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Inducible Defences in *Daphnia* Depend on Latent Alarm Signals from Conspecific Prey Activated in Predators

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Abstract

Some water fleas (*Daphnia* spp.) undergo phenotypic changes when exposed to chemical signals from predators. The chemical signals have been assumed to be of predator origin (i.e. kairomones), since juices of crushed *Daphnia* have been found ineffective. We speculated that latent alarm signals could be present in *Daphnia*, to be activated in predators following ingestion. Accordingly, fish predators were fed earthworms for 10 weeks to remove *Daphnia* remains from their gastrointestinal tracts. Following another 6 days of earthworm feeding, water conditioned by fish induced no morphological changes in *D. galeata*. When fish were alternatively fed *Daphnia* for 6 days, changes were induced with fish-conditioned water. Extracts made from intestines of earthworm-fed fish, homogenized with earthworms, gave no morphological changes, but intestines of the same origin homogenized with *Daphnia* did. Similar results were found when earthworms and *Daphnia* were homogenized with fish liver. Freshly frozen extracts of homogenized *Daphnia* gave no detectable changes at first instar stage in test animals, whereas extracts of *Daphnia* that had been kept at room temperature did induce such changes. Our results suggest that *Daphnia* respond to latent conspecific alarm signals (i.e. 'dormant' pheromones) that are activated by intestinal or bacterial enzymes in predators or in the water.

Key words: fish odour, growth, kairomone, morphology, phenotypic changes, pheromone

Introduction

By the end of the nineteenth century, it had become clear that the seasonal variation in appearance of waterfleas (*Daphnia* spp.) observed in lakes and ponds was not due to the succession of many species, but resulted from seasonal intraspecific changes in morphology (i.e. cyclomorphosis). A 'floating-theory' was proposed to explain the variation in *Daphnia* morphology during changing seasons (Wesenberg-Lund, 1900). According to this theory, *Daphnia* develops enlarged spines and 'helmets' as floating devices to counteract increased sinking rates associated with warmer summer waters. Many investigators opposed the theory and several alternative hypotheses have later been proposed to explain the phenomenon of cyclomorphosis (Jacobs, 1987).

Dodson (Dodson, 1974) observed that the morphology of *Daphnia* varied as a result of predation and suggested that the changes that took place during cyclomorphosis were adaptive. Rotifers (genus *Brachionus*) had at that time been shown to develop protective spines as a response to chemical cues released by *Asplanchna*, a rotifer predator (Gilbert, 1966). In *Daphnia*, however, the importance of chemical cues associated with predation was not recognized until some years later, when two papers appeared simultaneously (Grant and Bayly, 1981; Krueger and Dodson, 1981). In the first paper, a predator-induced 'helmet' was reported in individuals of the *D. carinata* complex (Grant and Bayly, 1981). The changes in these animals were induced by a chemical stimulus released by the invertebrate notonectid predator *Anisops calcaratus*. The authors suggested that crest development was a predation-avoidance mechanism, with the crested morphs being better at escaping predator attack. In the second paper, Krueger and Dodson (Krueger and Dodson, 1981) reported 'neckteeth' in *Daphnia pulex* embryos following exposure to chemical cues released by the predatory midge larva *Chaoborus americanus*. Their results also suggested that the induced form experienced reduced mortality, implying that the neckteeth of *Daphnia* were probably a defence against predators. The superiority of crested morphs compared to typical morphs of *D. pulex* in

escaping the grasp of predatory *Chaoborus* larvae has later been confirmed in behavioural studies (Havel and Dodson, 1984).

Following the two initial reports, morphological changes induced by chemical stimuli have been reported for a number of *Daphnia* spp. The shifts have been found with chemical cues from both invertebrate predators and fish (Hebert and Grewe, 1985; Havel and Dodson, 1987; Dodson, 1989; Vuorinen *et al.*, 1989; Walls and Ketola, 1989; Hanazato, 1990, 1991; Tollrian, 1990, 1993, 1994; Lüning, 1992; Tollrian and Dodson, 1999). The morphological changes caused by chemical cues from predators are commonly termed 'inducible defences' (Harvell, 1990; Tollrian and Harvell, 1999).

Tail spine length is one morphological trait that varies during changing seasons in *D. galeata* (Primicerio, 2003). For that species, the timing of increase in tail spine length corresponds with the seasonal dietary switch in stickleback, the main fish planktivore in many lakes at high latitudes. Spaak and Boersma (Spaak and Boersma, 1997) have shown that chemical stimuli associated with fish induce the elongation of the tail spine in *D. galeata*, as previously shown for other congeneric species (Tollrian and Dodson, 1999). The defensive roles of tail spines of *Daphnia* with regard to small fish predators has been demonstrated (Kolar and Wahl, 1998).

In addition to the morphological changes mentioned above, *Daphnia* also display behavioural and life-history adaptive responses to predators (Larsson and Dodson, 1993; Boersma *et al.*, 1998; Tollrian and Harvell, 1999). Following early findings (Dodson, 1988) of chemically induced predator-avoidance in *Daphnia*, predator-induced behaviour has later been found to be triggered by chemical cues from a number of invertebrates and fish predators (Ringelberg, 1991; Dawidowicz and Loose, 1992; De Meester, 1993; Watt and Young, 1994; Kleiven *et al.*, 1996; Lauridsen and Lodge, 1996; De Meester and Cousyn, 1997; Stirling and Roff, 2000). Responses in life-history traits, such as size and age at first reproduction and the production of males and resting eggs (i.e. ephippia), have also been found triggered with predator-associated stimuli (Dodson and Havel, 1988; Ketola and Vuorinen, 1989; Machácek, 1991, 1993, 1995; Lüning, 1992, 1994, 1995; Stibor, 1992; Weider and Pijanowska, 1993; Stibor and Lüning, 1994; Tollrian, 1995; Pijanowska and Stolpe, 1996; Burks *et al.*, 2000). It has previously been implied that the various response types (i.e. behavioural, morphological and life-history changes) may result from the same set of chemical cues (Ketola and Vuorinen, 1989; Lüning, 1992; Tollrian, 1995; Ringelberg and Van Gool, 1998). It may therefore be helpful to consider functional properties released by chemical cues for all three types of responses combined.

Chemical signals that are detected by a prey organism may have several sources. The signals may be interspecific messengers, originating in the predator or another prey species and termed 'kairomones' (Brown *et al.*, 1970). Alternatively, the signals may have their origin in injured conspecific prey. In that case, they function as intraspecific alarm substances—i.e. 'Schreckstoff' (Pfeiffer, 1963)—and should be classified as 'pheromones' (Karlson and Lüscher, 1959). With regards to inducible defences in *Daphnia*, the active chemical cues have generally been considered as kairomones since juices of crushed *Daphnia* have been found ineffective (Walls and Ketola, 1989; Parejko and Dodson, 1990). Additional support for the view that the active compounds do not originate in conspecific prey comes from the fact that the signals appear to possess predator-specific properties (Dodson, 1989; Stibor and Lüning, 1994).

Alarm signals have been demonstrated in a number of aquatic animal species (Pfeifer, 1963; Howe and Sheik, 1975; Atema and Stenzler, 1977; Parker and Schulman, 1986; Smith, 1992; Wilson and Lefcort, 1993). With respect to predator–prey interactions in aquatic environments, it was long assumed that prey animals were detecting chemical cues of predator origin. However, several studies of prey behaviour, carried out with careful control of the predators' diet, have revealed that predators may be 'labelled' with chemical alarm signals from previously ingested prey. Predator labelling has been shown in prey species as diverse as sea anemones, fish, amphibians, insect nymphs, marine snails and sea urchins (Howe and Harris, 1978; Mathis and Smith, 1993; Wilson and Lefcort, 1993; Chivers *et al.*, 1996; Jacobsen and Stabell, 1999; Hagen *et al.*, 2002). Predator labelling has also been demonstrated with regard to morphological defences induced by alarm signals in fish (Stabell and Lwin, 1997).

The concept of predator labelling postulates that a predator is being chemically disclosed by its choice of prey, but remains undetectable by distance chemoreception as long as its diet does not include recognizable prey (Howe and Harris, 1978; Mathis and Smith, 1993; Stabell and Lwin, 1997). Alarm signals from ingested prey are probably released from the digestive system of predators together with urine and faecal material (Wilson and Lefcort, 1993). Therefore, to distinguish alarm signal responses from true kairomone responses requires experimental evidence from research with proper control for predator diet.

We speculated that chemical cues from predators (i.e. kairomones) must be regarded as 'unreliable' with regard to predation risk for the prey. This is because the intensity of fish predation on *Daphnia* in a lake is not constant, but varies seasonally due to phenological changes in fish diet. The smell of fish can only inform *Daphnia* of fish presence, not of fish diet. Another objection to the reliability of predator 'kairomones' as cues of predation risk stems from evolutionary considerations. Predators that release easily detectable chemical cues might be expected to end up as losers in the long run. It can therefore be argued that the development of an advanced defence system in a prey, based on predator cues, does not represent a plausible evolutionary

scenario (Parker, 1984). Intriguing support for our speculations comes from a survey of the literature. In almost every paper dealing with defences in *Daphnia* that are supposedly induced by predator kairomones, the predators were fed the prey species of study.

Based on the knowledge listed above, we expanded the idea of how predator labelling may take place and proposed the presence of latent alarm substances in *Daphnia* that require activation by digestive enzymes or bacteria within the gastrointestinal tract of the predator. Such chemicals would provide reliable signals for the assessment of predation risk by *Daphnia*. Here we present evidence for the presence of such latent alarm signals in *Daphnia*.

Material and methods

Collection and rearing of animals

The water fleas (*Daphnia galeata*) used in the experiments were collected from Lake Lombola, located in the inner part of Troms County, North Norway (69° 07′ N). Another cladoceran prey (*D. pulex*) used in some treatments was collected from a pond (<100 m^2) on the main island of Tromsø. Clonal lines of *D. galeata* were reared at room temperature in the laboratory, kept in a synthetic zooplankton medium made from glass-distilled water with 0.5 g unrefined salt and 0.1 g $CaCO_3$ per litre (Hobæk and Larsson, 1990). The salt was heated to 450°C for 12 h, dissolved and filtered through glass fibre filters (Whatman GF/F), before the final dilution. A mixed stock of *D. pulex* was reared in 20 l glass jars, using filtered stream water from the college campus. A freshwater green alga (*Scenedesmus acutus*) was used as food for the *Daphnia*.

Three-spined sticklebacks (*Gasterosteus aculeatus*), a native predator fish, were trapped in a stream pond on the college campus and Malawi cichlids (*Nimbochromis venustus*), an alien predator fish, were obtained from an aquarium shop in Tromsø. The fish were fed either one of the two *Daphnia* species, or earthworms (*Lumbricus* spp.), depending on the experimental protocol. The rearing of sticklebacks took place in a cold-room at ~6°C, whereas the cichlids were reared in the laboratory at ambient room temperature. In addition, crucian carp (*Carassius carassius*), raised in the aquarium facilities on commercial fish feed and rolled oats, were used as donors of fish liver.

Preparation of stimuli

To produce predator-conditioned water, sticklebacks and cichlids were initially fed earthworms for 10 weeks. This was done to secure removal of possible remains from previously ingested *Daphnia* in the gastrointestinal tracts of the fish. Thereafter, three groups of sticklebacks and three groups of cichlids were each placed in separate 5 l aquaria and fed alternatively (a) *D. galeata*, (b) *D. pulex*, or (c) earthworms for 6 days. To produce fish-conditioned water, two fish from each species and treatment series were allowed to swim in 3 l of zooplankton medium for 24 h. During this period the fish were not fed and the water was continuously aerated by air-stones. Following the removal of fish, the fish-conditioned water was filtered (Whatman No. 1) and subsequently frozen in 350 ml plastic ice-cube bags at –18°C for storage (Stabell and Lwin, 1997). To avoid contamination, different nets were used for each treatment to transfer fish between the rearing aquarium and the glass container used for conditioning of water. Short names for the various types of predator-conditioned water used in the study are listed in Table 1 (1a–c, 2a–c).

Tissue extracts to be used as chemical stimuli were made in three different ways. In the first case, *Daphnia* and earthworms were each homogenized with intestines of predator fish that had been kept on an earthworm diet for a minimum of 10 weeks (Table 1, 3a,b). Extracts were prepared by homogenizing five adult individuals of *D. galeata*, or small pieces of earthworms (~0.3 g) with pieces of intestine from sticklebacks (~0.2 g) in 5 ml of zooplankton medium. Following a 2.5 h respite at room temperature to allow for bacterial and enzymatic activity to take place, the homogenates were centrifuged. The supernatants were diluted in a 1:100 ratio by volume with zooplankton medium and frozen in plastic ice-cube bags. Secondly, a sample extract was made from five adult specimen of *D. pulex* that were homogenized with ~0.1 g of crucian carp liver in 5 ml zooplankton medium. Extract of earthworms, homogenized with crucian carp liver and zooplankton medium, was used as a control sample (Table 1, 4a,b). Also in these cases, the homogenates were left at room temperature for 2.5 h, followed by centrifugation, dilution and freezing of the extracts until usage. Thirdly and finally, samples were made from *Daphnia* homogenized with zooplankton medium only (Table 1, 5a,b). Altogether, 10 adult specimen of *D. galeata* were used and half the homogenate was immediately centrifuged, diluted and frozen. The other half was left at room temperature for 2.5 h before centrifugation, dilution and freezing of the stimulus sample.

Experimental protocol

All experiments were conducted with a single clonal line of *D. galeata* and took place at room temperature (19–20°C) under conditions of constant light in glassware that had been thoroughly washed, water rinsed and autoclaved. At the onset of each series, 100 ml of zooplankton medium was added to each glass, with five replicate glasses for each treatment. The glasses were labelled according to treatments and placed at random on the bench. Two egg-bearing individuals of *D. galeata* were put in each glass (with the aim of obtaining a sufficient number of offspring for each parallel) and 1 ml of zooplankton medium was replaced by 1 ml of the stimulus solution prepared for each treatment. The relevant stimulus was thereafter added once a day during the experimental period using the above replacement procedure.

Table 1 Overview of short names, sample types, pre-treatments, preparation methods and storage procedures for chemical stimuli used in the study

Test no.	Short name	Sample type	Pre-treatment	Preparation method	Storage procedure
1a	galeata–stickleback	water conditioned by sticklebacks	sticklebacks fed *D. galeata*	water conditioned for 24 h	filtered and frozen fresh
1b	pulex–stickleback	water conditioned by sticklebacks	sticklebacks fed *D. pulex*	water conditioned for 24 h	filtered and frozen fresh
1c	earthworm–stickleback	water conditioned by sticklebacks	sticklebacks fed earthworms	water conditioned for 24 h	filtered and frozen fresh
1d	blank	zooplankton medium	none	none	frozen fresh
2a	galeata–cichlid	water conditioned by cichlids	cichlids fed *D. galeata*	water conditioned for 24 h	filtered and frozen fresh
2b	pulex–cichlid	water conditioned by cichlids	cichlids fed *D. pulex*	water conditioned for 24 h	filtered and frozen fresh
2c	earthworm–cichlid	water conditioned by cichlids	cichlids fed earthworms	water conditioned for 24 h	filtered and frozen fresh
2d	blank	zooplankton medium	none	none	frozen fresh
3a	galeata–intestine	homogenized *D. galeata* and fish intestine	sticklebacks fed earthworms	homogenate 2.5 h on bench	extract diluted and frozen
3b	earthworm–intestine	homogenized earthworm and fish intestine	sticklebacks fed earthworms	homogenate 2.5 h on bench	extract diluted and frozen
4a	pulex–liver	homogenized *D. pulex* and fish liver	crucian carp fed fish feed	homogenate 2.5 h on bench	extract diluted and frozen
4b	earthworm–liver	homogenized earthworm and fish liver	crucian carp fed fish feed	homogenate 2.5 h on bench	extract diluted and frozen
5a	galeata-fresh	homogenized *D. galeata*	zooplankton medium added	extract made immediately	diluted and frozen fresh
5b	galeata-aged	homogenized *D. galeata*	zooplankton medium added	homogenate 2.5 h on bench	extract diluted and frozen

Each glass was followed for 24 h to register offspring release. In general, this took place in a minimum of three glasses during the surveillance period. The 24 h limit for awaiting offspring production was set to produce homogeneous time series. Following offspring release, the mothers were removed and the developmental stages of the progeny followed. At regular time intervals after birth (days 1, 3, 5 and 7) one individual was taken at random from each replicate glass and measured. On day 1, the offspring were sampled within 12 h after birth (i.e. instar 1); by day 7, some individuals were carrying eggs (i.e. instar 5). Growth takes place stepwise in *Daphnia*, during shedding of the exoskeleton to establish the next instar stage.

Altogether, five series of experiments were conducted. In series 1, *D. galeata* were exposed to water conditioned by sticklebacks that had been fed (a) *D. galeata*, (b) *D. pulex*, or (c) earthworms. In addition, five replicates with added zooplankton medium (d), were run as blank treatments. The short names used for the exposure types of this series were 'galeata–stickleback', 'pulex–stickleback', 'earthworm–stickleback' and 'blank', respectively (Table 1, 1a–d). In series 2, exposure took place with water conditioned by three groups of Malawi cichlids that had been given the same three feed types (a–c) as in the series with sticklebacks. Also in this series, a blank treatment (d) of zooplankton medium was used. The short names for the exposure types of this series were 'galeata–cichlid', 'pulex–cichlid', 'earthworm–cichlid' and 'blank', respectively (Table 1, 2a–d). In series 3, *D. galeata* were exposed to extracts of intestine from earthworm-fed sticklebacks that had been homogenized with either (a) *D. galeata*, or (b) earthworms. The short names used in this series were 'galeata–intestine' and 'earthworm–intestine' (Table 1, 3a, b). In series 4, exposure took place with extracts of crucian carp liver homogenized with (a) *D. pulex*, or (b) earthworms. The short names used in this series were 'pulex–liver' and 'earthworm–liver' (Table 1, 4a,b). Finally, in series 5, *D. galeata* were exposed to extracts of either (a) homogenized *D. galeata* frozen fresh, or (b) homogenized *D. galeata* left for 2.5 h at room temperature before freezing. The short names used for the exposure types of this series were 'galeata-fresh' and 'galeata-aged' (Table 1, 5a, b).

Morphometric measurements and data analysis

Morphological measurements were carried out using a microscope equipped with an ocular micrometer. The

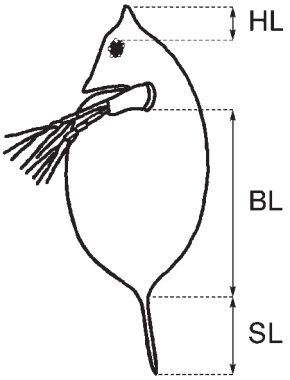

Figure 1 Morphological parameters measured on individuals of *Daphnia galeata*. HL, helmet length; BL, body length; SL, spine length.

measured parameters were helmet length (HL), body length (BL) and tail spine length (SL) (Hebert and Grewe, 1985; Hanazato, 1990; Pijanowska, 1990). HL was measured as the distance between the anterior margin of the compound eye and the tip of the helmet (Figure 1). When individuals lacked a helmet, the distance measured was between the eye and the anterior margin of the head. BL was measured as the distance between the posterior margin of the insertion point of the second antennae and the posterior margin of the carapace. SL was measured from the posterior margin of the carapace to the tip of the spine. BL was measured at 40× magnification, whereas HL and SL were measured at 100×.

To adjust for variation in size among individuals, data are presented as 'tail spine index' (SI), or 'helmet index' (HI), given by the formulae SI = SL/BL and HI = HL/BL (Hanazato, 1990; Pijanowska, 1990; Tollrian, 1990). To test for statistical differences between treatments ($a = 0.05$), the data were analysed using Student's *t*-test. Statistical calculations were carried out using the computer program Statview (SAS Institute, Cary, NC).

Results

Growth patterns

To visualize the general patterns of growth in *D. galeata* in the presence and absence of chemical cues from predators, the absolute lengths of body, tail spine and 'helmet' from hatching (instar 1) until the first observed stage of egg production (instar 5) are presented in Figure 2. Water conditioned by Malawi cichlids previously fed *D. galeata* was used as the predator cue in this example, whereas zooplankton medium was used as control cue. Individuals of *D. galeata* exposed to water conditioned by predators demonstrates a pattern of length increment for body, tail spines and 'helmets' different from that obtained following exposure to control treatments. During the first 3 days, tail spines and 'helmets' were found longer in the presence of predator cues compared to when the predator cues were absent. For tail spines, the differences between treatment

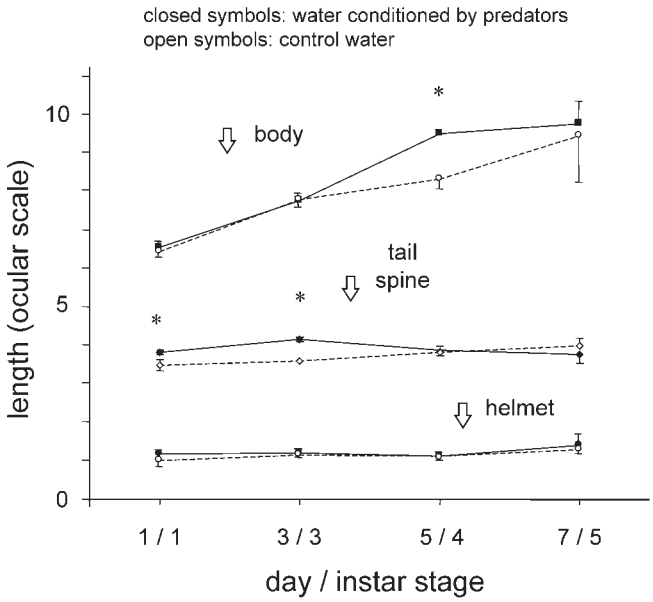

Figure 2 Changes in absolute length of body, tail spine and helmet in *Daphnia galeata*, measured for 7 days after hatching. The animals were either exposed to water conditioned by predator fish that had eaten conspecifics (closed symbols, solid lines) or zooplankton medium only (open symbols, hatched lines). At day 7 (i.e. instar stage 5) eggs could be observed in some individuals.

types were found at a statistically significant level ($P < 0.05$) on both day 1 and day 3. From day 5 on, body lengths were found to increase in the presence of predator cues compared to control treatment, whereas tail spine and 'helmets' tended to decrease. For body length, a statistically significant difference ($P < 0.05$) was found on day 5 with these treatments.

Exposure to predator-conditioned water

When *D. galeata* were exposed to the 'earthworm–stickleback' treatment (Table 1), both the tail spine and the helmet indices showed an almost horizontal pattern (slight decline) during the 7 days of exposure (Figure 3A, stippled lines). This means that the relative sizes of crests are only slightly decreasing with increasing instar stages. Almost identical patterns were found for *D. galeata* exposed to the 'blank' treatment (Figure 3B, stippled lines). No significant difference was found between the above treatments for either tail spine or helmet indices throughout the duration of the time series ($P > 0.05$). This result shows that predator-odour alone is not sufficient for induction of morphological changes in *Daphnia*.

When *D. galeata* were exposed to the 'galeata–stickleback' treatment (Figure 3A, solid lines) or the 'pulex–stickleback' treatment (Figure 3B, solid lines), however, another pattern of progress revealed for both indices. For both treatments, the tail spine index started at a higher value on day 1 than found with exposure to 'earthworm–stickleback' or 'blank' treatments and it declined throughout the experimental

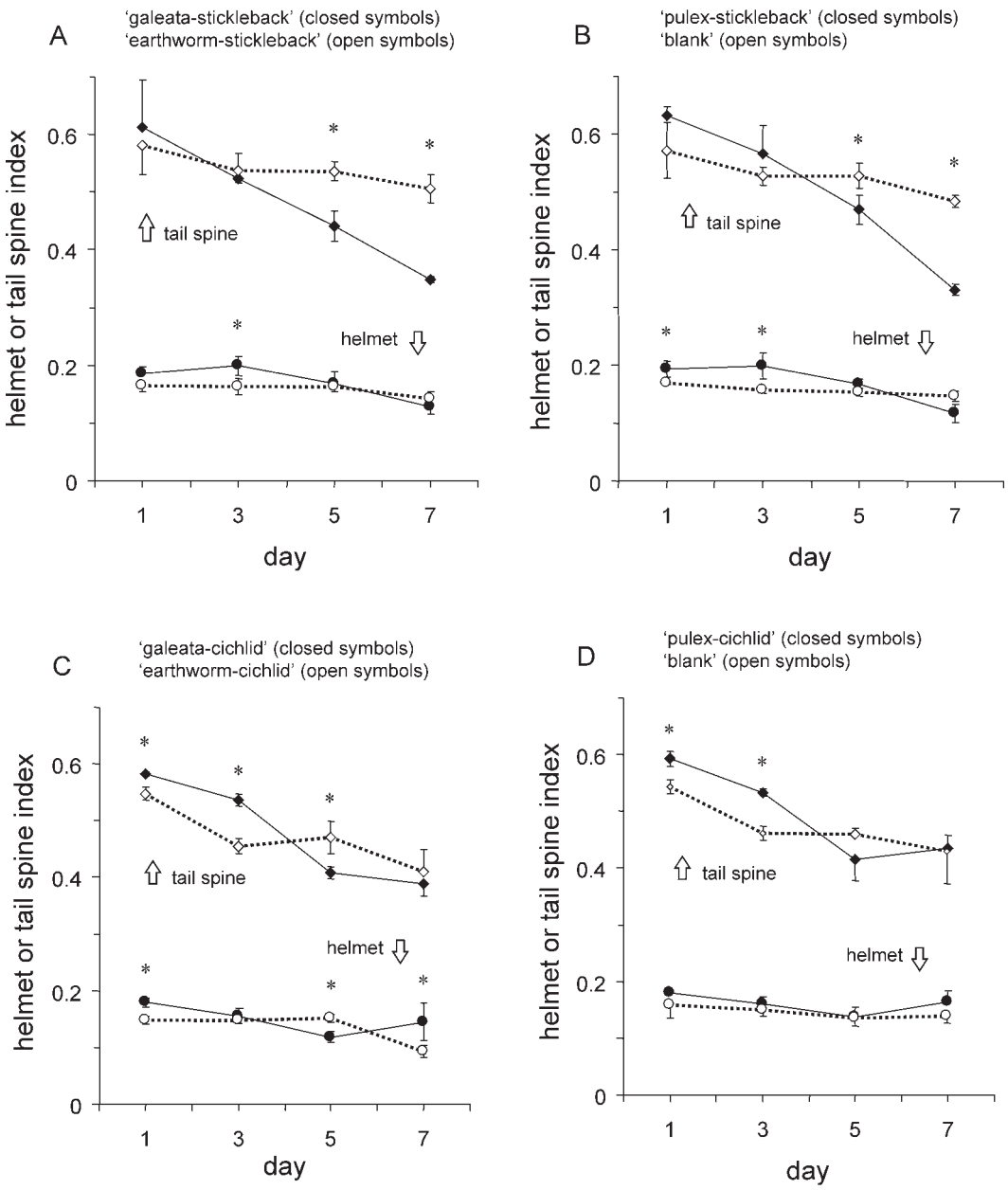

Figure 3 Pattern of development in relative size of tail spines and helmets in *Daphnia galeata* following exposure to predator odours. Measurements from the first 7 days after hatching are presented as mean (±SD) tail spine and helmet indices (ratio of crest size relative to body size) to adjust for differences in body size during development. The chemical stimuli applied were: **(A)** zooplankton medium conditioned by sticklebacks previously fed either *D. galeata* ('galeata–stickleback' treatment, closed symbols) or earthworms ('earthworm–stickleback' treatment, open symbols); **(B)** zooplankton medium conditioned by sticklebacks previously fed *D. pulex* ('pulex–stickleback' treatment, closed symbols), or zooplankton medium presented alone ('blank' treatment, open symbols); **(C)** zooplankton medium conditioned by Malawi cichlids previously fed either *D. galeata* ('galeata–cichlid' treatment, closed symbols) or earthworms ('earthworm–cichlid' treatment, open symbols); **(D)** zooplankton medium conditioned by cichlids previously fed *D. pulex* ('pulex–cichlid' treatment, closed symbols), or zooplankton medium presented alone ('blank' treatment, open symbols). *$P < 0.05$.

period to end at a lower value than obtained with those treatments on day 7. This result is in accordance with what is found in nature in the presence of fish predators, where increase in spine length develops for small *Daphnia*, followed by decrease in spine length when the animals are approaching mature stages, i.e. instar 5 or higher (Primicerio, 2003). Also, the helmet index was found to be larger than controls on day 1 for the 'galeata–stickleback' and the 'pulex–stickleback' treatments. The helmet index, however, increased slightly towards day 3 for both treatments, and subsequently decreased towards lower values than controls on day 7. For both treatments, statistically significant differences from

'earthworm–stickleback' and 'blank' treatments were found for the tail spine index on days 5 and 7, whereas statistically significant differences were found for the helmet index on days 1 and 3 for the 'pulex–stickleback' treatment and on day 1 for the 'galeata–stickleback' treatment. These results strongly suggest that native predators must have ingested specimen of *Daphnia* beforehand to induce morphological changes in *D. galeata*.

The general patterns obtained for the tail spine and helmet indices with stickleback-conditioned water were also found with water conditioned by Malawi cichlids (Figure 3C,D). No statistically significant differences were found in pattern of development between the 'earthworm–cichlid' and 'blank' treatments (Figure 3C,D, stippled lines). The major difference revealed with cichlid-conditioned water compared to stickleback-conditioned water, were the higher indices found for both tail spines and helmets on day 7 with water conditioned by fish fed *Daphnia* (Figure 3C,D, solid lines). However, direct statistical comparison is not possible between cichlid-conditioned waters and stickleback-conditioned waters, since these treatment groups result from different time series. For both the 'galeata–cichlid' and the 'pulex–cichlid' treatments, statistically significant differences from 'earthworm–cichlid' and 'blank' treatments were found for the tail spine index on day 1 and 3. This was also the case for the tail spine index on day 5 with the 'galeata–cichlid' treatment. For the helmet indices, however, only the 'galeata–cichlid' treatment resulted in statistically significant differences from control treatment (i.e. 'earthworm–cichlid' treatment), as given on days 1, 3 and 7. The above results demonstrate that also an alien predator will induce morphological changes in *D. galeata* if specimens of *Daphnia* have been ingested beforehand.

Exposure to extracts of homogenates

When *D. galeata* were exposed to the 'galeata–intestine' treatment (Figure 4A, solid line) or 'earthworm–intestine' treatment (Figure 4A, stippled line), the observed patterns of response were almost similar to those obtained with predator-conditioned waters (Figure 3). In this case, however, more pronounced differences were obtained between treatments, revealing significant differences on days 1, 3, 5 and 7 for the tail spine indices and on days 1, 3 and 7 for the helmet indices. The larger differences displayed may in part be due to the fact that the 'earthworm-intestinal' curve was slightly increasing during the surveillance period compared to 'earthworm–stickleback' and 'earthworm–cichlid' treatments. However, data representing these mentioned treatment curves were obtained from different time series, and different procedures for producing stimuli (conditioned water versus tissue extraction) may have influenced the result. Accordingly, the only conclusion to be drawn from these data is that extracts of predator intestinal tissue mixed with tissue of alien prey do not possess alarm signal properties. The tissue from predators must be mixed with tissue from *Daphnia* to release signals that trigger morphological responses.

Exposure of *D. galeata* to 'pulex–liver' treatment (Figure 3B, solid line) or 'earthworm–liver' treatment (Figure 3B, stippled line), showed the same patterns as found with predator-conditioned waters and intestinal extracts. Significant differences between the two series were found on days 1 and 3 for the tail spine index, but no differences were apparent for the helmet indices throughout the duration of the experiment. In the series with extracts of homogenized liver, there were not enough *Daphnia* offspring available to obtain data on day 7 for the 'earthworm–liver' treatment. However, the results from the first 5 days of the series with liver homogenates support the previous conclusion that the chemical substances in question do not originate in the GI tract of predators. Combined, the results obtained with homogenates of intestine and liver tissue strongly suggest that the active compounds should be searched for in the prey itself, i.e. in the tissue of *Daphnia*.

When live specimens of *D. galeata* were exposed to extracts of homogenized conspecifics, statistically significant differences were found on day 1 (i.e. at 1st instar) between the 'galeata-aged' and the 'galeata-fresh' treatments. The differences on day 1 were evident for both the tail spine and the helmet indices (Figure 3C). For both indices, the data obtained with bench-stored extract followed a pattern similar to that found for other *Daphnia*-containing homogenates. However, the data obtained with the 'galeata-fresh' treatment displayed a shift in the pattern, from a start similar to that generally found with 'blank' treatments on day 1, to a pattern similar to that found with bench-stored extracts containing *Daphnia* from day 3 on. This result reveals that the fresh extract was initially inactive, but displayed active signal properties after >12 h in the water.

Discussion

Daphnia galeata, the test species of the study, displayed a crest increment in the early instar stages of their life when exposed to predator signals. For later instars, body growth was given priority, presumably to prepare for egg production. The morphometric patterns for tail spine and body length displayed in our data are in accordance with results from field studies and their ecological implications are treated in detail elsewhere (Primicerio, 2003). In this study, fish predators were used initially to mimic the production of the active chemical signals that hitherto have been termed 'kairomones' in the literature. This was done by feeding fishes with earthworms for 10 weeks, resulting in loss of active chemical cues responsible for induction of crest development in *Daphnia*. When fed *Daphnia*, however, predators again released chemical cues with active signal properties. In this way also, an alien fish species was labelled

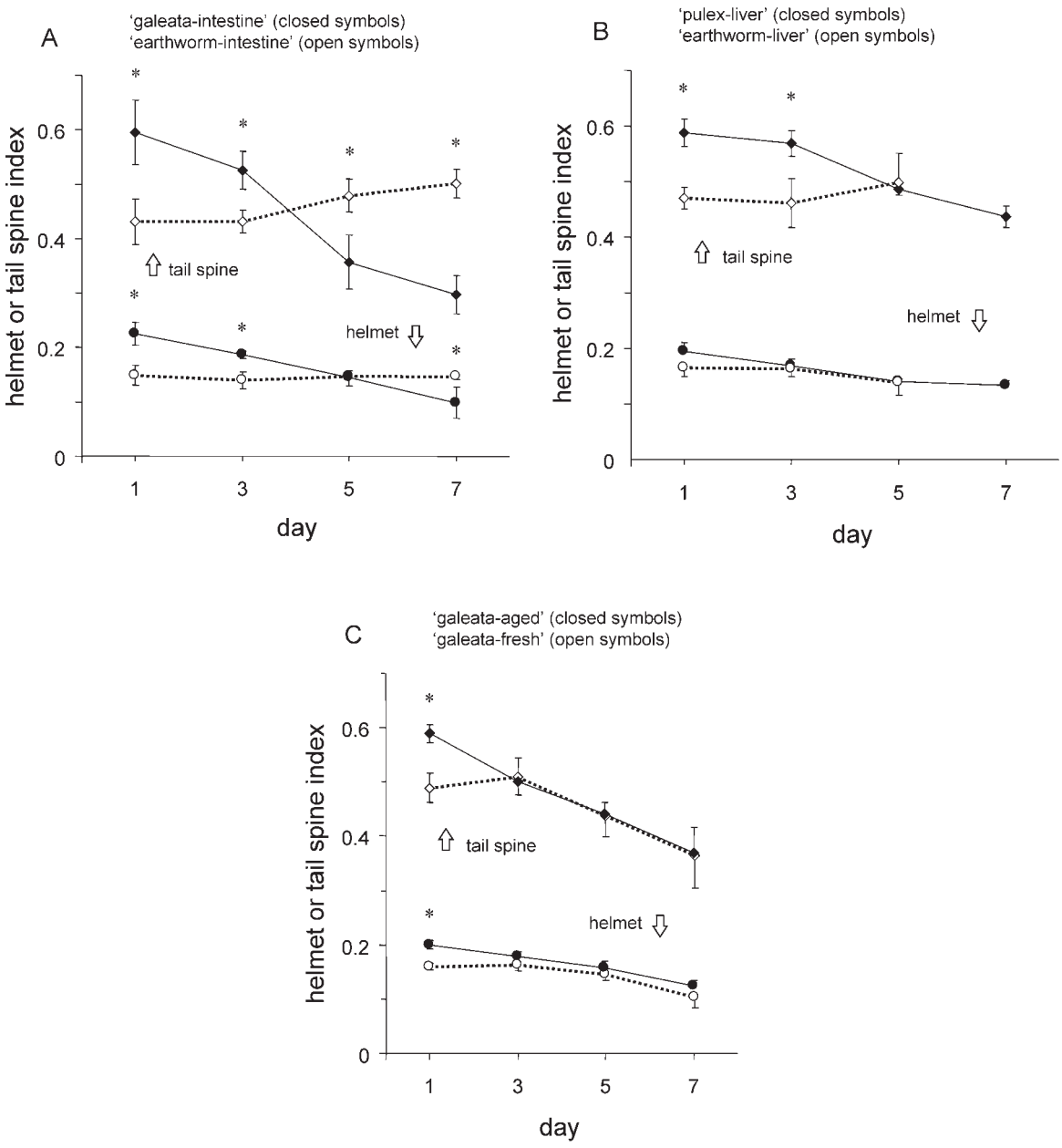

Figure 4 Pattern of development in relative size of tail spines and helmets in *Daphnia galeata* following exposure to extracts of various homogenates. Measurements from the first 7 days after hatching are presented as mean (±SD) tail spine and helmet indices (ratio of crest size relative to body size) to adjust for differences in body size during development. The chemical stimuli applied were prepared from: **(A)** intestine of sticklebacks homogenized with either *D. galeata* ('galeata–intestine' treatment, closed symbols), or earthworms ('earthworm–intestine' treatment, open symbols); **(B)** liver of crucian carp homogenized with either *D. pulex* ('pulex–liver' treatment, closed symbols), or earthworms ('earthworm–liver' treatment, open symbols); **(C)** homogenates of *D. galeata* aged at room temperature for 2.5 h ('galeata-aged' treatment, closed symbols), or homogenates of *D. galeata* frozen fresh ('galeata-fresh' treatment, open symbols). *$P < 0.05$.

by prey specific signals, the result being the release of active chemical signals by a presumably unknown predator.

The active chemical signals were subsequently made by homogenizing *Daphnia* with various types of tissues from predators. Both intestines and liver, taken from fish that previously had been fed earthworms, were found to release active chemical signals when homogenized with *Daphnia*. When homogenized with earthworm tissue, similar fish tissues displayed a lack of active cues. Even *Daphnia* homogenized alone were found to contain active chemical signals, but in all cases the homogenates needed a 2.5 h incubation time to release the active signals.

Freshly made homogenates of *Daphnia* were initially found inactive when tested for crest induction in *D. galeata*. The fresh extract, however, displayed a shift in signal properties after some hours in the water. This shift in pattern

of crest induction suggests that inactive compounds were present in the extracts of freshly homogenized *Daphnia* and were chemically altered in the water after >12 h, resulting in active substances that triggered prey responses. Bacteria present in the rearing water may have caused such a shift in chemical properties of the extract.

In the following, the origin of the novel chemical signals will be considered and the necessity of pre-treating predators to detect latent alarm signals will be addressed. The possible ways in which signal activation may take place will be evaluated and the implication for explaining species specificity of signals released by various predators will be presented.

The origin of signals

One initial concern in this study was to ensure that any possible *Daphnia* remains were removed from the predators. Accordingly, a thoroughly pre-treatment of the predators was carried out to ensure that they were not labelled by chemical cues presumably present in *Daphnia*. The pre-treatment of predators was carried out by feeding fish for a prolonged period of time with taxonomically distant prey (i.e. earthworms). A lack of predator pre-treatment may help explain the conflicting results obtained by other investigators. For instance, Dodson (Dodson, 1988, 1989), found in behaviour experiments that *Daphnia* responded only to those predators that had been a source of *Daphnia* mortality in nature, whereas the effect of predator signals on morphology was less conclusive. In addition, Loose *et al.* (Loose *et al.*, 1993) found in studies of vertical migration that it did not matter whether the predator fish was hungry, fed *Daphnia* or fed artificial food; the tested water was positive in all cases. However, our results suggest that the previous feeding of predators could represent a serious source of error and that predators can be made undetectable in an alarm signal context by controlling their feeding history.

For cyprinid fishes, it has been found that the alarm signals from one single club cell, diluted in 180 l of water, can be detected by conspecifics (Smith, 1992). Due to such low sensory thresholds in prey animals for detection of alarm signals, a complete removal of *Daphnia* residues from the intestine of predators should be a matter of concern in experimental design. The time needed to remove chemical signals of prey origin should be expected to be longer than that needed for the bulk passage of food through the intestine of a predator. Hypothetically, a predator could even be marked for life following consumption of a single prey individual. This could be the case since chemical signals may possess hydrophobic properties (Stabell 1987). Prey odour could then be stored in the adipose tissue of a predator, to leak slowly into the environment over time. Intermediate hydrophobic properties of the chemical signals affecting *Daphnia* have already been demonstrated (Parejko and Dodson, 1990; Tollrian and Von Elert, 1994; Von Elert and Loose, 1996; Von Elert and Pohnert, 2000).

Stirling (Stirling, 1995) considered the possibility of predator labelling, but ended up rejecting the hypothesis. However, the conclusion was based on an experimental design that involved repeated switching of food sources, with a risk of undesired labelling in the treatments. Since, otherwise, predator labelling has not been a concern in studies with *Daphnia*, the time needed to remove *Daphnia* residues from a predator has not been studied. However, by feeding fish predators for at least 10 weeks with earthworms in the current study, a complete removal was obtained with regard to chemical alarm cues. In this way it was shown that fish predators must eat *Daphnia* in order to release the active chemical signals, implying involvement of alarm signals of prey origin and predator labelling as the functional mechanism. We suggest that similar results will be found also with invertebrate predators if these are subjected to a proper pre-treatment procedure.

In many aquatic animal species, vertebrates as well as invertebrates, behavioural and morphological responses to predators cues have been reported, whereas conspecific alarm signals are seemingly absent. In light of the results of the present study and the subsequent discovery of latent alarm signals in sea urchins and marine snails (Hagen *et al.*, 2002; Jacobsen and Stabell, 2003), we suspect that latent alarm signals may be a common phenomenon among aquatic animals.

Possible mechanisms of signal activation

The basic idea behind this study was that digestive enzymes in the gut of predators act on engulfed *Daphnia* tissue to activate latent chemical signals. Parejko and Dodson (Parejko and Dodson, 1990) suggested that the chemical cues necessary to induce 'neckteeth' in *D. pulex* originated in the intestinal tract of the predator. Our data demonstrate that intestinal tissue of predators contain neither active signals nor any chemical precursors. However, when intestinal tissue from earthworm-fed predators was homogenized with *Daphnia*, then active chemical signals were found to be present. In fact, intestinal tissue was not required, since active signals were also obtained when *Daphnia* was homogenized with enzyme rich tissue such as fish liver, or even when homogenized *Daphnia* tissues were left alone. In all cases, homogenates required incubation time to ensure that activation of the latent alarm signals had taken place.

It is interesting to note that a total absence of predator influence on the activation of latent alarm signals would make it impossible for the predator to mask its presence, making the chemical signals evolutionarily reliable. How then, does the activation take place? Homogenized material was left at room temperature for 2.5 h to allow enzymes to act, but this procedure was also open to bacterial influence. Evidently, bacteria were abundant in all types of material

used, especially from within the gut of predators. Freshly frozen extract of homogenized *Daphnia* was activated after being introduced into the water, releasing a response in *Daphnia* after >12 h. Bacteria in the water seem the most likely candidates for such signal activation. However, under natural conditions, signal activation should be expected to take place within the gut of a predator. Ringelberg and Van Gool (Ringelberg and Van Gool, 1998) found that the behavioural responses of *Daphnia* to water conditioned by perch was significantly decreased if the fish was treated with the antibiotic ampicillin and suggested that bacteria were the true source of kairomones. Our findings give support to the idea of bacterial involvement, but the data reveal that bacteria can only be mediators in the activation process.

Earlier experiments, reporting various effects of exposure to extracts of crushed *Daphnia*, can be interpreted in light of our new findings. Walls and Ketola (Walls and Ketola, 1989) tested juices of crushed *D. pulex* in morphological experiments with negative results. Fresh extracts were added to new jars daily and the animals were transferred, while number of neckteeth was counted. By this procedure, sufficient bacterial activation of latent cues from *Daphnia* in the water may have been obstructed. It is interesting to note, however, that the crushed *Daphnia* treatment used by Walls and Ketola (Walls and Ketola, 1989) gave moderate responses in some of their series. The data provided by Pijanowska and Kowalczewsky (Pijanowska and Kowalczewsky, 1997b) also seem to support the idea that bacteria in the water may activate latent alarm signals. In that study, too, the growth medium was changed daily, but a significant effect of crushed *Daphnia* was observed from instar VI on. Further, Parejko and Dodson (Parejko and Dodson, 1990) tested for neckteeth development in *D. pulex* and reported a lack of effect from extracts of conspecifics. However, their fresh extracts were subjected to an extensive filtering procedure with a final cut-off at a mol. wt of 500 Da (Herbert and Grewe, 1985). The signal precursors, which must be assumed to be larger in molecular size than the active signals, may simply have been removed by the filtering procedure. Slusarczyk (Slusarczyk, 1999) also prepared fresh media daily when testing for production of ephippial eggs in flow-through chambers, but no responses were found with crushed *Daphnia* alone. However, when crushed *Daphnia* was added in combination with water conditioned by fish (fed chironomids), similar responses to those obtained with water conditioned by *Daphnia*-fed fish were detected. In this case, bacteria from fish in the water may have accelerated the activation of latent alarm signals from *Daphnia*.

Stirling (Stirling, 1995) prepared the chemical cue by grinding a portion of *D. galeata*, followed by dilution in spring water. The water was then mixed into the observation chambers containing *D. galeata*. The vertical distribution of individuals in the water column was then followed for 1 h, but no behavioural effects were observed. In that case, the time lapse between production and use of homogenates may have been insufficient for bacterial activation to take place in the water. On the other hand, Pijanowska (Pijanowska, 1997) reported a significant difference in vertical distribution after 10 h between *Daphnia* homogenate and control treatment, and this difference further increased until 50 h after start. A significant difference in aggregation behaviour between treatments was already present after 2 h, but the time lapse between production and use of homogenates was not stated. From the above, it may be concluded that the presence of alarm signals has previously been proposed, but their basic properties and mode of action seem not to have been fully appreciated and accounted for.

Specificity and distribution of signals

In the present study, both *D. galeata* and *D. pulex* induced morphological changes in *D. galeata* when used as feed for the purpose of labelling predators. Similar results were found when extracts were prepared from homogenates of the two species. However, this result does not necessarily mean that the alarm signals produced by the two species represent identical chemical compounds. It is still possible that the responses obtained result from functional overlap of species-specific signals in closely related species. Such signal overlaps are known to exist among cyprinid fishes (Schutz, 1956; Pfeiffer, 1962) and have also been demonstrated in snails (Stenzler and Atema, 1977). Accordingly, experience from other taxonomic groups gives reason to believe that common signal features are present among related species, but the apparent similarity in signal function between *Daphnia* species does not rule out species specificity in signal properties. However, no definite conclusions can be drawn from the current data on differences in signal properties between *Daphnia* spp. and a final answer to this problem must await more detailed functional and chemical investigations.

It appears also that the inactive precursors may adopt different alarm signal properties following passage through the gut of different predator species. Evidence for such signal specificity in *Daphnia* has emerged from data presented by other investigators. Dodson (Dodson, 1989) measured the predator-induced morphological responses of three common predators (phantom midge larva, *C. americanus*; adult backswimmer, *Notonecta undulata*; sunfish, *Lepomis macrochirus*) in seven species of *Daphnia*. Each *Daphnia* species responded with predator-specific morphological changes. Some of these *Daphnia* species could also distinguish different predator stimuli by opposing behavioural responses, i.e. sinking or rising in the water column (Dodson, 1988). Support to this finding was given by Loose *et al.* (Loose *et al.*, 1993), who found it unlikely that fish and *Chaoborus* release identical chemical cues and by Stibor and Lüning (Stibor and Lüning, 1994) who demonstrated that chemical cues from fish and invertebrate predators influence life-history traits of *Daphnia* differently. It is common

textbook knowledge that the microbial life of the gut varies between animal species, both between phyla as well as between species occupying different ecological niches. Various strains of bacteria may possess enzyme systems specific for their kind and could, accordingly, produce different alarm signals from a common chemical precursor. Such a mechanism would explain how taxonomically different predators might possess signal properties specific to their species. If this proves to be true, our work may represent a fascinating new gateway into the world of chemical communication.

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