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Toad tadpoles associate preferentially with siblings

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Highly structured social systems can involve close kinship groups (eusocial insects[1], lion prides[2], helpers-at-the-nest in birds[3]). However, kinship generally has not been considered to be important or necessary for what seem to be merely gregarious phenomena, such as schooling behaviour[4]. In fact, Fisher first proposed kinship theory to explain the evolution of noxious taste in certain gregarious insect larvae. He reasoned that traits which at first may not be directly beneficial to the individual, such as distastefulness or warning coloration, might be selected for if they conferred advantage to siblings in the swarm[5]. Like some insect larvae, the tadpoles of many toads (genus *Bufo*) are conspicuously coloured, distasteful to predators and highly gregarious, forming densely packed schools in open areas along pond shores[6]. The tadpoles' aggregative behaviour may be important for feeding efficiency[7,8], thermoregulation[9] or predator deterrence[8], but their conspicuousness coupled with their distastefulness suggest that schooling may also serve an aposematic function. Consistent with the predictions of a kin selection model, we report here that tadpoles of the American toad (*Bufo americanus*) preferentially associate with siblings in laboratory conditions.

During May and June 1977, we collected amplectant pairs of toads from several breeding congregations. Each pair was held in the laboratory in a separate 10-l bucket, and most females oviposited in these buckets within 24 h. Each clutch of eggs was transferred to a separate aquarium, where tadpoles were reared on boiled spinach for about 45 d, until they reached developmental stage 35 (ref. 10). Tadpoles were then placed for 24 h in 0.00025% aqueous solutions of either methylene blue or neutral red. This staining technique resulted in minimal mortality and marked the translucent fins of the tadpoles for several weeks, allowing accurate identification of sibling groups by visual inspection.

To determine whether tadpoles aggregate with siblings, for each test, 25 tadpoles from one clutch were marked blue, and 25 tadpoles from another clutch were marked red. The 50 tadpoles were then released at randomised positions and allowed to acclimatise for 24 h in an indoor test pool filled to a depth of 3 cm with water. Subsequently, for test periods ranging from 4 to 7 consecutive days, the positions of all tadpoles in the pool and their clutch-identifying colours were recorded twice each day, in the morning and afternoon; thus, each test consisted of 8–14 trials. During each trial a grid of sixteen 0.05-m^2 partitioned sections was lowered into the pool and the positions of all individuals were marked on an acetate sheet fastened over the grid. The partitions limited the movement of the tadpoles during the 90-s period in which data were recorded.

The coordinate positions thus obtained were analysed by comparing the distance of each tadpole to its nearest sibling neighbour with the distance to its nearest non-sibling neighbour. If they aggregate preferentially with siblings, one would expect that tadpoles should be closer to siblings than to non-siblings. This prediction was confirmed in all six experimental tests, using different groups of tadpoles in each test (Table 1). For each test, the mean nearest-neighbour distance between siblings was significantly less than the distance between non-siblings (Fig. 1). Analysis of variance showed this sibling effect to be highly significant ($P < 0.0001$, F-test), whereas variation within experimental tests, among tests and in interactions between effects was not significant (Table 2). The among-tests effect approaches significance, perhaps due to different aggregative tendencies among tadpoles used in different tests.

The results of separate analyses of individual trials further substantiate these findings (Table 1). In the majority of the experimental trials (65%; $n = 62$), the nearest-neighbour distance between siblings was significantly less than that between non-siblings (Table 1). Although discrete aggregations of sibling groups were not always apparent, in 37% of all trials the mean coordinates of the two groups differed significantly (Table 1), indicating that the sibling groups separated out into different areas of the pool. Moreover, the tadpole groups were not simply attracted to different fixed regions of the pool, as their positions changed in sequential trials (Fig. 2). This suggests that the tendency for tadpoles to be closer to siblings than to non-siblings cannot be explained by differential responses of the two groups to possible environmental gradients in the pool.

To control for the possibility that these results were caused by either the marking or testing procedures, we carried out tests in which all 50 tadpoles were from the same clutch. Half of these individuals were stained blue and half red; all other procedures were followed precisely as in the experimental tests. In the two control tests the nearest-neighbour distances between same-coloured tadpoles were not significantly less than those between

Table 1 Group identity and analysis of separate trials within each test

Test No.	Sibling groups Red	Sibling groups Blue	No. of trials per test	No. of trials where sibling distance < non-sibling distance ($P < 0.05$)	No. of trials where mean coordinates differ ($P < 0.05$)
Experimentals					
1	B	A	8	6 (75%)	2 (25%)
2	A	C	12	7 (58%)	1 (8%)
3	A	B	14	8 (57%)	5 (36%)
4	D	A	10	8 (80%)	6 (60%)
5	E	D	10	5 (50%)	4 (40%)
6	D	E	8	6 (75%)	5 (62%)
				No. of trials where same-colour distance < different-colour distance ($P < 0.05$)	
Controls					
7	D	D	8	0 (0%)	0 (0%)
8	D	D	8	0 (0%)	1 (12%)

In the experimental tests, egg clutches were obtained from five different pairs of parents (A–E) from four separate ponds (Tompkins County, New York). Pairs A and D were from the same pond; linear distances between the ponds of origin for the other pairs of parents were: A to B, 11 km; A to C, 3.7 km; D to E, 2.6 km. In the control tests, all groups of tadpoles were obtained from the same clutch. Unlike Fig. 1, where whole tests are compared, the individual sequential trials within each test were analysed with respect to (1) whether the distance between siblings was less than that between non-siblings (Wilcoxon signed-ranks test, one-tailed[19]), and (2) whether the mean coordinates of the two sibling groups differed (Mann–Whitney U-test, two-tailed[19]). For example, test 1 consisted of eight trials, in six of which siblings were significantly closer to each other than to non-siblings. In two of the eight trials in test 1 the centres of density (=centroids) of the two groups differed. For this analysis, the centres were considered different if their positions along either the north–south or east–west axis differed significantly.

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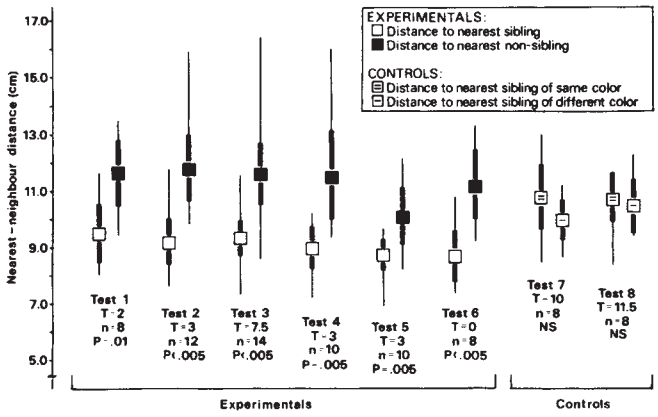

Fig. 1 Mean nearest-neighbour distance between sibling and non-sibling tadpoles (experimentals) and between siblings of the same and different colours (controls). Rectangular bars around means (symbols) represent 95% confidence intervals; vertical lines indicate ranges. Probability levels are given below the data for each test (T, n and P represent the test statistic sample size [number of pairs of trials] and level of significance, respectively). In all six experimental tests, individuals were significantly closer to siblings than to non-siblings ($P \leq 0.01$; Wilcoxon signed-ranks test). In control tests all tadpoles were siblings; same-coloured individuals were not significantly closer to each other than they were to differently coloured tadpoles ($P > 0.05$; Wilcoxon signed-ranks test).

differently coloured tadpoles (Fig. 1). Thus, the possibility that tadpoles in the experimental tests merely positioned themselves near same-coloured individuals can be discounted.

These results provide the first experimental evidence that a lower vertebrate is capable of sibling recognition. The tadpole distributions we found could arise if (1) siblings are directly attracted to one another, or (2) tadpoles avoid individuals they perceive to be non-siblings, perhaps due to aggressive encounters. We do not know what sensory modalities tadpoles use in making this discrimination, nor do we know how genetic and experiential factors effect this response. Siblings develop within a continuous jelly egg mass, and on hatching remain near the remnants of the jelly mass for several days. During these critical early developmental stages, tadpoles might 'learn' to identify siblings. Indeed, a facilitative social environment may be fundamental to the development of most normal sibling interactions[11]. Recognition of siblings might also be modifiable by experience later in life[12]. Clearly, sibling recognition need not be based solely on genetic factors.

Preferential association of tadpoles with siblings in ponds could result in the formation of sibling schools. Within schools, individuals can 'hide' from predators, thus decreasing the likelihood that they will be preyed on[13,14]. Yet to gain this advantage, individuals need not school with siblings. Indeed, the more adaptive strategy for the individual would seem to be to surround itself with non-siblings so that neither it nor its siblings are taken by a predator. However, if tadpoles associate with siblings, as our results suggest, their schooling behaviour may represent more than just 'selfish' cover-seeking. A predator that strikes a schooling individual may learn that tadpoles are distasteful, and thus it would avoid preying on other members of the school. The tadpoles' black body colour might function as a warning signal to further reinforce this 'lesson'. These traits, although not directly beneficial to the prey individual if it is eaten, may be selected for if they provide protection to related individuals in the school which are likely also to carry alleles for these altruistic traits[15]. The alarm pheromone released by injured *Bufo* tadpoles may represent another kin-selected trait, as the signalling individual is always wounded[16,17].

A sibling recognition mechanism could evolve in the following manner. In one individual, a mutation arises for distastefulness. The individual metamorphoses, survives to reproductive age, and produces young, several of which now carry the allele for distastefulness. As tadpoles associate in sibling groups for several days after hatching, because of their relative immobility, the loss of any of these distasteful individuals to a predator will benefit siblings, and thus distastefulness can spread by means of kin selection. Even if the unpalatability of a single larva is not enough to deter a predator, the cumulative noxious effect resulting from eating several larvae might be strong enough to cause future predator aversion to the school[18]. In a similar manner, aposematic coloration could spread. At this stage the benefits of distastefulness are high, but as the tadpoles begin actively swimming, and mixing with non-siblings becomes possible, the benefits become reduced or even negative. Next, a mutation arises for a sibling-association mechanism and is favoured because it prolongs the benefit of distastefulness and

Table 2 Analysis of variance for experimental tests

Source of variation	Degrees of freedom	Sum of squares	F Value	P
Sibling effect	1	77.39	44.06	<0.0001
Time effect (within tests)	7	14.49	1.15	0.36
Test effect (among tests)	5	21.97	2.44	0.06
Sibling × Test	5	5.29	0.59	0.71
Time × Test	35	71.54	1.13	0.36
Sibling × Time	7	6.88	0.55	0.79
Residual error	35	63.07	—	—

Three sources of variation and their interactions were considered. 'Sibling' refers to variation in nearest-neighbour distance dependent on whether or not that neighbour was a sibling. 'Time' refers to variation among sequential trials within single tests; only the first eight trials per test were used. 'Test' refers to variation among the six experimental tests.

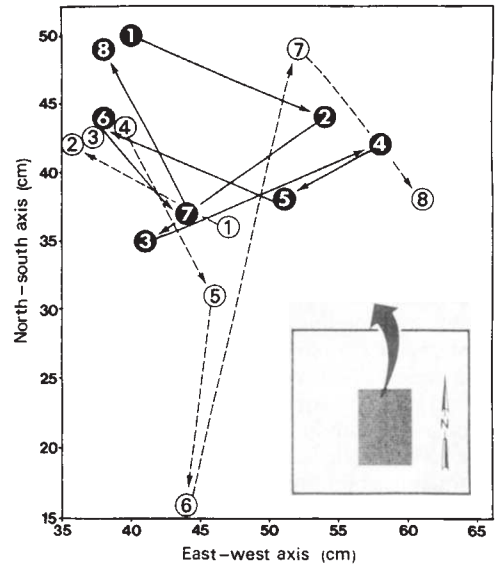

Fig. 2 Movement patterns of two sibling groups of tadpoles in a representative test. Centres of density given here are from test 6; the two sibling groups are group D (solid symbols and lines) and E (open symbols and dashed lines). The numbers inside symbols and the arrows show the sequence of trials in which the test was carried out. The two groups differed significantly in position along either the north–south or east–west axis during trials 1, 2, 3, 4 and 6 ($P < 0.05$; Mann–Whitney U-test, two-tailed). The inset shows the test pool, 88 × 96 cm, and the region of the pool in which the centres of density of the two groups were located (shaded area). A more detailed inspection of the sequential centres of density (trials 1–8) for the two sibling groups shows that each sibling group moved throughout the pool. Tadpoles were not fed during a test, nor were conditions in the pool altered. There was no evidence in this test or in the others that the separate groups preferred particular fixed regions of the pool.

aposematism into later developmental stages. Those individuals which discriminate siblings from non-siblings can preferentially associate with siblings, and thus can effectively direct their altruism towards siblings. This evolutionary scenario does not necessitate an error-free discrimination system; individuals which show any preferential sibling aggregative tendencies should have greater fitness than those which school indiscriminately with siblings and non-siblings. Although work is still in progress to identify the mechanisms by which sibling recognition is accomplished, our results are consistent with the predictions of a kin selection model.

We thank R. Altig, T. R. Halliday, W. D. Hamilton, G. Hausfater, B. Hölldobler, M. J. Ryan and R. Wassersug for comments on the manuscript and L. Nadler for assistance with the experiments. The research was supported by NSF (BNS 75-18693) and US Department of Agriculture funds.

Received 9 March; accepted 28 September 1979.

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Ca^{2+} is essential cofactor for trypanocidal activity of normal human serum

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Normal human serum has been known to exert a cytotoxic effect on *Trypanosoma brucei* subspecies for nearly 80 yr[1]. But in spite of many attempts, no trypanocidal factor was found in human or baboon serum[2–6], until Rifkin demonstrated a high density lipoprotein (HDL) in normal human serum with trypanocidal activity[7]. The conclusion that this was the trypanocidal factor was supported by the report that serum from patients with Tangier disease, characterised by a severe deficiency of HDL, lacked trypanocidal activity[7]. We report here that Ca^{2+} is an essential cofactor for the trypanocidal activity of normal human serum, in which α_2 macroglobulin (α_2) might function as a Ca^{2+}-carrier. We further show that D-glucose, D-fructose and D-mannose can suppress the trypanocidal action of normal human serum, whereas glycerol has the opposite effect.

We used four clones of *T. brucei* subspecies. Clones of the variable antigen types (VATs) AnTat 1 and 8 were derived from a stock of *T. brucei brucei*[8]. A serum sensitive (S) and a serum resistant (R) clone of VAT ETat 2 were derived from a stock of *T. brucei rhodesiense*[8–11]. A large number of cryo-stabilates of each clone was prepared by passage through mice (female OF1 mice) and stored under liquid nitrogen[12]. Trypanosomes were collected from mice 48 h after infection, purified by passage through a DEAE-cellulose column[13], washed and resuspended in PSG (50 mM sodium phosphate buffer, *p*H 8, 36.4 mM NaCl and 83 mM glucose) or BSG (48 mM sodium borate buffer, *p*H 8, 77 mM NaCl and 83 mM glucose). Human serum samples were collected from healthy volunteers, pooled and used immediately for assays or stored at −70 °C until use. *In vitro* assay for the trypanocidal activity of serum was performed by incubating parasites (10^6 cells per ml) at 36 °C in PSG or BSG (unless otherwise noted) in the presence of human serum at the concentration specified in the figure legends. Percentage cell lysis was determined by counting the cell ghosts among at least 500 cells under a phase contrast microscope.

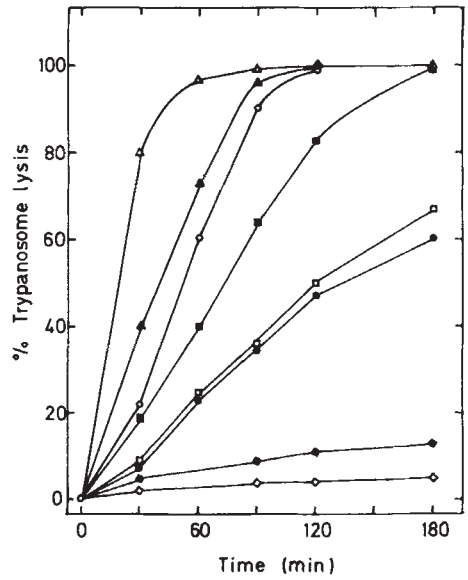

Fig. 1 Effect of dialysis, temperature and Ca^{2+} on trypanocidal activity of normal human serum. Serum was dialysed against PSG or BSG at 4 °C for 24 h with a minimal change of the volume of dialysate. The column-separated *T. b. brucei* AnTat 8 (10^6 cells per 20 μl) was incubated with 980 μl serum at 36 °C for the length indicated in the figure and cell lysis was followed as described in the text. When added, 50 μl of a stock solution of Ca^{2+} or Mg^{2+} was used in 930 μl serum to give a final concentration of 3.2 mM. ○, Undialysed serum; △, plus Ca^{2+}; ●, PSG-dialysed serum; ▲, plus Ca^{2+} or □, plus Mg^{2+}; ■, BSG-dialysed serum; ◇, heat-treated serum (60 °C, 45 min); ◆, plus Ca^{2+}.

Normal human serum, after dialysis against PSG for 24 h, had considerably less trypanocidal activity on *T. b. brucei* AnTat8 than did undialysed control serum, reaching only about 60% cell lysis even after 3 h incubation (Fig. 1). However, the addition of 3.2 mM Ca^{2+} to dialysed serum resulted in a marked restoration of the trypanosidal activity (Fig. 1). The extent of restoration was clearly dependent on the concentration (1.6–12.8 mM) of Ca^{2+} added (Fig. 2). Dialysis against PSG caused a more effective reduction of serum trypanocidal activity than did that against BSG, further implying an involvement of Ca^{2+} in this cytotoxic activity (Fig. 1). Ca^{2+} stimulated the trypanocidal activity, of not only dialysed but also undialysed serum, eliminating an initial lag period at high concentrations of Ca^{2+} (Figs 1 and 2). Another divalent cation, Mg^{2+}, had a negligible effect on the trypanocidal activity of PSG-dialysed normal serum (Fig. 1).

Furthermore, serum deprived of its trypanocidal activity by heat inactivation (45 min at 60 °C) could not be reactivated to a significant extent by the addition of 3.2 mM Ca^{2+} (Fig. 1). Thus the restoration of trypanocidal activity by Ca^{2+} requires the

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0028–0836/79/490613—03$01.00