hara and Blaustein 1982), *Rana sylvatica* (Waldman 1984; Gamboa *et al* 1991) and *Rana aurora* (Blaustein and O' Hara 1986b) preferentially associate with siblings. *Rana cascadae* and *B. americanus* tadpoles reared in

socially isolated or socially enriched environments discriminate between unfamiliar siblings and unfamiliar non-siblings (Blaustein and O' Hara 1981, 1982b; O' Hara and Blaustein 1981; Waldman 1981, 1991). Also, they can recognize individuals of various degrees of genetic relatedness (i.e. full siblings, maternal and paternal half siblings; Waldman 1981; Blaustein and O' Hara 1982a). *Rana sylvatica* tadpoles recognize full siblings over non-siblings; also those reared in isolation recognize unfamiliar siblings (Cornell *et al* 1989; Gamboa *et al* 1991). The froglets of *R. cascadae* and *R. sylvatica* preferentially associate with their kin (Blaustein *et al* 1984; Cornell *et al* 1989; Waldman 1989). In *R. aurora* recognition disappears during later stages of larval development (Blaustein and O' Hara 1986b). The tadpoles of *Rana pretiosa*, *Hyla regilla*, *Pseudacris crucifer* and *Rana pipiens* did not show any kin bias (O' Hara and Blaustein 1988; Fishwild *et al* 1990). Thus, kin recognition systems in anuran larvae show both similarities and differences among species.

**Keywords.** *Bufo melanostictus*; kin recognition; tadpoles; toad

The toad *Bufo melanostictus* is common in southern India. The toad tadpoles are found in aggregations, and both kinship and density affect growth and metamorphosis (Saidapur and Girish 2001). However, it is not known whether tadpoles of *B. melanostictus* possess the ability to discriminate between siblings and non-siblings. Hence, the present study on the toad tadpoles was undertaken to elucidate the ontogeny of kin recognition. The toad tadpoles were therefore reared (i) in isolation, (ii) with siblings, and (iii) in association with non-siblings, and then tested for their ability to associate preferentially with kin (familiar or unfamiliar) over non-kin (familiar or unfamiliar) using a choice tank.

## 2. Materials and methods

### 2.1 Materials

Various water bodies in Dharwad city (latitude 15°17'N, longitude 75°3'E) were surveyed to locate breeding sites and to study larval aggregation behaviour of *B. melanostictus* during March–July of 1996 and 1997. The eggs are laid in the form of two long strings by each female toad. We collected strings of eggs from three different ponds (situated 2 km apart) soon after oviposition. If two or more pairs were preparing to breed in a pond, we transferred the pairs to nearby ponds to prevent multiple paternity or any ambiguity about the sibship identity. During May–June 1997 we collected 12 egg clutches from different breeding pairs (i.e. full siblings/clutch). Eggs of each

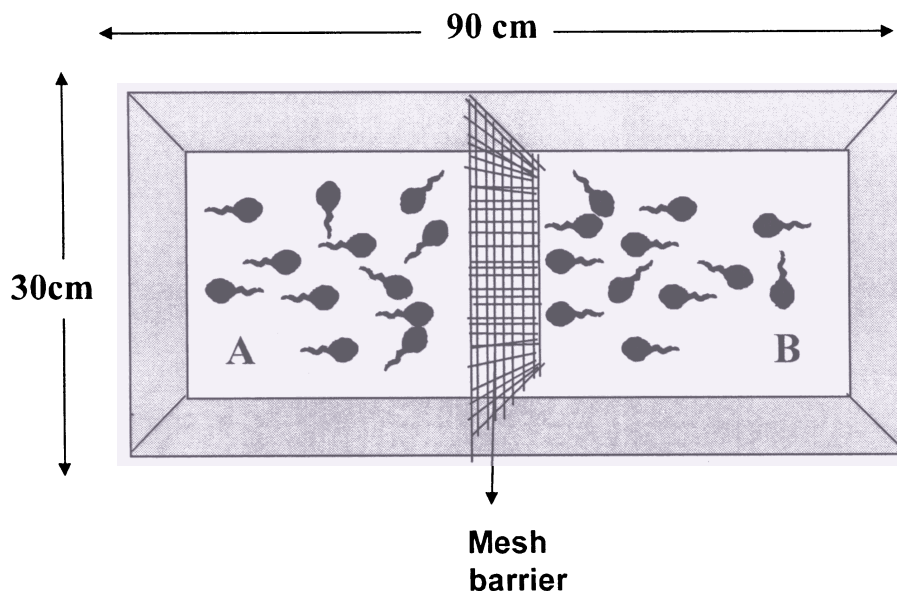
clutch were placed separately in plastic containers with 5 litres aged (dechlorinated) tap water until hatching or until subjecting them to different rearing conditions. The eggs usually hatched the next day at stage 19 when the heart begins to beat (Gosner 1960). The tadpoles were provided boiled spinach *ad libitum* except during the trials. They were staged as per the description given by Gosner (1960).

### 2.2 End-bias test

At the start, end-bias tests were run to check the potential bias of test tadpoles towards one or the other end of the choice tank or procedure by keeping both end compartments empty. One hundred trials were performed using one test tadpole each time. Ten tadpoles were randomly selected from each parental line per replicate. A total of 10 parental lines were used.

### 2.3 Rearing with kin

Hatchlings (stage 19) of each clutch were reared with siblings at a density of 60/25 litres water/aquarium so as to allow social contact between them. They were used to investigate the association preference between familiar siblings and unfamiliar non-siblings. The tests were performed at stages 28–32—characterized by the hind limb bud formation (early tests), and at stages 36–40—characterized by toe differentiation and complete formation of hind limbs (late tests). For early tests 200 individuals from 10



**Figure 1.** Design of a mixed rearing tank used to familiarize *B. melanostictus* tadpoles of one clutch (A) with tadpoles of another clutch (B).

different parental lines (20/egg mass) and for the late tests 120 individuals from 6 parental lines (20/egg mass) were used as test tadpoles.

#### 2.4 Mixed rearing

Tadpoles in stage 19, 75 each from two different egg clutches were placed in the two compartments of a rectangular tank (90 l × 30 b × 18 h cm) having a central 2 mm wire mesh partition (figure 1). The tadpoles in this regime could familiarize themselves with kin and non-kin through chemical(s) borne in water, visually and to a limited extent by tactile contact without being mixed. Sixty individuals from three different parental lines (20/egg mass) were tested against familiar siblings and non-siblings at stages 32–36.

The water was not mixed in the rearing tanks but a uniform coloration of the water within a few minutes ( $17.3 \pm 0.8$  min;  $N = 6$ ) of adding a few crystals of  $KMNO_4$  indicated diffusion of the chemical from one end to the other end of the rearing tank. Because mixed rearing involved several days, chemical cues emanating from tadpoles, if any, would easily diffuse throughout the tank.

#### 2.5 Rearing in isolation

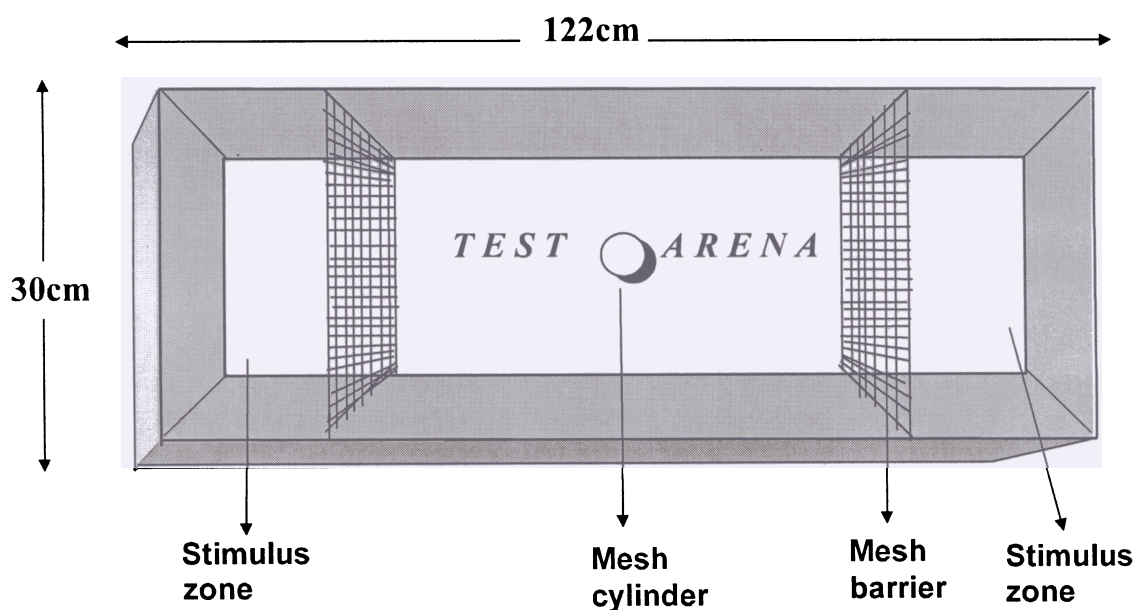
Embryos from each of the five different clutches were separated at stage 12 (yolk plug formation) and reared individually in separate plastic bowls (17.5 cm diameter). From each group 20 tadpoles at stage 30–34 were used to

test for their preference to associate with kin or non-kin, both unfamiliar to them.

#### 2.6 Choice tank and experimental design

A rectangular choice tank made of a galvanized iron sheet measuring 122 l × 30.5 b × 18 h cm was partitioned into three compartments using a 2 mm mesh screen (figure 2). The end compartments (each 16 cm) housed 20 stimulus tadpoles at a time that were either familiar or unfamiliar siblings or non-siblings depending upon the experiment. The central compartment (90 cm) serving as the test arena was divided into three equal sized zones by lines at the bottom of the tank. The most central (neutral) zone was used to introduce a chosen test tadpole. The zones adjacent to the neutral zone served as stimulus zones 1 and 2. The basic design is similar to that described earlier (Blaustein and O' Hara 1982a, b; 1986a; Hall *et al* 1995).

Before each test the tank was freshly filled with 6 cm fresh water, and the location of stimulus tadpoles was changed between trials. Stimulus tadpoles were renewed after 10 trials. Size and developmental stages of stimulus and test tadpoles were always matched, a given test tadpole was used once only. The test tadpole was introduced in the centre of the neutral zone using an open-ended mesh cylinder of 9 cm diameter, released after 10 min by gently lifting the cylinder, and allowed to acclimate itself to the test arena for 1 min. As a measure of association preference we recorded the total time (in seconds) spent by the test tadpole in each zone of the test arena during



**Figure 2.** Design of the choice tank used to study kin recognition ability in *B. melanostictus* tadpoles.

the subsequent 10 min. The test tank was cleaned after each test. Twenty tadpoles were used per replicate.

### 2.7 Statistical analysis

The number of tadpoles spending the majority of time in each stimulus zone was compared with binomial tests. The Wilcoxon signed-rank test was used to analyse the total time spent by tadpoles in the sibling zone. We tested differences in time spent near the sibling zone of test tank from a hypothetical random time expected (200 s) under the null hypothesis that tadpoles would spend equal amounts of time in each zone of the test arena. Therefore, only one score per animal was used in comparison with the expected mean to not violate the independence of the data. All tests were 2-tailed. The data were judged significant at  $P < 0.05$ . SPSS (version 6.1.3) was used for analysis. Meta-analysis of data of different test groups of the same rearing type was performed with Fisher's procedure to combine probabilities (Sokal and Rohlf 1995) from independent tests of significance for an overall result. Fisher's procedure was applied on probabilities obtained from the binomial test and Wilcoxon signed-rank tests.

## 3. Results

### 3.1 Field observations

The toads bred in various kinds of puddles, rain-filled ditches and man-made cement cisterns in parks both indi-

vidually and in groups (normally during the monsoon season). Tadpoles remained attached to the egg jelly for 3–4 days after hatching, and swimming began at stage 25. The toad tadpoles are poor swimmers and they remain in polarized aggregations of thousands of individuals. Such aggregations often break into small groups of few hundred tadpoles that forage at the substratum. Larval dispersal is low and they remain together until metamorphosis.

### 3.2 End-bias test

We tested the hypothesis that in the choice tanks with empty end compartments, the test tadpoles will occupy different sections of the test arena randomly. Accordingly, 100 test tadpoles of 10 parental lines showed that the number of tadpoles spending the majority of their time as well as the time spent by individual tadpoles were random with respect to the neutral and stimulus zones (table 1).

### 3.3 Kin recognition

**3.3a Early tests (stage 28–32):** In the experiments in which the two end compartments housed siblings and non-siblings respectively, the test tadpoles preferentially (159 out of 200) spent most of their time near siblings (table 2).

**3.3b Late tests (stage 36–40):** The test tadpoles in late developmental stages also showed a preference to associate with siblings over non-siblings. Significantly greater number of test tadpoles (87 out of 120) preferentially spent most of their time near siblings (table 2).

**Table 1.** Results of end-bias tests in *B. melanostictus* tadpoles in the absence of stimulus in the end compartments of the choice tank.

Group tested	Number spending most <sup>a</sup> time near		Time (s) spent in zones <sup>b</sup> (Mean ± SE)	
	Stimulus 1	Stimulus 2	Stimulus 1	Stimulus 2
E <sub>1</sub>	3	7	211 ± 27	260 ± 31
E <sub>2</sub>	5	5	161 ± 19	233 ± 32
E <sub>3</sub>	4	6	183 ± 37	198 ± 37
E <sub>4</sub>	4	6	203 ± 41	214 ± 51
E <sub>5</sub>	4	6	178 ± 22	249 ± 43
E <sub>6</sub>	7	3	206 ± 22	153 ± 25
E <sub>7</sub>	9*	1	269 ± 28*	95 ± 32
E <sub>8</sub>	5	4	200 ± 47	136 ± 26
E <sub>9</sub>	4	6	186 ± 34	171 ± 32
E <sub>10</sub>	7	3	227 ± 40	223 ± 36

E<sub>1</sub>–E<sub>10</sub> represents test tadpoles from 10 different parental lines. <sup>a</sup>Compared using binomial tests. <sup>b</sup>Time spent in sibling zone was compared with a random expectation using Wilcoxon signed rank test. \* $P < 0.05$ .

Fisher's procedure of combining probabilities for overall result:  $-2\sum \ln P = 24.02$ ,  $c_{[20]}^2$ ;  $P > 0.1$  (number data);  $-2\sum \ln P = 20.46$ ,  $c_{[20]}^2$ ;  $P > 0.1$  (time data).

### 3.4 Effect of mixed rearing on kin recognition

These tests were conducted to determine whether interaction with non-siblings from an early developmental stage had any influence on the development of kin discrimination behaviour. The test tadpoles preferred to associate with familiar siblings over familiar non-siblings. Significantly more test tadpoles (48 out of 60) spent most of their time near siblings when given a choice between association with either familiar siblings or familiar non-siblings (table 3).

### 3.5 Effect of rearing in isolation on kin recognition

A great majority (83 out of 100) of test tadpoles, reared in isolation from stage 12 onwards and tested in developmental stages 30–34, spent significantly greater amount of time near unfamiliar siblings compared to near unfamiliar non-siblings (table 4).

**Table 2.** Results of kin recognition ability in early (stages 28–32) and late tests (stages 36–40) in tadpoles of *B. melanostictus*.

Group tested	Number spending most <sup>a</sup> time near		Time (s) spent in zones <sup>b</sup> (Mean ± SE)	
	Familiar siblings	Unfamiliar non-siblings	Familiar siblings	Unfamiliar non-siblings
Early tests (stages 28–32)				
K <sub>1</sub>	17*	3	271 ± 20*	155 ± 24
K <sub>2</sub>	15*	4	287 ± 31*	149 ± 27
K <sub>3</sub>	13*	7	229 ± 25*	167 ± 23
K <sub>4</sub>	18*	2	255 ± 23*	121 ± 15
K <sub>5</sub>	17*	3	319 ± 22*	141 ± 21
K <sub>6</sub>	17*	3	320 ± 34*	113 ± 22
K <sub>7</sub>	18*	2	289 ± 19*	131 ± 25
K <sub>8</sub>	16*	3	256 ± 30*	130 ± 27
K <sub>9</sub>	16*	3	287 ± 27*	132 ± 34
K <sub>10</sub>	12	8	230 ± 32	190 ± 39
Late tests (stages 36–40)				
K <sub>1</sub>	10	8	281 ± 32*	144 ± 28
K <sub>3</sub>	13	6	284 ± 36*	158 ± 34
K <sub>4</sub>	17*	3	315 ± 24*	129 ± 28
K <sub>7</sub>	15*	5	277 ± 42*	95 ± 39
K <sub>8</sub>	16*	3	328 ± 35*	114 ± 28
K <sub>9</sub>	16*	4	320 ± 36*	122 ± 33

K<sub>1</sub>–K<sub>10</sub> represent test tadpoles from 10 different parental lines. <sup>a</sup>Compared using binomial test. <sup>b</sup>Time spent in sibling zone was compared with a random expectation using Wilcoxon signed rank test. \**P* < 0.05.

Fisher's procedure of combining probabilities for overall result:  $-2\sum \ln P = 186.4$ ,  $c_{[32]}^2$ : *P* < 0.0001 (number data);  $-2\sum \ln P = 184.2$ ,  $c_{[32]}^2$ : *P* < 0.0001 (time data).

## 4. Discussion

Tadpoles of *B. melanostictus* show a clear-cut association preference for siblings over non-siblings as reported in *R. cascadae* (O' Hara and Blaustein 1981, 1985; Blaustein and O' Hara 1983), *R. sylvatica* (Waldman 1984; Gamboa *et al* 1991) and *B. americanus* (Waldman 1981, 1982, 1986). Familiarity with siblings is not a prerequisite for kin recognition in *B. melanostictus* and it is interesting to note that the toad tadpoles preferentially associate with kin throughout the larval development.

Even after mixed rearing, *B. melanostictus* tadpoles could discriminate between familiar siblings and familiar non-siblings indicating that social enrichment does not affect kin recognition ability in this species. In contrast, *B. boreas* tadpoles reared with kin and non-kin lost the ability to discriminate between familiar siblings and familiar non-siblings (O' Hara and Blaustein 1982). *Bufo melanostictus* tadpoles may use cues that originate from themselves (for example in those reared in isolation) or from nearby individuals (reared with siblings). Apparently, sib preferences are established very early in development, and therefore exposure to cues emanating from non-sibs as in the mixed rearing tank might not alter kin discrimination ability of the toad tadpoles. This type of kin discrimination is explained by the phenotypic matching mechanism (Waldman 1981, 1998; Blaustein 1983; Lacy and Sherman 1983). In *B. americanus* there exists a sensitive period in the larval development during which sib preferences are established. *Bufo americanus* tadpoles that were initially reared in sibling groups, and were then transferred to flow tanks containing mixed groups, subsequently assorted preferentially with their siblings (Waldman 1981). But if their early experience included both siblings and non-siblings, they later failed to recognize

**Table 3.** Preferential association of *B. melanostictus* tadpoles near familiar siblings over familiar non-siblings (stages 32–36).

Group tested	Number spending most <sup>a</sup> time near		Time (s) spent in zones <sup>b</sup> (Mean ± SE)	
	Familiar siblings	Familiar non-siblings	Familiar siblings	Familiar non-siblings
M <sub>1</sub>	15*	4	272 ± 23*	172 ± 24
M <sub>2</sub>	16*	3	262 ± 20**	172 ± 17
M <sub>3</sub>	17*	3	250 ± 20*	181 ± 27

M<sub>1</sub>–M<sub>3</sub> represent test tadpoles from 3 different parental lines. <sup>a</sup>Compared using binomial test. <sup>b</sup>Time spent in sibling zone was compared with a random expectation using Wilcoxon signed rank test. \**P* < 0.05; \*\**P* < 0.01.

Fisher's procedure of combining probabilities for overall result:  $-2\sum \ln P = 28.74$ ,  $c_{[6]}^2$ : *P* < 0.005 (number data);  $-2\sum \ln P = 22.62$ ,  $c_{[6]}^2$ : *P* < 0.005 (time data).

**Table 4.** *B. melanostictus* tadpoles (stages 30–34) reared in isolation recognize unfamiliar siblings over unfamiliar non-siblings.

Group tested	Number spending most <sup>a</sup> time near		Time (s) spent in zones <sup>b</sup> (Mean ± SE)	
	Unfamiliar siblings	Unfamiliar non-siblings	Unfamiliar siblings	Unfamiliar non-siblings
I <sub>1</sub>	15*	3	342 ± 27*	109 ± 30
I <sub>2</sub>	20*	0	369 ± 27**	103 ± 22
I <sub>3</sub>	17*	3	381 ± 30*	110 ± 27
I <sub>4</sub>	16*	2	323 ± 26**	117 ± 27
I <sub>5</sub>	15*	3	279 ± 43*	158 ± 31

I<sub>1</sub>–I<sub>5</sub> represent test tadpoles from 5 different parental lines. <sup>a</sup>Compared using binomial test. <sup>b</sup>Time spent in sibling zone was compared with a random expectation using Wilcoxon signed rank test. \* $P < 0.05$ ; \*\* $P < 0.01$ . Fisher's procedure of combining probabilities for overall result:  $2\sum \ln P = 54.48$ ,  $c_{[10]}^2$ :  $P < 0.0001$  (number data);  $-2\sum \ln P = 64.42$ ,  $c_{[10]}^2$ :  $P < 0.0001$  (time data).

siblings. This narrow time window when sib preferences are established is 2 weeks in *B. americanus* (Waldman 1981). On the other hand *B. melanostictus* tadpoles exposed to siblings and non-siblings since stage 19 and later tested during mid-developmental stages (30–34) still associated preferentially with familiar siblings rather than with familiar non-siblings. The experiment clearly shows that the sib preferences of *B. melanostictus* tadpoles are established very early in development and that exposure to non-siblings does not alter the sib preferences.

The fact that *B. americanus*, *R. cascadae* and *R. sylvatica* (Waldman and Adler 1979; Blaustein and O'Hara 1981; Gamboa *et al* 1991) and *B. melanostictus* tadpoles reared in social isolation can discriminate siblings from non-siblings on first encounter lends support to the self-learning hypothesis. Tadpoles restricted from interacting with conspecifics until testing presumably experience some facets of their own phenotype and use it to discriminate unfamiliar kin against non-kin (Gamboa *et al* 1991; Waldman 1991). Further, maternally inherited (jelly components) or environmentally acquired cues (e.g. dietary cues) may also allow isolated individuals to recognize unfamiliar kin (Waldman 1981, 1987, 1988; Gamboa *et al* 1991).

Kin discrimination in anuran tadpoles might also occur as an epiphenomenon (Waldman 1984; Grafen 1990; Pfennig 1990). For example, kin association in tadpoles of *Scaphiopus multiplicatus* appears to result from habitat selection in response to diet based environmental cues rather than sib preferences (Pfennig 1990). Similarly, *Scaphiopus intermontanus* tadpoles use dietary cues for kin recognition (Hall *et al* 1995). In *R. sylvatica* both environmental (dietary) and genetic components are involved in kin recognition (Gamboa *et al* 1991). Grafen (1990) suggested that kin bias demonstrated in anuran tadpoles could be explained by a species recognition sys-

tem. That is, all members of the species may have the same smell, but it is likely that there is some genetic variation in the smell. This genetic variation, and the matching mechanism of species recognition, will produce discrimination by genetic similarity. Individuals will acquire a standard, which is more like their relatives than it is like that of the conspecifics in general. So when an individual uses its species recognition ability to join an aggregation of tadpoles, it will have a slight tendency to join a more related aggregation because it is perceived as closer to the acquired standard. In the present experiments tadpoles reared in isolation or with kin/non-kin were fed exclusively on spinach. Therefore, dietary cues would be common to tadpoles of all the groups. If dietary cues are important in kin recognition then *B. melanostictus* tadpoles fed on spinach alone should have lost their ability to discriminate between kin and non-kin. Yet, they exhibited a clear-cut kin bias indicating that despite similarity in dietary cue, kin discrimination ability was not abolished. Hence, involvement of dietary cues is unlikely to be the prime mechanism of kin association in *B. melanostictus*.

The tadpoles of *B. melanostictus* generally grow and metamorphose rapidly because of ephemeral water bodies where they occur and often under crowded conditions. Therefore, a short larval duration (25–30 days) and a kin recognition system that persists throughout the larval period might enhance inclusive fitness. Indeed, benefits of kinship are evident from the fact that *B. melanostictus* tadpoles raised as pure kin group in either crowded or uncrowded conditions grew better, metamorphosed at a larger size and completed their larval life within 25 days. In contrast, tadpoles reared as mixed (kin and non-kin) groups had retarded growth, smaller size at metamorphosis and they took longer to metamorphose (Saidapur and Girish 2001). Therefore, the kin recognition behaviour of

*B. melanostictus* tadpoles might be associated with growth regulation as in *R. temporalis* which performed better in growth and metamorphosis when reared with siblings than with non-siblings (Girish and Saidapur 1999).

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