Novel strategies in the complex defense repertoire of a stonefly (*Pteronarcys dorsata*) nymph

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Moore, K. A. and Williams, D. D. 1990. Novel strategies in the complex defense repertoire of a stonefly (*Pteronarcys dorsata*) nymph. – *Oikos* 57: 49–56.

The nymph of *Pteronarcys dorsata* Say, a large and primitive stonefly, exhibits a range of behavioural defenses. We studied predator-prey interactions between stonefly nymphs and co-existing pelagic (trout) and benthic predators (sculpins, suckers and crayfish) to determine whether the defenses used by *P. dorsata* were predator specific. Nymphs of *P. dorsata* distinguished between different predator types and applied an appropriate defense mechanism to effectively avoid predation. To benthic and pelagic fish predators, *P. dorsata* responded primarily by freezing and thanatosis (feigning death). Survival was lower in the stonefly species *Agneta capitata* which did not display thanatosis after attacks by trout. Nymphs of *P. dorsata* responded to benthic tactile crayfish predators by retreating and reflex bleeding (autohemorrhaging) which allowed for escape in all observed encounters. Reflex bleeding has never before been demonstrated as a defense mechanism for insects in aquatic environments. Risk of predation increased in nymphs deprived of their behavioural defenses. Stonefly nymphs were aggressive to conspecifics and experiments suggested that this behaviour may be a mechanism to avoid predation through spacing. Complex anti-predator defenses are generally unknown in aquatic insects; their discovery in such a primitive insect indicates that such behaviours may be evolutionarily ancient.

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Introduction

Prey defense mechanisms range from being general where they are directed towards all enemies, to specific where they enable the prey to cope with each predator type in a different way. The latter solution may be favoured by natural selection when predators vary in their hunting pattern such that a general protective mechanism is rendered ineffective. Prey with a variety of specific defense mechanisms must distinguish between different predators to apply the appropriate and more effective defense response to each situation (Curio et al. 1983). Specific defense responses to different predators have been reported extensively for birds (Curio et al. 1983, Eibl-Eibesfeldt 1975) and mammals (Struhsaker 1967) but rarely in aquatic invertebrates, although varied, non-specific responses have been

shown in a neustonic insect (Brönmark et al. 1984). Few studies have shown that wholly aquatic invertebrates are capable of distinguishing between different predators. Those species that have been shown to distinguish predators usually react by changing the frequency or intensity of a single defense response, as the risk of predation increases (Sih 1987) rather than by applying a different defense mechanism specific to each type of predator. For example, several species of mayfly have been found to differentiate between predatory and non-predatory stoneflies and to avoid predation by changes in distribution, activity levels or behavioural response (Peckarsky 1980, Williams 1987). Phillips (1978) demonstrated that sea urchins can distinguish between foraging and inactive starfish predators and alter the frequency of their defense responses. Amphipods are capable of distinguishing between different predators and

Accepted 1 August 1989

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respond by increasing activity to avoid encounters with benthic predators or decreasing activity to avoid visually-hunting pelagic predators (Williams and Moore 1985). Some species of gastropod can distinguish between two species of starfish and respond with defenses of differing intensity (Fishlyn and Phillips 1980).

In this study we determined: (1) whether nymphs of *Pteronarcys dorsata* Say, a large (4–5 cm long), primitive stonefly found in cool streams across North America can distinguish between different predator types; (2) whether nymphs can distinguish between predators and non-predators; (3) whether nymphs can apply predator specific defense mechanisms to effectively avoid predation against co-existing benthic and pelagic predators; (4) whether the risk of predation changes when specific defensive behaviours are disrupted; and (5) whether nymphs can detect predators using contact or non-contact cues. In addition, we explored the use of chemical defenses (autohemorrhaging) by the nymphs as examples of chemical defenses in aquatic larval arthropods are lacking in the literature (see recent reviews by Allan et al. 1987, Scrimshaw and Kerfoot 1987).

Methods

Pteronarcys dorsata nymphs were collected from riffle areas of Duffin Creek, Ontario (48°58'N; 79°05'W), where they occur in densities of less than 1 m⁻².

To determine the range of defenses used by this species against co-existing benthic predators we used two fish species, sculpins (*Cottus baerdi* Girard) and suckers (*Catostomus commersoni* Lacepede), and a crayfish (*Cambarus robustus* Girard). As a pelagic predator we used rainbow trout (*Salmo gairdneri* Richardson). All co-occur with this stonefly in local streams.

Two days before experimentation, predators were placed singly into aquaria (35 × 70 × 35 cm, in which there was a slight current created by aeration) containing gravel substrate (mean diameter 11.0 mm) sufficient to provide a refuge for the stoneflies. Experiments were carried out under low light conditions (4.2 × 10¹⁴ quanta s⁻¹ cm⁻²). During this time the predators were not fed. Glass barriers were inserted into the aquaria immediately before introducing the stonefly nymphs to initially separate the prey from the predator. Two late instar nymphs were placed into the predator-free end of each aquarium and allowed to acclimate for 30 min before the barrier was removed. The stoneflies' reactions to the predators were observed from behind a black plastic screen for 2 h.

To examine the effectiveness of the defenses used by nymphs of *P. dorsata* against fish predators, comparisons between *P. dorsata* (n = 24) and *Agneta capitata* (Pictet) (n = 20) (a species that does not feign death [thanatosis] and lives in similar habitats to *P. dorsata*) were made in experiments where stoneflies were dropped into tanks each containing a trout. Nymphs of *P. dorsata* ranging in length from 1.0 cm to 5.0 cm were

used to determine whether size was a factor in stonefly risk of predation by trout. The numbers of stoneflies of both species eaten and rejected by trout were recorded. To determine if the risk of predation changes when specific defensive behaviours are disrupted, we dropped freshly killed *P. dorsata* nymphs (with an extended body posture different from the curled up posture, characteristic of thanatosis) into tanks containing trout and recorded the number of nymphs eaten. In addition, nymphs were made to autohemorrhage (by squeezing with forceps) until their secretion was exhausted and then were presented to crayfish to determine if these predators would eat nymphs after capturing them (n = 7). As increased activity of the stoneflies was observed in the presence of crayfish, the total time each nymph spent moving in the presence and absence of a crayfish was recorded (n = 15) to determine whether the stonefly nymphs were detecting crayfish using contact or non-contact (chemical odour) cues.

Since considerable aggression between *P. dorsata* nymphs was observed, further experiments were conducted to determine whether aggressive behaviour functioned in defense of resources such as food or shelter. Eight aerated aquaria (17 × 30 × 20 cm) were set up each containing two dead maple leaves (one large [approximately 98 cm²] and one small [approximately 24 cm²]) as shelters and food resources. One stonefly nymph was put into each tank. After 24 h its position was noted and a second stonefly was introduced. Behaviour was observed for the next hour and the positions of each stonefly were noted after 24 h. Observations were also made in aquaria containing an abundance of leaves where food and shelter were not limited.

Scanning electron microscopy (SEM) was used to locate the specific sites of fluid release during the stonefly's reflex bleeding response and to determine if the secretion released by the nymphs affected the crayfish antennae. The external surfaces of stonefly nymphs were prepared for SEM by fixing whole animals in a glutaraldehyde fixative and dehydrating them using ethanol and acetone. The animals were then subjected to critical point drying in acetone. Crayfish antennae exposed to autohemorrhaging stonefly nymphs were prepared for the SEM by simply drying them in a desiccator since fixing and dehydration in organic liquids may have broken down lipid components which may have affected the properties of the secretion. Treated and untreated antennae were mounted on stubs and coated with gold for viewing.

To determine whether the fluid expelled by the stonefly was haemolymph or a specific chemical repellent secreted from glands, microscopic comparison was done using Wright's stain (a fixative and stain used for examining blood) applied to both the secretion and a control sample of haemolymph, the latter obtained by clipping a segment off the antennae.

The lipid components of both the haemolymph and secretion were extracted by mixing samples in a metha-

FREQUENCY OF BEHAVIOURAL RESPONSES (%)

Fig. 1. Specific behavioural responses of *P. dorsata* nymphs to benthic and pelagic fish, crayfish and conspecific nymphs showing that responses differ according to the point of contact. N values denote the number of encounters.

Fig. 2. Crayfish attacking nymph (A), and the nymph responding by autohemorrhaging. Arrows indicate the edge of the cloud of secretion (B). The crayfish then immediately releases the stonefly (C) and retreats (D).

nol and chloroform mixture (1:2 ratio). The chloroform interface containing the lipid components was then analyzed on a Carle 111 gas chromatograph.

To test for possible toxic properties of the secretion, pieces of dried bread were soaked either in the secretion or in water and subsequently fed both to trout and crayfish (following the technique of Hepburn et al. 1973). This was repeated 10 times.

Results

Response to benthic fish

The behavioural responses of *P. dorsata* nymphs to sculpins and suckers were not significantly different ($p > 0.05$, Chi square Goodness-of-fit analysis) therefore the responses were combined.

When active nymphs touched any of the benthic fish species with their antennae the nymphs froze, clinging upright to a rock surface, in 76.2% of the encounters (Fig. 1 A). If stoneflies were attacked by a benthic fish they responded by feigning death in a curled up position (thanatosis; 17.4% of all encounters). When fish touched a passive stonefly the nymphs response differed

depending on the contact point. For example, when the legs were touched, the nymph moved away (100%) (Fig. 1). Nymphs usually froze if their antennae were touched (86.2%) and most frequently displayed thanatosis if the body or cerci were touched (53.8%) (Fig. 1).

Response to pelagic fish

Pteronarcys dorsata nymphs behaved differently in response to pelagic fish than to benthic fish ($p < 0.001$, Chi square Goodness-of-fit analysis). Nymphs responded to a trout encounter by freezing (46.2%) or by thanatosis (45.3%). Thanatosis was seen most frequently during an attack when a stonefly was lifted off the substrate, whereas the nymph froze when the trout attacked but failed to loosen the nymph's grip on the substrate. The nymphs displayed thanatosis most frequently when the body or cerci were touched by trout (56.3% of encounters) but froze when the antennae or legs were touched (70.8% and 83.3% respectively) (Fig. 1 B). The time spent in thanatosis increased significantly between successive attacks when *P. dorsata* was attacked repeatedly by a trout (an average of 2.03 [1 SE = 0.51] minutes after first attack and 22.4 [1 SE = 5.89]

A

B

Fig. 3. Scanning electron micrographs showing the viscous film coating the sensilla on the surface of a crayfish antenna that has been exposed to an autohemorrhaging nymph (A) compared with a normal unexposed, antenna (B).

minutes after second attack) ($p < 0.05$, paired t-test). In the presence of trout, all stoneflies became motionless after 3–4 h and frequently buried themselves in the gravel, regardless of whether an encounter had occurred. When trout attempted to swallow a stonefly, the nymph responded by curling up tightly into a ball. Nymphs of *Agneta capitata* were all eaten by trout ($n = 20$), whereas 18 out of 24 nymphs of *P. dorsata* were rejected and subsequently ignored after an attack. *Agneta capitata* did not exhibit thanatosis. Trout easily swallowed all *P. dorsata* nymphs, head first, when nymphs were not curled up. However small and large nymphs were rejected with equal frequency when exhibiting thanatosis (9 out of 12 small nymphs, 10 out of 12 large nymphs). All dead nymphs (in which the defence response was absent) were eaten head first by trout ($n = 8$).

Reflex bleeding was observed only once during all of the trout attacks ($n = 111$) and it did not deter the fish.

Response to crayfish

Pteronarcys dorsata responded differently to the presence of crayfish than to benthic fish ($p < 0.001$, Chi square Goodness-of-fit analysis) and trout ($p < 0.001$, Chi Square Goodness-of-fit analysis). If an active or passive stonefly antenna was touched by a crayfish the nymph responded by moving away in 92.3%, and 90.0% of the encounters, respectively, whereas if the stonefly was contacted on its legs, body or cerci its response varied depending on where it was touched (Fig. 1 C). Stoneflies were infrequently captured by crayfish (6.5% of encounters) since they usually detected them first and retreated. If a crayfish did capture a stonefly, the nymph responded in all cases ($n = 44$) by autohemorrhaging. The fluid was forcibly expelled into the water as a milky cloud. The crayfish then immediately released the stonefly and retreated (Fig. 2) whereupon the crayfish spent between 30 s and two minutes cleaning its mouth parts, chelae and antennae. Crayfish that had been squirted by stoneflies would ignore or back away from nymphs presented to them for an average of 36.6 min (1 SE = 2.76, $n = 10$) before re-attempting to capture one. Crayfish readily ate all nymphs that were incapable of expelling further haemolymph ($n = 7$).

SEM showed that the defensive secretion from the nymphs is expelled through pores located on the trochanteral segments of the metathoracic pair of legs. The crayfish antennae exposed to autohemorrhaging nymphs were coated with a viscous film that covered mechanoreceptors, spines and hairs (Fig. 3).

The microscopic comparisons of the secretion and haemolymph showed similar structural elements. The haemolymph contained large granular and hyaline blood cells as well as smaller prohaemocytes and plasmatocytes (Arnold 1966) whereas the secreted fluid contained only the smaller blood cells, mainly prohaemocytes with some plasmatocytes. Gas chromatography showed similar lipid profiles. We conclude that the secretion is derived from haemolymph but is apparently strained as it is being expelled.

Since the bread soaked in water and soaked in the secretion were eaten readily by both fish and crayfish ($n = 10$), we suggest that there are no toxic substances in the secretion thus its repellent action must be through a cloaking effect on the crayfish's antennal sense organs.

Intraspecific aggression

The stonefly nymph that was introduced first into the observation aquaria always crawled under the largest maple leaf, after which it became less active, moving only occasionally to feed. When the second nymph was introduced, encounters with the first nymph occurred

while the second was searching for a retreat. When either nymph touched the conspecific with its antennae the most frequent response was mutual beating with these appendages (Fig 1 D). If retreat was not immediate, this behaviour was accompanied by biting until one of the nymphs retreated. Passive nymphs (first introduced) retreated in 49.2% of encounters whereas active nymphs (second introduced) retreated in 36.9% of encounters ($p = 0.013$, $n = 64$, Chi-square Goodness-of-fit analysis done only on the retreat response). The nymph that was attacked was not always the one that retreated. Autohemorrhaging was observed in one aggressive encounter but it had no visible effect on the attacker. Although fights lasted several seconds, we never observed any injuries. Occasionally nymphs withdrew without aggression. After 24 h, both stoneflies generally were found on the large leaf but spaced as far apart as possible. Aggression was also noted but not quantified during encounters in aquaria containing an unlimited source of food and shelter. Both stoneflies were observed to feed on leaves during the experiments. Nymphs showed increased activity after physically contacting crayfish and this promoted more intraspecific aggression due to increased encounter rates (a mean of 9.0 encounters/2 h observation period in the presence of crayfish compared with 1.2 encounters/2 h in the absence of crayfish) (t-test, $p < 0.01$, $n = 10$). During a two hour observation period, nymphs were more active after physically contacting crayfish ($\bar{x} = 29.7$ min of activity/observation period compared with a mean of 6.3 min in the absence of crayfish) (t-test, $p < 0.0001$, $n = 15$ observation periods).

Discussion

Prey may reduce their risk of predation by using tactics that reduce pre-contact predator-prey encounters, or by tactics that reduce attack and capture success (Allan et al. 1987, Sih 1987). The large rocks in streams under which the nymphs of *P. dorsata* live are a good refuge against fish predators such as trout. The nymphs' cryptic colour (grey-brown) also provides camouflage from such visual predators. These precontact defenses enable nymphs to avoid encounters with pelagic fish by reducing their proximity to these predators and by reducing the probability of detection. Several authors have shown that prey decrease movement in the presence of predators (Chapman 1966, Stein and Magnuson 1976, Gillett and Gonta 1978, Caldwell et al. 1980, Peckarsky 1980, Bauwens and Thoen 1981, Sih 1982, Williams and Moore 1982, 1985, Main 1987). Prey motion and size had the greatest influence on prey choice by the stickleback *Spinachia spinachia* L. (Kislalioglu and Gibson 1976). In our study, defenses such as thanatosis and reduced motion (freezing) were the dominant behaviours in nymphs exposed to fish predators. The risk of being preyed on by trout increased when these defensive behaviours were removed. Nymphs curled up dur-

ing thanatosis, projecting cerci outwards as spines, reduced the handling success by fish. Otto and Sjöström (1983) showed that stonefly nymphs with intact cerci were rejected more frequently by trout than those with cerci removed. The presence of spines is often an anti-predatory strategy (Edmunds 1974) and has been demonstrated for pike (*Esox lucius* L.) feeding on sticklebacks (*Pygosteus pungitius* L. and *Gasterosteus aculeatus* L.) (Hoogland et al. 1957) and for invertebrates feeding on zooplankton (Kerfoot et al. 1980, Greene 1983). Since *P. dorsata* nymphs rarely use reflex bleeding as a defense against trout and in the few instances when it did occur (Fig. 1) it did not deter the fish, it seems that this defense response is not effective for these vertebrate predators. This result agrees with observations on reflex bleeding in terrestrial insects (Cuenot 1896, Happ and Eisner 1961, Wallace and Blum 1971, Benfield 1974).

Since the preferred habitats of *P. dorsata* are the same large rocks as those inhabited by crayfish as well as by sculpins and suckers, encounter rates may be more frequent than with trout. However, throughout most of its aquatic life *P. dorsata* is too large to be preyed upon by benthic fish thus, presumably, crayfish pose a greater threat to nymphs and therefore more varied and costly (in terms of energy investment) defenses are required to avoid predation. Crayfish are tactile predators and nymphs have therefore evolved appropriate defenses such as withdrawal, deflection of an attack by reflex bleeding, and spacing. Sedentary prey have been found to be more vulnerable than mobile prey to predation by benthic tactile predators (Allan and Flecker 1988) therefore, upon encountering a crayfish, *P. dorsata* rapidly retreats rather than freezing to minimize the risk of being caught. Autohemorrhaging is used as a defense only when retreat from the predator fails and capture occurs. In plants, high energy investments in chemical defenses are associated with low growth rates and high predation stress (Coley et al. 1985). Although the nymph is able to secrete haemolymph 3-4 times in succession, all our observations indicated that once was sufficient to effect an escape. The risk of being preyed upon by crayfish clearly increases when the nymphs are not capable of reflex bleeding.

Autohemorrhaging results from increased blood pressure due to muscular contractions during traumatic stimuli (Happ and Eisner 1961, Wallace and Blum 1971). In terrestrial insects, the haemolymph clots rapidly following release, becoming sticky and viscous. Attacking invertebrate predators, such as ants, quickly become entangled in the secretion (Cuenot 1896, Happ and Eisner 1961, Wallace and Blum 1971, Benfield 1974). The bleeding reflex in nymphs of *P. dorsata* might be similarly effective against other tactile benthic predators such as predaceous stoneflies which may prey upon the stonefly's smaller instars. Until now, evidence of chemical defences among aquatic larval insects has not been demonstrated and this contrasts with the strong devel-

opment of glands and noxious substances in terrestrial counterparts and in the more exposed, mobile adult stages (Brönmark et al. 1984, Allan et al. 1987, Scrimshaw and Kerfoot 1987). Autohemorrhaging does occur in adult *P. dorsata* (pers. obs.) and has been noted in two other species of adult stoneflies (Arnold 1966, Benfield 1974).

Although prey aggregation may reduce predation by a confusion or dilution effect, sparser spacing has occasionally been shown to be a better defense (Tinbergen et al. 1967). Nymphs of *P. dorsata* displayed aggressive behaviour in a manner consistent with maintaining "personal space" (Biggs 1972) rather than of defending a fixed territory or resource. Since intraspecific aggression between *P. dorsata* nymphs does not appear to be related to territoriality, it may serve to space nymphs in their environment thus reducing the risk of predation by foraging crayfish.

Like many aquatic insects, *P. dorsata* lives the majority of its life as an aquatic nymph (2–4 yr) (Hilsenhoff and Narf 1972, Dosdall and Lehmkuhl 1979, Barton 1980) with a 3–6 wk period spent as a terrestrial adult (Lechleitner and Kondratieff 1983). It therefore risks predation for a greater period of time during its immature stages. Correspondingly, defenses against predation are probably more important in the larval instars. Studies need to be done to determine if the variety of behavioural defenses seen in terrestrial insects, and to a more limited degree in adult aquatic insects (Brönmark et al. 1984), are matched similarly in the larval stages of aquatic species.

The behavioural repertoire of defenses for *P. dorsata* range from freezing and feigning death in response to fish predators, to reflex bleeding and retreat in response to crayfish predators. Such complexity of behavioral defenses is well known in birds and mammals but not in a primitive aquatic species. We have shown for the first time, a chemical defense (reflex bleeding) that exists in a nymph of an aquatic insect, and that it is as effective in aquatic systems as has been shown for insects in terrestrial systems (Cuenot 1896, Hingston 1927, Maluf 1939, Happ and Eisner 1961, Wallace and Blum 1971, Benfield 1974). The recent stoneflies are thought to have evolved during the Permian (270 million years B. P.) and *P. dorsata* belongs to one of the most primitive families, the Pteronarcidae (Harper 1979). Our findings therefore suggest that complex behaviours are not necessarily restricted to more evolutionary derived animals but have their origins in primitive forms as well.

Acknowledgements – We thank I. M. Campbell for his suggestions and for the use of his gas chromatograph in the analysis of the haemolymph lipid profile. The manuscript also benefited from the comments of B. W. Feltsmate and J. M. Eadie. The Natural Sciences and Engineering Research Council of Canada funded the project through an operating grant to D.D.W.

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