Predator-specific reversibility of morphological defenses in *Daphnia barbata*

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Inducible defenses are a common phenotypically plastic response to a heterogeneous predation risk. Once induced, these defenses cannot only lose their benefit, but even become costly, should the predator disappear. Consequently, some organisms have developed the ability to reverse their defensive traits. However, despite extensive research on inducible defenses, reports on reversibility are rare and mostly concentrate on defensive behavior. In our study, we investigated the reversibility of morphological defenses in the freshwater crustacean *Daphnia barbata*. This species responds to *Notonecta glauca* and *Triops cancriformis* with two distinctively defended morphotypes. Within the numerous defensive traits, we found both trait- and predator-specific reversibility. Body torsion and tail-spine-related traits were highly reversible, whereas helmet-related traits remained stable, suggesting different physiological constraints. However, in general, we found the defenses against *Triops* mostly reversible, while *Notonecta*-induced defenses were persistent and grew further, even in the absence of a predator.

INTRODUCTION

A phenotype is determined both by its genotype and by its environment. While the genotype of an organism is usually determined at the beginning of its life, the environment can change extremely rapidly, even multiple times within its lifespan. In order to flexibly cope with these changes, almost every organism is in some way phenotypically plastic (Whitman and Agrawal, 2009). One example is the plastic adaptation to a heterogeneous predation risk, termed inducible defenses. Nearly all organisms are exposed to predation, whether predation *sensu strictu*, grazing or parasitism (Begon *et al.*, 2005). As a result, inducible defenses are extremely widespread in taxa ranging from bacteria (Fiałkowska and Pajdak-Stós, 1997) to protozoa (Kuhlmann *et al.*, 1999),

plants (Mcnaughton and Tarrants, 1983; Maleck and Dietrich, 1999; Franceschi *et al.*, 2005; Mithöfer and Boland, 2012) and animals (Lass and Spaak, 2003; Kishida and Nishimura, 2005; Touchon and Warkentin, 2008; Kishida *et al.*, 2009; Ángeles Esteban, 2012; Gómez and Kehr, 2012; Miner *et al.*, 2013; Kerfoot and Savage, 2016). However, when predators disappear or change an inducible defense that once held a benefit, this could then lead to a disadvantage and be costly. An extreme example are "survival trade-offs", that can appear, when the adaptation to one predator makes the prey more susceptible to another one (Benard, 2006; Hoverman and Relyea, 2009). Consequently, some organisms have the ability to change back again. This ability is referred to as "reversibility" of inducible defenses. While the last four decades of research have uncovered a range of inducible defenses (for reviews, see e.g. Harvell and Tollrian, 1999; Lass and Spaak, 2003; Chen, 2008; Donk and Ianora, 2011), in comparison only few studies have addressed and reported reversibility (but see Brönmark and Pettersson, 1994; Relyea, 2003; Mikulski, Czernik and Pijanowska, 2005; Kishida and Nishimura, 2006; Hoverman and Relyea, 2007; Orizaola, Dahl and Laurila, 2012; Miner *et al.*, 2013). Therefore, it is difficult to estimate how common and widespread this phenomenon is, based on the available experimental data. Thus, mostly theoretical models give explanations for this phenomenon (Gabriel, 1999; Gabriel *et al.*, 2005; Fischer *et al.*, 2014; Utz *et al.*, 2014). The necessary conditions for the evolution of reversibility of inducible defenses are only slightly different from those of the expression of inducible defenses. For inducible defenses, they consist of the heterogeneity in predation risk, the ability to form effective defenses, information about the predation risk and costs involved with the defense, which can offset the benefit in periods with no or low predation risk (Harvell and Tollrian, 1999). For reversibility, the main difference in these conditions lies in the costs. Reversibility differs in the need for maintenance costs, which remain after the establishment of a defense and can be saved by reversion. The ability to reverse an inducible defense is often associated with small developmental windows (Relyea, 2003; Hoverman and Relyea, 2007; Fischer *et al.*, 2014). This is especially the case for defenses with small developmental windows themselves, e.g. defenses which are only expressed during larval stages (Relyea, 2003; Kishida and Nishimura, 2006; Hoverman and Relyea, 2007). Furthermore, a young and (rapidly) growing organism might have better chances to reduce defenses by overall or compensating growth. Additionally, the sooner a predator disappears or changes within the lifetime of a prey organism, the worse is the relation between the time a defense provides a benefit versus the time it is disadvantageous. Consequently, the importance of reversibility of a defense seems stronger for juveniles than for adults.

The ability to reverse induced defenses has been shown in amphibians, mollusks, fish and plants, but so far in *Daphnia*, apart from the generally reversible diel vertical migration (e.g. Beklioglu *et al.*, 2008), reversibility has only been studied in the case of life history defenses (Mikulski *et al.*, 2005) and one morphological defense (Vuorinen *et al.*, 1989). Since 1974, when Dodson proposed that cyclomorphosis, the seasonal changes in morphology, might actually be an adaptation to a heterogeneous predation risk (Dodson, 1974), this group of planktonic freshwater crustaceans has been extensively studied for their inducible defenses. As a consequence, it is now known that they are able to change their behavior (e.g. diel vertical migration, Dodson, 1988a; Lampert, 1989) and their life history (Weber and Declerck, 1997; Riessen, 1999) in addition to morphology (Dodson, 1988b; Laforsch and Tollrian, 2004a) as defenses against a variety of predators, including fish (Kolar and Wahl, 1998), *Chaoborus* larvae (Riessen and Trevett-Smith, 2009), tadpole shrimps (Petrusek *et al.*, 2009; Rabus and Laforsch, 2011), notonectids (Barry, 2000; Herzog and Laforsch, 2013) and other invertebrates (Laforsch and Tollrian, 2004b). Mostly known for morphological defenses, the repertoire of *Daphnia*'s defense structures ranges from elongated spines (Kolar and Wahl, 1998; Laforsch and Tollrian, 2004a; Rabus and Laforsch, 2011), crests (Barry, 2000), helmets (Dodson, 1988b; Laforsch and Tollrian, 2004a) to structures like a crown of thorns (Petrusek *et al.*, 2009) or even a body torsion (Herzog *et al.*, 2016). *Daphnia barbata* in particular shows an extraordinarily large number of morphological defensive traits within its genus (Herzog and Laforsch, 2013). Furthermore, this African species, which predominantly populates temporary freshwater ponds and lakes (Benzie, 2005), exhibits predator-specific responses by reacting to *Triops cancriformis* and *Notonecta glauca* with specialized morphotypes (Herzog and Laforsch, 2013). These specialized defenses are based on the same structures (e.g. helmet, tail-spine and dorsal ridge), but built in a different shape (e.g. elongated versus curved). In the same study, it has been claimed, that the evolution of this specialization can only be explained, if the prey organisms face times with either one *or* the other predator, but not both at the same time. This hypothesizes a highly heterogeneous environment in which predators also disappear or change, promoting the ecological relevance for the development of reversible defenses. The predator-specific responses combined with the numerous defensive traits *D. barbata* possesses, and provide the opportunity to differentiate between physiological and ecological factors in a laboratory experiment: defenses sharing the morphological basis are very likely to share physiological constraints for reversibility and to have comparable

physiological costs. Consequently, physiological factors and/or constraints would be the probable explanation for trait specific but predator unspecific reversibility or irreversibility. However, predator-specific reversibility would suggest dependence on ecological factors directly or indirectly connected with predation.

To study the reversibility of morphological defenses in *Daphnia*, we exposed adult *D. barbata* to chemical cues of either *N. glauca* or *T. cancriformis* until primiparity. Then, the cues were removed and responses were compared to continuously induced daphnids, a non-induced control group and to each other.

METHOD

An Ethiopian clone (Eth 1) of *D. barbata* was used for the experiment, which has been used in a previous study (Herzog and Laforsch, 2013) and was originally provided by Joachim Mergeay. The predator *T. cancriformis* derived from a clonal line provided by Dr E. Eder from the University of Vienna, whereas adult *N. glauca* were caught in water tanks outside the faculty of biology in Martinsried, Germany, and subsequently treated against bacteria and fungi (TetraMedica General Tonic, *Tetra* GmbH, Germany) prior to the experiments. The whole experiment was conducted in a climate chamber at $20 \pm 0.5°C$ under a constant period of fluorescent light (15 h day:9 h night).

Phase I: Induction

The experiment started with three initial treatments (control, *Triops* induced and *Notonecta* induced) and eight replicates each. A replicate consisted of a 2-L beaker containing 1-L semi-artificial medium and a 125-µm mesh net-cage, which was either empty (control) or contained a single adult predator (*T. cancriformis* or *N. glauca*) and 100 neonates (<12 h old) *D. barbata*. Every day, daphnids were fed with $1 \, mg \, C \, L^{-1}$ of *Scenedesmus obliquus*, whereas predators were given 5–10 *D. barbata* and 3 Chironomid larvae. Feces and impurities caused by the predators were removed with glass pipets every other day. The daphnids were checked daily and removed upon reaching primiparity. A proportion of 25% of the removed daphnids of each treatment was preserved in 70% EtOH (p.a.) and the remaining daphnids were transferred to fresh beakers continuing Phase II (see below).

Phase II: Reversibility

The remaining control daphnids, which were removed from Phase I, were transferred into one fresh beaker (1 L size, 0.5 L medium) for each day and replicate. The remaining daphnids of the predator treatments were divided equally into two separate beakers for each day and replicate, one containing a net-cage with the corresponding predator and the other one with an empty net-cage. This resulted in five final treatments, control (*C*), *Triops* induced (T_{ind}), *Triops* removed (T_{rem}), *Notonecta* induced (N_{ind}) and *Notonecta* removed (N_{rem}), and an increased number of replicates. *Daphnia* and predators were fed the same concentration/amount of food as in Phase I. After 3 days (~1 molt), 6 days (~2–3 molts) and 13 days (~5–6 molts) daphnids were removed (number of removed daphnids = number of available daphnids in the replicate/number of remaining samplings) and preserved in 70% EtOH for later measurements. The resulting sample sizes were *C*: $n_{+3days} = 26$, $n_{+3days} = 22$, $n_{+3days} = 15$; T_{ind}: $n_{+3days} = 20$, $n_{+3days} = 18$, $n_{+3days} = 16$; T_{rem}: $n_{+3days} = 17$, $n_{+3days} = 18$, $n_{+3days} = 16$; N_{ind}: $n_{+3days} = 21$, $n_{+3days} = 16$, $n_{+3days} = 9$ and N_{rem}: $n_{+3days} = 18$, $n_{+3days} = 13$, $n_{+3days} = 8$.

Measurements

We used a digital image analysis system (cell^P software and Altra 20 camera, Olympus, Hamburg, Germany), mounted on a stereo microscope (Olympus SZX12), to measure (corresponding to the definition and findings of Herzog and Laforsch, 2013) body length, helmet length, helmet angle, absolute tail-spine length (ventral edge of the tail-spine), effective tail-spine length (direct line between base and tip of the tail-spine) and spine angle. The curvature of the tail-spine was calculated as the ratio between absolute tail-spine length and effective tail-spine length. Relative helmet length (*helmet length/body length*) and relative spine length (*absolute spine length/body length*) were also calculated. Additionally, from a dorsal view, further measurements were taken. The density of microspines on the helmet was measured as the distance between the 1st and the 10th dorsal microspine. Furthermore, dorsal ridge width, the longest dorsal microspine on the helmet and the angle of the fifth microspine relative to the dorsal ridge were measured. Body torsion was recorded and defined as the distance between the tip of the head to the tail-spine orthogonal to the body axis.

Statistical analysis was performed using SPSS 24.0 (IBM, Armonk, USA). The data were tested for normal distribution using a Shapiro–Wilk test and for homoscedasticity using a Levene's test. If all assumptions were met, data were analyzed using a one-way analysis of variance and Tukey's honest significant difference (HSD) test for *post hoc* analysis. In the case of heteroscedasticity, we used Welch tests and Tamhane's T2 tests for *post hoc*

analysis. Data, which were not normal distributed, were tested using a Kruskal–Wallis test with Bonferroni corrected Mann–Whitney-U tests for *post hoc* analysis.

RESULTS

Phase I: Induction

Daphnids of the T_{ind} and N_{ind} treatment showed almost identical responses compared to the previous descriptions of inducible defenses in *D. barbata* ([Herzog and Laforsch, 2013; Herzog *et al.,* 2016](#)), both in differences between the treatments and magnitude of response. The *Notonecta*-induced morph had an elongated helmet, elongated tail-spine as well as elongated microspines in the head-region of the dorsal ridge compared to the other treatments (see Table I, Fig. 1 and SI for detailed statistics). The *Triops*-induced morph showed an intermediate helmet, a curved tail-spine, both bent backwards, an increased dorsal ridge width and a higher density of elongated microspines, which were pointing sideways (see Table I, Fig. 1 and SI for detailed statistics). In addition to these known traits, we found *Triops*-induced daphnids to show a body torsion, which was characterized by the back of the helmet pointing to the right and the tail-spine pointing to the left of the helmet (see Fig. 2). The same orientation of the body torsion was found in all daphnids of the T_{ind} treatment and 95% of the T_{rem} treatment (in 5% no visible torsion could be identified).

Phase II

All measured parameters (helmet length, relative helmet length, body length, body width, tail-spine length, relative tail-spine length, tail-spine curvature, tail-spine angle, helmet angle, dorsal ridge width, maximum microspine length, microspine angle, microspine density and body torsion) showed significant differences between the groups ($P < 0.001$, see SI for detailed statistics and Fig. 3 for illustration).

During the experiment, significant differences between the T_{ind} and the T_{rem} treatment were found in relative helmet length, curvature, body width, body torsion, dorsal ridge width, maximum microspine length and microspine angle (see Table I and SI).

Helmet traits

Compared to continuously induced daphnids, those with the predator *Triops* removed showed a significantly smaller relative helmet length 3 days, 6 days and 13

Table I: Pairwise comparisons between treatments of traits showing reversibilities.[a]

Trait	Comparison	Primiparity	+3 days	+6 days	+13 days
Shared-induced traits					
Relative helmet length	C/T_{ind}	****	****	****	****
	T_{ind}/T_{rem}		**	**	**
	C/T_{rem}		****	****	****
	C/N_{ind}	****	****	****	****
	N_{ind}/N_{rem}		n.s.	n.s.	n.s.
	C/N_{rem}		****	****	****
Max. microspine length	C/T_{ind}	**	*	n.s.	n.s.
	T_{ind}/T_{rem}		n.s.	**	n.s.t.
	C/T_{rem}		n.s.t.	n.s.	n.s.
	C/N_{ind}	****	****	****	****
	N_{ind}/N_{rem}		n.s.t.	n.s.	n.s.
	C/N_{rem}		**	****	**
Body width	C/T_{ind}	n.s.	n.s.	n.s.	n.s.
	T_{ind}/T_{rem}		n.s.	*	n.s.
	C/T_{rem}		n.s.	n.s.	n.s.
	C/N_{ind}	****	****	****	**
	N_{ind}/N_{rem}		n.s.	**	n.s.
	C/N_{rem}		****	****	*
***Triops*-induced morph specific**					
Curvature	C/T_{ind}	**	n.s.	****	n.s.
	T_{ind}/T_{rem}		n.s.	**	n.s.
	C/T_{rem}		n.s.	n.s.	n.s.
Body torsion	C/T_{ind}	**	**	**	**
	T_{ind}/T_{rem}		n.s.	n.s.	**
	C/T_{rem}		****	****	n.s.
Dorsal ridge width	C/T_{ind}	****	****	****	****
	T_{ind}/T_{rem}		n.s.	****	****
	C/T_{rem}		****	****	****
Microspine angle	C/T_{ind}	*	****	****	****
	T_{ind}/T_{rem}		n.s.	****	n.s.
	C/T_{rem}		****	****	****
***Notonecta*-induced morph specific**					
Body length	C/N_{ind}	****	****	****	n.s.
	N_{ind}/N_{rem}		n.s.	n.s.	n.s.
	C/N_{rem}		***	**	n.s.

[a]Compared treatments are shown on the left, whereas points in time are marked on top of the matrices. Levels of significance are indicated by either asterisks (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$) or n.s. for "not significant" and n.s.t. for a non-significant trend ($P < 0.1$). Treatments are abbreviated with C for control, N_{ind} for *Notonecta* induced, N_{rem} for *Notonecta* removed, T_{ind} for *Triops* induced and T_{rem} for *Triops* removed.

days after the removal (Bonferroni corrected Mann–Whitney-U test, all $P < 0.01$, Fig. 1A).

Tail-spine traits

Significant differences in tail-spine curvature between T_{ind} and T_{rem} were only found after 6 days (Bonferroni corrected Mann–Whitney-U test, $P = 0.002$). The average curvature decreased continuously with age in the T_{ind} treatment and 13 days after the removal, no significant differences between induced and control daphnids could be found (Bonferroni corrected Mann–Whitney-U test, $P > 0.999$, see Fig. 1B).
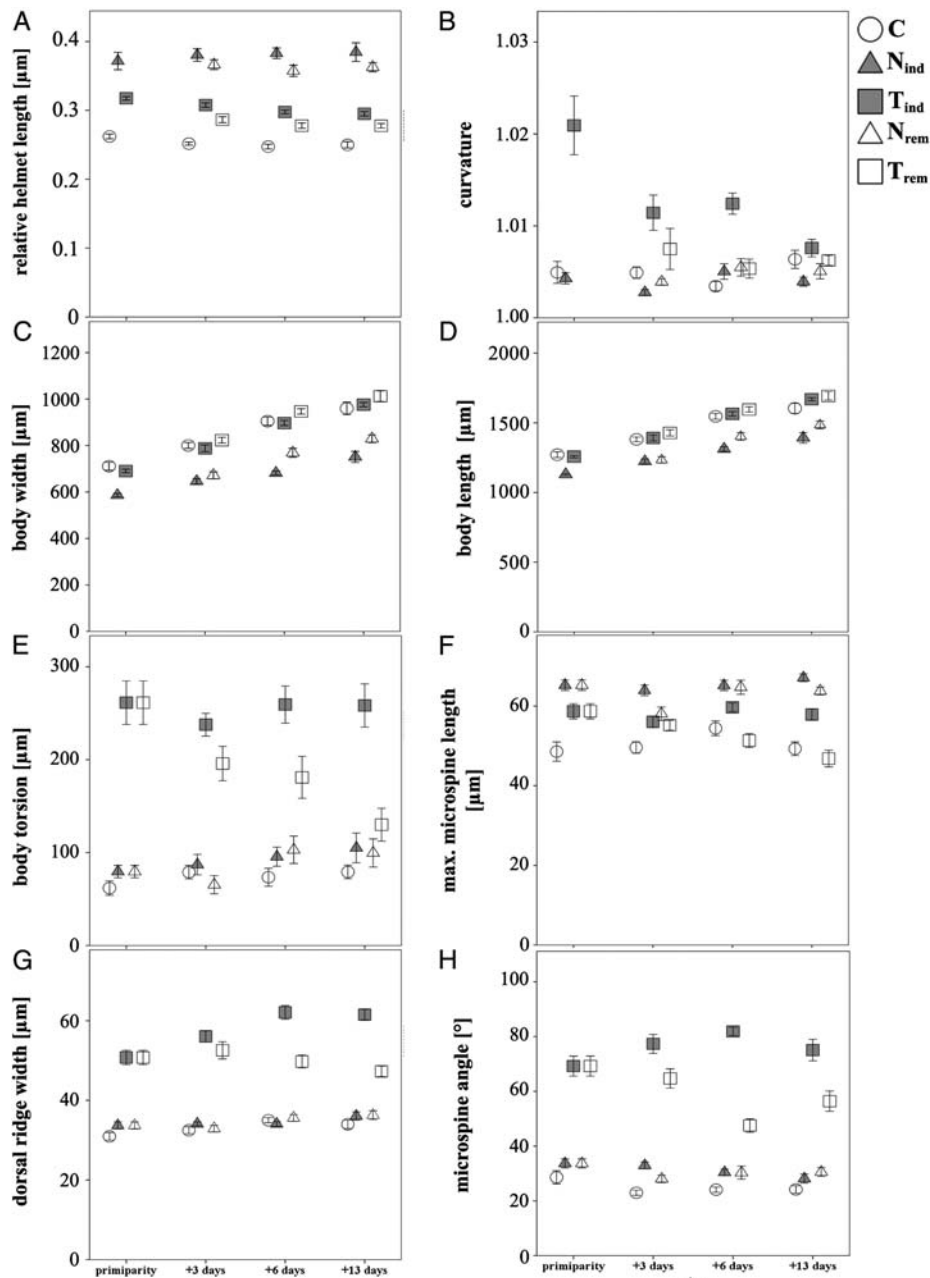
Fig. 1. Development of reversible traits. The graphs show mean trait values during the four stages of the experiment (primiparity, 3 days later, 6 days later and 13 days later), including: relative helmet length (**A**), curvature (**B**), body width (**C**), body length (**D**), body torsion (**E**), maximum microspine length (**F**), dorsal ridge width (**G**) and microspine angle (**H**). The error bars indicate the standard error of mean. Symbols represent the treatment control (circles, C), *Notonecta* induced (black triangles, N_{ind}), *Notonecta* removed (white triangles, N_{rem}), *Triops* induced (black squares, T_{ind}) and *Triops* removed (white squares, T_{rem}).

General body traits

There was also a significant difference in body width between T_{ind} and T_{rem} after 6 days (Tamhane's T2 test, $P = 0.034$ and Fig. 1C) with on average slightly wider continuously induced daphnids. Throughout the experiment, body torsion was significantly greater in T_{ind} and initially (after 3 and 6 days) T_{rem} daphnids, compared to the control (see Table I and Fig. 1E). After 13 days, T_{ind} daphnids showed a significantly stronger body torsion compared to T_{rem} daphnids (Kruskal–Wallis test, $P = 0.007$). At that time, no more significant differences were found between T_{rem} and the control (Kruskal–Wallis test, $P = 0.353$).
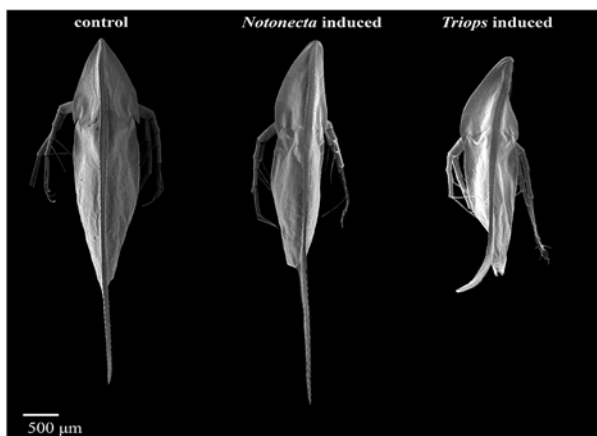
Fig. 2. Scanning electron microscope images of adult *D. barbata* (dorsal). The body torsion of *Triops*-induced daphnids compared to either control or *Notonecta*-induced daphnids is shown.
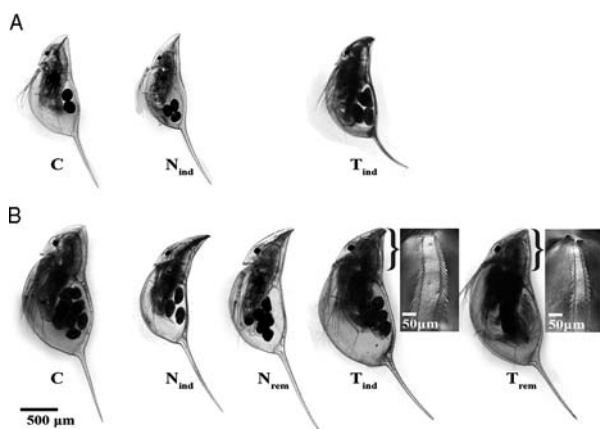

Fig. 3. Lateral view of *D. barbata* through the experiment, sorted by treatment control (C), *Notonecta* induced (N_{ind}), *Notonecta* removed (N_{rem}), *Triops induced* (T_{ind}), *Triops removed* (T_{rem}) at the end of phase I (**A**) and at the end of the experiment/phase II (**B**), respectively. For T_{ind} and T_{rem}, images of the dorsal ridge are included next to the lateral view.

Dorsal ridge-related traits

The maximum microspine length on the backside of the helmet was on average greater in the T_{ind} treatment (see Fig. 1F) compared to the control and significantly different throughout the experiment (see Table I). After 6 and 13 days, daphnids of the T_{rem} treatment showed no significant differences compared to the control (Tukey-HSD test, $P = 0.607$, Bonferroni corrected Mann–Whitney-U test, $P < 0.999$, respectively) but differed significantly from T_{ind} daphnids after 6 days (Tukey-HSD test, $P = 0.003$), with a non-significant trend after 13 days (Bonferroni corrected Mann–Whitney-U test, $P = 0.056$). Dorsal ridge width was significantly larger in T_{ind} compared to T_{rem} after 6 (Tamhane's T2 test,

$P < 0.0001$, see Table I and Fig. 1G) and 13 days (Tukey-HSD test, $P < 0.001$), but daphnids from the T_{rem} treatment still had significantly wider dorsal ridges than the control daphnids (Tukey-HSD test, $P < 0.001$). Microspine angle of T_{rem} was significantly smaller than in T_{ind} daphnids after 6 days (Tukey-HSD test, $P < 0.0001$, see Table I and Fig. 1H), but remained different from the control treatment throughout the experiment (see Table I).

The only significant differences between N_{ind} and N_{rem} daphnids were found after 6 days, when comparing body width (Tamhane's T2 test, $P = 0.007$), with the permanently induced daphnids showing an on average smaller body width (see Fig. 1C). Differences between N_{ind} and control daphnids (Tamhane's T2 test, $P = 0.002$), which were not found between N_{rem} and control daphnids (Tamhane's T2 test, $P = 0.232$) occurred only once, after 6 days for microspine angle. For body length, absolute helmet length, absolute tail-spine length, helmet angle, tail-spine angle and microspine density, no significant differences between the treatments with removed predators and their respective positive control were found (see Supplementary data). Similarly, differences to the negative control remained significant throughout the experiment for these parameters (see Supplementary data).

DISCUSSION

Regarding the reversibility of defensive traits, our results show that already 3 days after the removal of the predators, first differences of T_{rem} daphnids compared to the T_{ind} treatment appear in the relative helmet length. After 3 more days, these differences remain and additional ones appear, such as a lessened curvature, a narrower dorsal ridge and smaller microspines. Another week later, the daphnids from the T_{rem} treatment become "untwisted", with no more significant differences compared to the control. In contrast, the *Notonecta*-induced morph seems to be much more stable. The only significant change after the removal of *Notonecta* was a larger body width after 6 days. However, the question is, whether these changes represent reversibility or not. Most commonly, a defense is called reversible, if the inducible defenses disappear completely, leaving no differences between a phenotype that once experienced predators and a phenotype that never experienced one (Kishida and Nishimura, 2006; Utz *et al.*, 2014). Very often this is equivalent to a simple end of the expression of the trait in question, such as ceasing a specific behavior (Orizaola *et al.*, 2012) or a cessation in the reduction of clutch size (Mikulski *et al.*, 2005). This is usually not

the case for morphological defenses. An organism that ceases to grow a specific morphological trait as defense does not necessarily lose what has already grown. The differences between induced and non-induced phenotypes would remain as rudiments after the predator disappears and thus the result would not be called reversible. This shows a dilemma in the use of the term "reversibility", as rudiments, gradual or incomplete changes do not fit its definition. To solve this dilemma, we propose the differentiation between full reversibility, which means a complete disappearance of differences between formerly induced and non-induced phenotypes, and partial reversibility, which should include all phenotypic changes to inducible defenses that are caused by the disappearance of predators. For morphological traits, this can include counter-balancing growth (e.g. an induced increase in body width is countered by an increased growth in body length), active reduction of the defense (e.g. through apoptosis) or a discontinued growth, where the aforementioned rudiments may or may not remain. These rudimentary defenses can get smaller relative to the overall body size through further growth of the organism (e.g. in fish, Brönmark and Pettersson, 1994). Furthermore, it should be noted that the disappearance of a defense may not necessarily be a sign of phenotypic plasticity (thus reversibility) but can be the result of fixed changes during an organism's ontogeny. This fixed reversion can occur when predation risk or adaptive value of the defense declines with the prey organisms' growth, age or its metamorphosis. This is, for example, known for *Chaoborus* exposed *Daphnia pulex*, which develop so-called neckteeth only during juvenile instars when they are threatened by this size-limited predator (Riessen and Trevett-Smith, 2009). Regardless if the predator is present or not, the neckteeth are not built in later instars. Since this change (not the induction) is genetically predetermined and does not depend on the environment encountered, it does not describe reversibility in a sense of phenotypic plasticity. However, within the same species of *D. pulex*, a clone was described that possessed neckteeth in the first instar, even in the absence of any predator cue, but lost them subsequently in the second instar (Vuorinen *et al.*, 1989). While this defense was not induced by a predator, it certainly showed reversibility, since the disappearance was phenotypically plastic as kairomone-exposed daphnids retained their neckteeth for two to three more instars. Furthermore, in a transfer experiment in the same study, four neonate daphnids were transferred from kairomone-medium to uncontaminated water and exhibited neckteeth in their second instar (indicating an induction), but lost them in the third instar, which suggests reversibility of an early induced defense.

Applying these definitions, *D. barbata* shows reversibilities which are furthermore both trait and predator specific. The changes in body torsion in the T_{rem} treatment result in a morph showing no significant differences to the control morph, hinting at a full reversibility. As the absolute value of the body torsion decreased over time, it seems that it is actively reduced in a step-by-step (or molt-by-molt) process. The changes in dorsal ridge width of the T_{rem} daphnids reflect another example of a gradual reduction. However, the dorsal ridge width in the T_{rem} treatment remained wider than in the control morph, showing only a partial reversibility. The maximum length of the microspines on the dorsal ridge in the T_{rem} treatment seems to be fully reversible, as the average length drops even below the control after 6 and 13 days. While both *Triops* treatments showed no significant difference to the control in microspine length, there still were differences between T_{rem} and continuously *Triops* exposed daphnids even after 6 days (see Table I). The very fast response probably reflects a discontinued expression of the defense, as the microspines are built completely anew with each molt (personal observation). Interestingly, the expression of this trait was not stopped, when *Notonecta* was removed, even though it induces even larger microspines in *D. barbata*. The only significant change, an increase in body width compared to continuously *Notonecta* exposed daphnids, was also found between T_{ind} and T_{rem} daphnids. As brood chamber volume can limit clutch size (Bartosiewicz *et al.*, 2015), it is possible that this change is related to an increase in number or size of offspring. To increase the investment in offspring after a sudden change in the environment could be a viable strategy for *D. barbata*. Offspring, which developed after the removal of the predator, would show a phenotype fitting the new environment. Within 7–9 days, this new, perfectly adapted generation would have matured, being ready to replace the maladapted parents. For fast and clonal reproducing organisms like *D. barbata*, this could be an alternative to reverting defenses.

With the exception of body length, all phenotypic differences between *Notonecta* induced and control daphnids seem to be continuously expressed in N_{rem} daphnids. The helmet and the tail-spine do not stop growing, thus they continue to increase in size. The differences to the T_{rem} treatment, where all traits at least decrease their further growth, are apparent. This leads to the picture of a fast responding, but not fully reversible *Triops*-induced phenotype and an almost completely stable *Notonecta*-induced phenotype. The predator-specific differences in reversibility between both morphotypes do not seem to be a matter of physiological constraints, such as, for example, narrow developmental windows. If

one morphotype is able to reverse the size of micro-spines, so should the other, genetically identical morphotype. The same should apply for the helmet growth, which was decreased in the T_{rem} treatment but not in the N_{rem} treatment. It is known for *Daphnia magna* that the absolute tail-spine length gets smaller with each molt once the animals are adult (Rabus and Laforsch, 2011) both for induced and non-induced daphnids. Therefore, it is unlikely that a tail-spine reduction would be physiologically impossible for *Notonecta*-induced *D. barbata* after the removal of the predator. In contrast, the *Triops*-induced morphotype does not possess elongated tail-spines, but curvature and the tail-spine-related body torsion show at least partial reversibility. Still, it should be noted that curvature decreased in continuously *Triops*-induced daphnids as well, only slower, indicating a fixed ontogenetic change. The apparently high phenotypic plasticity of tail-spine-related traits in adult *Daphnia* stands in contrast to the differences in reversibility of the two predator-induced morphotypes. A difference in costs is possible and has already been stated as likely an explanation for the evolution of the two distinct morphotypes (Herzog and Laforsch, 2013). Despite the finding that the *Notonecta* defense offers the same or better protection against both predators, a specialized *Triops* defense exists. Supposedly, a specialized *Triops* defense would only provide an advantage under *Triops* predation in comparison to the *Notonecta* defense, if it was less costly than the latter. A costlier defense seems to be more beneficial to revert as more costs can be saved by this process. Consequently, we expected a higher reversibility in the *Notonecta* removed treatment, but the opposite is the case. Considering that the *Triops* removed treatment shows reversibilities, it seems possible to rule out that there are no (perpetual) costs involved with the morphological defenses of *Triops*-induced *D. barbata* and thus the same should be true for the other induced morphotype. At the very least, the costs for elongated microspines should be comparable in both induced morphotypes, but they only reverse in one case. This hints that the costs of the defenses against *Notonecta* cannot be saved, at least not under natural conditions. The reason would have to lie in factors, which relate to the ecology of predator and prey rather than their physiology: the heterogeneity of predation risk and the information about it. Even though the cues were removed for both predators, the quality of this information, especially in terms of reliability might be different. Predators may not only disappear, but reappear or even change. The chances for each change can differ between predators. *Notonecta* is able to fly and migrate freely between different ponds (Hutchinson, 1933). *Triops*, however, hatches from resting eggs in temporary ponds

(Takahashi, 1977), just as *D. barbata* does, and is bound to its habitat. Missing kairomones of *Notonecta* might not give a reliable prediction about the predator regime, given the chance that the waterbugs could return at any given time. In comparison, missing kairomones could be safer information regarding *Triops* predation. An alternative explanation is that even after the disappearance of *Notonecta*, the induced defense could have a benefit against other (remaining or following) predators. Similar defenses (long helmet and elongated tail-spine) in *D. cucullata* act as a general defense against multiple invertebrate predators (Laforsch and Tollrian, 2004b) and the same might be the case for *Notonecta*-induced *D. barbata*, as suggested by earlier findings (Herzog and Laforsch, 2013). Furthermore, it is possible that the threat caused by *Notonecta* is simply more permanent than that of *Triops* under natural conditions. Without the actual possibility to save costs associated with induced defenses, it is unlikely that reversibility will evolve. Unfortunately, with no field data available, it can only be speculated how predators appear, disappear and change in *D. barbata*'s natural habitats. Consequently, our results cannot provide a final explanation for the predator-specific reversibility of inducible defenses in *D. barbata*, but emphasize the importance of ecological factors for the evolution of phenotypic plasticity.

CONCLUSION

We report on the ability of adult daphnids to react morphologically to sudden environmental changes and that the loss of body symmetry as response to *Triops* is reversible. In this context, physiological constraints seem to be relevant, as suggested by differences in structure dependent reversibility. Nevertheless, our findings of predator-specific reversibilities underline the high importance of ecological factors, such as composition and seasonal abundance of predators. For a further understanding and a weighting of the interaction and importance of these factors, field studies are essential. The large number of inducible traits and the high predator specificity both in induction and reversibility show that the *D. barbata*–*Notonecta*–*Triops* complex provides an excellent study system for phenotypic plasticity. We hope that our study helps to construct a framework for this system as a basis for future research.

SUPPLEMENTARY DATA

Supplementary data can be found online at http://plankt.oxfordjournals.org

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