

Kinship affects innate responses to a predator in bluegill *Lepomis macrochirus* larvae

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Naïve kin groups and mixed-family groups of bluegill *Lepomis macrochirus* larvae were exposed to a novel predator cue. The larvae responded by increasing shoal cohesiveness in kin groups but not in mixed-family groups; moreover, larvae sired by males of the ‘cuckolder’ life history tended to have an enhanced ability to respond to direct cues of kinship *v.* larvae sired by males of the ‘parental’ life history, which instead appeared to respond to cues of life history rather than relatedness *per se*. The increased shoal cohesion among related individuals probably confers a survival benefit and indicates that the antipredatory shoaling response is innate in *L. macrochirus*. © 2009 The Authors

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The formation of animal groups has long been of interest to biologists (Welty, 1934; Tinbergen, 1953). Groups may form for the purpose of increasing the efficiency of foraging or mate choice, but perhaps most importantly, groups improve predator defence (Bertram, 1978). Improved predator defence may come from risk dilution, improved overall vigilance or increased predator confusion (Hamilton, 1971; Pitcher & Parrish, 1993; Godin, 1997). For example, in silvery minnows *Hybognathus nuchalis* Agassiz, solitary individuals were readily captured by largemouth bass *Micropterus salmoides* (Lacépède), whereas capture time was considerably longer when the *H. nuchalis* were in shoals (Landeau & Terborgh, 1986). Furthermore, when one or two *H. nuchalis* were experimentally manipulated to look different from the other individuals in the shoal, the *M. salmoides* took less time to capture the prey (Landeau & Terborgh, 1986). This result highlights the importance of looking similar to shoalmates and the resultant confusion effect that the similarity can have on predators.

Grouping with relatives may be especially effective in minimizing the susceptibility of individuals to predation. Antipredator behaviours such as predator-searching and predator-inspection involve co-operation among group members (Pitcher & Parrish, 1993; Godin, 1997), and kin selection theory predicts that groups of related individuals will be more co-operative than groups of unrelated individuals (Hamilton, 1964). Kin groups should also benefit from an increased predator confusion effect

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because related individuals tend to have more similar phenotypes than unrelated individuals (Rajakurana *et al.*, 2006).

The mechanisms individuals use to form groups in response to a predator have also received considerable attention. Broadly, these mechanisms can be innate or learned. For example, Chinook salmon *Oncorhynchus tshawytscha* (Walbaum) have an innate component to their behaviour as naïve individuals show characteristic antipredator behaviours when exposed to predator cues for the first time (Berejikian *et al.*, 2003; Hawkins *et al.*, 2007; Scheurer *et al.*, 2007). On the other hand, in steelhead trout *Oncorhynchus mykiss* (Walbaum), individuals reared in isolation of predators have poor antipredator responses when first exposed to a predator, but show an improved response after being exposed to a combination of a predator chemical extract and conspecific alarm cues (Berejikian *et al.*, 1999). These data suggest that antipredator behaviour can also have a significant learned component.

In this study, the antipredator behaviour of bluegill *Lepomis macrochirus* Rafinesque in response to a novel predator cue was investigated. *Lepomis macrochirus* are endemic to North America and have paternal care of the eggs and larvae (Lee *et al.*, 1980). Once the larvae leave the nest, however, they are subjected to predation by other fishes as well as cnidarians, *e.g.* *Hydra canadensis* (Elliott *et al.*, 1997; Gross & MacMillan, 1981). In response to a threat of predation, juvenile *L. macrochirus* can form shoals and it has been shown that tighter shoal cohesion reduces susceptibility to predation (Chipps *et al.*, 2004). Here, the role of kinship on shoal cohesion in naïve larval *L. macrochirus* in response to a predator cue was examined. These data allow the importance of kinship on predator defence and whether or not *L. macrochirus* larvae have an innate antipredator behaviour response to be determined.

The study was conducted using the *L. macrochirus* population found in Lake Opinicon, Ontario, Canada (44°16' N; 76°30' W). The Lake Opinicon population has a long history of behavioural studies (Gross & Charnov, 1980; Hain & Neff, 2006). In Lake Opinicon, adult males are characterized by a discrete life history termed 'parental' and 'cuckolder' (Gross & Charnov, 1980). Parentals mature at 7 years of age, construct nests, court and spawn with females, and provide sole parental care to the developing larvae. In contrast, cuckolders mature precociously at the age of 2 years and opportunistically steal fertilizations from the nest-tending parental. The offspring of cuckolders have previously been shown to actively discriminate between odours of kin and non-kin and prefer to associate with kin, but offspring of parentals do not appear to use a direct kin recognition mechanism to discriminate between kin and non-kin (Hain & Neff, 2006).

In June 2006 and 2008, swimmers equipped with snorkelling gear conducted daily surveys of *L. macrochirus* breeding activity in Lake Opinicon. When spawning was discovered, mature parentals, cuckolders and females were collected opportunistically using snorkelling gear and dip nets and transported by boat to the aquarium facilities at the Queen's University Biological Station, which sits on the lake's shore. These fish were used to create families using *in vitro* fertilization techniques as described in Neff & Lister (2007). Briefly, sperm was collected in 2 ml syringes from cuckolders and parentals by applying pressure to the gonad region of the abdomen. Eggs were collected from gravid females by applying gentle pressure to her abdomen. Eggs were then fertilized with sperm from either a cuckolder or a parental and then reared in 500 ml glass jars filled with lake water and equipped with a small airstone.

Each female and each male was used only once to form a family. Fifty per cent water changes were conducted daily until larval swim-up (5–8 days post-hatch), which signals the onset of exogenous feeding.

Antipredator response trials were conducted in brown translucent tanks measuring 409 mm × 282 mm × 150 mm filled to a depth of 8.0 cm with water from Lake Opinicon. Each tank was visually divided using a horizontal grid of 28 equally sized rectangles (arranged as 4 × 7) positioned beneath the tank. On the first day of exogenous feeding, 'pure' and 'mixed' groups were formed by transferring larvae of known pedigree to trial tanks using a small plastic pipette. Pure groups consisted of 10 full-siblings sired by either a parental or a cuckold. Three types of mixed groups of 10 larvae were created: a group of the 'mixed parental' type consisted of five full-siblings sired by a parental and five full-siblings sired by a second parental; a group of the 'mixed cuckold' type consisted of five full-siblings sired by a cuckold and five full-siblings sired by a second cuckold; and a group of the 'mixed life history' type consisted of five full-siblings sired by a parental and five full-siblings sired by a cuckold. Within all mixed groups, the two sets of full-siblings were themselves unrelated (*i.e.* the sets had different fathers and mothers). Families used in the trials were used once to form pure groups and a maximum of twice to form mixed groups. The larvae were then allowed to acclimate for *c.* 24 h. One hour before a trial began, a male pumpkinseed *Lepomis gibbosus* (L.), a known predator of *L. macrochirus* larvae: Gross & MacMillan, 1981; Neff, 2003) was placed in a tank filled with 10 l of lake water. This water conditioned by the *L. gibbosus* served as a predator cue in the trials. A trial began by recording the grid co-ordinates of each larva. Then, for 'control' treatments, 100 ml of unconditioned lake water was added to the centre of the tank and for 'predator' treatments, 100 ml of predator-conditioned water was added to the centre of the tank. Pilot trials were conducted to determine that a volume of 100 ml was small enough that the introduction of the cue would not disturb the fish in the tank, but was large enough to induce a response (Hain & Neff, 2006). The grid co-ordinates of each larva were read by a naïve observer to a stenographer at 20 and 80 s after the addition of the cue. The times when the location of the fish was recorded was chosen to give one initial measure after sufficient time for the larvae to respond to the odour cue, and another measure 1 min later to give an indication of how stable these groups were over time. The locations of the most active larvae were recorded first, followed by the location of less active or stationary larvae, and this process typically took no more than a few seconds per time step. All groups were subjected to both treatments on consecutive days with approximately half of the trials ($n = 31$) starting with the control treatment and the other half ($n = 30$) starting with the predator treatment. Fifty per cent water changes were performed between trials using water from Lake Opinicon.

The distance between pairs of larvae was calculated as the square root of the sum of the square of the horizontal grid reference position of larva A (H_A) minus the horizontal grid reference position of larva B (H_B) and the square of the vertical grid reference position of larva A (V_A) minus the vertical grid reference position of larva B (V_B): $[(H_A - H_B)^2 + (V_A - V_B)^2]^{0.5}$. For each individual, the distance to the third-nearest neighbour was determined and then averaged across each individual in a trial to determine the trial's shoal dispersion index (D_1). A group with a high shoal dispersion index can be interpreted as being more spread out than a group with a low shoal dispersion index.

For the analysis of shoaling behaviour, two paired *t*-tests were used to compare D_1 at the beginning of trials (*i.e.* days 1 and 2) for trials that began with either the control or predator treatments. This analysis was performed to test if conducting the predator treatment first had any residual effect on shoal dispersion the following day when the control treatment was conducted. For the analysis of shoaling behaviour, separate repeated-measures ANOVA were conducted on the control and predator treatments. To test the hypothesis that kinship affects shoal cohesiveness in response to a predator, the D_1 at each of the three time steps (before, 20 s after and 80 s after the introduction of the cue) was entered as the repeated measure, and degree of kinship (pure or mixed) and day (first or second day of trials) were entered as fixed factors. Day was included in the ANOVA as a fixed factor to statistically control for any effect of trial day (and treatment order) on shoaling dispersion. Within each time step, a *post-hoc* comparison between pure and mixed groups was made using a one-tailed independent sample *t*-test. For the predator treatments, two additional repeated-measures ANOVA were performed to compare mixed groups to pure groups for both the parental and cuckolder life histories, using kinship and day as fixed factors. Statistics were performed using SPSS version 16.0 (SPSS Inc.; www.spss.com) or JMP version 4.0.4 (SAS Institute; www.sas.com).

The first analysis revealed that there was no residual effect of the predator cue on shoaling behaviour at the beginning of trials on day 2; when the predator treatment was performed first, there was no significant difference between days 1 and 2 in the D_1 before the addition of the cue (paired *t*-test, d.f. = 29, $P > 0.05$). Similarly, when the predator treatment was performed first, there was no significant difference between days 1 and 2 in the D_1 before the addition of the cue (paired *t*-test, d.f. = 30, $P > 0.05$). Thus, being exposed to the predator cue on day 1 did not result in groups that were more clumped on day 2 at the beginning of the trial.

The results of the repeated-measures ANOVA are summarized in Table I. In the control treatment, the addition of the cue had no significant effect on D_1 [Table I and Fig. 1(a)]. Pure and mixed groups all responded similarly to the addition of the cue. In the predator treatment, however, there was a significant effect of timing on D_1 , indicating that the addition of the predator cue resulted in the larvae associating more closely [Fig. 1(b)]. There was a significant main effect of the level of kinship on D_1 , with pure groups less dispersed than mixed groups [Table I and Fig. 1(b)]. There was a trend towards an interaction between timing and kinship on D_1 , which can be explained by pure groups becoming less dispersed than mixed groups over time following the addition of the odour cue (one-tailed *t*-test comparing pure and mixed groups; before addition of cue: d.f. = 59, $P > 0.05$; 20 s after: d.f. = 59, $P > 0.05$; 80 s after: d.f. = 59, $P < 0.05$). Additionally, parental and cuckolder-sired larvae responded to the predator cues differently when in mixed groups *v.* pure groups. Specifically, there was no difference in D_1 for parental-sired larvae when in mixed groups *v.* pure groups [Table II and Fig. 1(b)], but there was an interaction effect of time and kinship on D_1 for cuckolder-sired larvae (Table II). Both mixed and pure cuckolder-sired groups tended to become less dispersed immediately following the addition of the predator cue, but the mixed cuckolder-sired groups tended to become more dispersed than the pure cuckolder-sired groups 80 s after the addition of the predator cue [Fig. 1(b)]. There was also a significant interaction of time and day on D_1 for cuckolder-sired larvae (Table II). This interaction seemed to be driven by the small number of pure trials conducted on day 1, which become less dispersed

TABLE I. Summary of repeated-measures ANOVA for shoal dispersion index D_1 in *Lepomis macrochirus* larvae exposed to a predator cue. The cohesiveness of shoals was measured at three time steps in response to the control treatment (no predator cue) and the predator treatment (predator odour cue). The fixed factors in the analysis are kinship (full-siblings or mixed relatedness) and day that the treatment was performed

Treatment	Variable	<i>F</i>	d.f.	<i>P</i>
Control	Time (repeated measure)	0.57	2, 56	>0.05
	Kinship	0.065	1, 57	>0.05
	Day	1.09	1, 57	>0.05
	Kinship × day	1.00	1, 57	>0.05
	Time × day	1.01	2, 56	>0.05
	Time × kinship	0.46	2, 56	>0.05
	Time × kinship × day	0.10	2, 56	>0.05
	Predator	Time (repeated measure)	3.27	2, 56
Kinship		4.11	1, 57	<0.05
Day		1.72	1, 57	>0.05
Kinship × day		2.37	1, 57	>0.05
Time × day		0.29	2, 56	>0.05
Time × kinship		2.68	2, 56	>0.05
Time × kinship × day		2.63	2, 56	>0.05

Significant variables in bold.

TABLE II. Summary of repeated-measures ANOVA for shoal dispersion index D_1 in parental and cuckolded-sired *Lepomis macrochirus* larvae exposed to a predator cue. The cohesiveness of the shoals was measured at three time steps in response to the control treatment (no predator cue) and the predator treatment (predator odour cue). The fixed factors in the analysis are kinship (full-siblings or mixed relatedness) and day that the treatment was performed

Treatment	Variable	<i>F</i>	d.f.	<i>P</i>
Parental	Time (repeated measure)	0.37	2, 19	>0.05
	Kinship	0.17	1, 20	>0.05
	Day	0.03	1, 20	>0.05
	Kinship × day	0.16	1, 20	>0.05
	Time × day	0.20	2, 19	>0.05
	Time × kinship	0.32	2, 19	>0.05
	Time × kinship × day	0.17	2, 19	>0.05
	Cuckolder	Time (repeated measure)	2.75	2, 18
Kinship		1.49	1, 19	>0.05
Day		0.03	1, 19	>0.05
Kinship × day		0.43	1, 19	>0.05
Time × day		4.67	2, 18	<0.05
Time × kinship		4.55	2, 18	<0.05
Time × kinship × day		3.38	2, 18	>0.05

after the addition of the cue [mean dispersion index, \bar{D}_1 before cue = 2.09 *v.* \bar{D}_1 80 s after cue = 1.11, $n = 3$], while in contrast, on day 2 the pure group tended to become more dispersed after the addition of the cue (\bar{D}_1 before cue = 1.66 *v.* \bar{D}_1 80 s after cue = 1.94, $n = 8$).

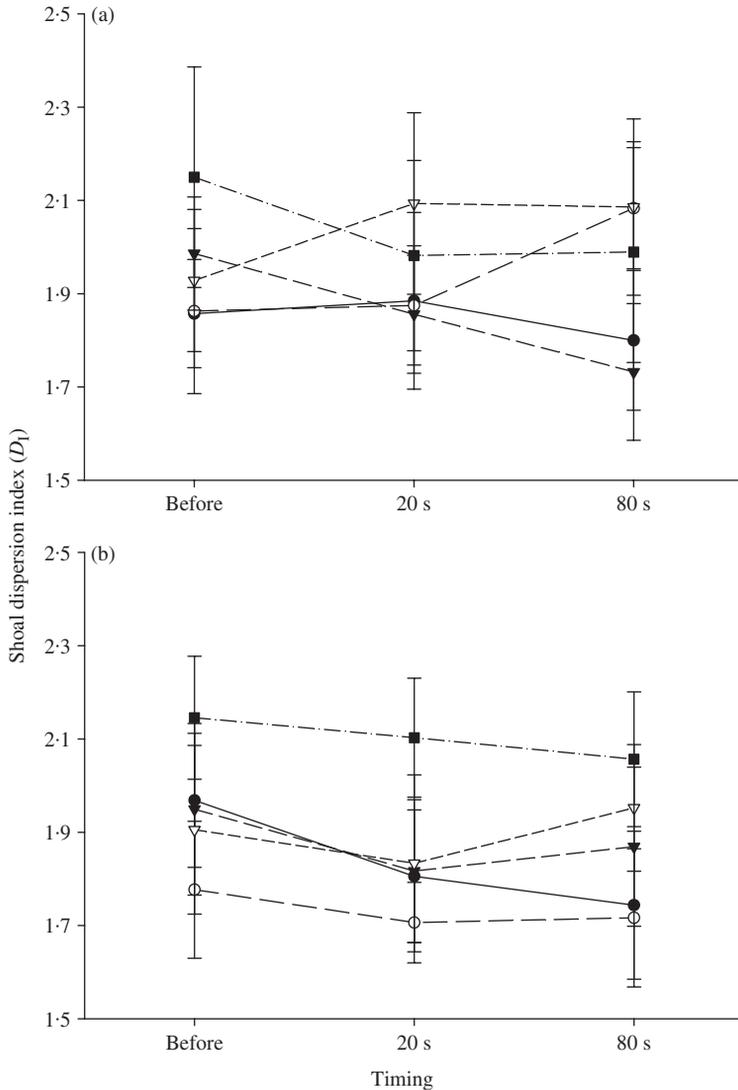


FIG. 1. Shoal dispersion index (D_1) for *Lepomis macrochirus* larvae [pure parental-sired groups ($n = 13$; —●—), pure cuckolder-sired groups ($n = 11$; --○--), mixed parental-sired groups ($n = 11$; --▽--), mixed cuckolder-sired groups ($n = 11$; --▽--), and mixed life history groups ($n = 15$; -■-)] after the addition of (a) lake water or (b) a predator cue. In the lake water (control) treatment, there were no significant differences among groups. In the predator cue treatment, pure full-sibling groups associated more closely than mixed groups. Values are means \pm S.E.

Thus, *L. macrochirus* larvae shoal more cohesively when subjected to an odour cue of a potential predator (*L. gibbosus*). Given that these larvae had never before been exposed to *L. gibbosus*, or any other predator, these results are strong evidence of an innate ability of *L. macrochirus* larvae to recognize the odour and respond as if it is a potential predator. Furthermore, full-sibling groups of *L. macrochirus* larvae, but not groups of mixed relatedness, reacted to the predator cue by increasing their

shoal cohesiveness. Interestingly, the effect of kinship was dependent on the sire's life history (cuckolder *v.* parental).

Numerous fishes have been shown to increase shoal cohesion in response to a predator (Botham *et al.*, 2006; Pink *et al.*, 2007). Presumably, this increased cohesion reduces predation efficiency and leads to increased survivorship of shoal members (Chipps *et al.*, 2004; Ruxton *et al.*, 2007). In *L. macrochirus*, for example, individuals in open water habitats tend to shoal more closely together than those in littoral habitats, and the increased cohesion reduces predation intensity by *M. salmoides* (Chipps *et al.*, 2004). Because *L. macrochirus* larvae move to open waters once they leave the nest (Garvey *et al.*, 2002), the increased shoal cohesion detected after the introduction of a predator odour no doubt is an effective antipredator response. Interestingly, unlike pure kin groups, mixed-family groups did not appear to reduce their shoal dispersion. Although the hypothesized benefits from an increase in shoal cohesion in the presence of a predator exist for both kin and mixed groups, additional kin selective benefits may exist for kin groups from predator inspection because kin benefit from performing the behaviour even in the absence of reciprocity (Croft *et al.*, 2006; Griesser *et al.*, 2006). Furthermore, the confusion effect is enhanced when members of a shoal are composed of similar phenotypes (Ranta *et al.*, 1994; Godin, 1997). Given that closely related individuals tend to look and smell more similar than unrelated individuals (Rajakurana *et al.*, 2006), shoaling with kin would serve to increase the confusion effect for predators.

The difference between parental and cuckolder-sired larvae in their reaction to the predator cue is interesting and consistent with previous studies. Here, the shoal dispersion of parental-sired larvae was the same regardless of whether the shoal was composed entirely of full-siblings or was of mixed parentage, but the dispersion of cuckolder-sired larvae differed based on the relatedness of the group. Previous work has shown that *L. macrochirus* larvae sired by cuckolders but not parentals discriminate between the odour of full-siblings and unrelated conspecifics using a mechanism referred to as self-referencing (Hain & Neff, 2006). In contrast, larvae sired by parentals tend to be highly related to their nestmates; they could form kin groups simply by continuing to associate with nestmates after swim-up, which is a form of indirect kin recognition (Mateo, 2004; Hain & Neff, 2006). The data in the present study suggest that parental-sired larvae may identify kin based on odour cues related to their sire's life history as opposed to kinship *per se*. This mechanism would be reliable in nature because multiple parentals rarely spawn eggs in the same nest (Neff, 2001). In contrast, cuckolder-sired larvae can differentiate between groups of full-siblings and groups of mixed relatedness, and associate more closely with full-sibling groups. This direct recognition mechanism would be required in nature to associate with kin because multiple cuckolders routinely spawn in a single nest (Stoltz & Neff, 2006).

There has been much debate, particularly among conservation, biologists, over the relative importance of learning *v.* an innate ability in recognizing predators. Early evidence suggested that fishes were unable to recognize a predator innately (Thompson, 1966; Goodyear, 1973; Berejikian, 1995). More recent studies, however, have shown that several fishes can recognize a predator innately and respond defensively (Alemadi & Wisenden, 2002; Berejikian *et al.*, 2003; Hawkins *et al.*, 2007; Scheurer *et al.*, 2007), and that there may be an additional learned component to predator recognition (Olla & Davis, 1989; Berejikian, 1995). In this study, there

was evidence of an innate response to predator odours by *L. macrochirus* larvae. In Lake Opinicon, the larvae hatch in the littoral region and on swim-up head to deeper, open water (Garvey *et al.*, 2002). Switching habitats exposes the larvae to novel predators (Keast & Harker, 1977) and thus it is probable that survival of the larvae depends on their ability to recognize a predator and respond appropriately to the type of threat presented even when they have never before been exposed to that threat. *Lepomis macrochirus* larvae may additionally modify their innate antipredator response after repeated exposure to a predator in a way that is consistent with learning, but this has yet to be examined. Conceivably, as has been shown in other fishes such as *O. mykiss* (Berejikian, 1995) and fathead minnows *Pimephales promelas* Rafinesque (Ferrari *et al.*, 2005), *L. macrochirus* larvae may have both innate and learned components to their antipredator shoaling response.

In summary, kin selection has been a major advancement in biologists' understanding of social behaviour (Hamilton, 1964). Although many animals have been shown to recognize kin (Mateo, 2004), the particular benefits gained by fishes are only beginning to be understood (Greenberg *et al.*, 2002; Ward & Hart, 2003; Gerlach *et al.*, 2007). The results of this study suggest that not only is an antipredator response innate in *L. macrochirus*, larvae use kin recognition to increase shoal cohesion when exposed to a predator cue. Specifically, cuckolded-sired larvae use a direct recognition mechanism whereas parental-sired larvae appear to use a life-history-based recognition mechanism. The increased cohesion should lead to reduced predation intensity (Chippis *et al.*, 2004). Thus, kin selection appears to influence antipredator behaviour in *L. macrochirus* larvae.

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