Hatching with the enemy: *Daphnia* diapausing eggs hatch in the presence of fish kairomones

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Summary. Infochemicals are known to play a key role in mediating predator-prey interactions, both in aquatic and terrestrial communities. However, state-dependent variation may exist in how effectively individuals can use this information, depending on genotype, life stage and experience. For our study, we used the predator-prey model system fish-waterflea *Daphnia magna* Straus (Cladocera, Daphniidae). Adult *Daphnia* use fish-derived infochemicals, so-called kairomones, as indicators of predation risk, and exhibit a spectrum of morphological, behavioural and life-history responses to the presence of fish kairomones. Here, we investigate whether diapausing eggs, an embryonic resting stage in the life cycle of *D. magna*, also use fish kairomones and tune their hatching to the risk of fish predation, as reported for diapausing stages of dinoflagellates. In two laboratory experiments, we studied hatching proportion and time until hatching of *D. magna* diapausing eggs in the absence and presence of fish kairomones. *D. magna* families differed significantly in their response to the presence of fish kairomones; some families reduced hatching proportion, whereas others increased it. Our results imply genotype-dependent differences in the hatching reactions to fish kairomones as observed for other traits in adult *Daphnia*.

Key words. inducible defences – resting egg – ephippia – infochemical – diapause

Introduction

The ability to detect potential consumers via chemical cues, so called kairomones (Dicke & Sabelis 1988; 1992), and the capability to react by employing inducible defences are widespread phenomena in the plant (e.g. Palo & Robbins 1991; Karban & Baldwin 1997) and animal kingdoms (e.g. Stoddart 1980; Roitberg & Isman 1992; Tollrian & Harvell 1999). Numerous theoretical and experimental studies have demonstrated the effects of predator kairomones on prey behaviour, morphology and life history (for review see Kats & Dill 1998). However, fewer studies have examined

the effect of predator kairomones on life-history switch points such as those that are related to the timing of hatching from eggs and diapausing stages (e.g. Sih 1987; Li 2002). Theory predicts that organisms should switch to the next life-history stage when their mortality/ growth ratio is lower in the later stage than in the previous one (Werner 1986).

Diapause is a widespread feature and especially common in organisms inhabiting variable environments such as freshwater habitats (Cáceres 1997). Diapause serves a number of adaptive functions, but is traditionally regarded as an adaptation to escape from unfavourable environmental conditions in space and time (Southwood 1978; Cáceres 1997). Entering and terminating diapause are important life-history switch points, because the success of this strategy depends on when dormancy is entered and broken.

Diapause is a common feature in the cladoceran *Daphnia* and has received attention in this genus for more than a century (e.g. Weismann 1879). Most *Daphnia* species are cyclical parthenogens, producing asexual subitaneous (immediately hatching) eggs throughout the growing season and sexual diapausing eggs to outlast unfavourable environmental conditions (Zaffagnini 1987). These diapausing eggs are protected by an ephippial case against harsh conditions. Ephippia, i.e. ephippial case with diapausing eggs, of different *Daphnia* species survive the passage through fish guts (Mellors 1975) implying that mortality due to fish predation is higher for hatchlings than for diapausing eggs. Thus, diapausing eggs are expected to reduce and delay hatching in the presence of zooplanktivorous fish.

Such a reduced hatching response of diapausing stages as a reaction to predator presence, indicated by kairomones, has been observed in dinoflagellates (Rengefors *et al.* 1998). Here, we investigate if *Daphnia* diapausing eggs also adjust their hatching characteristics (hatching proportion and time until hatching) to the presence of potential predators. Several studies found evidence for local adaptation in plastic responses to predation risk (Parejko & Dodson 1990; Leibold & Tessier 1991; De Meester 1996; Boersma *et al.* 1999). Here, we further aim to understand if diapausing eggs of *Daphnia* clones from habitats with fish react stronger to fish kairomones than clones from fishless ponds.

Materials and methods

Origin of the *Daphnia*

The *Daphnia magna* Straus (Cladocera, Daphniidae) clones used for our experiments originated from four different freshwater habitats. *Daphnia* derived from two lakes with planktivorous fish (Grosser Binnensee, Northern Germany; Lake Blankaart, Belgium) and from two fishless ponds (Rixdorfer Pferdetränke, Northern Germany; Citadelpark, Belgium). Sixteen clonal lineages, four from each of the four habitats, were established from ephippia collected in the different habitats and kept in the laboratory for five years prior to our experiment. Previous studies have shown that all clones are capable of sensing and reacting to fish kairomones (Boersma *et al.* 1998; Boersma *et al.* 1999).

Media

Lake water from eutrophic Greifensee (Switzerland) was used as medium for the production of ephippia, their storage and for the hatching experiments. This lake water had been circulating over a biological sandfilter at 25 °C for at least 24 h to stimulate bacterial breakdown of possible kairomones. Fish water was continuously prepared throughout both hatching experiments, and only fresh (< one hour after removal of fish) fish water was used for media exchange. To prepare the fish water for the first hatching experiment, we kept five bitterlings, *Rhodeus sericeus amarus* Bloch (Cypriniformes, Cyprinidae, length approx. 5 cm) for 46 h at 20 °C in 5 L Greifensee water that had circulated over the sandfilter. For the second hatching experiment we used a different fish species to prepare the fish water: one golden orfe, *Leuciscus idus* L. (Cypriniformes, Cyprinidae, length approx. 10 cm) was kept for 21 h at 20 °C in 5 L Greifensee water. Kairomones of both fish species have been shown to induce reactions in *Daphnia* (Boersma *et al.* 1998; Lass *et al.* 2001). Prior to use the control water (Greifensee water circulated over the sandfilter) and the fish water were filtered (0.45 µm) and aerated to adjust oxygen concentration.

Ephippia production, sampling and storage

Prior to both hatching experiments, we obtained ephippia in two separate attempts by inducing intraclonal sexual mixis in populations of *D. magna*. For each attempt, we established 16 clonal populations by transferring eight juvenile females of each clone into glass beakers containing 1 L filtered lake water. Cultures were kept at 19 °C and a photoperiod of 12 h bright light: 12 h dim light and fed regularly with chemostat-grown *Scenedesmus obliquus* (Turp.) Kütz (Chlorococcales, Scenedesmaceae). We added 1 mg C L⁻¹ of algae food every second day for two weeks and thereafter 2 mg C L⁻¹ algae daily. We sampled ephippia on day 37 and 60 in the first attempt and after 60 days in the second. From the first attempt, we obtained ephippia of four clones from Grosser Binnensee, of three from Lake Blankaart, of three from Citadelpark and of two from Rixdorfer Pferdetränke. From the second attempt, we obtained ephippia of one clone from Grosser Binnensee, of one from Lake Blankaart, and of four clones from Citadelpark. We had different numbers of ephippia per clone, which we kept separately for each clone in 1.5 ml-tubes filled with 1 ml filtered lake water and stored at 4 °C in the dark for 3 months prior to the first hatching experiment and for 22 months for the second experiment. Diapausing eggs of other *Daphnia* species exhibited an increased hatching success after being kept under such diapause conditions (Barbara Keller, personal communication). Ephippia of the first attempt were used for the first hatching experiment, and those of the second attempt for the second hatching experiment.

Hatching experiments

We carried out two hatching experiments whereby environmental parameters, such as temperature and media exchange were more

Table 1 Details of the two hatching experiments, carried out with *Daphnia magna* diapausing eggs enclosed in ephippia. Prior to both hatching experiments, we induced intraclonal mixis in 16 *D. magna* populations in two separate attempts. Both attempts yielded a different number of families producing diapausing eggs and a different number of ephippia

	Experiment 1	Experiment 2
# Ephippia	756	482
# Clones	12	6
Experimental temperature (°C)	11	10
Media cooled prior to use	No	Yes
Duration (days)	24	16
Media exchange	Every second day	Daily
Last hatchlings (day)	13	14

constant in the second experiment (Table 1). We started both hatching experiments by placing all ephippia obtained into six-well culture plates. We incubated ephippia from the same clone, hereafter referred to as ephippia from one family since they do not represent a single clone, in batches of five whenever possible, otherwise they were equally divided over the treatments. Half of the batches from one family was kept in control water whereas the other half was exposed to fish water. Batches of ephippia of different families were randomly assigned to different wells but each culture plate contained either fish water or control water to avoid mixing of different media during media exchange. Each batch was kept in 7 ml of medium under permanent light. These conditions have been shown to break diapause in *Daphnia* (Weider & Hebert 1987; Weider *et al.* 1997). We monitored the exposed ephippia daily immediately removing and counting the hatchlings. After each hatching experiment, all ephippia were immediately decapsulated and the remaining viable diapausing eggs, defined as the ones that were intact and had a typical olive greenish colour, were counted. Each *Daphnia* ephippium can potentially contain two eggs, i.e. has two loculi. By adding the number of hatchlings to the number of remaining eggs, we estimated the number of diapausing eggs present at the beginning of the hatching experiments.

Hatching characteristics and statistical analysis

We measured two hatching characteristics: (1) the cumulative hatching proportion, defined as the number of living hatchlings divided by the number of viable eggs incubated, and (2) the time until hatching, defined as the mean number of days, after which incubated eggs hatched. For the first experiment, we evaluated differences in hatching characteristics among treatments, in different families from different lakes with a different background of fish predation risk, for significance by mixed model ANOVAs with random and fixed effects. Family was nested in lake and in predation background, and lake was nested in predation background. ANOVAs were carried out with kairomone (=fish water) and predation background as fixed effects, family and lake as random effects, hatching proportion and time until hatching as dependent variables.

Results of the second experiment could not be analysed in the same way, because we had ephippia of only six families and thus, could not include lake and background as factors. We therefore analysed the data with two two-way ANOVAs, with families (as random factor) and kairomone (as fixed factor) as independent and hatching proportion and time of hatching as dependent factors, respectively. For both experiments, hatching proportion was angular transformed, and for the time until hatching we used a Box-Cox transformation prior to analyses (Sokal & Rohlf 1995). All data, including the replicates in which no hatching was observed, were included in the analyses of hatching proportions, whereas for the analyses of the time until hatching, we could only include those replicates, in which hatching occurred. All ANOVAs were done using JMP (SAS Institute Inc. 2002).

Table 2 Results of the nested ANOVAs on hatching characteristics of diapausing eggs from different *D. magna* families in the first hatching experiment. Family was nested in lake and in background, and lake was nested in background. Background and kairomone were analysed as fixed effects, whereas family and lake were random effects. Hatching proportion (after angular transformation) and time until hatching (after Box Cox-transformation) were used as dependent variables. Significant p-values are bold

Effect	Hatching proportion					Time until hatching				
	Df	SS	MS	F	p	Df	SS	MS	F	p
Background	1	11861.4	11861.4	1.168	0.396	1	6.869	6.869	3.352	0.198
Lake	2	25945.1	12972.5	3.346	0.087	2	5.411	2.705	3.388	0.150
Family	8	27346.5	3418.3	8.514	0.003	5	5.189	1.038	4.199	0.071
Kairomone	1	674.7	674.7	0.742	0.475	1	0.371	0.371	2.642	0.153
Kair. × Backgr.	1	108.2	108.2	0.119	0.761	1	0.001	0.001	0.008	0.931
Kair. × Lake	2	2223.6	1111.8	2.899	0.109	2	0.216	0.108	0.443	0.661
Kair. × Fam.	8	3211.8	401.5	2.158	0.036	5	1.236	0.247	1.110	0.359
Error	118	21957.4	186.1			107	23.824	0.223		

Table 3 Results of the two-way ANOVAs on hatching characteristics of diapausing eggs from different *D. magna* families in the second hatching experiment. Families was included as a random and kairomone as a fixed effect. Hatching proportion (after angular transformation) and time until hatching (after Box Cox-transformation) were used as dependent variables. Significant p-values are bold

Effect	Hatching proportion					Time until hatching				
	Df	SS	MS	F	p	Df	SS	MS	F	p
Kairomone	1	23.427	23.427	0.069	0.799	1	0.166	0.166	0.606	0.442
Family	5	43165.300	8633.070	26.869	0.001	5	11.206	2.241	11.649	0.009
Kair. × Fam.	5	1606.470	321.295	0.770	0.574	5	0.962	0.192	0.525	0.756
Error	82	34210.062	417.200			69	25.277	0.366

Fig. 1 Hatching proportion of intraclonal diapausing eggs from different *D. magna* families in the absence and presence of fish kairomones. Graphs present results obtained in the first (a) and the second (b) hatching experiment. Filled symbols represent families with a fish background, whereas open symbols mark families from fishless ponds. Error bars indicate 95 % confidence limits

Fig. 2 Time until hatching of intraclonal diapausing eggs from different *D. magna* families in the absence and presence of fish kairomones. Graphs present results obtained in the first (a) and the second (b) hatching experiment. Filled symbols represent families with a fish background, whereas open symbols mark families from fishless ponds. Error bars indicate 95 % confidence limits

Results and discussion

The 756 ephippia investigated in the first experiment contained 1098 eggs, meaning that 73 % of the loculi contained fertilised eggs, whereas in the second experiment about 65 % of the loculi contained fertilised eggs (628 eggs in 482 ephippia). Hatching proportion averaged over all families was 0.60 ± 0.01 (95 % c.l.) for the control and 0.62 ± 0.01 for the fish treatment in the first experiment and 0.59 ± 0.01 for the control and 0.60 ± 0.02 for the fish

treatment in the second experiment. Overall mean time until hatching of all families was 5.1 ± 0.7 days (95 % c.l.) for the control and 5.1 ± 0.5 days for the fish treatment in the first experiment and 9.5 ± 0.4 days for the control and 9.7 ± 0.3 days for the fish treatment in the second experiment. Differences in time until hatching between both experiments are explained by the experimental conditions. The media were cooled to the experimental temperature prior to media exchange only in the second and not in the first experiment,

in which media overall had a higher temperature. This allowed a faster development of the diapausing eggs in the first experiment as compared to the second.

Ephippia from different families differed significantly in their hatching responses (Tables 2 and 3, Figures 1 and 2). Such genotypic differences in ephippial hatching characteristics have been observed before (Schwartz & Hebert 1987; De Meester & De Jager 1993a). Differences in hatching characteristics may reflect differential responses to the hatching conditions rather than a differential capability of hatching (De Meester & De Jager 1993a). Eggs, which did not hatch under the given conditions and were found to be intact after opening the ephippia, are likely to respond to different or a second hatching cue (De Meester & De Jager 1993b).

In the first experiment, we found a significant interaction between fish kairomone and family (Table 2). Diapausing eggs of some families decreased their hatching proportion in the presence of fish kairomones whereas others increased their hatching proportion (Figure 1). These differences did not depend on the background or on the lake the families originated from (Table 2, Figure 1). Thus, our results provide no evidence for local adaptation in the hatching response to fish kairomone presence.

We did not find an overall effect of fish kairomone on the hatching characteristics of the diapausing eggs (Tables 2 and 3, Figs 1 and 2). It may be that under natural conditions, the effect of fish kairomone interacts with other environmental factors, such as temperature and light, which we did not take into account in our experiments. Interaction effects of grazer kairomones and temperature are relevant for the termination of dinoflagellate resting cysts (Rengefors *et al.* 1998). Dinoflagellate cysts use the chemical presence of potential grazers in addition to temperature as a cue to determine when to excyst (Rengefors *et al.* 1998). The temperature we used in our study is similar to the spring situation in temperate lakes. In spring, juvenile fish may play a minor role as *Daphnia* predators (Boersma *et al.* 1996) because they reach their maximum abundance normally in late summer (Spaak & Hoekstra 1997). Therefore, one might argue that ephippia should exhibit strongest responses to fish kairomones under summer conditions and thus at higher temperatures rather than under spring conditions at low temperatures. However, until the late eighties Baltic herring used to enter the Grosser Binnensee in spring (Lampert 1991) and consequently, the *Daphnia* populations from Grosser Binnensee were already exposed to fish predation each spring. Furthermore, several studies suggest that *Daphnia* ephippia in temperate lakes mostly hatch in spring (Carvalho & Crisp 1987; Wolf & Carvalho 1989; Müller & Seitz 1994; Hairston *et al.* 2000). For all those reasons, we expect diapausing eggs to be able to react to fish kairomones under spring as well as under summer conditions.

Photoperiod and light intensity seem to be among the most important hatching stimuli for *Daphnia* diapausing eggs (Pancella & Stross 1963; Stross 1966; Cáceres & Schwalbach 2001). In our experiments, we exposed ephippia to 24 h constant light, which has been shown to induce hatching (e.g. Pancella & Stross 1963). However, these light conditions do not mimic natural conditions in the temperate habitats of the *Daphnia* families under study. High light intensity and a long photoperiod may be most efficient

stimuli for hatching in *Daphnia* diapausing eggs and fish kairomones may have an effect only under low light intensity and a shorter photoperiod than applied in our study. A recent study (Cáceres & Schwalbach 2001) has shown that hatching success of ephippia in the laboratory is higher than that under field conditions which may result from differences in light intensities, chemical stimuli, etc. In our experiments, possible effects of fish kairomones on hatching traits may have been obscured by the artificial conditions we applied.

We studied ephippia resulting from clonal selfing. Inbreeding can have deleterious effects in *Daphnia* (e.g. Innes & Hebert 1984; De Meester 1993; Haag *et al.* 2003). Hatching rates of diapausing eggs of intrapopulation crosses have been found to be higher than those of selfed families (De Meester 1993). Furthermore, there are hints that clonal selfing is avoided and not the rule in natural *Daphnia* populations (De Meester 1993; De Meester & Vanoverbeke 1999). The diapausing eggs we used in our experiments might therefore not be representative for naturally diapausing egg assemblages. However, we have no reason to expect antipredator defences to disappear completely in selfed *Daphnia*.

Daphnia hatchlings certainly face a higher mortality from fish predators than diapausing eggs, which are likely to survive passage through fish and other vertebrate guts (Proctor 1964; Mellors 1975). Thus, reducing or delaying hatching in the presence of fish kairomones is likely to be adaptive for diapausing eggs. Under high fish predation, *Daphnia* will most likely reproduce only once before being eaten (Lampert 1991; 1993). For relatively large zooplankton species, such as *D. magna*, the probability of successful reproduction in the presence of visually hunting fish predators might be even smaller (Hall *et al.* 1976). Diapausing stages of dinoflagellates reduce germination in the presence of predator kairomones (Rengefors *et al.* 1998) suggesting that diapause provides an effective temporal escape from predation.

In *Daphnia*, diapause emerges as a strategy that primarily tunes *Daphnia*'s presence in time to food conditions, as indicated by light and temperature cues (e.g. Stross 1966; Carvalho & Hughes 1983; Kleiven *et al.* 1992). Diapause induction by fish kairomones has been shown in a single *D. magna* clone (Slusarczyk 1995; Pijanowska & Stolpe 1996; Slusarczyk 1999), but it remains to be studied how important this induction is in general. Our results imply that there are genetic differences in the ability of *Daphnia* diapausing eggs to adjust their hatching in the presence of predator kairomones. For some families, fish kairomones seem to be a cue indicating predator presence and they reduce their hatching, whereas others increase hatching or do not react at all. How such genotype-dependent variation in information processing and reaction affect the structure and functioning of aquatic and terrestrial food webs is not well understood and deserves further investigations.

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