

Neckteeth formation in two species of the *Daphnia curvirostris* complex (Crustacea: Cladocera)

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ABSTRACT

Cladocerans of the genus *Daphnia* show different morphological adaptations against invertebrate predation. Among those, the formation of neckteeth has attracted substantial attention. Morphotypes exhibiting neckteeth better resist predation from larvae of phantom midges *Chaoborus* (Diptera). These morphological structures are known from several species of the *Daphnia longispina* and *D. pulex* complexes; recently they have also been reported in the *D. curvirostris* complex, within which they are well documented from the Far East species *D. sinevi* and from Central European *D. hrbaceki*. Much scarcer are indications of the formation of these structures in the widespread species *D. curvirostris*. Careful inspection of samples from pools with *Chaoborus* larvae nevertheless revealed that a small necktooth in the first few instars of *D. curvirostris* is not uncommon, but probably has been mostly overlooked in the past. Occasionally, even adult *D. curvirostris* males may carry this feature. We provide documentation, particularly by scanning electron micrographs, of neckteeth in field-collected *D. curvirostris*, and in juvenile individuals of its sister species *D. hrbaceki*. In addition, we tested the response of three clones each of *D. curvirostris* and *D. hrbaceki* to *Chaoborus kairomones* in laboratory experiments. Two clones of the former species and all three of the latter responded to this predator cue with neckteeth formation. First-instar juveniles of *D. hrbaceki* also occasionally carried neckteeth in control treatments without *Chaoborus kairomones*, but second and third instars did not. We also observed strong interclonal variation in neonate length in the presence of *kairomones* in this species. We provide a summary table listing all *Daphnia* species presently known to exhibit neckteeth, and propose that the ability to form these structures may be more widespread among common *Daphnia* species than previously assumed.

Key words: *Daphnia hrbaceki*, *Chaoborus*, interclonal variability, inducible defences, neckteeth, predation

1. INTRODUCTION

Inducible morphological defences are among the most interesting antipredator adaptations, as they are often very conspicuous traits. They have been documented in most groups of organisms, ranging from bacteria to vertebrates (Tollrian & Harvell 1999). In aquatic environments, prey responses are often initiated by detecting predator kairomones, i.e., infochemicals associated with a particular predator (Dicke & Sabelis 1988). In cladocerans of the genus *Daphnia*, phenotypic plasticity in antipredator defensive traits has been frequently studied. Various *Daphnia* species show striking protective morphological structures, such as helmets of various shapes in *D. cucullata* Sars, 1862 (Tollrian 1990) or *D. longicephala* Hebert, 1977 (Grant & Bayly 1981), sharp spines in *D. lumholtzi* Sars, 1885 (Sorensen & Sterner 1992; Tollrian 1994; Dzialowski *et al.* 2003), or spiny head lobes called the "crown of thorns" in the *D. atkinsoni* complex (Laforsch *et al.* 2009; Petrusek *et al.* 2009). Other morphological antipredator defences are much less obvious. In the presence of predatory phantom-midge (*Chaoborus*) larvae, a number of *Daphnia* species form neckteeth (Tab. 1), characteristic small spines on the dorsal part of their cara-

pace. Although it had been shown that neckteeth efficiently increase the resistance of *Daphnia* to *Chaoborus* predation (Havel & Dodson 1984; Repka *et al.* 1995), the mechanism of this protective effect remained unclear. Laforsch *et al.* (2004) nevertheless showed that the phenotypic changes accompanying neckteeth formation are much more complex, and involve not only superficially visible structures but also substantial strengthening of the carapace.

Neckteeth can be formed by various species of the subgenus *Daphnia* (*sensu* Johnson, 1952; i.e., including both *D. longispina* and *D. pulex* groups), especially in juvenile individuals (Colbourne *et al.* 1997; Kotov *et al.* 2006). The presence of neckteeth also recently received attention in the *D. curvirostris* complex, shown to contain several lineages in the Palaearctic region (Ishida *et al.* 2006; Kotov *et al.* 2006; Juračka *et al.* 2010). Species of this complex often live in small fishless pools where invertebrate predation is usually strong (Arnott & Vanni 1993) and *Chaoborus* larvae are common (e.g., Kvam & Kleiven 1995; Sell 2006). Despite this, an observation of neckteeth in a member of the *D. curvirostris* complex was pointed out in the literature only recently, for *D. sinevi* Kotov, Ishida & Taylor, 2006, a species newly described from the Russian Far East (Kotov *et al.* 2006). This discovery provided additional

Tab. 1. List of *Daphnia* lineages known to produce neckteeth, arranged according to their phylogenetic relationships. Species complexes are labelled according to Adamowicz *et al.* (2009), their phylogenies are provided in Adamowicz *et al.* (2009) and Juračka *et al.* (2010). Nomenclature of *D. longispina* follows Petrussek *et al.* (2008). Nomenclature of the *D. pulex* complex is not resolved (see, e.g., Mergeay *et al.* 2008); the lineage indicated as FLO9 was named *D. arenata* in Hebert (1995) and several subsequent publications but has never been formally described; different lineages are labelled *D. pulicaria* and *D. pulex* in the Old and the New World.

Species	Neckteeth type	Habitat	Distribution	References
<i>D. pulex</i> complex				
<i>Daphnia</i> sp. FLO9	several teeth in a row	coastal ponds	Western Nearctic	Hebert (1995); Benzie (2005); Juračka <i>et al.</i> (2010)
<i>D. pulex</i> Leydig	rosette	pools, ponds, lakes	Palaeartic and Ethiopian	Tollrian (1993); Sell (2000); Laforsch <i>et al.</i> (2004)
<i>D. "pulex"</i> (American lineage)	rosette	ponds, lakes	Nearctic, Panarctic	Havel (1985); Parejko & Dodson (1991); Riessen & Trevett-Smith (2009)
<i>D. pulicaria</i> Forbes	N/A	ponds, lakes	Nearctic, alpine lakes in Europe	Luecke & Litt (1987)
<i>D. "pulicaria"</i> (European lineage)	single tooth	ponds, lakes	Palaeartic	V. Kořínek, pers. observation
<i>D. catawba</i> complex				
<i>D. catawba</i> Coker	single tooth	ponds, lakes	Eastern Nearctic	Haney <i>et al.</i> (2010)
<i>D. minnehaha</i> Herrick	single to multiple teeth in a row or rosette	ponds	North Eastern Nearctic	Colbourne <i>et al.</i> (1997); Benzie (2005); Riessen & Trevett-Smith (2009); Juračka <i>et al.</i> (2010)
<i>D. obtusa</i> complex				
<i>D. obtusa</i> Kurz	single to multiple teeth in a row	puddles, pools	Western Palaeartic	P. J. Juračka, pers. observation
<i>D. longispina</i> complex				
<i>D. dentifera</i> Forbes	rosette	ponds, lakes	Nearctic	Benzie (2005)
<i>D. longispina</i> O.F. Müller (including <i>hyalina</i> and <i>rosea</i> forms)	single to multiple teeth in a row or rosette	ponds, lakes	Palaeartic and Ethiopian	Negrea (1983); Boronat & Miracle (1997); Benzie (2005)
<i>D. curvirostris</i> complex				
<i>D. curvirostris</i> Eylmann	single to multiple teeth	ponds, pools, various temporary water bodies	Palaeartic, Ethiopian, Nearctic	Glagolev (1986); Kirdyasheva (2010); Hudec (2010, this study)
<i>D. hrbackeki</i> Juračka, Kořínek & Petrussek	single tooth	pools	Western Palaeartic	Juračka <i>et al.</i> (2010)
<i>D. sinevi</i> Kotov, Ishida & Taylor	single tooth	ponds	Eastern Palaeartic	Kotov <i>et al.</i> (2006)

support for the conclusions of Colbourne *et al.* (1997) that neckteeth in *Daphnia* originated several times independently.

Another species of the *D. curvirostris* complex recently described from Central Europe, *Daphnia hrbackeki* Juračka, Kořínek & Petrussek, 2010, exhibits this protective structure as well (Juračka *et al.* 2010). Juračka *et al.* (2010) observed neckteeth in both male and female juveniles and even in adults in the *D. hrbackeki* type locality, which was inhabited by *Chaoborus* larvae. In some natural populations, adults carrying neckteeth had a conspicuous hump-shaped dorsal body outline, presumably a phenotype accompanying the formation of inducible antipredator structures under certain environmental conditions. Similar forms are known from the North American species *D. minnehaha* Herrick, 1884 (Hebert 1995). *D. hrbackeki* escaped recognition and formal description for a long time, although its hump-shaped phenotypes strikingly differ from other European *Daphnia* species. Apparently, this is due to the fact that such morphs occur only under specific environmental conditions: while observ-

ing the population at the species' type locality for several years, we noted that hump-shaped phenotypes slowly disappeared, despite the continuing presence of *Chaoborus* larvae (Juračka *et al.* 2010).

Even the most widespread member of the species complex, *Daphnia curvirostris* Eylmann, 1887, which has been known for more than a century, may apparently form neckteeth. However, this has been largely overlooked. When comparing *D. hrbackeki* to *D. curvirostris* to elucidate species-specific traits, we observed a single necktooth in juveniles and even adult males in some Central European populations of the latter species; we therefore searched for evidence for this feature in the available literature. To our knowledge, the only drawings of *D. curvirostris* with one necktooth have been given by Glagolev (1986) and Kirdyasheva (2010) from a Russian population. Additionally, Matile (1890) provided documentation of neckteeth formation in adult *Daphnia* specimens of a taxon described by him as *D. dentata*, which might belong to the *D. curvirostris* complex, from the vicinity of Moscow. Apart from formation of a single neckteeth in *D. curvirostris*, Hudec

Tab. 2. Material analyzed morphologically in this study. Abbreviations of collector names: AGK: A.G. Kirdyasheva, DV: D. Vondrák, PJJ: P.J. Juračka, VK: V. Kořínek, VKr: V. Kraslová. All localities except Borok (Russia) are in the Czech Republic. *Chaoborus* was identified to species level if material was available; otherwise its presence is noted. NA indicates a pre-sorted sample where presence of this predator could not be evaluated.

Locality	Coordinates	Locality type	Date	<i>Chaoborus</i>	Collector
<i>Daphnia curvirostris</i>					
Tupadly	N 50°26'16" E 14°28'20"	experimental pools	23 October 2007, 6 October 2008	<i>C. crystallinus</i> <i>C. obscuripes</i>	DV
Vrbno, near Smyslov pond	N 49°25'07" E 13°48'10"	temporary pool	4 June 2010	present	VK
Tchořovice, near Radov pond	N 49°25'17" E 13°49'22"	forest pool	May 2010	present	VK
Tvrdonice	N 48°44'54" E 17°01'25"	temporary pool	15 April 2008	<i>C. pallidus</i>	VKr
Borok (Russia)	N 58°03' E 38°13'	temporary puddles	11 June 2004	present	AGK
<i>Daphnia hrbackeki</i>					
Nosálov (type locality)	N 50°28'54" E 14°41'10"	pool	7 samples between May 2005 and November 2007	<i>C. crystallinus</i>	PJJ
Nosálov	N 50°29'11" E 14°41'24"	pool	10 November 2006	<i>C. crystallinus</i> <i>C. flavicans</i>	PJJ
Drásov	N 49°41'37" E 14°06'19"	temporary pool	3 July 1995	NA	VK
<i>Daphnia obtusa</i>					
Nosálov	N 50°29'11" E 14°41'24"	pool	4 July 2005, 13 October 2005	<i>C. crystallinus</i>	PJJ
<i>D. "pulicaria" (European lineage)</i>					
Pole	N 49°25'23" E 13°48'03"	pool	30 July 2010	present	VK

(2010) documented a juvenile female ascribed to this species with multiple neckteeth from a Slovakian population, and Kirdyasheva (2010) reported that some juveniles from one of the Russian populations also carried three or more neckteeth. Several independent observations therefore confirm that *D. curvirostris* is able to form neckteeth; unfortunately, the above-cited works are mostly difficult to access.

The present study has two aims: 1) to provide light and scanning electron microscopy documentation of neckteeth in *D. curvirostris*, and compare them with those of its sister species *D. hrbackeki*; 2) to experimentally test whether neckteeth formation in both of these European members of the *D. curvirostris* complex can be induced by *Chaoborus* kairomones under laboratory conditions. Neckteeth induction has been successfully demonstrated in laboratory experiments with other *Daphnia* species (e.g., Havel & Dodson 1987; Tollrian 1995; Sell 2000; Riessen & Trevett-Smith 2009); we therefore hypothesized that both species would be responsive to *Chaoborus* cues.

2. METHODS

2.1. Material examined

The studied populations of *Daphnia*, particularly *D. curvirostris* and *D. hrbackeki*, used for neckteeth documentation and for laboratory experiments, are listed in table 2. If present in the samples, *Chaoborus* was identi-

fied to species level according to Rozkošný *et al.* (1980).

For the first laboratory experiment, each species was represented by three different clones, distinguishable from each other by alleles at seven microsatellite loci (described in Brede *et al.* 2006): Dp281NB, DaB17/17, SwiD14, Dgm105, Dgm112, SwiD4, and SwiD18 (A. Thielsch, unpublished data). Two of those clones per species, together with a clone of *Daphnia pulex* Leydig, 1860 known to be well responsive to predator cues, were used in the second experiment. The *D. pulex* clone was included as a control for neckteeth formation; it was provided by Ralph Tollrian and has been cultured in the laboratory for several years.

2.2. Documentation of neckteeth from field samples

To document neckteeth from natural populations, we used both light and scanning electron microscopy (SEM). Photographs were taken by a Nikon D300 digital camera attached to an Olympus BX51TF optical microscope. A selected specimen was photographed 10 times with different depths of focus, and the resulting image was merged to gain extended depth of field with Helicon Focus 5.1.2. and Adobe Photoshop CS3 software.

Specimens used for SEM were dehydrated in a graded acetone series and then dried with organic volatile matter hexamethyldisilazane (Laforsch & Tollrian

2000). Dehydrated specimens were gold-coated in a BAL-TEC Sputter Coater SCD 050 for 5-7 minutes in argon plasma at 10^{-1} millibar vacuum. Then, they were imaged with a JEOL JSM-6380 LV scanning electron microscope.

2.3. Experimental design

We used three clones each of *D. curvirostris* and *D. hrbackei*, sampled in late August 2006, to test their response to *Chaoborus* kairomones. *D. curvirostris* clones originated from shallow temporary pools near Přerov nad Labem (N 50°10', E 14°49'), *D. hrbackei* from its type locality near Nosálov (see Tab. 2). The animals were reared in the laboratory under constant conditions (20 °C ± 0.5, 16 hours of light per day) in artificial medium (according to Jeschke & Tollrian 2000); local groundwater from Planegg-Martinsried was used instead of tap water. Daphnids were fed daily with *Scenedesmus obliquus* (1.5 mg carbon L⁻¹).

For the first experiment, we randomly selected six juvenile females of each clone and placed them into separate beakers (volume 1.5 L). Into each of these beakers, we put a small plastic cage with the bottom made from a 200 µm mesh, allowing the flow of infochemicals but not physical contact with the predator. In three beakers, the cage contained five specimens of the 4th larval instar of *Chaoborus crystallinus*. The other three beakers containing daphnids of each clone served as control treatments without the predator presence. *Chaoborus* were fed with *Daphnia* neonates of the same clone as in the respective beaker to maximize the expression of morphological defences, as predators consuming conspecific prey are known to increase the formation of inducible defences (Stabell *et al.* 2003; Laforsch *et al.* 2006). To ensure sufficient mixing of predator kairomones and prey alarm substances with the culture medium, each cage with *Chaoborus* larvae was raised almost out of the medium and lowered back down twice a day. The medium in each beaker was changed with every reproductive event.

Daphnia individuals with which the experiment started (the "mother generation") were exposed to the predator cues to take maternal effects into account (Agrawal *et al.* 1999). We then used individuals from the third clutches of these females to evaluate the response of the next-generation juveniles to predator kairomones (neonates of the first and second clutch were removed and used as feed for *Chaoborus*). The third clutch neonates were counted, individually photographed to measure body and spine length (see below), and checked for the presence of neckteeth. Immediately afterwards, we randomly selected five individuals from the clutch (or less in cases of smaller clutches) and transferred them to separate 0.1 L beakers (the smaller flask volume was used due to space limitations) to follow the life history and morphological changes of each daphnid individually. The media were changed twice a

day in each beaker. In *Chaoborus* treatments, the beakers contained culture medium with predator-conditioned water prepared as described for the mother generation (see above). The control medium contained only algal food but no predator or prey infochemicals. We took a second measurement of morphometric parameters of each individual *Daphnia* at the age of first reproduction, and evaluated the number of offspring in their first clutch.

To compare neckteeth formation among first three juvenile instars, we performed a second experiment using two clones of each species tested in the first experiment (*D. hrbackei* clones 2 and 3, and *D. curvirostris* clones 1 and 3). In addition, we also exposed a clone of *D. pulex* to *Chaoborus* kairomones to test for the efficiency of the predator cue, as this species is known to exhibit distinct neckteeth in response to *Chaoborus* (e.g., Tollrian 1995). The experimental design was similar to our first experiment, with the exception that we did not transfer the juveniles of the third brood of preconditioned mothers separately into small beakers but kept them in the original vessel to constantly expose the animals to predator cues. In addition, we used ten *Chaoborus* larvae per litre to increase the concentration of predator cues. We randomly selected 20 individuals (if available) in three consecutive days to collect animals of the first three instars. We checked for presence or absence of neckteeth in these instars under a Leica M10 stereomicroscope.

2.4. Measurements and statistical analyses

Photographs of each measured individual from the first experiment were taken by an Olympus ALTRA20 digital camera mounted on a Leica M10 stereomicroscope. Subsequently, we measured two morphometric parameters in the software Olympus cell[^]P: body length (defined as the length between the upper edge of the compound eye to the base of the tail spine) and tail spine length (a straight line between the base of the tail spine and its top). Occasional juvenile individuals that were substantially larger than the others were removed from the dataset, as we suspected them of already being in the second instar. We also measured the body length of randomly selected neckteeth-carrying individuals from one population of each species (*D. curvirostris*: Tvrdonice, 15 April 2008; *D. hrbackei*: type locality near Nosálov, 17 August 2006), to evaluate their size distributions and thus check whether neckteeth are present in different instars.

We used Pearson's Chi-square test to compare ratios of induced (i.e., with neckteeth) and uninduced specimens within each species in both experiments. Since we used 3 tests in the second experiment, we applied consequent manual Hochberg's p-value adjustment (Benjamini & Hochberg 1995) for multiple testing. The morphometric parameters were compared between individuals in *Chaoborus* and control treat-

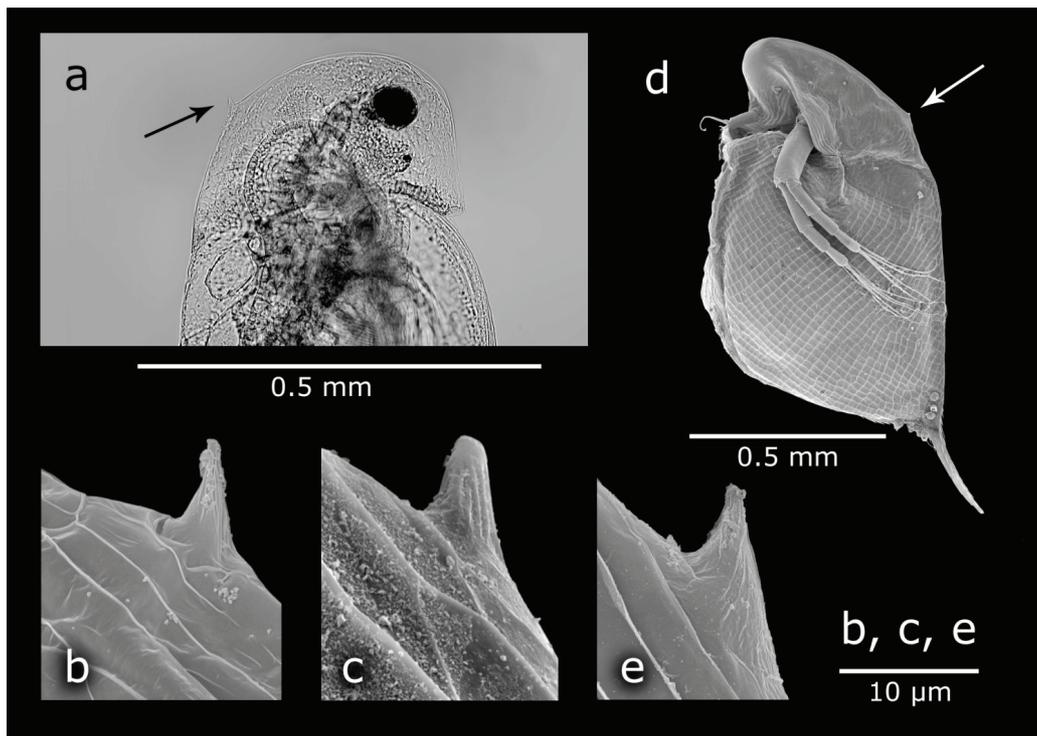


Fig. 1. Neckteeth in Czech populations of the *Daphnia curvirostris* complex. *D. curvirostris* from Tupadly: head (A) and necktooth detail (B) of juvenile females; an adult male in lateral view (D); detail of an adult male necktooth (E). *D. hrbaceki*: necktooth of a juvenile female from Nosálov (C). More figures of *D. hrbaceki* with neckteeth are available in Juračka *et al.* (2010). Arrows indicate neckteeth.

ments by a series of non-parametric Wilcoxon's signed-rank test with consequent manual Hochberg's *p*-value adjustment. As the sizes of different specimens within one clutch cannot be considered independent replicates, we averaged them for each clutch, and used a single value for the whole clutch. Wilcoxon's signed-rank test was also used for comparing the size of clutches from controls and *Chaoborus* treatments.

3. RESULTS

In the samples of *Daphnia curvirostris* originating from Czech and Russian pools with *Chaoborus* larvae, most juveniles carried a small (5–10 µm) necktooth (Fig. 1a, b). A necktooth of approximately the same size was also occasionally retained in adult males (Fig. 1d, e), as seen in field samples from Tupadly, Czech Republic (but also documented from Borok, Russia; Kirdyasheva 2010). Neckteeth of juvenile *D. hrbaceki* (Fig. 1c) were of a similar morphology as those of *D. curvirostris*. In both species, neckteeth were carried by a wide size range of juveniles, clearly indicating that the structure is present in several juvenile instars: the size of measured neckteeth-carrying individuals ranged between 0.61 mm–1.26 mm in *D. curvirostris* from Tvrdonice, and between 0.52 and 1.04 mm in *D. hrbaceki* from its type locality.

Juveniles of both *D. curvirostris* and *D. hrbaceki* also formed neckteeth during the laboratory experiments (Tab. 3); these individuals did not differ phenotypically

from those in the natural populations. In the first experiment, *D. hrbaceki* had a much stronger tendency to form these structures: in all three tested clones, all first-instar juveniles carried a necktooth in the treatment with *Chaoborus* kairomones. Interestingly, some first-instar juveniles with neckteeth were also found in the control treatments; their proportion was nevertheless significantly lower than in the *Chaoborus* treatments. A small proportion of individuals from one of the three tested *D. curvirostris* clones also formed neckteeth; however, there was no significant difference between controls and *Chaoborus* treatments. In the second experiment, however, almost all specimens of all three instars of both species produced neckteeth in the presence of *Chaoborus*, while those not exposed to predator cues only formed these structures in the first instar in *D. hrbaceki* (Tab. 3). No specimen of *D. hrbaceki* with a hump-shaped carapace (as found in the wild) was observed in the laboratory experiments.

Differences in daphnid morphometric and life history traits measured in the first experiment were not consistent between the *Chaoborus* and control treatments, either between the two tested species or among clones within species. We did not observe any clear trends or significant differences in size at first reproduction, clutch size, or relative spine length. The neonate size, however, showed interesting patterns (Fig. 2). *D. hrbaceki* clones 1 and 3 formed significantly larger neonates in the presence of *Chaoborus* than in controls

Tab. 3. Ratios of induced (with neckteeth) and uninduced neonates of *Daphnia hrbaceki* and *D. curvirostris* in the laboratory induction experiments. *D. pulex* served as a control for the efficiency of the predator cue in the second experiment. Significances of differences between *Chaoborus* and control treatments were tested by the Pearson's Chi-square test (adjusted *p*-values are given for the second experiment).

	Instar	Clone	% with neckteeth (total N)		Chi-square tests	
			Control	<i>Chaoborus</i>	χ^2	<i>p</i> -value
Experiment I						
<i>Daphnia hrbaceki</i>	1	1	100% (10)	100% (19)	12.2	<0.001
		2	50% (8)	100% (14)		
		3	71% (17)	100% (8)		
<i>Daphnia curvirostris</i>	1	1	0% (5)	15% (27)	2.46	0.12
		2	0% (10)	0% (3)		
		3	0% (16)	0% (23)		
Experiment II						
<i>Daphnia hrbaceki</i>	1	2	67% (6)	100% (1)	1.66	0.6
		3	100% (18)	100% (15)		
	2	2	0% (14)	100% (5)	63.43	<0.001
		3	0% (20)	94% (32)		
	3	2	NA	100% (1)	23.03	<0.001
		3	0% (20)	67% (30)		
<i>Daphnia curvirostris</i>	1	1	0% (20)	100% (20)	70	<0.001
		3	0% (20)	100% (10)		
	2	1	0% (20)	100% (20)	80	<0.001
		3	0% (10)	100% (20)		
	3	1	0% (20)	70% (20)	36.52	<0.001
		3	0% (20)	NA		
<i>Daphnia pulex</i>	1		0% (17)	100% (30)	47	<0.001
	2	1	0% (20)	100% (50)	70	<0.001
	3		0% (20)	100% (20)	40	<0.001

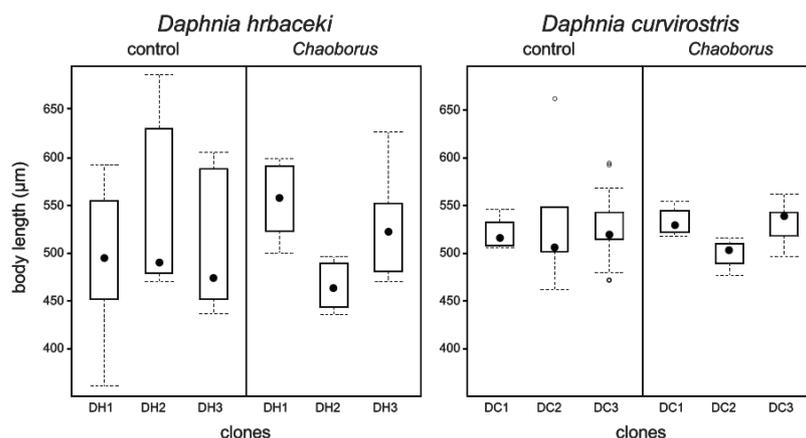


Fig. 2. Body length of *Daphnia hrbaceki* and *Daphnia curvirostris* third-clutch neonates in absence and presence of *Chaoborus* kairomones in the laboratory induction experiment. Median (dark circle), interquartile ranges (box) and non-outlier ranges (whiskers) are shown by the box-and-whisker plot; outliers are indicated by empty circles. *D. hrbaceki* clones 1 and 3 were significantly larger in the kairomone treatment than in the control, clone 2 was significantly smaller. In *D. curvirostris*, differences in neonate lengths from kairomone and control treatments were not significant.

(Wilcoxon's signed-rank tests with Hochberg's *p*-value adjustment; adjusted *p* = 0.036, *W* = 0 and 0.027, *W* = 0, respectively); on the contrary, clone 2 neonates were significantly smaller under the same conditions (adjusted *p* = 0.027, *W* = 58). *D. curvirostris* clones did not exhibit any difference in neonate size between treatments (adjusted *p* > 0.376, *W* ≤ 7 in all three comparisons). We also did not observe any trade-off between neonate size within the clutch and clutch size in either of the tested species.

4. DISCUSSION

Our study confirms that both studied European species of the *Daphnia curvirostris* complex are able to form neckteeth in the field as well as under laboratory conditions, and in several juvenile instars. However, we observed neckteeth formation in the *D. hrbaceki* first instar not only in the presence of *Chaoborus* kairomones but also in the treatments without predator cues. Similar observations are known from some lineages of

the *D. pulex* complex, including European *D. pulex* Leydig, 1860 (Tollrian 1993) as well as the North American *D. "pulex"* (H. Riessen, personal communication); additionally, Kirdyasheva (2010) reported neckteeth in juvenile instars of *D. curvirostris* from a population where *Chaoborus* had not been observed. Spontaneous neckteeth development in neonates of these species may be explained by the fact that they occur in fishless habitats where invertebrate predation is often very strong. Hence, neckteeth development even in the absence or low concentration of *Chaoborus* cues may be a good start-up defence, as predation pressure may change rapidly.

Based on previous experimental work on other *Daphnia* species (Havel 1985; Tollrian 1993), we expected that our studied taxa should produce a higher ratio of neonates with neckteeth in *Chaoborus* treatments than in controls. The results were consistent with this hypothesis in both experiments (Tab. 3). In the first experiment, the trend was significant only for *D. hrbaceki*, in which all neonate individuals of all three tested clones carried neckteeth in the *Chaoborus* treatment. In the second experiment, the kairomone effect was much stronger, and differences between control and predator treatments were highly significant in almost all species and instars (with the exception of the *D. hrbaceki* first instar). The difference between the experiments may be explained by doubled kairomone concentration in the second one. This corresponds to results of previous studies reporting the influence of kairomone dose on the formation of protective traits (e.g., Tollrian 1993). In the first experiment that focused on neonates only, *D. curvirostris* formed neckteeth much less frequently than *D. hrbaceki* (only 15% of juveniles of a single clone in the *Chaoborus* treatment). This is in accordance with the infrequent field observations of *D. curvirostris* populations with neckteeth, and suggests that *D. hrbaceki* is more likely to respond with morphological defences under low kairomone concentrations.

In our experiments, individuals of both species showing neckteeth exclusively formed a single necktooth. We did not observe any rosette-like neckteeth formed by more dorsal spines, as documented in *D. curvirostris* by Hudec (2010) and Kirdyasheva (2010). Their field observations nevertheless suggest that the taxon is one of those *Daphnia* species that are plastic in their level of neckteeth expression (see Tab. 1).

In both experiments, we did not observe any hump-shaped morphs. The failure to produce inducible defences as strong as those seen in the wild is common in laboratory experiments (Dodson 1988; Tollrian 1994; Laforsch & Tollrian 2004; Tanner & Branstrator 2006). In our case, this may be due to various reasons. It could be due to an incomplete or insufficiently intense inducing stimulus. Tanner & Branstrator (2006) found a three-generation delay in *D. mendotae* Birge, 1918 producing a round helmet in reaction to the predatory

cladoceran *Leptodora*; possibly, a dorsal hump in *D. hrbaceki* may only be formed in an experiment spanning several generations. Riessen and Young (2005) suppose that similar hump-shaped phenotypes in North American *D. minnehaha* are induced by the predator only under low-food conditions. This synergistic interaction would correspond to the field observations of *D. hrbaceki* from its type locality, a newly excavated pool. Hump-shaped morphs were common there during the first years of habitat existence, but disappeared two to three years later when the trophic status of the habitat substantially increased (Juračka *et al.* 2010). In addition, it has been shown that small scale turbulence evoked by the movement of predators can act synergistically with chemical cues to induce maximal trait responses (Tollrian & Laforsch 2006). Hence, synergistic effects of kairomones and environmental conditions are well known within the *Daphnia* genus (e.g., Weber 2001; Weetman & Atkinson 2002; Tollrian & Laforsch 2006), and may also explain the absence of hump-shaped morphs in our experiments.

Daphnia are known to react to the presence of predators not only through morphological changes, but also by adaptive shifts in their life history (e.g., Schwartz 1984; Weber & Declerck 1997; Boersma *et al.* 1998). Among the most common changes are alterations in the number and size of offspring through maternal effects, depending on the specific predators (Tollrian 1995; Agrawal *et al.* 1999). In the presence of predators preferring larger prey (particularly fish), some species tend to produce smaller neonates (Reede 1997; De Meester & Weider 1999; Spaak *et al.* 2000; Mikulski 2001). On the other hand, the same prey species may follow the opposite strategy in the presence of predators which are gape-limited, including *Chaoborus* (Pastorok 1981). In this case, females exposed to predator kairomones usually tend to produce large neonates (Riessen & Sprules 1990; Lüning 1992; Spitze 1992; Tollrian 1995; but for exception, see Spitze, 1992).

As both studied species occur in small fishless pools with frequent strong predation pressure by *Chaoborus* (Mura & Brecciaroli 2003; Louette & De Meester 2005; Juračka *et al.* 2010), a tendency to increase neonate size in the kairomone treatments could have been expected. However, although all three clones of *D. hrbaceki* reacted to *Chaoborus* cues with a significant change in neonate size, the direction of this change varied among the clones. Two clones produced significantly larger neonates, while the third one produced smaller ones (Fig. 2). Strong interclonal variability in the reaction to predator kairomones is well known from previous laboratory experiments in *Daphnia* (Parejko & Dodson 1991; Weber & Declerck 1997; Boersma *et al.* 1998), including opposite reactions within one species (Spitze 1992; Boersma *et al.* 1998; Pauwels *et al.* 2005).

Based on field observations, we suppose that neckteeth are induced relatively often as a defence against

Chaoborus predation, probably also by other species of the subgenus *Daphnia* in which this feature is not known. It is generally assumed that neckteeth in *Daphnia* originated multiple times independently, and this hypothesis has been suggested by several authors. Colbourne *et al.* (1997) and Kotov *et al.* (2006) came to this conclusion because neckteeth had been documented only sporadically in distinct *Daphnia* species complexes. The potential independent origin of neckteeth in *D. longispina* and *D. pulex* groups (treated as distinct subgenera) was also discussed by Beaton and Hebert (1997) in their study of the cellular basis of *Daphnia* head morphology. Representatives of the two groups differed in the number of polyploid cells in the muscle attachment region, which might be responsible for neckteeth formation. Colbourne *et al.* (1997) also claimed, in support of the multiple-origin hypothesis, that some *Daphnia* species living mostly in small turbid habitats without *Chaoborus*, e.g., North American members of the *D. obtusa* complex, do not produce neckteeth even in experiments with *Chaoborus* kairomones. However, Beaton & Hebert (1997) proposed a potential for neckteeth formation in three species of that complex, although they lacked evidence of this ability from field samples or laboratory collections. This is in agreement with field observations from Europe: *D. obtusa* Kurz, 1874 (*sensu stricto*) does produce neckteeth in pools with high *Chaoborus* abundances (P.J. Juračka, personal observation).

The growing evidence that neckteeth are more common than previously assumed among various daphnids from both the *pulex* and *longispina* groups may also give some support to an alternative scenario of evolution of neckteeth defences. Ontogenetic mechanisms allowing neckteeth formation could be a plesiomorphic character, expressed only in taxa where selection by predators strongly favoured them. This is further supported by the fact that some species apparently exhibit different forms of neckteeth (ranging from single to multiple arranged in a row or a rosette-like fashion). Further research into the genomic basis of neckteeth formation may reveal whether the different forms of neckteeth are homologous in unrelated *Daphnia* species or not.

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