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RESEARCH ARTICLE

Phenotypic plasticity in three *Daphnia* genotypes in response to predator kairomone: evidence for an involvement of chitin deacetylases

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ABSTRACT

The genetic background of inducible morphological defences in *Daphnia* is still largely unknown. Dissolved infochemicals from the aquatic larvae of the phantom midge *Chaoborus* induce so-called 'neck-teeth' in the first three post-embryonic stages of *Daphnia pulex*. This defence has become a textbook example of inducible defences. In a target gene approach, by using three *Daphnia* genotypes which show a gradient of neck-teeth induction in response to equal amounts of kairomone, we report a high correlation of neck-teeth induction in *Daphnia pulex* and relative gene expression of two chitin deacetylases. Further, previous studies suggested genes from both the juvenoid and the insulin hormone signalling pathways as well as several morphogenetic genes downstream to be responsible for neck-teeth induction in *D. pulex*. However, these data were not supported by our study. None of the three *D. pulex* clones showed an upregulation of these previously proposed candidate genes as a response to predator kairomone, which is interpreted as the result of refined methods used for both RNA sampling and kairomone enrichment yielding unambiguous results compared with earlier studies. The assessment of a clonal gradient of *Daphnia* in the presence and absence of infochemicals provides a promising approach to identify further genes involved in the induction of morphological defences by correlating gene expression and morphology.

KEY WORDS: Predator, *Chaoborus*, Gene expression, Inducible defence, Hormone signalling, Infochemicals

INTRODUCTION

Defences against predation are a crucial issue for organisms throughout ecosystems and can be divided into two types: constitutive and inducible. Constitutive defences are deployed independent of any cue that indicates the presence of a predator and are thus expressed regardless of whether there is a threat of predation or not. Theory predicts constitutive rather than inducible defences in the presence of a fairly constant risk of predation or if the defence does not impose costs (Rose and Mueller, 1993).

In contrast, inducible defences are a means by which prey organisms respond to a varying risk of predation. Inducible defences are triggered by the perception of the predator through predator-associated cues, which, in freshwater ecosystems, are often waterborne chemicals, so-called kairomones. Kairomone-induced defences provide a fitness advantage to the receiver but a fitness disadvantage to the emitter (Dicke and Sabelis, 1988). Inducible defences should be adaptive if predation pressure is unpredictable and if the defence is associated with high costs (Harvell, 1990).

Daphnia, a keystone species and model organism for freshwater ecosystems, has been shown to respond to the presence of predators by a shift in life history, a change in behaviour or a modification of morphology (reviewed by e.g. Lampert et al., 1994; von Elert, 2012; Repka and Pihlajamaa, 1996; Laforsch and Tollrian, 2009; Tollrian and Harvell, 1999). *Daphnia* feeds unselectively on phytoplankton and thus links higher trophic levels to primary production, being preyed upon by both vertebrates like fish and a variety of invertebrates (e.g. Sommer et al., 1986; Müller-Navarra et al., 2000). Very important invertebrate predators of *Daphnia* are aquatic larvae of the phantom midge *Chaoborus* spp., which are characterized by a cosmopolitan distribution (Borkent, 1981) and which have been shown to reach a high abundance (e.g. Wissel et al., 2003; Voss and Mumm, 1999). Therefore, the interaction of *Daphnia* and *Chaoborus* has been studied in great detail. For *Daphnia pulex*, it has been shown that juveniles have a larger size at hatching when exposed to *Chaoborus* kairomone during embryonic development, which should be advantageous, as *Chaoborus* is a gape-limited predator, with a strike efficiency that is lower at larger prey sizes (Riessen and Trevett-Smith, 2009). Further, *D. pulex* migrates upwards in the water column as a response to *Chaoborus* kairomone (Boeing et al., 2006; Oram and Spitze, 2013). Finally, in the presence of kairomone from *Chaoborus* larvae, juveniles of *D. pulex* develop neck-teeth at the back of the head, a morphological defence that has been shown to reduce mortality due to *Chaoborus* predation (Havel and Dodson, 1984). In line with this, induction of neck-teeth occurs only in those instars that are vulnerable to gape-limited predation by *Chaoborus* sp. (Riessen and Trevett-Smith, 2009).

Although a thoroughly studied topic, regulation of the induction of neck-teeth on the level of hormones remains poorly understood. However, the deciphering of the underlying endocrinology poses a task that is hard to accomplish, as quantification of hormone titres by analytical and/or immunological approaches in small animals such as *Daphnia* sp. is rather challenging. Thus, molecular approaches have been used to investigate the endocrinology of morphological defences in response to kairomones from larvae of *Chaoborus*.

The juvenile hormone pathway offered a promising perspective, as it has been shown to be involved in the regulation of moulting in crustaceans (Chang et al., 1993) and thus is putatively connected to predator-induced changes in life history. Further, Olmstead and LeBlanc (2002) demonstrated that the juvenoid hormone methyl farnesoate was capable of inducing male formation in *Daphnia* embryos and thus was involved in the induction of a morphologically distinct *Daphnia* phenotype. Hence, it seemed plausible to assume a potential involvement of the juvenile hormone pathway in the induction of neck-teeth as a response to predator kairomones.

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In line with this, Miyakawa et al. (2010) investigated the differential expression of genes involved in the synthesis/degradation of juvenile hormones in *D. pulex* in the absence and presence of *Chaoborus* kairomone. A similar approach was conducted by Dennis et al. (2014) with genes of a network of nuclear receptors that were assumed to be specific for juvenoid endocrinology. Unfortunately, neck-teeth induction had not been monitored during the Miyakawa et al. (2010) study. Dennis et al. (2014) did monitor neck-teeth induction but only for second instar juveniles, while gene expression was measured during the first juvenile instar, right after the hatching of the animals. Thus, the changes in gene expression cannot be unambiguously attributed to an increase in neck-teeth induction as *D. pulex* juveniles take approximately 1 day from hatching until ecdysis to the second juvenile instar. However, identifying the genes responsible for neck-teeth induction is of great interest in order to obtain a deeper understanding of the complexity of predator–prey interactions of *Daphnia* and *Chaoborus*.

However, *D. pulex* is not the only *Daphnia* species known to exhibit inducible morphological defences as a response to the presence of predator kairomones. *Daphnia magna*, for example, has been shown to develop an increased bulkiness when directly exposed to *Triops cancriformis* or its chemical cues, which provides a protection against predation (Rabus and Laforsch, 2011). Beyond this, Laforsch et al. (2004) showed that the stability of the carapace of two *Daphnia* species was increased by up to 350% after kairomone exposure, offering protection from invertebrate predators that have to crush their prey in order to ingest it. Exposure of *T. cancriformis* kairomone has been shown to lead to upregulation of two chitin deacetylases (1 and 2A) at the protein level (Otte et al., 2014), which catalyse N-deacetylation of chitin. In *Tribolium castaneum*, several types of chitin deacetylases have been identified in the exoskeletal epidermis (Arakane et al., 2009), which suggests that these enzymes may be involved in exoskeletal development and thus morphological defence induction in *Daphnia*. These findings provide another approach to elucidate the network of responsible genes by providing candidate genes beyond hormonal pathways.

The present study used three different clones of *D. pulex*, which differ in responsiveness to *Chaoborus* kairomone, i.e. the clones express different intensities of neck-teeth induction at equal *Chaoborus* kairomone concentrations. With this 'clonal-gradient', gene expression levels in the absence and presence of kairomone were determined in order to correlate the strength of gene expression to neck-teeth induction throughout the clones. Therefore, gravid females of the three *D. pulex* clones were exposed to *Chaoborus* kairomone, and induction of neck-teeth in newly hatched animals was quantified and the same animals were subsequently used for analysis of gene expression by quantitative real-time PCR (qPCR). We determined the expression of five putative target genes from Miyakawa et al. (2010) that had shown the most marked upregulation. We further hypothesized that two chitin deacetylases that, using proteomics, have been shown to be involved in another morphological defence in *D. magna* (Otte et al., 2014) would also be involved in the morphological changes induced by larvae of *Chaoborus* in *D. pulex*. This study aimed to provide a deeper understanding of the involvement of several previously proposed candidate genes by applying a more sophisticated sampling method as well as incorporating data on the strength of neck-teeth induction and directly correlating this to clone-specific gene expression of those candidate genes. From this, it should be possible to differentiate between genes that are directly involved in neck-teeth induction and those which are part of a more general stress response to the presence of predator kairomone.

MATERIALS AND METHODS

Animals

All research was carried out within the framework of the DFG-Guidelines 'Sicherung guter wissenschaftlicher Praxis'. Three clones of *Daphnia pulex* were used in this study. *Daphnia pulex* clone TCO was isolated from a naturally inbred population inhabiting a permanent pond in the Siuslaw National Forest, near the Pacific coast in Oregon, USA; clone TCO was chosen for genome sequencing (Colbourne et al., 2011). *Daphnia pulex* clone Gerstel was isolated from a pond in northern Germany (Koch et al., 2009). *Daphnia pulex* clone Münster was originally isolated from a pond in Münster (Gievenbeck), Germany; it was used in a study by Kuster and von Elert (2013).

All clones have been cultivated in stock cultures for several years. All *D. pulex* were reared in 800 ml aged and aerated tap water with no more than 15 animals per glass with 2 mg particulate organic carbon (POC) l^{-1} of *Chlamydomonas klinobasis* as food and were transferred into fresh water and food every second day. Forth instar larvae of *Chaoborus obscuripes* were obtained from an internet pet shop (Interquaristik.de).

Food

The strain of the green algae *Chlamydomonas klinobasis* used in this work was originally isolated from Lake Constance. The alga was cultured in 5 l batch cultures in glass bottles at constant 20°C and continuous light conditions (3 fluorescent lamps with a photon flux density of 95 µmol s^{-1} m^{-2}) in sterile Cyanophyceae medium according to von Elert and Juttner (1997) with vitamins [thiamine hydrochloride 300 nmol l^{-1}, biotin 2 nmol l^{-1} and cyanocobalamin (vitamin B_{12}) 0.4 nmol l^{-1}] (modified according to Guillard, 1975).

Preparation of *Chaoborus* incubation water extract

Approximately 1000 *Chaoborus* larvae were incubated for 24 h in 1 l of aged tap water. The incubation water containing *Chaoborus* kairomones was filtered through membrane filters (pore size: 0.45 µm). For bulk enrichment of the kairomones, a C_{18} solid-phase cartridge (10 g of sorbent, volume 60 ml, end-capped, Varian Mega Bond Elut, Agilent Technologies) was pre-conditioned with 50 ml 1% methanol–water prior to adding the sample. Methanol was added to the filtered *Chaoborus* incubation water to obtain a 1% concentration, and 1 l of sample was passed through the cartridge. The loaded cartridge was washed with 50 ml of ultrapure water with 1% methanol and then eluted with 50 ml of methanol. The eluates originating from 20 l of *Chaoborus* incubation water were pooled, evaporated to dryness using a rotary evaporator and redissolved in 1 ml of methanol.

Bioassay

Daphnia pulex mothers with their third clutch just deposited in the brood pouch (yolk eggs) were used for all experiments. Neck-teeth induction has been shown to be maximized when the kairomone exposure starts during the second stage of embryonic development (Naraki et al., 2013). Bioassays were conducted in glass jars. Kairomone treatments contained 5 µl *Chaoborus* incubation water extract, which was evaporated to dryness before applying 100 ml aged tap water to the jars. Control jars contained 5 µl methanol as a negative control. Within the first hour of hatching, the neck-teeth induction of the first instar juveniles was quantified using a scoring method introduced by Tollrian (1993), and animals were sampled for RNA extraction. Each treatment (*Chaoborus* incubation water and control) was carried out with 15 replicates for all three clones.

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Table 1. Investigated candidate genes with respective primers and gene ID

Candidate gene (abbreviation)		Primer	*Daphnia pulex* gene ID
Chitin deacetylase 1 (*Cda1*)	F	TTCCCAACACTCCAGCTCTA	Dappu-309273
	R	GGAAGCGAGAAAAACGGTAAA	
Chitin deacetylase 2 (*Cda2*)	F	TTCTTCGTTTCGCACAAGTA	Dappu-327753
	R	TGCCATCGGTCCAGTATT	
Escargot (*esg*)	F	CGATTGCCACAAGTCGTACT	Dappu-50534
	R	GCCCAGCGAGACGTAGAC	
Extradenticle (*exd*)	F	GGGTAGTTCCTCGCCATACA	Dappu-219790
	R	ACCACCGTTGAGTCCCATAG	
Insulin-like receptor (*InR*)	F	GTGGAAAGCCAACGCACTA	Dappu-270048
	R	CGTTCCGTGGTCATCCTTAT	
Juvenile hormone acid methyl transferase (*JHAMT*)	F	CTTCGTTGACGGGCATAGAT	Dappu-300180
	R	CGAATCCATCGGGGAATACT	
Tyramine β-monooxygenase (*TBM*)	F	TGGACCGTGACAATCACTACA	Dappu-1839
	R	GCGTTCTCCTTTTCCGTAGA	

RNA extraction and reverse transcription

First instar *D. pulex* were stored at −80°C immediately after hatching. Later, approximately 50 animals were pooled and RNA was extracted using the RNeasy extraction kit from Qiagen. Concentrations of RNA were determined immediately after extraction using a Nanodrop (Spectrophotometer ND-1000, software v3.8.1, Thermo Scientific). RNA (1 mg) was reverse-transcribed using the high-capacity cDNA reverse transcription kit (Applied Biosystems). The cDNA was stored at −20°C.

qPCR

For normalization, two different endogenous controls [18S ribosomal RNA (18S) and 28S ribosomal RNA (28S)] were used in qPCR analysis. These endogenous controls were chosen from a given set of 10 reference genes (Heckmann et al., 2006). Stability (*M*) of these genes was below 0.5 and coefficient of variance (CV) was below 0.25, which are regarded as required values for stably expressed reference genes (Hellemans et al., 2007).

The candidate genes to be tested for response to *Chaoborus* kairomone were extradenticle (*exd*), escargot (*esg*), juvenile hormone acid methyltransferase (*JHAMT*), insulin-like receptor (*InR*) and tyramine β-monooxygenase (*TBM*) as published by Miyakawa et al. (2010), as well as the genes chitin deacetylase 1 and 2 (*Cda1* and *Cda2*). Primers are given in Table 1.

Primers were designed with Primer3 v2.3.5 and the quality checked with NetPrimer (Premier Biosoft; Table 1). Melting curve analyses confirmed specific amplification without primer dimer formation. The data acquisition for the relative expression was performed on a 7300 qPCR system (Applied Biosystems). Each reaction contained 5 ng of cDNA template, 10 µl SYBR green PCR master mix and 2.5 mmol l^{-1} of each primer in a final volume of 20 µl. Cycling parameters were 95°C for 10 min for the initial activation of the DNA polymerase, followed by 40 cycles of 95°C for 15 s, 55°C for 30 s, 68°C for 30 s, and a final dissociation step with 95°C for 15 s, 55°C for 30 s, 68°C for 30 s and 95°C for 15 s. The baseline and threshold for the cycle threshold (*Ct*) was set automatically, and each reaction was conducted in triplicate. Amplification efficiencies for every primer pair of each candidate gene were determined.

Statistics

After qPCR, raw data were analysed with qBasePLUS v2.0 (Biogazelle) based on qBase (Hellemans et al., 2007) and geNorm (Vandesompele et al., 2002). Data on neck-teeth induction in the three *D. pulex* clones were checked for homogeneity of variance (equal variance test) and for normality (Shapiro–Wilk). An analysis of variance (one-way ANOVA) was carried out, followed by a Tukey's HSD multiple comparison test. Relative expression was also checked for homogeneity of variance (equal variance test) and for normality (Shapiro–Wilk). *t*-Tests were calculated for each gene pair separately. Data were subsequently subjected to a Bonferroni correction. For the correlation of gene expression level and neck-teeth induction, the data were checked for both constant variance and normality, and subsequently a linear regression analysis was carried out for each gene separately. A significance level of $P<0.05$ was applied to all statistical analyses. All statistics were performed with SigmaPlot v11.0 (Systat Software).

RESULTS

Neck-teeth induction of *Daphnia* clones

Neck-teeth induction in the first juvenile instar of three differently inducible *D. pulex* clones, exposed to *Chaoborus* kairomone or control treatment (without kairomone), were compared (Fig. 1). A one-way ANOVA revealed significant differences between the treatments ($F_{5,84}=519.324$, $P<0.001$). Data were homoscedastic (equal variance test: $P=0.387$) and normally distributed (Shapiro–Wilk: $P=0.302$). *Daphnia pulex* TCO in the control treatment did not develop any neck-teeth at all and were thus excluded from all statistical analyses. A Tukey's HSD revealed neck-teeth induction to be significantly higher in *D. pulex* Münster exposed to kairomone (88% induction) when compared with all other treatments (Münster + kairomone versus all other treatments: $P<0.001$). *Daphnia pulex* Münster in the control treatment had the second highest neck-teeth induction among all treatments (49% induction) and were also significantly different from all other treatments (Tukey's HSD: $P<0.001$ for Münster control versus all other treatments). *Daphnia pulex* Gerstel in the kairomone treatment showed the third highest neck-teeth induction (20% induction) and was also significantly different from all other treatments (Tukey's HSD: $P<0.001$ for Gerstel with kairomone versus all other treatments). Neck-teeth induction in clone Gerstel without kairomone (7% induction) and clone TCO with kairomone (11% induction) did not differ from one another (Tukey's HSD: $P=0.425$) but both differed from all other treatments (Tukey's HSD: $P<0.001$ for all other pairwise comparisons of clone TCO with kairomone and clone Gerstel without kairomone).

Relative gene expression of candidate genes within one clone

RNA was extracted immediately after hatching (up to 1 h) from *D. pulex* that had been exposed to either control water or *Chaoborus* kairomone-containing water during embryogenesis. Relative gene expression was determined via qPCR. All gene expression levels in the kairomone treatments were normalized to

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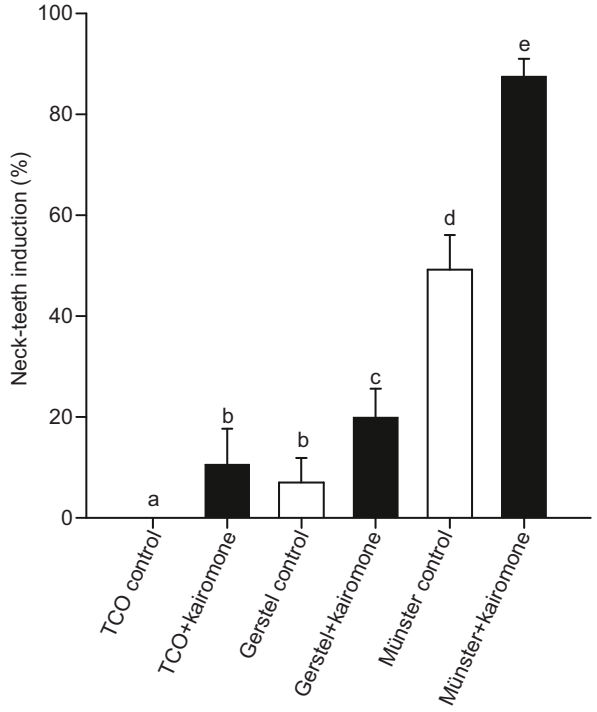

Fig. 1. Neck-teeth induction in first instar juveniles of *Daphnia pulex* clones TCO, Gerstel and Münster by *Chaoborus* kairomone. Data are means+s.d. (N=15) in the presence (black bars) and absence (white bars) of kairomone. Different letters indicate a significant difference within each clone (P<0.05).

their respective controls. For every clone, a separate one-way ANOVA was calculated. All data were homoscedastic. In the case of the *D. pulex* TCO clone, the data were not normally distributed. Thus, a one-way ANOVA on ranks was calculated. As the relative expression of each kairomone-induced gene was normalized to its respective control treatment, no cross-gene comparisons were taken into account.

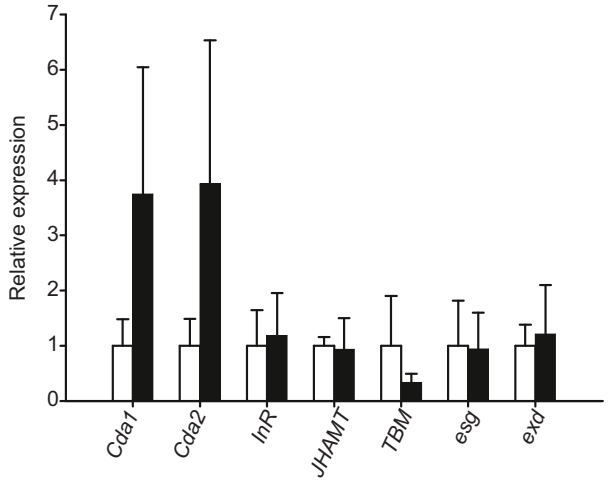

Fig. 2. Relative expression of candidate genes in *D. pulex* clone TCO. *Daphnia* were exposed to either water (control, white bars) or *Chaoborus* kairomone (black bars) during embryogenesis. Expression was determined in first instars, immediately after hatching (means+s.d., N=3). *Cda1*/*2*, chitin deacetylase 1 and 2; *InR*, insulin-like receptor; *JHAMT*, juvenile hormone methyl transferase; *TBM*, tyramine β-monooxygenase; *esg*, escargot; and *exd*, extradenticle.

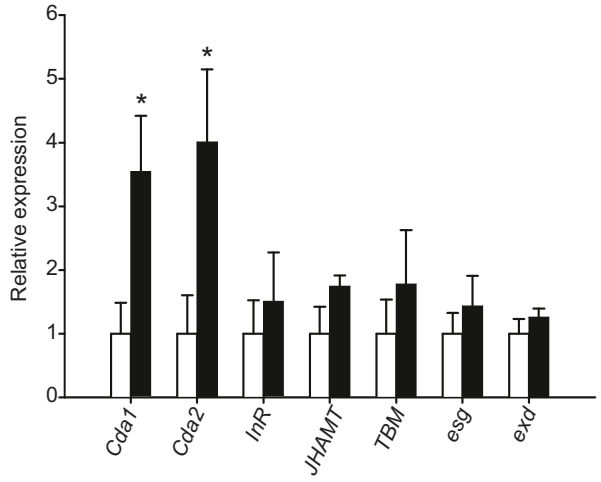

Fig. 3. Relative expression of candidate genes in *D. pulex* clone Gerstel. For details and abbreviations, see legend to Fig. 2. Control, white bars; *Chaoborus* kairomone, black bars (means+s.d., N=3). Asterisks indicate a significant difference between control and kairomone treatments (*t*-test: *P<0.05).

For clone TCO (Fig. 2), a one-way ANOVA on ranks showed no statistically significant differences for the relative expression of any of the candidate genes (P=0.095, H_{13}=20.017). For clone Gerstel (Fig. 3), a one-way ANOVA showed statistically significant differences in the relative expression of the candidate genes (P<0.001, $F_{1,28}$=7.897). Pairwise comparisons revealed a significant upregulation of the expression of *Cda1* (3.5-fold upregulation, Tukey's HSD: P=0.002) and *Cda2* (4-fold upregulation, Tukey's HSD: P<0.001) as a result of exposure to *Chaoborus* kairomone. The other candidate genes did not show any significant changes. For clone Münster (Fig. 4), a one-way ANOVA showed significant differences in the relative expression of the candidate genes (P<0.004, $F_{1,28}$=3.234). However, the pairwise comparison of the control and kairomone treatments for the respective candidate genes failed to show any significant upregulation or downregulation.

Cross-clone comparison of deacetylase genes

Gene expression levels of *Cda1* (Fig. 5A) were compared between all *D. pulex* clones with and without kairomone by means of a one-

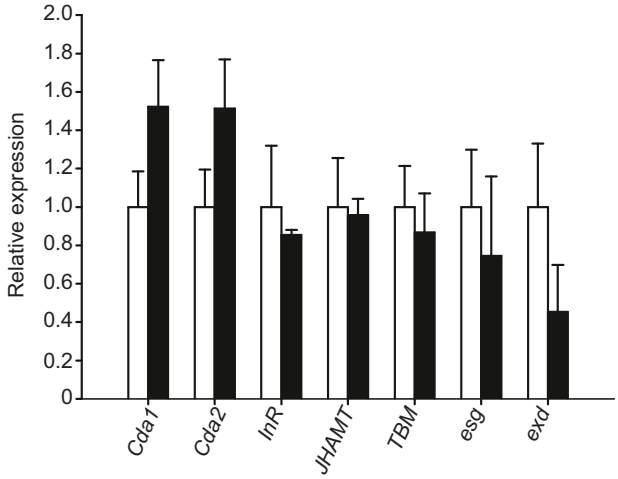

Fig. 4. Relative expression of candidate genes in *D. pulex* clone Münster. For details and abbreviations, see legend to Fig. 2. Control, white bars; *Chaoborus* kairomone, black bars (means+s.d., N=3).

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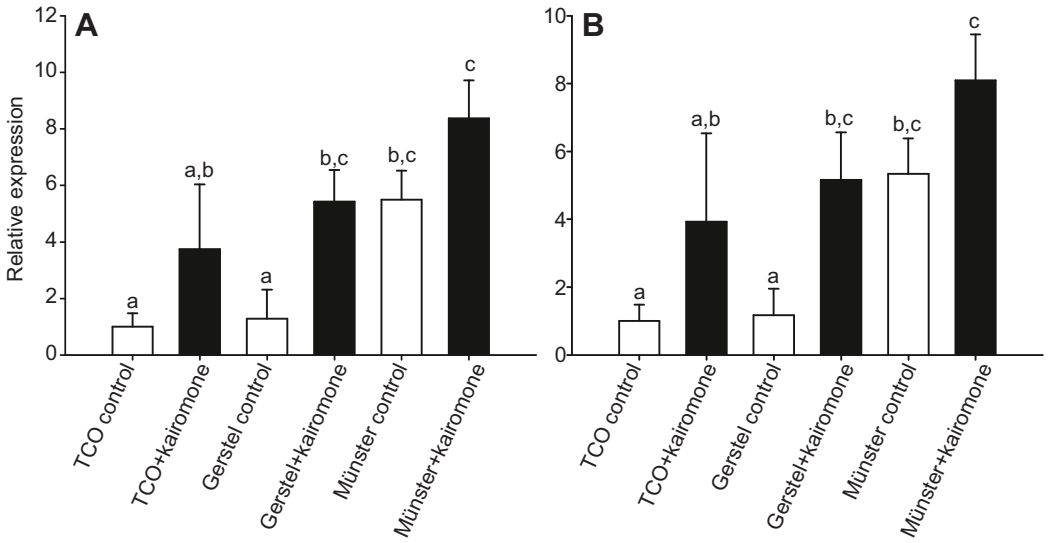

Fig. 5. Relative expression of chitin deacetylase genes in the three *D. pulex* clones. *Daphnia* clones TCO, Gerstel and Münster were exposed to either water (control, white bars) or *Chaoborus* kairomone (black bars) during embryogenesis. Expression of (A) *Cda1* and (B) *Cda2* was determined in first instars immediately after hatching (means+s.d., N=3). Different letters indicate a significant difference between groups (Tukey's HSD: P<0.05).

way ANOVA. All gene expression levels were normalized to that of the clone TCO control treatment. Normality test (P=0.641) and equal variance test (P=0.745) were passed. The ANOVA revealed significant differences between the treatments ($F_{5,12}$=13.333, P<0.001). The clone Münster kairomone treatment had the overall highest relative gene expression (8.4-fold increase compared with the clone TCO control treatment, Tukey HSD: P<0.001). Further, expression of *Cda1* in the clone Münster control treatment (5.5-fold increase) and the clone Gerstel kairomone treatment (5.4-fold increase) were also significantly upregulated compared with the clone TCO control treatment (Tukey HSD: P=0.014 for clone Münster control treatment and P=0.015 for clone Gerstel kairomone treatment). However, gene expression in the clone Gerstel control treatment (Tukey HSD: P=0.367) and in the clone TCO kairomone treatment was not upregulated (Tukey HSD: P=0.193).

The results for *Cda2* were quite similar (Fig. 5B). All gene expression levels were normalized to that of the TCO control treatment. Normality test (P=0.757) and equal variance test (P=0.621) were both passed. The ANOVA revealed significant differences between the treatments ($F_{5,12}$=10.581, P<0.001). The overall pattern was identical to that of *Cda1*, with only slightly differing expression levels and P-values. The clone Münster kairomone treatment showed the greatest upregulation (8.1-fold, Tukey HSD: P<0.001). Gene expression in the clone Münster control treatment (5.3-fold, Tukey HSD: P=0.029) and the clone Gerstel kairomone treatment was also upregulated (5.2-fold, Tukey HSD: P=0.038), whereas that in the clone Gerstel control treatment (Tukey HSD: P=1.000) and the clone TCO kairomone treatment (Tukey HSD: P=0.203) was not.

Relative gene expression as a function of neck-teeth induction

When the expression levels of *Cda1* and *Cda2* were depicted as a function of first instar neck-teeth induction values of the respective treatments across all *D. pulex* clones and treatments, a regression analysis revealed a significant linear correlation between both parameters for *Cda1* (Fig. 6A; $F_{1,5}$=17.914, P=0.013, r^2=0.82) and *Cda2* (Fig. 6B; $F_{1,5}$=16.534, P=0.015, r^2=0.81). A normal distribution of the data was obtained (Shapiro–Wilk normality test, for *Cda1*: P=0.505 and for *Cda2*: P=0.481).

DISCUSSION

The presence of a predator, indicated by a chemical cue, imposes stress on potential prey organisms. In *Daphnia*, this may result in an increased alertness of the prey (Brewer et al., 1999), a change in behaviour (Dodson, 1988), changes of life history (Sakwinska and Dawidowicz, 2005) or an altered morphology (Krueger and Dodson, 1981). However, these inducible defences are not exclusively expressed, but have been shown to co-occur in many different combinations (Boersma et al., 1998). Therefore, the attribution of an upregulation of a candidate gene to a single inducible defence poses a challenging task when several defences are expressed at the same time. By introducing the novel method of a gradient of morphologically differently responsive *D. pulex* clones and by correlating candidate gene expression profiles of several *D. pulex* clones with their respective strength of neck-teeth induction, new insights concerning the putative involvement of candidate genes can be obtained.

The three *D. pulex* clones used in this study differed significantly in their neck-teeth expression between kairomone and control treatments (Fig. 7). Further, all kairomone treatments differed from one another as did all control treatments, providing a clonal gradient of neck-teeth development suited for the envisaged endeavour.

Promising genes were chosen as candidates according to studies by Miyakawa et al. (2010) and Otte et al. (2014). Using a candidate gene approach, Miyakawa et al. (2010) proposed the involvement of juvenile hormone and the insulin signalling pathway in the regulation of neck-teeth induction. This suggestion was corroborated by the finding that juvenile hormone pathway genes (juvenile hormone acid methyltransferase and methoprene-tolerant) and genes of the insulin signalling pathway (insulin-like receptor and insulin receptor substrate-1) were upregulated in post-embryonic *D. pulex* in response to *Chaoborus* kairomone. An involvement of the juvenile hormone pathway was further supported by Miyakawa et al. (2013), who reported an increase of kairomone-dependent neck-teeth induction in the presence of juvenile hormone.

Previous work has provided a persuasive hypothesis on how these genes fit into the greater regulatory picture of neck-teeth induction. Beckerman et al. (2013) proposed a model of how juvenoid hormones are connected with chitin synthesis regulation after taking various arthropod-related studies into account, which

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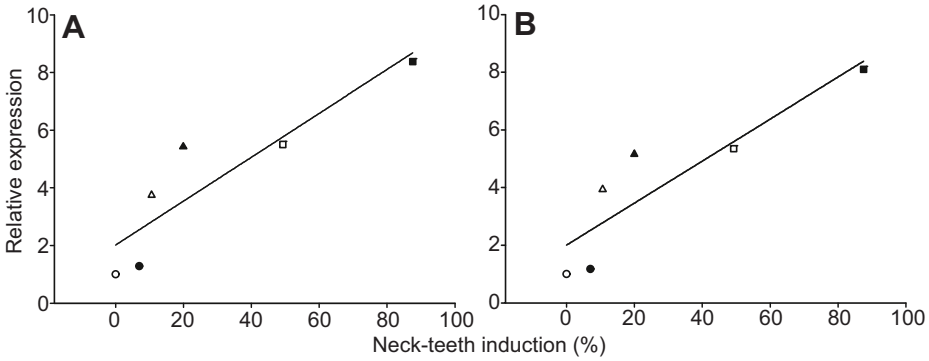

Fig. 6. Relative expression of chitin deacetylase genes as a function of neck-teeth induction in the three *D. pulex* clones. *Daphnia* clones TCO (circles), Gerstel (triangles) and Münster (squares) were exposed to either water (control, white symbols) or *Chaoborus* kairomone (black symbols) during embryogenesis. Expression of (A) *Cda1* and (B) *Cda2* was determined in first instars immediately after hatching (N=3). r^2 for the regression line is 0.817.

demonstrated that juvenile hormones as well as ecdysteroids are involved in morphological polyphenism. In *Daphnia*, juvenile hormones have been demonstrated to be involved in temperature-dependent sex determination (Oda et al., 2005), which suggests that juvenoid-related genes might also be involved in predator-induced polymorphism. Furthermore, Weiss et al. (2015) proposed that the neurohormone dopamine is, in concert with juvenoid signalling, not only indirectly involved in the regulation of synthesis and degradation of juvenile hormones (Gruntenko et al., 2005a,b) but also directly by enhancing sclerotization and even the development of morphological defences in *Daphnia* induced by kairomones of invertebrate predators.

Unfortunately, our data do not support the upregulation in the presence of *Chaoborus* kairomone reported by Miyakawa et al. (2010) of the genes *TBM*, *JHAMT*, *InR*, *esg* and *exd* in any one of the three clones used in this study, all of which are somehow involved in hormonal signalling. However, the present study and the study by Miyakawa et al. (2010) applied different methods of sampling, which may explain the diverging findings. Miyakawa et al. (2010) sampled animals over a time period of 24 h starting with the hatching of the juvenile *Daphnia* and subsequently pooled all RNA for measurements of relative gene expression, whereas in this study, animals were sampled within an hour of hatching. The narrow time window chosen here is in accordance with the finding that fish-borne kairomones have been shown to induce upregulation of a specific set of genes in *Daphnia* in a time frame of only 2 h (Effertz and von Elert, 2014). It was reasoned that, as neck-teeth induction takes place during the late phase of embryogenesis (Parejko, 1992), gene expression differences should be most prominent directly prior to the hatching of juveniles from the brood pouches of their mothers. As this time point is hard to pinpoint, in this study all juveniles were collected within 1 h of birth. However, the two sampling methods are not mutually exclusive, as both provide valuable information on predator kairomone-induced gene expression patterns in *Daphnia*.

Daphnia pulex clones have been shown to vary widely with respect to the strength of neck-teeth induction in response to *Chaoborus* kairomone (Lüning, 1995). Therefore, first instar *D. pulex* used for gene expression analysis in this study were examined for their strength of neck-teeth induction directly prior to RNA extraction. The *D. pulex* clone Münster has been shown to have very high neck-teeth induction even in the absence of kairomones. This might lead to highly expressed neck-teeth genes in general with no, or only a slight, upregulation in the presence of kairomone. Therefore, the clonal gradient of differently responsive genotypes developed here provides a new tool for investigating kairomone-dependent gene expression of candidate genes, where single-clone approaches could simply overlook important information or report false positive or false negative results. Further, it is of tremendous importance to measure neck-teeth induction hand in hand with gene expression as only a precise quantification of the strength of neck-

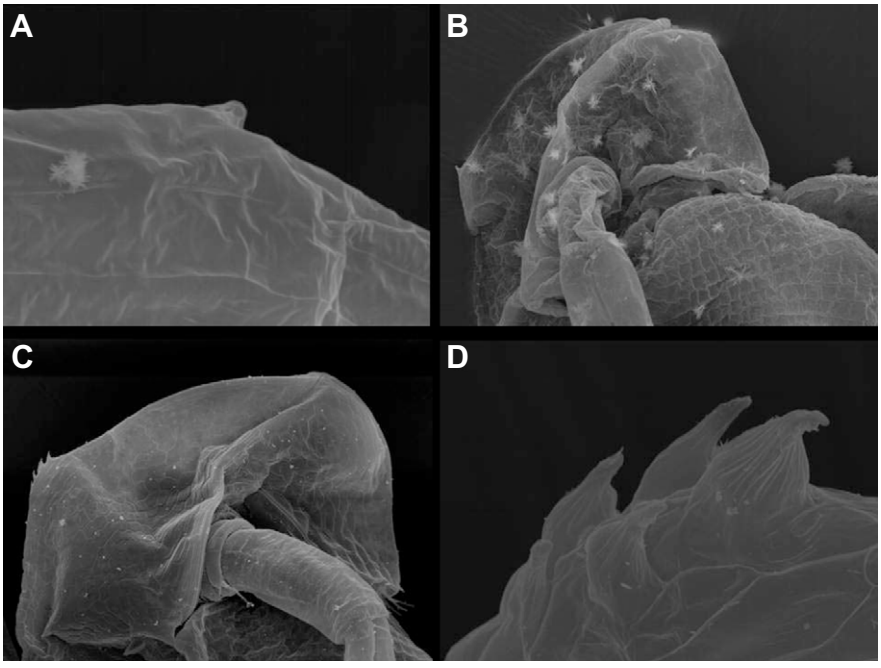

Fig. 7. First instar juveniles of different *D. pulex* clones. (A) Clone TCO with just a single tooth (10% induction); (B) clone Gerstel with one neck-tooth on a keel (40% induction); (C) clone Münster with five neck-teeth on a pedestal; (D) same individual as C, close-up of the back of the head.

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teeth induction allows for a correlation of relative candidate gene expression and morphological defence.

A similar approach was conducted by Dennis et al. (2014), though they did not use a clonal gradient but a gradient of induction in which a single *Daphnia* genotype was exposed to increasing concentrations of *Chaoborus* kairomone. RNA sampling was conducted directly after hatching of *D. pulex* from the brood pouches. This approach also addressed the involvement of the juvenile hormone pathway in neck-teeth induction and proposed a small network of genes. Using a single clone of *D. pulex*, Dennis et al. (2014) showed a dose-dependent upregulation of the genes *EcRb*, *HR3* and *HB2* in response to three different concentrations of *Chaoborus* kairomone; this represents a pattern of expression that is in good agreement with the juvenoid expression profile. *EcRb* and *HR3* have been shown to be essential for moulting in *Tribolium* (Tan and Palli, 2008) with *EcRb* and *HR3* being nuclear receptors and *HB2* being a haemoglobin gene. Even though our data do not support the involvement of juvenile hormone signalling in neck-teeth induction, *EcRb*, *HB2* and *HR3* were not investigated in the present study, which might explain diverging results. The method of applying a gradient of neck-teeth induction in a single *Daphnia* genotype as in Dennis et al. (2014) comprises a useful tool, like our clonal gradient, for future investigations of the genetic regulation of morphological defences.

However, for future experiments concerning juvenoid-related gene-expression changes, we propose refraining from drawing kairomone from crude *Chaoborus* larvae extract or from boiling frozen larvae, as this might lead not only to the release of kairomone but also to the liberation of a vast number of other substances that are naturally contained in the larval tissue, such as juvenile hormones. As these molecules belong to a group of non-polar substances, the subsequent solid-phase extraction would lead to an enrichment not only of the *Chaoborus* kairomone but also of juvenile hormones, as shown by Grossniklaus-Bürgin and Lanzrein (1990), which might affect patterns of gene expression of the juvenile hormone signalling pathway.

In conclusion, the data presented here can neither support nor deny an involvement of the juvenoid signalling pathway. It highlights once more the importance of the time point of sampling because of fast relative gene expression changes over the course of hours. More work needs to be done in order to unravel the interplay of morphological defences and the juvenoid signalling pathway.

In addition to the candidate genes that were used according to Miyakawa et al. (2010), we also investigated effects of *Chaoborus* kairomone on the expression of two chitin deacetylases. Expression of these two genes was found to be upregulated exclusively in *D. pulex* clone Gerstel in response to *Chaoborus* kairomone, which has an intermediate strength of neck-teeth induction among the three *D. pulex* clones used in this study. In *D. pulex* clone TCO, no neck-teeth were detectable in the control treatment, and only a very low induction was found in the kairomone treatment. In *D. pulex* clone Münster, in contrast, even first instars from the control treatment showed a strong neck-teeth induction, which was significantly higher than that resulting from kairomone treatment of *D. pulex* clones TCO and Gerstel. Kairomone treatment of *D. pulex* clone Münster caused the strongest neck-teeth induction of all treatments. Surprisingly, this strong morphological defence seemed not to be reflected at the gene level. However, when comparing chitin deacetylase gene expression in all treatments and in all three *D. pulex* clones, the normalized values revealed a positive correlation between strength of neck-teeth induction and gene expression along the clonal gradient. This was similar for the two chitin deacetylase genes with only minor differences. Generally, this pattern of expression of chitin deacetylase genes fits the picture proposed by Beckerman et al. (2013), who argued that the arthropod response to predator kairomones is based on the regulation of chitin synthesis and degradation. The chitin metabolism is centred on the temporal and spatial modifications of the carapace, which is known to be chitin rich. Chitin deacetylases are chitin-modifying enzymes, known to catalyse the N-deacetylation of chitin to influence the protein-binding affinity of chitin filaments. In *Tribolium castaneum*, chitin deacetylases of type 1 and 2 are mainly expressed in the exoskeletal epidermis (Arakane et al., 2009), which would be in accordance with an involvement of chitin deacetylases in the expression of morphological defences. Neck-teeth are not the only part of the carapace in *Daphnia*, which might be subjected to predator-induced morphological changes. As Laforsch et al. (2004) pointed out, some *Daphnia* species, among them *D. pulex*, also respond to predator kairomones by increasing their overall carapace stability. Unfortunately, the present study does not feature measurements of carapace stability, and other potential changes in the carapace of the individuals investigated above might have been overlooked. Laforsch et al. (2004) used daphnids from the second juvenile stage for their measurements, whereas we used juveniles from the first instar stage directly after hatching. It therefore remains unclear whether the increase in expression of chitin deacetylases in the first instar, as reported here, is associated with increased carapace stability in this ontogenetic stage or is a prerequisite for the increased carapace thickness in the second instar that has been reported by Laforsch et al. (2004). It will be interesting to investigate whether in *D. pulex* clone Münster the observed high level of deacetylase expression in the absence of *Chaoborus* kairomone is associated with a thicker carapace in the absence of predators. Nonetheless, it can be concluded that chitin deacetylases play an important role in inducible morphological defence and, as we argue here, in neck-teeth induction.

Conclusions

The results of this study indicate the importance of chitin-related genes for the development of morphological defences in *Daphnia*. Although chitin deacetylases are probably not the basal genes responsible for the regulation of neck-teeth induction, the more than 8-fold upregulation in the induced *D. pulex* clone Münster compared with the uninduced *D. pulex* clone TCO indicates the importance of these deacetylases. Further research is needed to completely understand the molecular mechanics of morphological defences in *Daphnia*, and this study offers a useful approach by introducing the use of a clonal neck-teeth gradient to differentiate expression of neck-teeth-specific genes from that of genes that are part of other defence-related pathways or are upregulated as a result of general predator-imposed stress.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

M.C., P.F. and E.v.E. made a substantial contribution to conception and design. M.C. and P.F. made a significant contribution to data acquisition. M.C. and E.v.E. were involved in data analysis and interpretation, and in drafting the manuscript and revising it critically for important intellectual content. All authors gave their final approval of the version to be published.

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