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Associative learning in hatchlings of the lizard *Calotes versicolor*: taste and colour discrimination

Bhagyashri A. Shanbhag*, Veena H.F. Ammanna, Srinivas K. Saidapur

Abstract. The study examined whether newly born hatchlings of *Calotes versicolor* discriminate between tastes and exhibit associative learning. The one-day-old hatchlings ate 2nd instar silk-moth larvae (prey) placed in non-painted, orange or green dishes without any bias for the background colour. They ate sucrose coated sweetened prey, but given a choice preferred natural larvae. But they spat bitter tasting chloroquine phosphate (CP)-coated prey and exhibited aversion behaviour. Hatchlings fed on natural larvae from non-painted or orange dishes for 10 days preferred food from the dish to which they were accustomed when choice of both colour backgrounds was given suggesting they also remember different tastes in association with background colour cues. Upon swapping sucrose and CP-coated prey/dish colours the hatchlings were misled and they attempted to eat from the dishes to which they were accustomed prior to the trials regardless of whether food/prey was coated with sucrose or CP. However, bitter prey was immediately spat and aversion behaviour followed. The study shows for the first time, taste discrimination and associative learning behaviour in new-born lizard hatchlings.

Keywords: association, background cues, behaviour, hatchling, lizard, prey, taste aversion.

Introduction

Both plants and animals evolve strategies like incorporation of toxic/noxious chemicals and rendering themselves distasteful and unpalatable to discourage their consumption by animals. Some animal species may evolve aposematic warning colours and patterns (seen in distasteful species) to dissuade predators from eating them (Harvey et al., 1982). Therefore, predators also develop abilities to select proper food items and avoid distasteful/harmful ones. This calls for learning behaviour. Animals may learn to select or avoid certain foods based on the visual, olfactory or gustatory stimuli arising from food source. Among amniotes, learning behaviour to avoid unpalatable food is well documented in birds and mammals. For instance, avian species rapidly associate a colour stimulus with palatability of food (Wilcoxon, Dragoín and Kral, 1971; Roper and Marples, 1977). Frugivorous monkeys associate olfactory cues of food with its palatability (Clutton Brock, 1977; Laska and Metzker, 1998). Similarly, squirrel monkeys and marmosets associate unpalatable food with colour and shape (Laska and Metzker, 1998). Learning is also reported in a few chelonians and lizards. Studies on chelonians, *Pseudemys scripta elegans* (Boycott and Guillery, 1962), *Chrysemys picta picta* (Kirk and Bitterman, 1965) and *Chelydra serpentina* (Burghardt and Hess, 1966) have shown that they can be trained to respond differentially to olfactory and visual stimuli. Lizards are also known to use visual and/or chemical cues to locate and identify food (Benes, 1969; Schall, 2000; Stanger-Hall et al., 2001). The whiptailed lizard, *Aspidoscelis* (formerly *Cnemidophorus*) *tigris* is known to discriminate between red and green colour and could be trained to associate food with reward or shock (Benes, 1969), indicating associative learning in these lizards. Similarly, *Anolis cristatellus* respond to instrumental conditioning in the field with food as reward (Shafir and Roughgarden, 1994). Furthermore, they were able to modify their foraging behaviour based on their previous ingestion of food (Shafir and Roughgarden, 1994). The ability to learn and reverse the task using position cues rather than feature cues like colour/texture is
reported in *Cnemidophorus inomatus* (Day, Ismail and Wilczynski, 2003). Only one study on *Cnemidophorus marinus* showed that they learn to distinguish between palatable and unpalatable food based on colour and location as cues (Schall, 2000). However, it is not known whether recently emerged hatchlings of lizards possess the ability to associate colour with taste of food, vis-à-vis, associative learning.

The agamid *Calotes versicolor* is a good model to determine whether it exhibits associative learning for the discrimination of colour and food taste as it has good vision, and also taste buds (Paranjape, 1974). However, it is not known whether it can discriminate between food of different taste and also associate taste through visual cues. The present study was undertaken to determine whether hatchlings of *C. versicolor* (1) discriminate between natural, bitter and sweet tastes, (2) learn to associate food with colours and (3) avoid distasteful food based on background colours vis-à-vis associative learning.

**Materials and methods**

The hatchlings of *C. versicolor* were obtained by incubating eggs as described earlier (Radder, Shanbhag and Saldapur, 2002) and hence were captive-born and naive for any exposure. They were fed with 2nd instar silk moth larvae (*Bombyx mori*), henceforth referred simply as larvae/prey. Glass petri dishes (60 mm diam.) were used to provide the prey to test hatchlings. The petri dishes used were both normal transparent ones (non-painted) and those painted orange or green translucent colour at their rim and bottom. Hereafter, these are referred as non-painted, orange and green dishes, as painted dishes to know whether they show preference or aversion for the colours used. Hatchlings (*n = 8*) were provided larvae in non-painted as well as painted dishes to know whether they exhibit preference or aversion for the colours used. Hatchlings (*n = 30*) in an orange dish. No competition or fights for food were observed among the hatchlings housed in the tank during pre-trial period. After they were accustomed to feed from orange dishes for 10 days, the hatchlings were used for the following trials to determine whether they exhibit preference to food in orange dish. The test tank used for the trials was identical to that used for rearing the hatchlings. For each trial fresh larvae were provided. Twelve trials were conducted for each kind of test described below using one test hatchling at a time:

**Experiment 1 — Tests to elucidate taste discrimination behaviour**

In this experiment, we made the food taste bitter by coating the larvae with Chloroquine phosphate (CP, Bayer's pharmaceutical, India) or sweet by coating with sucrose (Qualigens, India). 125 mg of CP/sucrose paste was prepared separately using 0.2 ml of water. Using a fine paint brush ~5 mg of paste was applied to each larva on all sides. The non-painted, orange and green dishes were presented to the hatchlings during the trials in an alternate manner. One-day old hatchlings (*n = 6*) of *C. versicolor* randomly chosen from 2 parental lines were maintained in glass tanks (600 × 300 × 300 mm) and fed natural larvae for a day. On the subsequent day, they were presented with sweet tasting larvae. On the 3rd day, they were presented with bitter-tasting larvae. On all occasions (day 1-3) larvae were presented in non-painted dishes. Feeding behaviour of hatchlings was recorded.

**Experiment 2 — Tests to elucidate associative learning behaviour**

In this experiment, hatchlings of *C. versicolor* were trained to feed on natural tasting larvae from orange dishes to test later whether they associate food with orange colour or feed from other colour dishes also. One-day-old hatchlings (*n = 12*) were randomly chosen from 12 parental lines and maintained in glass tank (600 × 300 × 300 mm) in groups of six. The floor of the terrarium was covered with a sand layer about 10 mm thick. They were provided larvae (*n = 30*) in an orange dish. No competition or fights for food were observed among the hatchlings housed in the tank during pre-trial period. After they were accustomed to feed from orange dishes for 10 days, the hatchlings were used for the following trials to determine whether they exhibit preference to food in orange dish. The test tank used for the trials was identical to that used for rearing the hatchlings. For each trial fresh larvae were provided. Twelve trials were conducted for each kind of test described below using one test hatchling at a time:

**Test 1** — To elucidate foraging choice of hatchlings with respect to orange and non-painted dishes.

**Test II** — To elucidate foraging choice of hatchlings with respect to orange and green dishes.

**Test III** — To elucidate foraging choice of hatchlings with respect to non-painted, orange and green dishes.

**Testing protocol** - In tests I and II each dish contained 3 larvae, while in test III, each dish housed 2 larvae. The dishes with larvae were placed in the centre along the length of one side of the test tank. The distance between the dishes was 20 mm. A test hatchling (SVL = 22.95 ± 0.26 mm) was then released on the opposite side of the test tank at a distance of ~230 mm from the dishes containing food. The experimenter sat 1 m away from the test tank and made observations through glass wall of the tank without any disturbance to the test hatchling. Feeding behaviour and the number of larvae consumed by a test subject from a given dish was recorded for 30 min. The position of dishes...
with different colours was interchanged between the trials to rule out bias for the position of the dishes. Each test was conducted with an interval 10 min from previous test and a fresh tank was used in each trial. The data were analyzed using Chi-square test under the null hypothesis that frequency of hatchlings feeding larvae from different dishes would be the same and considered significant if \( P < 0.05 \). The data on number of larvae consumed from different dishes were analyzed by Chi-square test of equality of proportions.

**Experiment 3 - Tests to elucidate feeding in relation to associative learning**

One-day-old *C. versicolor* hatchlings randomly chosen from 18 parental lines \( (n = 36) \) were maintained in glass tanks \( (600 \times 300 \times 300 \text{ mm}) \) in groups of five or six. They were provided natural larvae \( (25-30/day) \) in a non-painted dish so as to accustom them to eat from these dishes for 10 d unlike those in experiment 2 in which hatchlings were trained to feed natural larvae from orange dish.

To determine whether the hatchlings presented with larvae with different tastes in a chosen combination of dish colour can associate with food taste, following trials were conducted on day 11-14. The larvae \( (n = 6) \) coated with CP or sucrose were used as food in each trial. A total of \( 18 \times 2 \) trials were conducted for each type of taste. The number of larvae eaten or spit-out during a trial period was recorded. Behaviours such as tongue flicks and rubbing of snout against the wall of the dish were also recorded.

Trials with natural larvae. The hatchlings were given normal larvae (water-coated) placed in non-painted dish. The tests were carried out on two consecutive days.

Trials with sucrose-coated larvae. The trials were run on two consecutive days using larvae coated with sucrose and placed in orange dish to know whether the hatchlings remember the sweet taste even on the second day and feed on them.

Trials with CP-coated larvae. In these trials, the larvae coated with CP were placed in green dish.

The trials were run on two consecutive days to know whether on the second day test hatchlings remember bitter taste and avoid feeding on them. The hatchlings used in these trials also served as the test hatchlings for experiment 4 described below.

Data on the number of larvae consumed with a particular taste on day 1 and 2 by the hatchlings were analyzed by Student’s paired 't' test. Since the same hatchlings were provided larvae with 3 tastes on 3 different days, Cochran's 'Q' test was used to know whether there was a significant difference in the number of hatchlings that fed on larvae with different tastes. In order to know the hatchlings' choice of food (number of larvae eaten with each taste), repeated measures of ANOVA followed by least significance difference (LSD) test as a measure of post-hoc test were used.

**Experiment 4 - Tests to validate feeding in relation to associative learning**

In this experiment, two sets of tests were conducted. In the first set, hatchlings \( (n = 36) \) used in experiment 3, i.e., hatchlings that briefly experienced sweet or bitter larvae from orange and green dishes were used. The design involved presenting test hatchlings simultaneously with natural, sweet and bitter larvae \( (2/day) \) placed in non-painted, orange and green dishes and record their choice of food taste, dish colour and associative learning behaviour.

In the second set of experiments, conducted in the subsequent year, test hatchlings with similar training of feeding regimes as in the first set were used \( (n = 22; 5 \text{ clutches}) \). In these trials, larvae with a given taste (bitter or sweet) were not presented in the same coloured dish to which they were accustomed earlier to validate the results of first set of experiments. The test hatchlings were presented with bitter larvae in non-painted and natural larvae in orange dishes and sweet larvae in green dishes to know their ability to associate taste with colour of the dish.

In each trial of 30 min, number of larvae consumed by the test subjects from each container was recorded. The data were analyzed using Chi-square test to determine whether the test hatchlings preferentially fed or avoided the food in response to the colour of the dish, i.e., association of taste with the background colour. The number of larvae consumed was analyzed by Chi-square test of equality of proportions.

**Results**

**Experiment 1 - Tests to elucidate taste discrimination behaviour**

All hatchlings ingested natural and sucrose coated larvae without showing any sign of aversion, for latter type of prey. In contrast, when CP-coated larvae were provided the hatchlings \( (n = 6) \) took them inside the mouth and spat. This was followed by tongue flicking and rubbing the mouth to the rim or wall of the dish.

**Experiment 2 - Tests to elucidate associative learning behaviour**

In these trials, hatchlings accustomed to feed on larvae from orange dishes were used.

During test trials, most hatchlings preferred food from the orange dish rather than from the non-painted dish (table 1). In fact, they immediately moved to orange dish after releasing in the test tank. Similarly, when larvae were
Table 1. Feeding preference of *C. versicolor* hatchlings following their training to prey upon normal silk-moth larvae placed in orange dish for 10 days from hatching and given choice of different color background combination (*n* = 12 per test, hatchlings derived from different parental lines).

<table>
<thead>
<tr>
<th>Test</th>
<th>Choice between dish color</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Orange</td>
<td>Non-painted</td>
<td>Orange</td>
<td>Green</td>
</tr>
<tr>
<td>No. of hatchlings</td>
<td>10</td>
<td>2</td>
<td>11</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>($\chi^2 = 5.32; P &lt; 0.05)^a$</td>
<td>($\chi^2 = 8.33; P &lt; 0.001)^a$</td>
<td>($\chi^2 = 9.50; P &lt; 0.001)^a$</td>
<td></td>
</tr>
<tr>
<td>No. of larvae ingested</td>
<td>17</td>
<td>2</td>
<td>17</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>($\chi^2 = 10.41; P &lt; 0.001)^b$</td>
<td>($\chi^2 = 10.41; P &lt; 0.001)^b$</td>
<td>($\chi^2 = 22.47; P &lt; 0.001)^b$</td>
<td></td>
</tr>
</tbody>
</table>

Data analyzed by *a*Chi-square test; *b*Chi-square test of equality of proportions. In tests I and II, each dish contained 3 larvae each, while in test III each dish had 2 larvae each.

Table 2. Larvae ingested and regurgitated by *C. versicolor* hatchlings (*n* = 36) following their training for 10 days from hatching to prey upon normal silk-moth larvae placed in non-painted dish and subsequently provided natural larvae in non-painted dish, sweet larvae in orange dish and bitter larvae in green dish (experiment 2).

<table>
<thead>
<tr>
<th>Petri dish color and taste of prey</th>
<th>Day 1</th>
<th>Day 2</th>
<th>Paired <em>t</em> test</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Ingested larvae</td>
<td>Regurgitated larvae</td>
<td>Ingested larvae</td>
</tr>
<tr>
<td></td>
<td>No. of hatchlings</td>
<td>No. of larvae ingested</td>
<td>No. of hatchlings</td>
</tr>
<tr>
<td>Normal larvae in non-painted dish</td>
<td>36</td>
<td>0</td>
<td>80</td>
</tr>
<tr>
<td>Sucrose coated larvae in orange dish</td>
<td>35</td>
<td>0</td>
<td>59</td>
</tr>
<tr>
<td>CP-coated larvae in green dish</td>
<td>8</td>
<td>28</td>
<td>8</td>
</tr>
</tbody>
</table>

*Q* = 54.07, *F*$_{2,35}$ = 515.6, *Q* = 70, *F*$_{2,35}$ = 653.7. 

Provided in orange and green dishes, significantly more hatchlings fed on larvae from orange rather than from green dish (table 1). In experiments with all the three background colour choices, a greater number of hatchlings fed on larvae from the orange dish compared to those from green or non-painted dishes (table 1). During the trial period of 30-min, hatchlings did not switch to other dishes after ingesting larvae from a dish. The test hatchlings trained previously to feed from orange dishes showed clear preference for food placed in orange dishes when given a choice of food in non-painted or green dishes (table 1) vis-à-vis association of food with dish colour. The lizard hatchlings thus chose food by associating it with dish colour.

Experiment 3 – Tests to elucidate feeding in relation to associative learning

In trials with natural larvae provided in non-painted dishes, all the test hatchlings fed at a more or less uniform rate (2 larvae/hatchling) in the 30-min trial period. Further, the feeding rate of hatchlings was comparable on day 1 and 2 (table 2).
As many as 35 out of 36 hatchlings fed on the sucrose coated larvae placed in orange dish on day 1 of the trial and all 36 on the day 2. The number of larvae consumed on the two consecutive days was also similar (table 2).

In trials with CP-coated larvae, as few as 8 (22%) hatchlings ate bitter larvae on day 1 and the remaining 28 (88%) took the prey inside the mouth but then spat them out. On day 2, only one hatchling ingested one bitter larva while 3 hatchlings took them inside the mouth but spat and quickly engaged themselves in tongue flicking and rubbing their mouth to the rim or wall of the dish. The remaining 89% hatchlings (32 out of 36) did not even go near food. Total number of bitter larvae consumed on day 1 and day 2 differed significantly (table 2). On day 1 of trial, 78% hatchlings exhibited aversion to bitter prey and as many as 97% on day 2.

Cochran’s $Q$ test revealed that there was a significant difference in the number of hatchlings feeding on larvae with different tastes, both on day 1 and 2 (table 2). The number of hatchlings feeding on natural and sweet larvae was similar but very few hatchlings fed on bitter larvae ($P < 0.05$). Further, on day 1, 78% of the hatchlings swallowed and regurgitated bitter larvae, while on day 2, majority of hatchlings did not go near the green dish; only 8% of individuals swallowed and regurgitated bitter larvae (placed in green dish). There was a significant difference in the number of natural, sweet and bitter larvae ingested by test hatchlings on day 1 and also on day 2 (table 2). In general, the test hatchlings consumed maximum number of natural larvae followed by sweet larvae while they clearly avoided bitter tasting prey.

### Experiment 4 – Tests to validate feeding in relation to associative learning

The test hatchlings accustomed to feed on natural larvae for 10 days from non-painted dishes and subsequently fed on sweet larvae from orange dish or bitter larvae from green dish for 2 days were used in this experiment. In the first set of trials, 33 (92%) individuals ingested food from non-painted dish containing natural larvae, 3 ate from the orange dish containing sweet larvae while none ate from green dish containing bitter larvae (table 3). In the second set of trials ($n = 22$), when dish colour and larval taste were swapped with one another, 73% hatchlings ($n = 16$ out of 22) swallowed and regurgitated bitter larvae placed in non-painted dish, while 4 hatchlings fed natural larvae from orange dish and 2 hatchlings fed sweet larvae from green dish (table 4). The change in background colour thus confused test hatchlings in their selection of prey.

### Discussion

Avoidance of noxious/distasteful food is an important component of foraging in animals in general. However, this can be accomplished only by gustatory stimuli through taste buds on the tongue. Among several species of lizards, including $C$. versicolor, the occurrence of taste buds on the tongue is reported (Paranjape, 1974; Schwenk, 1985). In the present study, the hatchlings that ingested prey either from green dish or orange dish were significantly different ($P < 0.001$) (table 3).

<table>
<thead>
<tr>
<th>Number of Hatchlings that ingested prey</th>
<th>Dish color and prey taste</th>
<th>$\chi^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Natural + Non-painted</td>
<td>Sweet + Orange</td>
</tr>
<tr>
<td>Hatchlings that ingested prey(\text{*})</td>
<td>33</td>
<td>3</td>
</tr>
<tr>
<td>Prey ingested(\text{\text{*}})</td>
<td>56</td>
<td>6</td>
</tr>
</tbody>
</table>

*Data analyzed by: \(\text{*}\)Chi-square test; \(\text{\text{*}}\)Chi-square test for equality of proportions.
liable 4. Food ingestion in C. versicolor hatchlings with respect to color of dish and taste of larvae following their training to feed upon natural larvae from non-painted dish for 10 days and brief experience of feeding on sweet tasting larvae from orange dish and bitter larvae from green dish (experiment 4). Note that there is swapping of dish color and taste of larvae during trials.

<table>
<thead>
<tr>
<th>Number Dish color and prey taste</th>
<th>Natural + Non-painted</th>
<th>Sweet + Orange</th>
<th>Bitter + Green</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hatchlings that ate or regurgitated* prey</td>
<td>16*</td>
<td>4</td>
<td>2</td>
</tr>
<tr>
<td>Larvae ingested or regurgitated*</td>
<td>16*</td>
<td>7</td>
<td>2</td>
</tr>
</tbody>
</table>

\[ x^2 = 15.64, P < 0.001^a \]

\[ x^2 = 27.01, P < 0.001^b \]

Data analyzed by: *Chi-square test; \^{b} Chi-square test for equality of proportions.

lings of C. versicolor selectively fed on natural larvae, and almost to a comparable extent on sweet larvae but literally avoided the bitter ones. Their dislike for bitter taste was evident from the fact that they spat out bitter prey and engaged in tongue flicking and jaw rubbing to the walls of the dish in an attempt to rid of bitter taste. Therefore, C. versicolor hatchlings use taste buds to discriminate between food tastes. The behavioural observations on C. versicolor hatchlings to that reported in whiptail lizards (Cnemidophorus spp.). These lizards upon ingesting a lady bird beetle, quickly spit it out (Burt, 1938 cited in Benes, 1969). Then they were seen rubbing their jaws on the ground as if trying to remove something unpleasant. They were also seen tasting toadlets with their tongue and then rejecting them as food. Ingestion and then spitting of meloid beetles is observed in the lizard, Cnemidophorus sexlineatus (Hardy, 1962 cited in Benes, 1969). Anolis carolinensis preferred crickets treated with dextrose but avoided those treated with QHCl (Stanger-Hall et al., 2001).

In the present study naïve hatchlings of C. versicolor accustomed to eat silk moth larvae since hatching from an orange dish preferred to eat from such dishes when given choice from non-painted or green dishes containing the same food. Also, the hatchlings trained to eat food from a non-painted dish from hatching preferred to eat from non-painted dish rather than from orange or green. These observations rule out that hatchlings of C. versicolor have an innate bias for any particular colour. That the lizard hatchlings associated food with colour suggests their ability not only for training but also for associative learning. If hatchlings could associate the background colour with food they may use colour cues in the selection of food in their natural environment as well. The present findings are in conformity with those reported for adult lizards (Benes, 1969; Schall, 2000; Stanger-Hall et al., 2001) that can be trained to choose food based on background colour. Moreover, C. versicolor hatchlings can be trained, as they can learn rapidly to locate food using colour cues.

The present study also showed that C. versicolor hatchlings learn very rapidly to avoid bitter larvae and remembered bitter taste even after a lapse of 2 days. Further, they exhibit the ability for learning to avoid novel distasteful food in association with background colour. However, it must be noted that selection of natural tasting prey or avoidance of noxious prey by the C. versicolor hatchlings was chiefly based on the background colour used to present the prey. This view is strengthened by the fact that when natural tasting prey was provided in green dish, except 2 out of 22 hatchlings avoided them while most others (16 out of 22) were misled resulting in biting the bitter larvae placed in non-painted dish. However, bitter larvae were spat out quickly. Thus, in these experiments, hatchlings were fooled following placement of food in favourable background colours. Evidently, in nature, perception of colour in association with taste is vital in the evolution of foraging strat-
Associative learning in hatchlings of the lizard Calotes versicolor

egy and also aposematism. As A.R. Wallace stated "distastefulness alone would be insufficient to protect a caterpillar unless some outward sign indicated to its would-be destroyer that its prey was a disgusting morsel" (Darwin, 1871, p. 416). However, little is known about how predators avoid distasteful prey. One possible way is to learn through experience and remember it. The hatchlings of C. versicolor did exactly this. This strategy is reflected in the form of associative learning; naive hatchlings could associate colour of the dishes (background) with the larvae (prey, food) and use colour cues in selection or avoidance of food in the laboratory tests. The same could happen in their natural surroundings. It is known that predators often learn to avoid unsuitable prey by visual cues.

In summary, the present study shows for the first time the associative learning behaviour in new-born hatchlings of lizards by choosing palatable or rejecting distasteful foods in association with the background colour based on their prior experiences. The present study also shows that hatchlings of C. versicolor can distinguish between the colours and have a sense of taste and exhibit aversion for bitter taste, quite early in their life.

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Absence of kin discrimination in the hatchlings of a lizard, *Calotes versicolor* (Agamidae)

Veena H.F. Ammanna, Srinivas K. Saidapur and Bhagyashri A. Shanbhag*

Department of Zoology, Karnatak University, Dharwad-580 003, India

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### Abstract

Laboratory born *Calotes versicolor* hatchlings were reared for 15 days in pure or mixed sibships or singly in isolation and then tested for their kin discrimination ability. As *C. versicolor* orients itself visually, visual displays like push-ups, gular extensions, and distance between the test individuals were used as indicators of arousal/aggression towards the opponents (familiar vs. familiar/unfamiliar sib or non-sib). The hatchlings exhibited aggressiveness towards unfamiliar sibs as well as non-sibs in the form of push-ups and gular extensions; they also remained at a longer distance from each other. In contrast, they did not exhibit such behaviors against familiar individuals regardless of their relatedness, and stayed closer to each other. In tests involving once familiar individuals, sib, or non-sib reared together for 15 d from hatching and then separated for 7 or 30 d, the lizards showed aggressiveness towards each other following 30 d separation. A greater number of push-ups and gular extensions were exhibited by the test individuals after 30 d separation compared to those separated for 7 d. Thus, in this non-social lizard, there is no kin discrimination. An early dispersal of hatchlings in this species may have led to a loss of kin discrimination. However, familiar individuals are recognized as long as they continue to remain familiar thereby suggesting a 'dear enemy phenomenon'.

**Keywords**

Agamidae; *Calotes versicolor*, hatchling; kin discrimination; lizard

### Introduction

The phenomenon of kin recognition has been documented in a diverse array of animals (Waldman, 1991; Pfennig and Sherman, 1995; Saidapur and Girish, 2000; Bull et al., 2001; Gramapurohit et al., 2006) and also plants (Dudley and File, 2007). Kin recognition is considered to be based on phenotype matching and/or familiarity (Halpin, 1991; Mateo, 2004). The familiarity based recognition requires a period of

* Corresponding author; e-mail: bhagyashrishanbhag@gmail.com
association with one another to allow the individuals to learn distinctive cues of each other and treat them as 'kin or familiar' upon subsequent encounters. Phenotype matching also needs at least a short time of prior association, while only in self-referent phenotype matching an individual's own cues are sufficient to recognize kin members.

Among reptiles, the first systematic report on sibling recognition was made in the green iguana hatchlings (Werner et al., 1987). Subsequent studies in reptiles mainly focused on discrimination between parent-offspring in viviparous forms such as the female sleepy lizard (Tiliqua rugosa) and the gidgee skink (Egernia stokesii). Studies on these species showed that the mothers recognize their offspring even after their separation from the time of birth (Bull et al., 1994; Main and Bull, 1996). Interestingly, the separated juveniles also directed their attention to their real mothers rather than to foster mothers (Main and Bull, 1996). The juveniles of Lacerta vivipara are reported to recognize the odor of their mothers regardless of the familiarity (Lena and De Fraipont, 1998). However, they recognize familiar but not unfamiliar siblings. Absence of kin discrimination is reported in the hatchlings of Alligator mississippiensis (Passek and Gillinghan, 1999). In contrast, juvenile tree skinks (Egernia striolata) exhibit kin recognition and discriminate between related and unrelated individuals using olfactory cues (phenotypic similarity) and not familiarity (Bull et al., 2001). In contrast, a closely related species, Egernia saxatilis, exhibits kin discrimination based on the familiarity and not on the genetic relatedness (O'Connor and Shine, 2005). Among snakes, kin discrimination in Crotalus horridus is inferred based on association of related individuals (Clark, 2004), while in Coronella austriaca, a self-referent phenotype matching is proposed since their siblings discriminate between kin and non-kin using chemoreception (Pernett et al., 2009). In general, mother-offspring or sibling recognition in reptiles is documented in species that live in some sort of social structure where ability to discriminate kin from non-kin is beneficial. However, it is not known whether sibling recognition is encountered in lizards that do not live in social association.

Calotes vesicolor is a non-social lizard. Its hatchlings disperse within a week or so after hatching. However, the dispersal pattern and home range of these lizards are not known. These lizards orient themselves visually while chemoreception is poorly developed in juveniles (Ammanna, Saidapur and Shanbhag, unpublished observations). The agamid lizards (e.g., Amphibolurus muricatus, Psammophilus dorsalis, C. versicolor) are known to use vision for detection of food, predators, and communication between conspecifics (Husak, 2004; Radder et al., 2006; Shanbhag et al., 2010). Extensive studies on A. muricatus and the iguanid Sceloporus graciscus have revealed that they exhibit push-ups and gular extensions upon encounter with unfamiliar conspecifics (Martins, 1993; Ord and Evans, 2003; Van Dyk and Evans, 2007). Adult C. versicolor also displays push-ups and gular extensions behaviors before combat with conspecifics (Ammanna; Saidapur and Shanbhag, personal observations). Therefore, in the present study, visual displays like the push-ups and gular extensions are used as parameters of aggression or arousal between two indi-
individuals. Likewise, 'distance' between the test dyad in the test arena is considered to reflect their degree of familiarity or kin discrimination abilities as an additional parameter used in conjunction with the visual displays. Accordingly, a closer disposition of test individuals was considered to indicate more affinity between them as reported in the snake, *C. horridus* in which kin members settle closer to each other compared to the non-kin members (Clark, 2004). The present study was undertaken to determine whether *C. versicolor* hatchlings discriminate between kin and non-kin using push-ups and gular extensions, and proximity between test individuals to assess familiarity/kin discrimination abilities.

**Material and methods**

The hatchlings of *C. versicolor* (15-21 eggs/clutch; \( N = 30 \) clutches) were obtained by incubating eggs as described earlier (Radder et al., 2002). Hatchlings of each clutch were uniquely marked with tiny dots of 3 different colored paints either on their head or flank (right or left) or combinations to distinguish their sibship. They were reared in groups in glass terraria (60 \( \times \) 30 \( \times \) 30 cm) with 20 mm sand bed and mesh lid at the top. For isolated rearing, single hatchlings were reared in plastic boxes (26 \( \times \) 13 \( \times \) 22 cm) with 20 mm sand bed with mesh on the top. All terraria were kept in a well-ventilated area with only two walls opposite to each other and a roof. The other two sides of enclosure were open without walls. Therefore, the lizards experienced natural photoperiod and temperature. No temperature gradient was evident within and between terraria. Water was made available in small dishes. The glass walls of terraria facing the adjacent terraria were covered with paper to prevent visual familiarity between the hatchlings of different terraria. The hatchlings were fed daily on termites (*Odontotermes asmuthi*) and larvae of silkmoth (*Bombyx mori*). The new born hatchlings were reared in groups for 15 d, as shown below, prior to testing for their kin discrimination ability.

(a) Rearing with kin: Ten siblings/per terrarium were reared together. Six such sib groups were maintained.

(b) Rearing with non-kin: Members of two parental lines (5 sibs from each parental line) were reared together to facilitate familiarity among them. Six such mixed groups were maintained.

(c) Rearing in isolation: Five individuals from each of the 6 parental lines (whose sibs were reared in sib groups: Group a) were reared individually (\( N = 30 \)), i.e., in isolation.

(d) Rearing with kin or non-kin for 15 d and then in isolation: Ten siblings or 5 hatchlings of two different sibships were reared for 15 d. Twelve groups each of sib and mixed individuals from 18 parental lines were maintained. After 15 d of familiarity, the lizards were separated and reared singly in isolation for next 7 d (\( N = 60 \) for sib and mixed groups). Another set of lizards were reared
similarly in isolation but for 30 d ($N = 60$ for sib and mixed groups) in plastic boxes. These individuals were then used for various trials.

**Testing procedure**

Half an hour before the trials, the test hatchlings were placed individually in open plastic boxes ($26 \times 13 \times 22$ cm). Trials were run in a glass test tank ($60 \times 30 \times 30$ cm) devoid of any temperature gradient. Sides of the test tank at the base were marked using a glass marking pen at 5 cm interval which were further marked at 1 cm interval to facilitate recording the distance between two test hatchlings more accurately. Two test hatchlings, familiar or unfamiliar, either of the same sibship or different sibship (chosen arbitrarily) were released at the center of the test tank. They were allowed to settle in the test arena for 1 min. The laboratory raised hatchlings do not get disturbed even when a human subject approaches ~0.5 m distance from the test tank. One of the authors sat at ~1.5 m distance from the test tank and made observations on the dyad for 30 min. The number of push-ups and gular extensions by each test subject and also the number of individuals exhibiting these behaviors were manually recorded. We also recorded the distance nearest to ~2 cm between the positions of the test hatchlings (snout to snout) at every 5 min interval for 30 min trial period (six recordings for a pair). The assumption was that if the lizards recognize each other as kin/familiar, they would stay nearby or else they would stay away from each other. In order to rule out observer bias if any, a Sony handycam was fixed above the test tanks such that it covered the entire tank and set at 35 min to record the behavior of the test hatchlings. Five such video recordings were made for each test along with the manual observations. The videos were then transferred to a computer and an observer blind to experimental protocol recorded the behavior of each lizard. Since the camera was fixed on top of the test tank, the recordings clearly showed push-ups but not the gular extensions. The data on number of push-ups and distance between the two test hatchlings obtained manually and from the video recordings were comparable ruling out observer bias in manual recordings. The test tank was thoroughly cleaned with water and dried before using for a new trial. A test tank used in a trial was not used immediately for subsequent trials on the same day even though perception of chemical cues is poor in the hatchlings of *C. versicolor* (Ammanna, Saidapur and Shanbhag, unpublished observations). A test hatchling was used once only. After completion of trials the hatchlings were released in the botanical garden of the University Campus.

**Experiment 1**

This experiment was conducted to determine kin discrimination ability and possible mechanism of kin recognition (self-referent phenotype matching or familiarity) in *C. versicolor* hatchlings. The following tests were conducted using a pair of hatchlings comprising of (i) familiar sibs raised in a sib group, (ii) familiar sibs reared as a mixed group (iii) unfamiliar sibs: sibs raised in isolation, (iv) unfamiliar sibs:
individuals from sib group rearing and their sibs reared in isolation, (v) familiar non-sibs: reared in a mixed group, and (vi) unfamiliar and unrelated: individuals from different sibships. These tests allow us to know the responses of *C. versicolor* hatchlings to familiar and related, familiar and unrelated, unfamiliar related, and unfamiliar unrelated members. The behavioral responses, i.e., the number of push-ups and gular extensions exhibited by a dyad, and the number of hatchlings exhibiting push-ups and gular extensions were recorded in each trial. The distance between two test subjects were recorded at 5 min intervals for 30 min. Each test consisted of a total of 180 trials.

Experiment 2

The experiment was conducted to find out whether hatchlings familiar to each other (reared with sibs or non-sibs) can recognize each other following their separation for 7 and 30 d. The tests were conducted using a pair of hatchlings comprising of (i) familiar siblings separated for 7 d, (ii) familiar siblings separated for 30 d, (iii) familiar non-sibs separated for 7 d, and (iv) familiar non-sibs separated for 30 d. The behavioral responses, i.e., the number of push-ups and gular extensions exhibited by a dyad, and the number of hatchlings exhibiting push-ups and gular extensions were recorded. The distance between two test subjects were recorded at 5 min intervals for 30 min. Such tests reveal whether prior familiarity is essential in kin discrimination and if so, whether this memory (imprinting) remains after separation for 7 and 30 d. Each test consisted of a total of 120 trials.

The work was carried out following the approval of CPCSEA, New Delhi (Reg. No. 639/GO/02/a/CPCSEA).

Statistics

*Z* test of equality of proportions was used to test whether the number of hatchlings exhibiting push-ups and gular extensions was significant in a test group. The variations in the mean number of push-ups and gular extensions among the groups (Experiment 1 and 2) were analyzed using a Kruskal-Wallis test followed by a confidence interval post-hoc test (Miller, 1981). The data on distance between the test subjects (5-min interval during 30 min trial period) were analyzed using the Friedman test. Since there were no significant differences in the distance between the two individuals in a given trial, the data were pooled and the mean distance was used for further statistical analysis. The variation in the mean distance between the test hatchlings among different test groups was analyzed using a Kruskal-Wallis test followed by a confidence interval post-hoc test (for Experiment 1 and 2). In order to strengthen evidence in favor of our hypothesis, we fixed the *α* level at 0.01.
Results

Experiment 1

The test hatchlings pitted against familiar sibs (either reared with sibs or in mixed group) did not exhibit push-ups or gular extensions (fig. 1). When they encountered non-sibs but familiar lizards, only 3 out of 60 showed push-ups and gular extensions \( (Z = 1.79, N = 60, P > 0.01; \text{fig. 1}) \). In contrast, when unfamiliar individuals were tested against each other (sibs or non-sibs), significantly greater...
number of test individuals exhibited push-ups (unfamiliar sibs-sibs, reared in isolation: $Z = 11.33, N = 60, P < 0.001$; unfamiliar sibs-reared with sib vs. sib reared in isolation: $Z = 11.86, N = 60, P < 0.001$; unfamiliar non-sibs: $Z = 11.17, N = 60, P < 0.001$), and gular extensions (unfamiliar sibs-sibs reared in isolation: $Z = 11.86, N = 60, P < 0.001$; unfamiliar sibs-reared with sib vs. sib reared in isolation: $Z = 11.86, N = 60, P < 0.001$; unfamiliar non-sibs: $Z = 11.17, N = 60, P < 0.001$) indicating aggressive behavior towards the opponents (fig. 1).

Also, mean number of push-ups and gular extensions exhibited by the dyads with unfamiliar individuals was significantly greater than that exhibited by dyads with familiar individuals (push-ups: $H_{15} = 13.15, P < 0.01$; gular extensions: $H_{15} = 15.63, P < 0.01$; fig. 2) irrespective of whether they were sibs or non-sibs.

Throughout the 30 min trial period the familiar hatchlings stayed closer than the two unfamiliar individuals regardless of whether they are sibs or non-sibs. In contrast, mean distance between the unfamiliar test subjects was significantly higher ($H_5 = 26.77, P < 0.001$; fig. 2) than that found between familiar subjects regardless of their relatedness (sib/non-sib).

**Experiment 2**

In trials following 7 d separation from the familiar individuals there was no significant response in terms of push-ups and gular extensions upon their encounter with each other regardless of whether the opponent was sib (push-ups: $Z = 1.79, N = 60, P > 0.01$; gular extensions: $Z = 1.43, N = 60, P > 0.01$) or non-sib (push-ups: $Z = 1.79, N = 60, P > 0.01$; gular extensions: $Z = 1.79, N = 60, P > 0.01$; fig. 3). However, encounter after a lapse of 30 d separation, irrespective of whether the opponent was a sib or non-sib resulted in a significantly greater number of hatchlings exhibiting push-ups (sibs: $Z = 16.67, N = 60, P < 0.001$; non-sibs: $Z = 17.29, N = 60, P < 0.001$) and gular extensions (sibs: $Z = 16.67, N = 60, P < 0.001$; non-sibs: $Z = 17.29, N = 60, P < 0.001$; fig. 3). Further, the mean number of push-ups and gular extensions exhibited by the lizards after 30d separation was significantly greater than that exhibited following 7 d separation (push-ups: $H_3 = 17.95, P < 0.001$; gular extensions: $H_3 = 18.04, P < 0.001$; fig. 4). Throughout the trial period, the test hatchlings (familiar sibs or non-sibs) isolated for 7 d stayed closer to each other than those separated for 30 d. The mean distance between the test subjects following 7 d separation was significantly lower compared to that seen after 30 d isolation from the group mates ($H_3 = 17.45, P < 0.01$; fig. 4).

**Discussion**

The present study shows that *C. versicolor* hatchlings exhibit push-ups and gular extensions when they encounter unfamiliar conspecifics irrespective of whether they are sibs or non-sibs demonstrating their arousal/aggressiveness towards each other. Also, they prefer to stay away from the unfamiliar individuals. Interestingly,
Figure 2. Mean number of push-ups and gular extensions, and mean distance between the test hatchlings of *C. versicolor* in trials with sibs and non-sib. Note that in tests with familiar sibs the aggressive behavior (push-ups or gular extensions) was absent and it was very low against familiar non-sibs. Dissimilar letters on the bars indicate a significant difference between the test groups. *N* = 30 trials/test.
familiarity greatly reduced or even abolished arousal of push-ups and gular extension behaviors. These findings agree with those reported for *L. vivipara* (Lena and De Fraipont, 1998) and *E. saxatilis* (O’Connor and Shine, 2005), where juveniles distinguished between familiar and unfamiliar individuals. These studies used detection of chemical cues as the criteria for deciphering the discriminatory behavior while the present study used visual displays to understand their discrimination between familiar and unfamiliar individuals. The present study also shows that remembrance of familiarity is short lived (7 d) and disappears by 30 d. This is evident since the hatchlings exhibit a greater number of push-ups and gular extensions after 30 d separation compared to 7 d separation from the cage mates. Apparently, discrimination of individuals as familiar or unfamiliar in hatchlings of *C. versicolor* is based on their proximity to each other. A separation even for a month results in the loss of familiarity between the cage mates. Loss of familiarity based kin discrimination is also observed in *L. vivipara* following rearing in isolation (Lena and De Fraipont, 1998).
Figure 4. Mean number of push-ups, gular extensions, and mean distance between the test hatchlings of C. versicolor reared in isolation for 7 d or 30 d after their initial familiarity with sibs or non-sibs (mixed rearing) for 15 d. Dissimilar letters on the bars indicate significant difference between the groups. N = 30 trials/test.
Though a mechanism of kin recognition is not clear in most cases, the benefits of kin recognition are evident. For animals that live in social groups, kin discrimination is advantageous for parents in taking care of their offspring or to reduce kin cannibalism and avoid inbreeding (Waldman, 1988). Theoretically, maintenance of any physiological mechanism including kin recognition is expected to incure some energy costs. If so, in species that disperse early in the neonatal life maintaining a kin recognition mechanism is hardly beneficial. The hatchlings of *C. versicolor* disperse soon after hatching. An early dispersal is likely to reduce kin encounter later on. Hence, maintaining kin recognition mechanism may be of less importance in *C. versicolor*. Interestingly, they discriminate between familiar and unfamiliar individuals. Territorial lizards are known to discriminate between familiar and unfamiliar conspecifics having overlapping territories. Presumably, this helps the residents to reduce temporal and energetic costs of resource defense and the risk of injury. Further, in such species aggressiveness during encounter between rivals varies with the degree of familiarity (Husak and Fox, 2003; Van Dyk and Evans, 2007). This pattern of differential aggression is popularly known as the ‘dear enemy’ phenomenon (Fisher, 1954). The present study also revealed a differential aggressiveness in *C. versicolor* towards familiar and unfamiliar conspecifics (kin or non-kin), a behavior akin to the ‘dear enemy’ phenomenon.

In summary, hatchlings of *C. versicolor* do not discriminate between kin and non-kin per se. In this species dispersal of hatchlings early in life may have lead to a loss of kin discrimination ability. Further, the study shows a familiarity based absence of aggressiveness towards conspecifics even at juvenile stages. The study is also first in its kind to assess kin discrimination in a visually-oriented ‘non-social’ lizard using behavioral displays as indicators of recognition between familiar and unfamiliar conspecifics.

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References


