Ontogenetic shifts in tadpole kin recognition: loss of signal and perception

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Abstract. Although numerous studies of the ontogeny of kin recognition behaviour have been conducted, large gaps in our knowledge remain concerning the dynamics of the individual components of the recognition system. A series of laboratory experiments were conducted to investigate the signal/perception system in tadpoles of the red-legged frog, Rana aurora, a species whose kin discrimination ceases at a particular stage of larval development. Laboratory choice tests suggest that the phenotypic marker used in discrimination diminishes and the ability to perceive the marker ceases at a precise stage of larval development. After this stage, tadpoles do not discriminate between kin and non-kin. The phenotypic recognition marker is chemically based and is probably perceived by olfaction.

A growing body of evidence suggests that individuals representing numerous species can discriminate between related and unrelated individuals (commonly known as kin recognition; e.g. reviews by Holmes & Sherman 1983; Fletcher & Michener 1987; Porter 1987; Blaustein et al. 1988; Waldman 1988; Barnard 1989; Hepper 1991). Studies of kin recognition have enhanced our knowledge of how behaviour patterns develop and have provided important information concerning social behaviour (see reviews in Blaustein et al. 1987a; Fletcher & Michener 1987; Hepper 1991). Kin recognition is especially germane to kin selection theory and to the concept of inclusive fitness (Hamilton 1964a, b) and investigations of kin recognition have provided heuristic tests of these theories. Moreover, kin recognition may play an important role in mate choice (e.g. Bateson 1983). Optimal outbreeding (Bateson 1983) may be enhanced by individuals who discriminate between distant relatives and close ones and between kin and non-kin.

Whereas progress has been made in understanding the ontogeny of kin recognition behaviour, large gaps in our knowledge remain concerning critical components of recognition systems (Beecher 1988). It is possible that the phenotypic marker(s) (i.e. cues, signals, signatures) by which an individual is identified, and/or the perception of the marker may change ontogenetically with shifts in social or ecological conditions. Yet, detailed investigations of the signature/perception system have been made only rarely.

Recent theoretical models and empirical research concerning the nature of the signature/perception system by Beecher (1988, 1989) have provided important, unique insight into how individuals may be identified and the potential variability of kin recognition mechanisms. Beecher (1988) pointed out that selective pressures and costs and benefits to the sender and to the receiver may not coincide. Under certain conditions, the sender does not benefit by reliably identifying itself. Beecher (1988, 1989) suggests that under changing environmental and social conditions, individuals may indeed turn their signatures off and on. Of course, while intermittent signature-onset of this nature is possible within certain phenotypic traits (e.g. calls in birds), other discriminable traits (e.g. whole body odours, conspicuous visual features) are continuously accessible (Porter & Blaustein 1989).

To understand more fully the ecological and evolutionary significance of a kin recognition system, it is important to study how the signature/perception system develops. For several reasons, anuran amphibian larvae have been a model taxon for investigating the ontogeny of kin recognition and the proximate cues used for recognition (e.g. see reviews by Blaustein 1988; Waldman 1991; Blaustein & Waldman 1992). Anuran larvae are easy to manipulate experimentally in the laboratory and in the field (e.g. Blaustein 1988; Waldman 1991; Blaustein & Waldman 1992). Compared with most other vertebrates, interspecific comparisons of kin recognition have been most comprehensive.
for anurans (Blaustein et al. 1991; Waldman 1991; Blaustein & Waldman 1992). The complex life histories (sensu Wilbur 1980) of anurans make them especially useful for investigating the ontogeny of kin recognition behaviour. By following the development of kin recognition as larvae grow, develop, and eventually metamorphose into adult forms, any behavioural changes that occur may reveal important clues as to the ecological and evolutionary significance of the behaviour.

Ontogenetic changes in kin recognition behaviour have been documented in three vertebrate species (see also Kiseleva 1989), all amphibians: red-legged frogs, *Rana aurora* (Blaustein & O’Hara 1986a), wood frogs, *R. sylvatica* (Waldman 1989; Rautio et al. 1991); salamanders, *Ambystoma opacum* (Walls 1991). In *R. aurora*, developmental changes in kin recognition behaviour occurred during the larval stage (Blaustein & O’Hara 1986a). *Rana aurora* tadpoles discriminate between kin and non-kin in early developmental stages but fail to do so after they reach a particular stage in larval development (Blaustein & O’Hara 1986a). *Rana aurora* is the only vertebrate known to lose its affinity to associate with kin within the same life-history stage as it grows and develops.

In this paper we investigate in detail the ontogenetic shifts in kin recognition in *R. aurora* tadpoles with the primary purpose of determining whether the cessation of kin discrimination is due to loss of signal production or loss of ability to perceive the signal. We also examine the cues used in discrimination.

**METHODS**

**Animals and Rearing Conditions**

We collected eight clutches of *R. aurora* (A, B, D, E, F, G, H and X) from one site and one clutch (R) from another site in Lincoln County, approximately 112 km southeast of Corvallis, Oregon, U.S.A. About 200 larvae were reared in each aquarium under two basic regimes: (1) animals were reared exclusively with siblings in aerated 38-litre aquaria; (2) animals were reared with a mixture of siblings and non-siblings (mixed rearing regime) by placing an equal number of tadpoles from two sibling groups on opposite sides of an aerated 38-litre aquarium divided by 1.5-mm plastic mesh. Partitioning the aquarium allowed the two sibling groups to be reared together and aeration allowed for complete water mixing. There was visual and some tactile contact between groups (see details of this rearing regime in O’Hara & Blaustein 1981).

All eggs except for half from each of clutches E, G and H were reared under a 14:10 h light:dark cycle at 20–22°C (warm regime). The remaining half of the eggs from clutches E, G and H were reared under an LD 14:10 h photoperiod at 5–7·2°C (cold regime). Eggs under cold regimes develop more slowly than those under warmer regimes. By rearing animals from the same sibling simultaneously in warm and cold regimes, it was possible to have related individuals of the same age but at different developmental stages at the same time. Therefore, it was possible to examine kin recognition in tadpoles that differed only in stage of development. By testing animals at different developmental stages it was possible to determine whether the signal used in identification and/or the ability to perceive the signal changed as tadpoles developed.

Water was changed in all rearing aquaria every 3–5 days. Tadpoles were fed rabbit pellets and Hartz Min tropical fish food daily.

**Apparatus and Standard Testing Procedure**

A tank measuring 122 × 44 × 30 cm was used to test two tadpoles simultaneously for sibling group preferences (figures of this apparatus have been published previously; e.g. O’Hara & Blaustein 1981; Blaustein & O’Hara 1986b). A pencil mark was drawn width-wise to delineate the two halves of the tank. To create end compartments for holding stimulus tadpoles, a partition of 1.5-mm plastic mesh was placed 15 cm from each end of the tank. The remaining central portion of the tank was divided longitudinally by an opaque water-tight partition allowing us to test two tadpoles simultaneously (but independently; see Results).

Prior to each test, the tank was filled to a depth of 6 cm with 34 litres of dechlorinated tap water (at room temperature). After 2–4 min, stimulus tadpoles of one sibling group and those from a second sibling group were placed in opposite end compartments and left undisturbed for 15 min. In all experiments, except for experiment 4 (see below), 25 tadpoles were placed in each stimulus compartment. One test tadpole was released at the tank centre on each side of the longitudinal partition and allowed to acclimatize for 10 min. The time spent in seconds by each tadpole in sibling and
non-sibling halves (as demarcated by the pencil mark) of the tank was recorded for four 5-min trials (total = 1200 s) at 10-min intervals. Previous tests of numerous tadpoles using these and other techniques indicate that tadpoles spend the majority of the time near the stimulus ends of the tank and avoid the central portion (e.g. Blaustein & O'Hara 1983). Observations were taken from behind a plastic blind. Following each test, the tank was drained and thoroughly rinsed. The two tadpoles tested simultaneously were always members of the same sibling group. No test tadpole was tested more than once, and the same stimulus animals were used in no more than five tests. We alternated stimulus groups from one end to the other between tests. Other controls for the apparatus and testing procedures have been reported elsewhere (e.g. Blaustein & O'Hara 1981; O'Hara & Blaustein 1981). All stimulus tadpoles and test individuals were matched for body size. Except for tadpoles in experiments 7–8, test tadpoles and stimulus tadpoles were also matched for developmental stage. We determined the developmental stages of tadpoles using Gosner's (1960) criteria. The relationships of the test animals to stimulus animals are reported in Tables I–IV.

Tests Conducted

We modified the standard testing regime according to the type of experiment that we were conducting. At least 20 different tadpoles were used per replicate in each experiment. The detailed methods for each experiment are outlined below. Except for experiments 7–9, tests were conducted 'blind' (the researchers did not know the sibling composition of the test animals or the stimulus groups).

Experiments 1, 2 and 3: do R. aurora tadpoles discriminate between siblings and non-siblings?

To corroborate the earlier findings of Blaustein & O'Hara (1986a), experiments 1, 2 and 3 (see Table I for details) were replicates of their earlier work. The initial study of kin recognition in R. aurora tadpoles (Blaustein & O'Hara 1986a) showed that individuals can discriminate between siblings and non-siblings only in early larval stages and only if the test animals are reared with siblings. Test animals reared in mixed rearing regimes did not discriminate between siblings and non-siblings.

Test procedures were similar to those described in Blaustein & O'Hara (1986a) and above for standard tests.

Experiments 4, 5 and 6: what is the sensory basis for kin recognition in R. aurora tadpoles?

Amphibians that can discriminate between kin and non-kin do so by chemical cues (reviewed in Blaustein & Waldman 1992). We used techniques similar to those of Blaustein & O'Hara (1982a) to determine whether chemical cues alone are sufficient for discriminating between kin and non-kin.

In experiment 4, 30 stimulus tadpoles were placed in each end compartment of the test tank within small chambers (12.5 × 5.5 × 8 cm: 15 tadpoles per chamber; two chambers on each end; figures of this apparatus were published in Blaustein & O'Hara 1982a, 1986b). The chambers were composed of a 3-mm-thick opaque plastic front (facing test animals) and sides. The top, back and bottom were composed of 1.5-mm plastic mesh and several 1.5-mm diameter holes were bored in the plastic sides. These chambers were designed to permit the continuous diffusion of chemical cues from stimulus animals while blocking visual cues. Chambers were centred within end compartments, and the top portion of the chambers rose just above the surface of the water level. After each test, all chambers were thoroughly rinsed and alternated from one end to the other. The testing procedure was identical to that described above for the standard tests.

In experiment 5, we investigated the role of chemical cues in kin recognition in greater detail by testing tadpoles in standard tests whose external nares were blocked with Orabase plain oral protective paste (Colgate-Hoyt Laboratories, Canton, Massachusetts, U.S.A.). A small amount of Orabase was placed inside the nares of test animals using a blunt needle while tadpoles were in a net under a dissecting microscope. This technique has been used successfully by Waldman (1985a) in his investigation of olfaction and kin recognition in B. americanus tadpoles. Orabase has no pharmacological effects and prevents water and odourants from entering the nares (Waldman 1985a). However, some odourants may still reach olfactory receptors through the oral cavity (Waldman 1985a).

We conducted a sham control (experiment 6) for experiment 5 by following the same procedures but
without placing Orabase into the nares of test subjects. Test animals were placed under the microscope and a blunt needle without Orabase was placed inside the nares while tadpoles were in a net. Tadpoles appeared to swim in a normal fashion after being treated with Orabase and after being subjected to handling in the sham controls.

Experiment 7: can tadpoles in later stages of development discriminate between siblings and non-siblings that are in early development?

Because *R. aurora* tadpoles can discriminate between siblings and non-siblings in early developmental stages (Blaustein & O'Hara 1986a), the cues used in discrimination must be present at these stages. If developmentally advanced tadpoles fail to discriminate between siblings and non-siblings that are in early stages of development (i.e. those whose developmental stage has been experimentally altered), then it is likely that the ability to perceive the cues is diminished.

For this experiment, we followed the standard testing regime. However, test tadpoles were at relatively late stages of development and the stimulus animals were at early stages of development.

Experiment 8: can tadpoles in early stages of development discriminate between siblings and non-siblings that are in later stages of development?

If tadpoles in early stages of development fail to discriminate between siblings and non-siblings in later stages of development, we can assume that the cues used in kin recognition are altered or that their production is lowered as tadpoles develop.

We followed the standard testing regime. However, test animals were at early stages of development and stimulus animals were at later stages of development.

Experiment 9: can tadpoles discriminate between siblings and non-siblings after they have been reared under a cold regime?

It is possible that rearing tadpoles under a relatively cold regime altered their ability to discriminate between kin and non-kin. The perception ability and/or the cues used in recognition could have been altered. Therefore, experiment 9 was used as a control to determine whether tadpoles reared under cold conditions could still discriminate between kin and non-kin.

Test tadpoles were at early developmental stages, had been reared with siblings only, and were reared under the cold regime. Stimulus tadpoles were at the same developmental stage as test tadpoles and were also reared under the cold regime.

Experiments 10, 11 and 12: do test tadpoles prefer to associate with conspecifics?

Relatively little is known about the larval aggregation tendencies of *R. aurora* tadpoles in nature (Blaustein 1988). Therefore, we tested *R. aurora* tadpoles to examine their propensity to associate with conspecifics under laboratory conditions. Based on previous results (Blaustein & O'Hara 1986a), we expected that *R. aurora* tadpoles would tend to spend most of their time in the portion of the test tank containing conspecifics over an empty compartment on the opposite end of the test tank.

Standard testing techniques were employed. In experiments 10 and 11, test animals and stimulus animals were at early stages of development. The stimuli presented to test individuals in experiment 10 were familiar siblings (reared together) and an empty test compartment. The stimuli presented in experiment 11 were unfamiliar non-siblings (reared apart) and an empty test compartment. In experiment 12, test tadpoles and stimulus tadpoles were at late stages of development. The stimuli presented to test animals in experiment 12 were familiar siblings and an empty test compartment (replicate 1) and unfamiliar non-siblings and an empty test compartment (replicate 2).

Statistical Tests

We used the binomial test to determine whether the number of tadpoles spending most of their time on the sibling side (or conspecific side in experiments 10, 11 and 12) differed from random expectation. We used one-tailed statistical tests because previous experiments (Blaustein & O'Hara 1986a) demonstrated preferential association among siblings during early, but not later, stages of development in *R. aurora* tadpoles.

For comparison with similar studies, we calculated a 'ratio of preference' (sensu Egidi & Lenington 1985; Lenington & Egidi 1985; see also Waldman 1989; Fishwild et al. 1990) for each trial (*N* = 20) for the various test groups. We defined preference as the ratio of the amount of time (s) that a test tadpole spent near the end of the chamber containing a sibling stimulus group to that spent near the end containing a non-sibling stimulus group. Similarly, the
preference of a test tadpole for a stimulus group (composed of either siblings or non-siblings) versus an unoccupied (control) end of the chamber was calculated as the ratio of time spent near the end of the chamber that housed the stimulus group to that spent near the unoccupied end of the chamber.

**RESULTS**

As in previous studies of kin recognition in anuran larvae conducted in our laboratory (e.g. Blaustein & O'Hara 1981, 1982b, 1983, 1987; O'Hara & Blaustein 1982, 1988), test tadpoles swam from end to end within the test tank. Test tadpoles spent relatively more time near the stimulus compartments rather than in the middle of the test tank (see Blaustein & O'Hara 1983). Moreover, as in previous tests (above) stimulus tadpoles constantly swam within the stimulus compartments and generally schooled around the circumference of the stimulus compartments providing continuous visual and chemical stimuli to the test individuals.

**Experiments 1, 2 and 3**

When reared with siblings only, test animals in early developmental stages showed a preference to associate in the portion of the test tank nearest siblings, whereas those in later developmental stages or those reared in mixed rearing regimes did not (Table 1). The number of tadpoles that spent most of their time on the sibling portion of the test tank was significantly different from random (experiment 1). *Rana aurora* tadpoles that were reared with siblings, but tested in later developmental stages, did not show a preference for either side of the test tank (experiment 3). The number of animals spending most of their time on the sibling side of the test tank did not differ from random expectation in any replicate or for all replicates combined. Test tadpoles reared in mixed rearing regimes displayed a random association within the test tank.

**Experiments 4, 5 and 6**

The results of experiments 4–6 illustrate that tadpoles can distinguish between kin and non-kin when provided solely with chemical cues (Table II). Moreover, the results of experiment 5 strongly suggest that the chemical cues are olfactory. Test tadpoles in the sham control behaved normally and preferentially spent most of their time nearest kin. Therefore, the application procedures used in experiments 5 and 6 probably did not affect the behaviour of test individuals.

**Experiments 7, 8 and 9**

In experiment 7, test tadpoles at late stages of development did not discriminate between siblings and non-siblings at early stages of development (Table III). In experiment 8, tadpoles at early stages of development did not discriminate between tadpoles at late stages of development (Table III). In experiment 9 (Table III), tadpoles in early developmental stages that were reared under a cold regime associated most often in the sibling portion of the test tank when given a choice between siblings and non-siblings. Thus, the cold regime did not influence the tadpoles' ability to discriminate or to exude chemical cues. Tadpoles can discriminate between siblings and non-siblings at stage 27 (experiment 1) but fail to do so at stage 29 (experiment 3).

**Experiments 10, 11 and 12**

*Rana aurora* tadpoles associated nearest to the stimulus side of the test tank containing conspecifics rather than the side containing no tadpoles (Table IV). These results were obtained when tadpoles were in early stages of development (experiments 10 and 11) and in later stages of development (experiment 12). Moreover, these results were obtained with kin and non-kin as conspecific stimuli. This suggests that *R. aurora* tadpoles are positively attracted to conspecifics regardless of whether they are siblings or non-siblings. Therefore, kin recognition in *R. aurora* tadpoles that are in an early stage of development is probably based on attraction to siblings rather than an aversion to non-siblings.

**DISCUSSION**

Results of the standard tests (experiments 1–3) corroborate the earlier findings of Blaustein & O'Hara (1986a) that *R. aurora* tadpoles can discriminate between kin and non-kin in early developmental stages. Like other tadpoles, chemical cues are used by *R. aurora* to discriminate between kin and non-kin (experiments 4–6; see Blaustein & O'Hara 1982a; Dawson 1982; Waldman 1985a).
<table>
<thead>
<tr>
<th>Test group</th>
<th>Stimuli</th>
<th>Development stage of test tadpoles and stimuli</th>
<th>Number spending majority of time nearest</th>
<th>Binomial P</th>
<th>Mean time spent nearest siblings (SE)</th>
<th>Mean ratio of time spent nearest siblings (SE)</th>
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<tr>
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<td></td>
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</tr>
<tr>
<td>Replicate 1</td>
<td>A (Familiar siblings)</td>
<td>26–27</td>
<td>14</td>
<td>6</td>
<td>ns*</td>
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<td></td>
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<td>(18.3)</td>
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<td>26–27</td>
<td>18</td>
<td>2</td>
<td>&lt;0.001</td>
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<td>(41.8)</td>
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<tr>
<td>Replicate 1</td>
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<td>14</td>
<td>6</td>
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<td>631.2</td>
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<tr>
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<td>Bₐ† (Unfamiliar siblings)</td>
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<td>13</td>
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<td>565.4</td>
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<td><strong>Experiment 3</strong></td>
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<tr>
<td>Replicate 1</td>
<td>A (Familiar siblings)</td>
<td>29–30</td>
<td>11</td>
<td>9</td>
<td>ns</td>
<td>610.8</td>
</tr>
<tr>
<td></td>
<td>B (Unfamiliar non-siblings)</td>
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<td>9</td>
<td>11</td>
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<td>29–30</td>
<td>12</td>
<td>8</td>
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<td>680.9</td>
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<td>B (Unfamiliar non-siblings)</td>
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*P < 0.0001 for the combined binomial for replicates 1–3 of experiment 1.

†Test animals were reared with siblings and non-siblings (mixed rearing regime).
Table II. Results of chemical cue tests

<table>
<thead>
<tr>
<th>Test group</th>
<th>Stimuli</th>
<th>Developmental stage of test tadpoles and stimuli</th>
<th>Number of tadpoles spending time nearest</th>
<th>Bitomial P</th>
<th>Mean time spent nearest siblings (st)</th>
<th>Mean ratio of time spent nearest siblings (st)</th>
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<tr>
<td>Experiment 4</td>
<td>X (Familiar siblings)</td>
<td>26–27</td>
<td>16</td>
<td>4</td>
<td>0·006</td>
<td>729·6 (46·5)</td>
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<td>Chemical cue tests</td>
<td>R (Unfamiliar non-siblings)</td>
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<td></td>
<td></td>
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<td>2·68 (0·74)</td>
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<tr>
<td>Experiment 5</td>
<td>X (Familiar siblings)</td>
<td>26–27</td>
<td>8</td>
<td>12</td>
<td>NS</td>
<td>560·7 (42·6)</td>
</tr>
<tr>
<td>Test tadpoles</td>
<td>X (Familiar siblings)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1·08 (0·168)</td>
</tr>
<tr>
<td>with Orabase</td>
<td>R (Unfamiliar non-siblings)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Experiment 6</td>
<td>X (Familiar siblings)</td>
<td>26–27</td>
<td>17</td>
<td>3</td>
<td>0·001</td>
<td>764·2 (44·2)</td>
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<tr>
<td>Sham controls for Orabase</td>
<td>R (Unfamiliar non-siblings)</td>
<td></td>
<td></td>
<td></td>
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<td>3·52 (1·21)</td>
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### Table III. Results of signal/perception tests

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<tr>
<th>Test group</th>
<th>Developmental stage of test group</th>
<th>Developmental stage of stimulus group and stimuli choices</th>
<th>Number spending majority of time nearest</th>
<th>Binomial P</th>
<th>Mean time spent nearest siblings (se)</th>
<th>Mean ratio of time nearest siblings (se)</th>
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<tr>
<td>Replicate 1</td>
<td>E</td>
<td>39–40</td>
<td>26–27 E (Familiar siblings) G (Unfamiliar non-siblings)</td>
<td>12 Siblings 8 Non-siblings NS</td>
<td>643:8 (21:4)</td>
<td>1:22 (0:094)</td>
</tr>
<tr>
<td>Replicate 2</td>
<td>G</td>
<td>38–41</td>
<td>26–27 G (Familiar siblings) E (Unfamiliar non-siblings)</td>
<td>11 Siblings 9 Non-siblings NS</td>
<td>604:2 (14:8)</td>
<td>1:04 (0:050)</td>
</tr>
<tr>
<td>Replicate 3</td>
<td>H</td>
<td>37–38</td>
<td>26–27 H (Familiar siblings) E (Unfamiliar non-siblings)</td>
<td>12 Siblings 8 Non-siblings NS</td>
<td>585:1 (40:5)</td>
<td>1:14 (0:163)</td>
</tr>
<tr>
<td><strong>Experiment 8</strong></td>
<td></td>
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</tr>
<tr>
<td>Replicate 1</td>
<td>E</td>
<td>26–28</td>
<td>37–41 E (Familiar siblings) G (Unfamiliar non-siblings)</td>
<td>9 Siblings 11 Non-siblings NS</td>
<td>607:0 (26:7)</td>
<td>1:11 (0:099)</td>
</tr>
<tr>
<td>Replicate 2</td>
<td>G</td>
<td>26–27</td>
<td>37–41 G (Familiar siblings) E (Unfamiliar non-siblings)</td>
<td>9 Siblings 11 Non-siblings NS</td>
<td>613:8 (26:5)</td>
<td>1:14 (0:515)</td>
</tr>
<tr>
<td>Replicate 3</td>
<td>H</td>
<td>27–28</td>
<td>37–41 H (Familiar siblings) E (Unfamiliar non-siblings)</td>
<td>12 Siblings 8 Non-siblings NS</td>
<td>621:4 (30:5)</td>
<td>1:19 (0:120)</td>
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<tr>
<td><strong>Experiment 9</strong></td>
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<tr>
<td>Replicate 1</td>
<td>E</td>
<td>26–27</td>
<td>26–27 E (Familiar siblings) G (Unfamiliar non-siblings)</td>
<td>14 Siblings 6 Non-siblings NS*</td>
<td>678:5 (32:9)</td>
<td>1:53 (0:207)</td>
</tr>
<tr>
<td>Replicate 2</td>
<td>G</td>
<td>26–27</td>
<td>26–27 G (Familiar siblings) E (Unfamiliar non-siblings)</td>
<td>16 Siblings 4 Non-siblings =0.006</td>
<td>653:1 (18:9)</td>
<td>1:25 (0:087)</td>
</tr>
<tr>
<td>Replicate 3</td>
<td>H</td>
<td>26–27</td>
<td>26–27 H (Familiar siblings) E (Unfamiliar non-siblings)</td>
<td>15 Siblings 5 Non-siblings =0.021</td>
<td>693:9 (42:2)</td>
<td>3:11 (1:604)</td>
</tr>
</tbody>
</table>

*P < 0.0001 for the combined binomial test for the three cold controls (experiment 9).
<table>
<thead>
<tr>
<th>Test group</th>
<th>Developmental stage of test/stimulus group and stimuli choices</th>
<th>Number spending majority of time nearest</th>
<th>Binomial ( P )</th>
<th>Mean time spent nearest siblings (SE)</th>
<th>Mean ratio of time spent nearest conspecifics (SE)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Experiment 10</strong></td>
<td></td>
<td></td>
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<tr>
<td>Replicate 1</td>
<td>E</td>
<td>27–28&lt;br&gt;E (Familiar siblings)&lt;br&gt;+ empty compartment</td>
<td>13&lt;br&gt;6*</td>
<td>ns†</td>
<td>687.6&lt;br&gt;(42.5)</td>
</tr>
<tr>
<td>Replicate 2</td>
<td>H</td>
<td>27–28&lt;br&gt;H (Familiar siblings)&lt;br&gt;+ empty compartment</td>
<td>17&lt;br&gt;3</td>
<td>=0.001</td>
<td>744.1&lt;br&gt;(43.7)</td>
</tr>
<tr>
<td><strong>Experiment 11</strong></td>
<td></td>
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<tr>
<td>Replicate 1</td>
<td>E</td>
<td>27–28&lt;br&gt;G (Unfamiliar non-siblings)&lt;br&gt;+ empty compartment</td>
<td>13&lt;br&gt;7</td>
<td>ns‡</td>
<td>663.8&lt;br&gt;(45.0)</td>
</tr>
<tr>
<td>Replicate 2</td>
<td>H</td>
<td>27–28&lt;br&gt;F (Unfamiliar non-siblings)&lt;br&gt;+ empty compartment</td>
<td>16&lt;br&gt;4</td>
<td>=0.006</td>
<td>787.0&lt;br&gt;(39.7)</td>
</tr>
<tr>
<td><strong>Experiment 12</strong></td>
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<td></td>
</tr>
<tr>
<td>Replicate 1</td>
<td>H</td>
<td>38–41&lt;br&gt;H (Familiar siblings)&lt;br&gt;+ empty compartment</td>
<td>16&lt;br&gt;4</td>
<td>=0.006</td>
<td>742.1&lt;br&gt;(45.6)</td>
</tr>
<tr>
<td>Replicate 2</td>
<td>H</td>
<td>38–41&lt;br&gt;E (Unfamiliar non-siblings)&lt;br&gt;+ empty compartment</td>
<td>13&lt;br&gt;7</td>
<td>ns§</td>
<td>711.8&lt;br&gt;(53.1)</td>
</tr>
</tbody>
</table>

*One individual spent an equal amount of time on each side of the tank and could not be included in statistical analysis.
†\( P < 0.0005 \) for the combined binomial of replicates 1 and 2 of experiment 10.
‡\( P < 0.003 \) for the combined binomial of replicates 1 and 2 of experiment 11.
§\( P < 0.003 \) for the combined binomial of replicates 1 and 2 of experiment 12.
The chemical recognition system is probably based on olfaction (results of experiments 4–6).

The results of experiments 7 and 8 suggest that both the cue(s) used in distinguishing kin from non-kin and the ability to distinguish kin from non-kin diminished near Gosner’s (1960) developmental stage 28, when the hindlimb bud is equal to or slightly greater than its diameter. Cold rearing regimes did not affect discrimination behaviour of tadpoles. Tadpoles reared in a cold regime and tested in early developmental stages discriminated between kin and non-kin that were also reared in the cold regime and that were also in early developmental stages (experiment 9).

The results of the conspecific association tests (Table IV) are similar to those obtained for *R. cascadae* tadpoles (Blaustein & O’Hara 1987). In tests similar to experiments 10–12 of this paper, *R. cascadae* tadpoles also associated nearest conspecifics over a stimulus compartment containing no tadpoles (Blaustein & O’Hara 1987). Thus, chemosensory attraction, rather than aversion to non-siblings, seems to serve as the basis for kin association in *R. cascadae* and *R. aurora* tadpoles. Conversely, other species display a negative response to non-kin. For example, in a Y-maze apparatus, toad tadpoles, *B. americanus*, preferentially oriented towards blank water in preference to water containing non-siblings (Waldman 1986). Thus, a chemosensory avoidance of non-siblings, rather than an attraction to siblings, appears to be the basis for kin association in *B. americanus*.

**Ontogenetic Changes in Behaviour**

Only a few studies of amphibians have investigated changes in behaviour patterns from early larval stages to later stages and through metamorphosis (e.g. McKeown 1968; Goodyear & Altig 1971; O’Hara 1974; Wassersug & Hessler 1971; Tomson & Ferguson 1972; Walls 1990). Moreover, few studies have documented shifts in kin recognition behaviour from early to later stages of development. In vertebrates, these changes have been documented only in amphibians (Cornell et al. 1989; Waldman 1989; Rautio et al. 1991; Walls 1991; see also Walls & Roudebeush 1991).

Regarding anurans, Waldman (1984) and Cornell et al. (1989) reported that wood frog, *R. sylvatica*, tadpoles can discriminate between kin and non-kin as larvae. However, *R. sylvatica* tadpoles in early stages of development do not show this discrimination behaviour (Rautio et al. 1991). Once kin recognition in *R. sylvatica* develops, it persists throughout the larval stage and for about 1 day after metamorphosis (Cornell et al. 1989) and fades within 4 days after metamorphosis (Waldman 1989). In contrast, *R. cascadae* kin recognition persists for at least 47 days after metamorphosis (Blaustein et al. 1984).

**Kin Recognition Mechanisms and the Ontogeny of Recognition Systems in Anuran Larvae**

The ontogeny of kin recognition differs among the various amphibian species examined (Blaustein 1988; Waldman 1991; Blaustein & Waldman 1992). In several species, the development of kin recognition is significantly influenced by the rearing regime. For example, American toad, *B. americanus*, tadpoles reared with siblings or in social isolation associated preferentially with siblings over non-siblings in laboratory studies (Waldman 1981). Tadpoles reared with siblings in early development and then exposed to siblings later in development showed similar tendencies to associate with siblings over non-siblings at later developmental stages. However, tadpoles reared with both siblings and non-siblings in early development and then exposed to siblings only in later development, failed to associate preferentially with familiar siblings over familiar non-siblings (Waldman 1981). These results suggest the existence of a sensitive period early in development during which *B. americanus* tadpoles familiarize themselves with other individuals (Waldman 1981).

The ontogeny of *B. boreas* tadpoles is also significantly influenced by the rearing regime. However, unlike *B. americanus*, their kin recognition abilities do not seem to be established during an early sensitive period. *Bufo boreas* tadpoles reared with siblings preferentially associated with siblings over non-siblings in laboratory choice tests (O’Hara & Blaustein 1982). However, individuals reared in mixed rearing regimes, or with non-kin only, displayed a random association with respect to siblings and non-siblings. Even when preferences were fully established after prolonged rearing with siblings, short-term exposure to non-siblings nullified these preferences (O’Hara & Blaustein 1982).

The development of kin recognition in *R. aurora* tadpoles is similar to that of *B. boreas* tadpoles. Like *B. boreas* tadpoles *R. aurora* tadpoles only discriminate between siblings and non-siblings if they have not been in contact with non-siblings (Blaustein &
O’Hara 1986a). Previous tests (Blaustein & O’Hara 1986a) of *R. aurora* tadpoles suggest that each tadpole kin group has a unique chemical signature that is retained when each kin group is reared by itself. However, when tadpoles are reared in mixed sibships, individuals seem to develop a composite ‘odour’ composed of the odour of each sibship because test tadpoles taken from mixed rearing tanks failed to discriminate between familiar siblings and familiar non-siblings. This chemical signal convergence masks the unique signatures of the individual kin groups (see discussion of the ‘Gestalt Model’ by Crozier & Dix 1979 and detailed discussion of the odour transference process in amphibians by Waldman 1985b).

Thus, in some anuran larvae such as *R. aurora*, *B. boreas* and *B. americanus*, there is a significant ‘familiarity’ component incorporated into the recognition system. Larvae of other anuran amphibian species discriminate between kin and non-kin throughout larval ontogeny whether or not they have been in contact with non-kin during development (see reviews by Blaustein 1988; Waldman 1991; Blaustein & Waldman 1992).

**Sensory Basis of Recognition**

Visual, auditory and chemical cues are used in vertebrate kin recognition (discussed in Blaustein et al. 1987b; Halpin 1991; Waldman 1991). More than one signal may be involved in the recognition system, a primary signal that enables an animal to orient towards a conspecific group or individual and a secondary signal that may allow an animal to fine tune its primary signal so that it can discriminate between classes of animals such as between kin and non-kin (Blaustein et al. 1987b).

For example, in aquatic species, both fish and anuran larvae may use the lateral line to orient towards and swim with conspecifics (Partridge & Pitcher 1980; Katz et al. 1981; Partridge 1982; Bleckmann 1986). Laboratory experiments of anuran larvae have shown that visual cues may be used as a primary signal for orientation and schooling (Wassersug & Hessler 1971; Wassersug 1973; O’Hara 1981; Foster & McDermid 1982). However, in fish and amphibian larvae, the more fine-tuned ability to discriminate between parents and offspring and between kin and non-kin appears to be mediated largely by chemical cues (e.g. McKay & Barlow 1976; Blaustein & O’Hara 1982a; Quinn & Busack 1985; Waldman 1985a; Olsen 1989).

These cues may be fixed or variable, depending upon the species (Blaustein et al. 1987b). In tadpoles, sound production appears to be unimportant in kin discrimination (Blaustein & O’Hara 1982a). Chemical cues are the basis for kin recognition in anuran larvae (*R. cascadae*, Blaustein & O’Hara 1982a; *B. americanus*, Dawson 1982; Waldman 1985a). In at least one species, *B. americanus*, visual cues may actually hamper the recognition process (Dawson 1982). In choice tests, *B. americanus* discriminated between siblings and non-siblings when the stimuli were chemical cues alone (Dawson 1982). But when tadpoles were provided with visual and chemical cues, they did not discriminate between siblings and non-siblings. This visual effect was not observed in *R. aurora* tadpoles in the present study. *Rana aurora* tadpoles can discriminate between siblings and non-siblings when provided with chemical cues alone or when they are given in conjunction with visual cues.

**The Ontogenetic Shift**

The abrupt shift in kin recognition behaviour in *R. aurora* tadpoles is difficult to explain. From a proximate point of view, an explanation may be found in the neuroanatomical and physiological changes that occur as larvae grow and develop. In general, the olfactory system in tadpoles becomes functional before the other sensory systems (e.g. Zwilling 1940; Khalil 1978; Spaeti 1978; Waldman 1985a). In some species it is the only functional sensory system immediately after hatching (Spaeti 1978).

The anatomical and physiological changes occurring as tadpoles develop may help us understand the functional significance of the change in kin recognition behaviour. Based on experiments reported in this paper, it is likely that *R. aurora* tadpoles ‘prefer’ to aggregate with conspecifics. Young larvae associate preferentially with siblings over non-siblings. After stage 28, tadpoles do not discriminate between siblings and non-siblings but still prefer to associate with conspecifics as stimuli over a stimulus with no tadpoles. It is possible that the benefits of being in a group are the overriding selective pressure for *R. aurora* tadpoles to join aggregations with conspecifics rather than with kin per se (see discussions in Waldman 1982; Blaustein et al. 1987a).

Sibling association may occur in the early larval stages because tadpoles are using their chemosensory system for aggregating, which is the only intact
sensory system in early stages. Tadpoles may form a template for those individuals that they are most similar with based on chemical cues (see discussion in O'Hara & Blaustein 1982; Blaustein et al. 1987b; Waldman 1991). If there is a strong genetic component to the recognition signature, then tadpoles will presumably associate most often with kin (see Waldman 1985b). There may also be a strong environmental component to the recognition signature. For example, some tadpole species may associate with individuals that have eaten the same types of food and thus their metabolic byproducts may permeate the environment with a 'familiar' food component that is identified by the tadpoles as they aggregate (Pfennig 1990; Gamboa et al. 1991).

Kin Recognition or Species Recognition?

For some species, kin recognition and kin association may be more easily explained as species recognition and association with conspecifics. Using data obtained from studies of tadpoles, Grafen (1990) suggested that a species recognition mechanism would work well, if, for example, all members of the species had essentially the same smell and some genetic variation in the smell. Then, individuals will 'acquire a standard (the smell of themselves) which is slightly more like relatives than it is like conspecifics in general' (page 44). Thus, when a species uses its species recognition capability to join a tadpole aggregation, it will be biased to join a more related group because such a group is perceived as being closer to the 'acquired standard' (as discussed in Blaustein et al. 1991).

O'Hara & Blaustein (1982) suggested that species recognition may be a parsimonious explanation for kin association in laboratory tests of toad tadpoles (B. boreas). They suggested that B. boreas may use the familiar cues emanating from conspecifics to seek optimal habitats or to aggregate with conspecifics to obtain the benefits of group living (see also discussion in Blaustein et al. 1990).

Although little is known about the larval ecology of R. aurora, there is no evidence that larval R. aurora form prolonged aggregations with conspecifics in nature. In some ponds, R. aurora tadpoles may disperse far from their sites of oviposition and mix freely with members of other kinships (see Calef 1973; Blaustein 1988). As discussed above, contact with non-kin during development causes R. aurora tadpoles to lose their preferences to associate with kin even in early larval stages (Blaustein & O'Hara 1986a). Therefore, it is unlikely that kin association in R. aurora tadpoles could be maintained unless they do not interact with non-kin in nature.

We cannot rule out the possibility that kin association and kin recognition could have been more important to R. aurora tadpoles in the evolutionary past, under different social and environmental conditions. A remnant of this behaviour may still exist in R. aurora that may enable larvae to discriminate between kin and non-kin under present environmental regimes. This may be especially possible if a single clutch is deposited in one area of a pond or a few clutches are deposited in distinct habitats such that some segregation between clutches is maintained.

Moreover, kin recognition and kin association seem to be context dependent (Beecher 1988, 1991; Blaustein et al. 1987b; Waldman 1991). For example, Beecher (1988, 1991) argued that discrimination is not always beneficial for both the discriminator and the recipient of the discrimination and therefore may not be manifested. Recognition 'failures' may reflect conservative decision making rather than perceptual inability (Beecher 1991). Thus, the discriminator may not initiate a recognition response unless there are benefits to doing so. In some species, the signature to be recognized may not be displayed by the signature holder unless there are benefits to being recognized (see discussions in Beecher 1988, 1991). It is also possible that under certain experimental conditions, animals may not be 'motivated' or behave in a way that reveals to researchers their ability to distinguish among kinship levels (Blaustein et al. 1987b). Finally, with regard to R. aurora tadpoles, it is possible that kin association and kin recognition are of selective value only during the early larval stages.

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