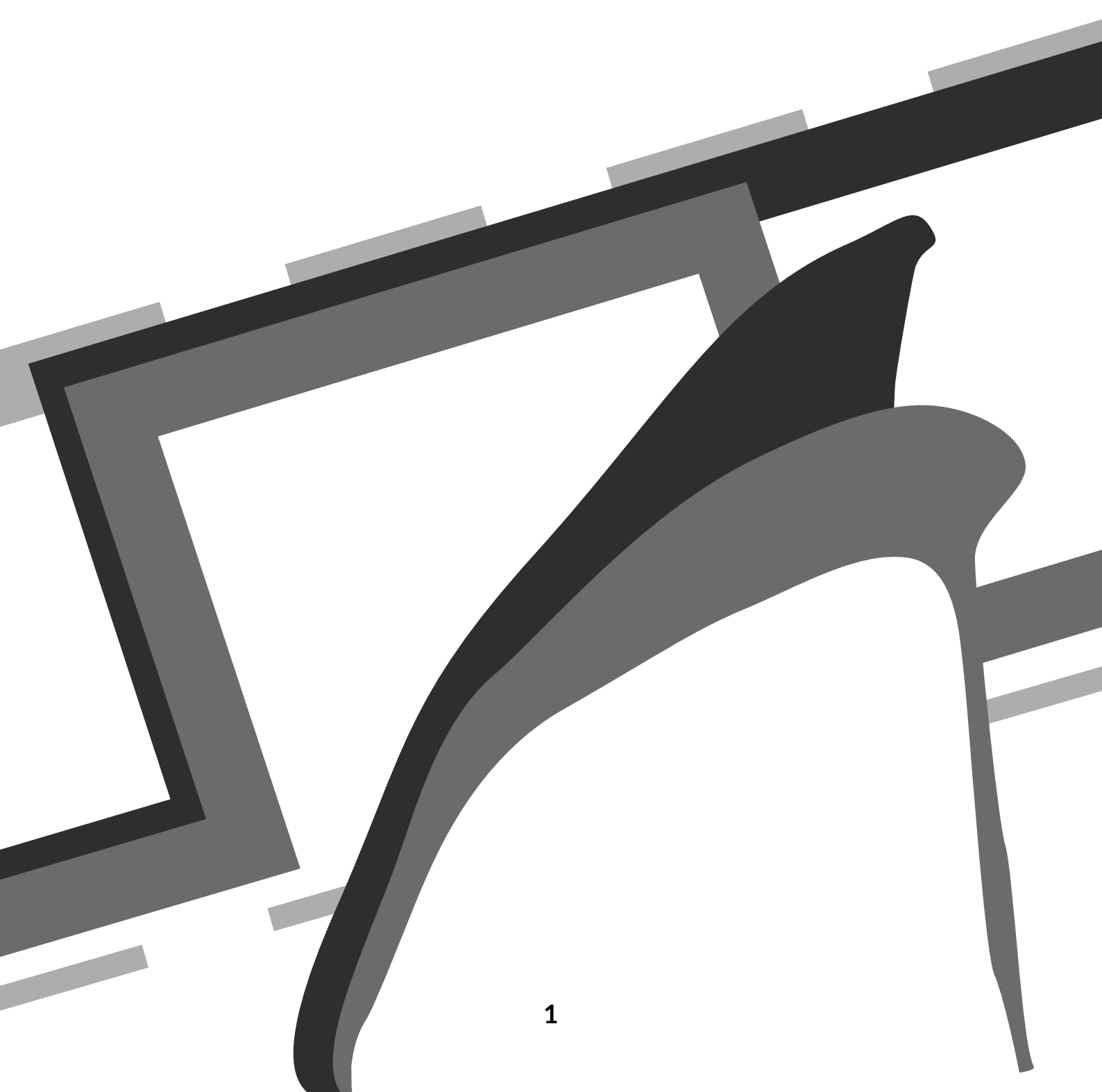# REVERSIBILITY OF INDUCIBLE DEFENSES IN *DAPHNIA*

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Nahezu jeder Organismus ist im engeren oder weiteren Sinne Prädation ausgesetzt, sei es durch Prädatoren sensu strictu, Weidegänger, Parasiten oder Parasitoiden. Da jedoch der Prädationsdruck häufig Schwankungen und Veränderungen unterliegt, werden eine große Anzahl an Anpassungen auf diese Bedrohung plastisch im Phänotyp ausgeprägt. Diese sogenannten induzierbaren Verteidigungen finden sich weit verbreitet in nahezu allen größeren Taxa. Sie ermöglichen einen Schutz bei vorhandener Bedrohung, während sie in Abwesenheit selbiger nicht ausgebildet werden und infolgedessen mit der Expression verbundene Kosten einsparen. Ein weiterer Schritt in der Plastizität ist das Zurückbilden einmal induzierter Verteidigungen. Essentiell dafür sind jedoch Kosten, die nach Ausbildung weiterhin bestehen, etwa durch Aufrechterhaltung der Verteidigung oder Maladaptation an die veränderte Umwelt. Theoretische Studien lassen vermuten, dass eine solche Reversibilität ein häufiges Phänomen darstellt. Bislang bestehen dafür allerdings mit Ausnahme von verhaltens-basierten Verteidigungen nur wenige experimentelle Nachweise. Dies trifft insbesondere auf die induzierbaren Verteidigungen innerhalb der Gattung *Daphnia* zu. Diese Gruppe von Modellorganismen der limnologischen, ökotoxikologischen und bio-medizinischen Forschung liefert zahlreiche Beispiele induzierbarer Verteidigungen durch Änderungen in Verhalten, Life-History sowie Morphologie. Insbesondere letztere sind als Phänomen der „Zyklomorphose" seit über hundert Jahren bekannt, mit einem starken Fokus der Forschung in den letzten Jahrzehnten und infolgedessen der Beschreibung zahlreicher, weiterer Beispiele. Dennoch ist über Reversibilität induzierbarer Verteidigungen innerhalb der Gattung *Daphnia* bislang kaum etwas bekannt. Selbiges gilt auch für den Einfluss von mit induzierbaren Verteidigungen assoziierten Kosten in Bezug auf die Reversibilität phänotypischer Anpassungen sowie möglicher physiologischer Einschränkungen. Die Rolle und Bedeutung ökologischer Faktoren, wie etwa Unterschiede in den Räubern, wurde darüber hinaus ebenfalls bislang nicht untersucht. Ziel dieser Promotion war daher die Identifikation von Reversibilität induzierbarer Verteidigungen innerhalb der Gattung *Daphnia* mit einem Fokus auf morphologische Parameter. Für die Unterscheidung von physiologischen und ökologischen Faktoren wurde die Etablierung eines neuen Systems mit mehreren Räubern angestrebt. Des Weiteren wurde Reversibilität ebenfalls in einem etablierten System untersucht. Darüber hinaus sollte die Bedeutung von Kosten induzierbarer Verteidigungen in Bezug auf die Reversibilität untersucht werden.

Basierend auf der afrikanischen Art *Daphnia barbata* sowie den beiden invertebraten Prädatoren *Triops cancriformis* und *Notonecta glauca*, wurde ein neues System entdeckt, beschrieben und für die Untersuchung auf Reversibilität herangezogen. Dabei konnten in einer Studie erstmals induzierbare Verteidigungen für diese Art nachgewiesen werden. Infolgedessen zeigte sich, dass *D. barbata* auf Basis derselben Strukturen, unterschiedliche, auf die jeweiligen Räuber angepasste, spezialisierte Verteidigungen ausbildet. Starke Maladaptationskosten konnten mittels direkter Prädationsversuche mit vertauschten Räubern jedoch bei keiner der beiden induzierten Morphotypen beobachtet werden. Einige der vorgefundenen Anpassungen stellen zudem bislang gänzlich unbeschriebene morphologische Verteidigungen innerhalb der Gattung *Daphnia* dar. Darunter fällt insbesondere die Körpertorsion durch *Triops*-Induktion, die zu einem Verlust der

Bilateral-Symmetrie führt. Darüber hinaus wurde ein theoretisches Konzept beschrieben, das den Einfluss multipler Prädatoren für die auf Beuteorganismen wirkende natürliche Selektion und die erwartete phänotypische Expression induzierter Verteidigungen beschreibt. Des Weiteren wurde mittels des Konzeptes eine Kategorisierung von Multi-Prädator-Systemen vorgenommen, basierend auf der Vektorisierung von Selektionsdrücken. In einer weiteren Studie wurde gezeigt, dass sowohl Torsion sowie, mit Ausnahme der Helmgröße, die Mehrzahl induzierter morphologischer Verteidigungen gegen *Triops* reversibel sind. Gegenüber *Notonecta* ausgebildete morphologische Veränderungen wurden hingegen nicht zurückgebildet, selbst solche nicht, die bei beiden induzierten Morphotypen auftraten. Damit konnte erstmalig experimentell gezeigt werden, dass zwar physiologische Faktoren die Reversibilität induzierbarer Verteidigungen in Daphnien beeinflussen können, dass aber ökologische Faktoren ebenfalls eine hohe Relevanz besitzen. Zur Identifikation von Reversibilität in einem etablierten System wurde die Art *Daphnia magna* als Beuteorganismus, sowie *T. cancriformis* als Räuber herangezogen. Hierbei konnte bei adulten induzierten Tieren eine starke Reversibilität von morphologischen sowie von Life-History Parametern innerhalb weniger Häutungen beobachtet werden. Ebenso zeigte sich erstmals, dass selbst bei adulten *D. magna* noch Verteidigungen induziert werden können. In einer weiteren Studie wurden mögliche kontinuierliche Kosten untersucht, die im Zusammenhang mit der Schwimmeffizienz stehen. Tatsächlich konnte gezeigt werden, dass induzierte *D. magna* eine deutlich erhöhte Absinkgeschwindigkeit aufweisen. Schwimmexperimente wiesen jedoch darauf hin, dass diese Unterschiede von induzierten Tieren vollständig kompensiert werden. Weitere Berechnungen legen zudem nahe, dass mit weniger als 0,1% des Energiehaushaltes der energetische Aufwand der Kompensation überaus gering ausfällt. In Bezug auf die dennoch beobachtete Reversibilität könnte dies auf weitere, bislang verborgene Kosten hinweisen, etwa durch das mit der Häutung verbundene zyklische Erneuern der Verteidigungen.

Die in dieser Dissertation beschriebenen Ergebnisse liefern damit die ersten klaren Nachweise von Reversibilität induzierbarer morphologischer Verteidigungen, eine Einschätzung zu den verbundenen Kosten und die Beschreibung bislang unbekannter induzierbarer Verteidigungen. Darüber hinaus wurde ein neues Multi-Räuber-Beute-System mit einer weiterführenden Grundlage zu proximaten und ultimaten Faktoren von Reversibilität beschrieben.

Almost every organism is subject to predation, may it be through predators sensu strictu, grazers, parasites or parasitoids. Since predation pressure often undergoes changes and variations, a large number of adaptations to this threat are plastic in their phenotype. These so-termed inducible defenses are widespread in almost all larger taxa. They allow protection if a threat is present, while they are not being expressed in its absence, thus saving associated costs. Another level of plasticity is the reversion of already induced defenses. However, for reversibility costs are essential, that remain after the expression of the defense, such as for its maintenance or through maladaptation to the new environment. Theoretical studies suggest, that reversibility is a common phenomenon. Still, with the exception of behavior-based defenses, only few experimental records exist. This applies especially to the genus *Daphnia*. This group of model organisms of limnological, eco-toxicological and bio-medical research provides numerous examples for inducible defenses through changes in behavior, life-history and morphology. The latter has been known as 'cyclomorphosis' for over a hundred years, with recent attention in the last decades, leading to a growing number of described inducible defenses. However, despite this focus, almost nothing is known about the reversibility of inducible defenses in the genus *Daphnia*. The same applies to the influence of costs and limitations on the reversibility of induced defenses and the underlying physiological and eco-logical factors. Therefore, the aim of this thesis was the identification of reversibility of inducible defenses within the genus *Daphnia*, with a focus on morphological parameters. Furthermore, the establishment of a new multi-predator-prey system was proposed, in order to achieve a differentiation between physiological and ecological factors influencing reversibility. An already established predator-prey system was to be used for additional tests of reversibility. Finally, within the scope of this thesis, possible costs of inducible defenses were assessed in relation to their reversibility.

Based on the African species *Daphnia barbata* and the two invertebrate predators *Triops cancriformis* and *Notonecta glauca*, a new system was discovered, described and used to test for reversibility. This led to the first description of inducible defenses in this species. Additionally, it was shown that *D. barbata* induces defenses based on the same structures, but distinctly adapted to each of the two predators. Strong maladaptation costs could not be found for either of the two defensive morphotypes, when exposing them to the mismatching predator in predation trials. Furthermore, some of the defensive traits of *D. barbata* are previously undescribed morphological defenses in the genus *Daphnia*, most notably the body torsion, which leads to an abolishment of bilateral symmetry. In addition, a theoretical framework was developed in order to explain how multiple predators affect the selection pressure acting on prey organisms as well as the resulting expression of inducible defenses in the phenotype. This framework was then used for a categorization of multi-predator systems based on the vectorization of selection pressures. In a further study, it was shown that with the exception of helmet size, the majority of morphological defenses against *Triops* – including body torsion - are reversible. On the contrary, induced morphological defenses against *Notonecta* were not reverted – not even traits, that both defended morphotypes shared. In consequence, for the first time it could be shown experimentally that, while physiological factors hold an influence, ecological factors have a high importance for the reversibility of inducible defenses or the lack

therof. Regarding the identification of reversibility in an established system, the species *Daphnia magna* was used as prey organism together with the predator *T. cancriformis*. It could be shown, that adult induced *D. magna* can revert both morphological and life-history defenses within a short timeframe. Similarly, an induction of previously non-induced adult daphnids could be described. Using the same predator-prey system, potential running costs associated with inducible defenses and swimming efficiency were tested for in a further study. It was determined, that induced *D. magna* possessed a larger drag and consequently higher sinking rate compared to non-induced daphnids of the same size. However, swimming experiments showed that induced *D. magna* compensate these differences fully. Furthermore, calculations suggest that the energetic costs of this compensation only amount to less than 0.1% of the general energy consumption of the daphnids. In relation to the observed reversibility in *D. magna*, this could hint at hidden costs, e.g. through the periodical renewal of inducible defenses in connection with the molting process.

In conclusion, this thesis provides not only the first substantial records of reversibility of inducible morphological defenses, but also an estimation of associated costs and the description of previously unknown inducible defenses. Finally, a new multi-predator-prey system is presented together with groundwork for the estimation of proximate and ultimate causes and conditions for the phenomenon of reversibility of inducible defenses.

CHAPTER I:
INTRODUCTION

CHAPTER

1

1.1   Phenotypic plasticity

Natural selection drives the evolution of organisms in order to maximize their fitness under the conditions they experience. When these conditions change, but the optimal phenotype stays the same, a process termed environmental canalization can lead to a robustness to these fluctuations (Waddington 1942; Debat & David 2001; Siegal & Bergman 2002). However, most ecosystems are dynamic and flexible, which leads to varying, sometimes even contrasting selection pressures for the organisms they contain (Benard 2006; Hoverman & Relyea 2009). In consequence, the phenotypes, which provide the highest fitness also change (Fordyce 2006; Whitman & Agrawal 2009). Depending on the degree, frequency and continuance of these changes, a genetically fixed phenotype might not only fail to provide the highest fitness, but also cannot adapt to the new conditions fast enough (Gabriel 1999; Gabriel *et al.* 2005). The result is, that almost all organisms possess the ability to develop variable phenotypes depending on the environment, which is termed phenotypic plasticity (Dewitt, Sih & Wilson 1998; Whitman & Agrawal 2009). For instance, many plants develop 'shade' or 'sun' leaves, depending on the experienced light conditions (Lichtenthaler *et al.* 1981; Sultan 1987), mice can shape their mandible characteristics accordingly to their diet (Anderson, Renaud & Rayfield 2014) or some caterpillars develop into different mimics, depending on the plant they feed on (Greene, Series & Feb 1989). Since this phenomenon is so widespread (Schlichting 1986; West-Eberhard 1989; Harvell & Tollrian 1999) and has many implications for ecosystems (Miner *et al.* 2005), populations (Kishida & Trussell 2010; Aranguiz-Acuna, Ramos-Jiliberto & Bustamante 2010), inter- and intraspecific interactions (Kopp & Tollrian 2003; Mougi, Kishida & Iwasa 2011) and genetics (Weber & Declerck 1997; Price *et al.* 2003; Tautz 2011; Miner *et al.* 2012), it has received a large attention in the last decades (West-Eberhard 1989; Dewitt *et al.* 1998; Whitman & Agrawal 2009; Auld, Agrawal & Relyea 2010). In consequence, the causes, conditions and results of its evolution have become central questions in the field of ecology, especially regarding predator-prey relations (Tollrian & Harvell 1999) .

1.2   Inducible defenses

Almost all organisms are subject to natural selection through one or more forms of predation, ranging from predation sensu strictu, grazing and parasitism to parasitoism (Begon, M. and Townsend, C.R. and Harper 2005). As predation pressure is rarely a stable condition, it is a common biotic factor for the evolution of phenotypically plastic adaptations (Harvell & Tollrian 1999). These adaptations are generally termed inducible (or induced) defenses and can be found in almost all types of lifeforms, including bacteria (Fialkowska & Pajdak-Stos 1997), protozoa (Kuhlmann & Heckmann 1985; Altwegg *et al.* 2004), plants (McNaughton & Tarrants 1983; Xing, Higgins & Blumwald 1996; Maleck & Dietrich 1999; Howe & Jander 2008; Mithöfer & Boland 2012; Meyer *et al.* 2014; Ballhorn, Kautz & Laumann 2015), mollusks (Lakowitz, Brönmark & Nyström 2008; Bourdeau 2009), crustaceans (van Donk, Ianora & Vos 2011; Kerfoot & Savage 2016), rotifers (Gilbert 2009, 2011), amphibians (Relyea 2001; Buskirk *et al.* 2002; Kishida & Nishimura 2005), birds (Parejo *et al.* 2013) and fish (Brönmark & Pettersson 1994). The forms of adaptations are equally diverse and often categorized into either three or four groups:

Behavioral defenses. In response to predation, prey organisms can react by temporally or spatially avoiding confrontation with the predator. Many zoo-plankton organisms for example exhibit a diel vertical migration (Lampert 1989). Through the day, the prey organisms sink to the darker depths in

order to avoid visually hunting predators and ascend to surface waters during the night to feed on algae (Lampert 1989). It is also known that some animals alter their activity, for instance decorator crabs reducing their feeding activity (Stachowicz & Hay 1999), or voles giving up on food resources in response to an estimated predation risk (Jacob & Brown 2000). Furthermore, the anti-predator vigilance behavior to screen for potential threats, found inter alia in many birds and mammals (Elgar 1989; Lima 1992; Yasukawa, Whittenberger & Nielsen 1992), can itself change in intensity based on previous experience (Wiedenmayer 2010) but also trigger other plastic behavioral defenses, such as alarm calls (Bergstrom & Lachmann 2001) and in consequence escaping (Mathot *et al.* 2011). A change in predation impact can also lead to an aggregation of prey organisms, e.g. in guppy fish (Botham *et al.* 2006) or cyanobacteria (Yang *et al.* 2006). Other defenses include feigning death (Honma, Oku & Nishida 2006; Santos *et al.* 2010), fending of the predator through intimidation (Vallin *et al.* 2005) and even actively fighting and killing predators (Koeniger *et al.* 1996) among many more behavioral changes, although the term 'inducible defenses' is rarely used in the literature to categorize them.

Life history defenses. The appearance of predators can impact the balance between reproduction and somatic growth, leading to shifts in the life history of organisms. For example, through delaying the first reproduction, the freshwater snail *Helisoma trivolvis* can invest more energy in somatic growth, leading to a larger mass at reproduction (Hoverman, Auld & Relyea 2005). Producing larger offspring is in addition a common response against size-limited predators, which often leads to fewer offspring (Lüning 1992; Riessen 1999). Similarly, an increase in the number of offspring can also counter large predator-associated mortality but is usually accompanied by a decrease in neonate size (Lüning 1992; Riessen & Trevett-Smith 2009). For organisms that go through metamorphosis or distinct life stages, an earlier transformation (usually associated with reaching maturity) can help avoiding predation targeting larval forms. Such life history defenses can for example be found in amphibians and holometabolous insects (Benard 2004; Relyea 2007). Other organisms such as sand dollar *Dendraster excentricus* are able to trigger asexual reproduction in larvae, leading to a higher density of smaller individuals, which are less susceptible to predation (Vaughn 2010).

Morphological defenses. Alterations of the appearance of an organism in response to predation can affect its coloration, structure, size and shape. A change in the pigmentation can be used as camouflage, for instance in the freshwater snail *Radix balthica*, that develops dark instead of translucent shells in response to predatory fish (Ahlgren *et al.* 2013) and kelps crabs that change their color accordingly to the algae they feed on (Hultgren & Stachowicz 2008), thus reducing predation. Another use for a change in pigmentation seems to be to redirect the predators' attention to a certain body part, for example the tails of gray treefrog tadpoles in order to increasing the chance to escape and survive an direct encounter with dragonfly larvae (McCollum & Leimberger 1997). Similarly, structural alterations mostly defend against direct confrontations with predators. They can work as body fortifications like a thicker or harder exoskeleton, often found in mollusks (Trussell & Smith 2000; Trussell *et al.* 2002; Newell, Kennedy & Shaw 2007; Lakowitz *et al.* 2008; Bourdeau 2009), but also in crustaceans (Laforsch *et al.* 2004; Rabus *et al.* 2013) and insects (Flenner *et al.* 2009). Other common defenses consist of thorns or spines, widespread in plants (Milewski, Young & Madden 1991), bryozoan (Harvell 1992), rotifers (Gilbert 1966, 2009, 2011), fish (Januszkiewicz & Robinson 2007), insects (Flenner *et al.* 2009) and crustaceans (Swaffar & O'Brien 1996). Changes in the body shape are often a mechanism to defend against gape limited predators, e.g. frog tadpoles changing into a bulkier morph to avoid salamander predation (Kishida & Nishimura 2005) and crucian carps, which increase their body depth in response to piscivorous pikes, rendering them too large to prey upon (Brönmark & Miner 1992; Brönmark & Pettersson 1994). However, some shape alterations interfere in other ways with the feeding mechanism of

the specific predator, as for example in snails, which can develop elongated shells (Bourdeau 2009) or shells with narrower whirls (Krist 2002) in response to shell-invading predators, thus reducing their attack success. Furthermore, defenses can cause a direct mechanical interference with the predator's feeding apparatus as observed in rotifers that induce spines that reduce the retention time within the filter structures of waterfleas, which prey upon them (Stemberger & Gilbert 1987).

Physiological defenses. Since all defenses are the result of physiological processes, this category is often not explicitly mentioned. However, there is quite a number of plastic defenses, which are solely expressed on the physiological level. The adaptive immune system, that gnathostomata, us humans included, possess (Pancer & Cooper 2006) is a prime example of a physiological inducible defense. In general, immune reactions, like the production of cecropins in insects (Morishima *et al.* 1990; Lemaitre, Reichhart & Hoffmann 1997) and other antimicrobial peptids like defensins in insects (Hoffmann & Hetru 1992), mammals (Lehrer, Lichtenstein & Ganz 1993) and plants (Broekaert *et al.* 1995; van Loon, Rep & Pieterse 2006) fall into this category. Other plastic physiologic reactions include chemical defenses like the production of toxins. They can often be found in algae (Paul & Van Alstyne 1992) and vascular plants (Piubelli *et al.* 2003; van Loon *et al.* 2006; Howe & Jander 2008; Ballhorn *et al.* 2015) as response to herbivory. Similar defenses are also present in insects, such as aposematic hemipterans (Williams, Evans & Bowers 2001) and ladybird beetles (Holloway *et al.* 1991), whichsecrete substances in order to fend of insectivores and even vertebrates like toads change the production of toxins when exposed to predators (Benard & Fordyce 2003).

1.3 Conditions for inducible defenses

Harvell and Tollrian formulated four basic requirements that have to be met in order for inducible defenses to evolve and persist (Harvell & Tollrian 1999):

> *(I) A heterogeneous predation risk*

> *(II) An effective way to defend that reduces risks and costs exerted by predation pressure*

> *(III) Reliable information about the predation risk*

> *(IV) Costs imbued with the defenses that offset the benefit in times with no or low predation*

The first and most basic requirement addresses the heterogeneous predation risk, i.e. the already mentioned environmental fluctuations. The second point, the ability to form an effective defense poses the limit of this phenotypic plasticity. An inducible defense has to provide a significant benefit under predation compared to non-defended phenotypes. For example, the ability to form flexible defenses could be limited by developmental constraints (Hoverman & Relyea 2007). The third point addresses the importance for an organism to correctly assess the right conditions, in order to phenotypically match the environment. This can be achieved in many ways. Often predators themselves provide the needed cues for the prey, such as through direct contact or through chemical information the predator releases (Hanazato & Dodson 1992; Gool & Ringelberg 2002; Stibor 2002; Weber 2003; Schoeppner & Relyea 2005). Such chemical cues that provide a disadvantage to the sender (predators), but help the receiver (prey organisms), are termed kairomones (Brown Jr, Eisner & Whittaker 1970). They can be accompanied or substituted by alarm cues (Schoeppner & Relyea 2005; Laforsch, Beccara & Tollrian 2006; Richardson 2006; Meuthen, Baldauf & Thünken 2012), chemical information from prey organisms themselves, which is only indirectly caused by the predator. These signals can be either actively released by prey organisms upon contact with the predator (e.g. secretion of pheromones by hemipterans (Lockwood & Story 1987)) or passively

through injuries resulting from an attack, e.g. in fish, where damaged 'club cells' located in the epidermis, release cues alarming conspecifics (Chivers *et al.* 2007). Cues that only correlate with the predation risk are a third possibility. If predation pressure shows regular seasonal patterns, co-occurring events might provide the necessary trigger for inducible defenses, such as a temperature change or light (Havel & Dodson 1985; Gool & Ringelberg 1998; Yurista 2000). The last requirement - costs - is essential for a defense to evolve or stay *plastic*. Inducible defenses have to incur costs, that are lower than the benefit under predation. Without (or under low) predation, these costs would negatively affect the organism's fitness and thus, by not expressing the defense, these costs can be saved. Without this trade-off, constitutive defenses would be expected. However, these costs are not to be misinterpreted as 'costs of plasticity' (Dewitt *et al.* 1998; Relyea 2002; Auld *et al.* 2010). This term refers to costs for the ability to flexibly react to the environment and not for costs that an organism experiences during the actual expression of the flexible response (e.g. a higher metabolism). Hence, 'costs of plasticity' decrease fitness of an induced phenotype compared to a permanently defended phenotype under predation (Pigliucci 2005). On the other hand, maladaptation costs of a defense under low or no predation increases fitness of an inducible, but non-induced organism compared to a permanently defended one (Gabriel *et al.* 2005). Therefore, 'costs of plasticity' hamper inducible defenses, whereas maladaptation costs of defenses promote them. However, when a predator disappears or changes, the same maladaptation costs can lead to an even further step of phenotypic plasticity:

1.4 Reversibility of inducible defenses

For the last five decades, since inducible defenses were defined, thousands of studies of inducible defenses accumulated. However, in comparison only a few experimental studies address reversibility (Brönmark & Pettersson 1994; Relyea 2003; Mikulski, Czernik & Pijanowska 2005; Kishida & Nishimura 2006; Hoverman & Relyea 2007; Orizaola, Dahl & Laurila 2011; Miner *et al.* 2013). For that reason, theoretical models still give the best explanations, when and under which conditions reversibility can be expected to occur (Gabriel 1999; Gabriel *et al.* 2005; Fischer *et al.* 2014; Utz *et al.* 2014). In contrast to the rare description of reversibility, theoretical models suggest the phenomenon to be common (Utz *et al.* 2014). In principle, the evolution of reversible inducible defenses follows the same four basic conditions as all inducible defenses. The main distinction lies in the nature of the maladaptation costs. Reversibility becomes evolutionary favorable, when an already induced phenotype possesses a decreased fitness under no or low predation, compared to non-defended phenotypes. The same is the case, when predators change and the inducible defense makes the prey more susceptible to the new predator. This effect is known as 'survival trade-off' (Benard 2006; Hoverman & Relyea 2009). In short: costs that an organism has to pay only once for the establishment of a defense results in a persistent defense, whereas running costs promote reversibility. Apart from the costs, the speed at which the phenotype can react in relation to the frequency the environment changes decide, whether reversibility is favorable or not. A long 'gap phase' and frequent changes in the predator regime inhibit reversibility and favor intermediate, generalist responses (Gabriel *et al.* 2005). Therefore, behavioral and physiological responses, which are known to change fast, are considered to be likely reversible, while the often slowly changing morphological defenses are considered to be less so (Gabriel *et al.* 2005; Utz *et al.* 2014). Furthermore, there is a higher chance for additional limits of plasticity, which can be caused by developmental windows (Hoverman & Relyea 2007; Fischer *et al.* 2014). They describe the timeframe within the ontogeny in which a specific phenotype can be expressed (Hoverman & Relyea 2007). Since many defenses themselves are only expressed within a limited phase (Relyea 2004; Kishida & Nishimura 2005; Riessen & Trevett-Smith 2009), the developmental window for

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the reversion becomes even narrower. Also, a delay in the first reproduction cannot be reversed after the event happened, only shortened beforehand. In consequence, reversibility is not only limited by the reaction speed of the defensive trait, but also by its continuance.

1.5 Daphnia – a group of model organisms

Within the large number of organisms, for which inducible defenses have been observed and described, *Daphnia* holds a special importance. Their wide distribution, large abundance and importance as key-stone species in freshwater ecosystems have made *Daphnia* a group of model organism in limnologic research (Lampert 2006; Miner *et al.* 2012). Other contributing factors were the easy cultivation within the laboratory and the short generation time of about one to two weeks, with adults reproducing approximately every three days. The fact that daphnids predominantly reproduce through parthenogenesis allows furthermore easy experimental set-ups for a separation of genetic and environmental influences. The widespread laboratory use of *Daphnia* has spread to eco-toxicological research (e.g. (Baird *et al.* 1991; Mirza, Pyle & Bay 2009; Kim *et al.* 2012)) and testing (OECD 1984, 2012). This and the publication of the eco-responsive genome of *Daphnia pulex* (Colbourne *et al.* 2011) even led to the recognition as model organisms for bio-medical research (NIH). Apart from the recent rise of attention, for more than a century, the genus *Daphnia* has been known for a phenomenon termed 'cyclomorphosis' (Wesenberg-Lund 1900; Coker & Addlestone 1938). These seasonal changes in the morphology were long known and have been observed in many species, especially those of the sub-genus *Hyalodaphnia*, like the lake-dwelling *D. cucullata* (Coker 1939; Brooks 1965), *D. retrocurva* (Brooks 1946) *D. cristata* (Coker 1939; Brooks 1965) and *D. galeata* (Brooks 1946), which usually exhibit helmets and elongated tail-spines during summer. The ultimate and proximate causes were widely speculated and originally thought to be related to the temperature changes and the resulting effect on the water viscosity (Woltereck 1913; Coker & Addlestone 1938). This view changed in 1974, when Dodson introduced his 'anti-lock and key' hypothesis and concluded that cyclomorphosis was actually a plastic response to predation (Dodson 1974). Numerous studies that followed confirmed this relationship and revealed additional inducible defenses in an ever-growing number of species, including daphnids from the *Ctenodaphnia* sub-genus (Barry 1998; Petrusek *et al.* 2009; Rabus & Laforsch 2011; Rabus *et al.* 2011) and *D. pulex*-group (Black 1993; Tollrian 1993; Juračka, Laforsch & Petrusek 2011). Despite the prevalent seasonal pattern of these phenotypic shifts, kairomones (Hanazato & Dodson 1992; Barry 2000; Lass & Spaak 2003) and alarm cues (Stabell, Ogbebo & Primicerio 2003; Laforsch *et al.* 2006) instead of temperature or light intensity have been identified as direct proximate causes for the induction. Kairomones often result in a specific defense against the emitting predator (Hebert 1978; Barry 1998; Rabus & Laforsch 2011), with alarm cues amplifying the response (Laforsch *et al.* 2006). Especially morphologic defenses are often only caused by particular predators and thus seem to be specialized adaptations, although exceptions exist (Laforsch & Tollrian 2004b) and most studies focus only on the exposition to one predator or one at a time.

The increased attention to inducible defenses in *Daphnia* led to a large list of morphological traits, such as helmets (Laforsch & Tollrian 2004a), large tail-spines (Kolar & Wahl 1998; Rabus & Laforsch 2011), crests (Barry 2000), neckteeth (Tollrian 1993; Juračka *et al.* 2011), fortifications of the carapace (Dodson 1984; Laforsch *et al.* 2004; Rabus *et al.* 2013) and a crown of thorns (Petrusek *et al.* 2009). With the exception of *D. lumholtzi*, which produces a spine-like helmet and an elongated tail-spine as response to fish predators (Kolar & Wahl 1998; Engel, Schreder & Tollrian 2014), all of these defenses are built against invertebrate predators, especially against tadpole shrimps (Petrusek *et al.* 2009; Rabus & Laforsch 2011; Rabus *et al.* 2011), backswimmers (Hebert

1978; Barry 1998) and *Chaoborus*-larvae (Hanazato & Dodson 1992; Tollrian 1993; Riessen & Trevett-Smith 2009; Weiss *et al.* 2012). Apart from morphologic traits, behavioral changes were also identified as predator related defenses. Examples include diel vertical migration to deeper and darker water layers, reducing predation risk by visually hunting fish (Lampert 1989; Pijanowska, Weider & Lampert 1993; Brewer, Dawidowicz & Dodson 1999) and reverse diel vertical migration as means to avoid *Chaoborus*-larvae hunting in deeper water layers (Nesbitt, Riessen & Ramcharan 1996; Boeing, Ramcharan & Riessen 2006). Increased alertness furthermore allows for faster reactions to predatory attacks (Pijanowska *et al.* 1993; De Meester & Pijanowska 1996). Even life-history shifts in age and size of first reproduction, clutch size and neonate size were found as anti-predator responses (Lüning 1992; Riessen 1999; Riessen & Trevett-Smith 2009). Moreover, a common defense mechanism seems to be a generally larger body size against invertebrate predators, which are considered to be gape-limited and a smaller body size against visually hunting vertebrate predators.

Despite the extensive research on inducible defenses in *Daphnia*, numerous questions remain. Among them is the matter, whether or not defenses once induced can also be reverted. With the exception of one study showing the reversibility of clutch size changes as a life-history defense in *D. magna* in relation to fish predation (Mikulski *et al.* 2005), another study broaching the reversibility of neckteeth in *D. pulex* (Vuorinen I., Ketola M. & Walls M. 1989)and the inherently reversible diel vertical migration (e.g. Beklioglu *et al.* 2008), the topic has been largely neglected.

1.6 Aims of this thesis

So far, a large gap of knowledge exists in regards to the reversibility of inducible morphologic defenses in the genus *Daphnia*. Without the identification of reversibility or persistence of inducible defenses, an assessment of the ultimate and proximate causes is even beyond speculation. The same applies for the implications this holds for predator-prey interactions. For these reason, my basic aim within this thesis was to test morphologic defenses for their ability to reverse. However, in addition I wanted to lay out a foundation for an assessment of the influence of several factors on the reversibility or irreversibility of induced defenses. This included the search for running costs of induced defenses and the distinction between physiological limitations and ecological reasons. In order to do so, I followed two approaches. One was to establish a new system with a species, that features numerous morphologic traits, that are easy to measure and respond differently to distinct predators and to use this system to differentiate between physiological and ecological reasons behind reversibility or irreversibility. The second was to use the already established predator-prey system (Rabus & Laforsch 2011; Rabus *et al.* 2011, 2013; Otte *et al.* 2014) of the predator *Triops cancriformis* together with *D. magna*, one of the most commonly used daphnid species in laboratory research, to test for possible costs and reversibility of morphologic traits.

In detail, my research included the following:

1.   *Establishment of a new dual-predator-prey system*. In most natural systems, prey is confronted with multiple predators, either one at a time or in succession. Despite this fact, research on inducible defenses in daphnia has mostly concentrated on single predators. I described the inducible defenses of a previously disregarded species, *D. barbata* in relation to two different predators in order to introduce a new model system (chapter II and III). Moreover, I developed a framework to describe the influence selection pressure caused by different predators can have on the development of inducible defenses (chapter II).

2.   *Identification of reversibility of inducible defenses.* So far, only one study has tested for the reversibility of an induced morphologic defenses in juvenile *Daphnia* (neckteeth in *D. pulex* (Vuorinen I. *et al.* 1989)). For the reversibility of all other morphological traits and the morphologic plasticity of adults no published data exists so far. Chapter IV presents the first data on the reversibility and irreversibility of numerous morphologic traits in adult *D. barbata*. Furthermore, using the established system of *D. magna-T. cancriformis* as reference point, a switch of adult *Triops*-induced daphnids in control conditions and an exposition of the respective non-induced daphnids to *Triops*-cues were conducted. In the following, the development of morphology and life-history were observed and checked for reversibility (chapter V).

3.   *Assessing the importance of ecological and physiological factors for reversibility in Daphnia.* Reversibility or irreversibility of inducible defenses is influenced by a number factors. Even by comparing the responses of closely related prey-species many variables change and impede an assessment of the underlying causes for similarity and dissimilarity in reversibility. Therefore, *D. barbata* was used to study reversibility in relation to the inducing predator and the induced morphologic structure (chapter IV). Such a 'single-prey-species' approach should lead to a more direct and accurate estimation and differentiation of the influence of physiological and ecological factors.

4.   *Identification of costs.* A basic condition for reversibility are running costs, that could be saved when predators disappear or change by reverting the defense. A possible source of such running costs could be a decrease in swimming efficiency. Studies have shown a causal relation between temperature and cyclomorphosis (Havel & Dodson 1985; Yurista 2000; Schalau *et al.* 2008). While it is now known that these morphological changes are not an adaptation to water viscosity, this could hint for running costs of inducible defenses that limit them (more) at lower temperature. In order to assess these potential costs, experiments were conducted that focused on the sinking rates of *D. magna* and a possible impairment of swimming behavior in relation to an induction with *T. cancriformis* (chapter VI).

Modality matters for the expression of inducible defenses: Introducing a concept of predator modality

CHAPTER

II

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Modality matters for the expression of inducible defenses: introducing a concept of predator modality

Herzog and Laforsch

BMC Biology

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Modality matters for the expression of inducible defenses: introducing a concept of predator modality

Quirin Herzog[1*] and Christian Laforsch[2*]

Abstract

Background: Inducible defenses are a common and widespread form of phenotypic plasticity. A fundamental factor driving their evolution is an unpredictable and heterogeneous predation pressure. This heterogeneity is often used synonymously to quantitative changes in predation risk, depending on the abundance and impact of predators. However, differences in 'modality', that is, the qualitative aspect of natural selection caused by predators, can also cause heterogeneity. For instance, predators of the small planktonic crustacean *Daphnia* have been divided into two functional groups of predators: vertebrates and invertebrates. Predators of both groups are known to cause different defenses, yet predators of the same group are considered to cause similar responses. In our study we question that thought and address the issue of how multiple predators affect the expression and evolution of inducible defenses.

Results: We exposed *D. barbata* to chemical cues released by *Triops cancriformis* and *Notonecta glauca*, respectively. We found for the first time that two invertebrate predators induce different shapes of the same morphological defensive traits in *Daphnia*, rather than showing gradual or opposing reaction norms. Additionally, we investigated the adaptive value of those defenses in direct predation trials, pairing each morphotype (non-induced, *Triops*-induced, *Notonecta*-induced) against the other two and exposed them to one of the two predators. Interestingly, against *Triops*, both induced morphotypes offered equal protection. To explain this paradox we introduce a 'concept of modality' in multipredator regimes. Our concept categorizes two-predator-prey systems into three major groups (functionally equivalent, functionally inverse and functionally diverse). Furthermore, the concept includes optimal responses and costs of maladaptions of prey phenotypes in environments where both predators co-occur or where they alternate.

Conclusion: With *D. barbata*, we introduce a new multipredator-prey system with a wide array of morphological inducible defenses. Based on a 'concept of modality', we give possible explanations how evolution can favor specialized defenses over a general defense. Additionally, our concept not only helps to classify different multipredator-systems, but also stresses the significance of costs of phenotype-environment mismatching in addition to classic 'costs of plasticity'. With that, we suggest that 'modality' matters as an important factor in understanding and explaining the evolution of inducible defenses.

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Background

Predation is a strong selective force which drives evolution of prey defenses. Due to its variable nature, it is known to cause adaptations in the form of plastic responses in phenotypes, termed inducible defenses. Since they were first described [1] extensive research has revealed that this phenomenon is extremely widespread in many taxa, including bacteria [2], plants [3-5], invertebrates [6] and vertebrates [7,8]. For inducible defenses to evolve, four prerequisites have to be met: (I) the ability to form effective defenses, (II) associated costs that can offset the benefit in times with no or low predation, depending on the environmental conditions, (III) reliable cues to assess the current state of predation and (IV) heterogeneity of predation impact [9]. To date heterogeneity has often been used synonymously with variation in predation intensity (that is, the quantity of prey consumed or density of predators), caused by the presence or absence of predators (for example, by seasonal patterns [10]). However, it is not only relevant how much prey is eaten. It is also of importance which predator consumes the prey. It is known that different predators often pose different threats to their prey [11] and that predators can change their impact throughout their own [12] or their prey's ontogeny [13]. Thus, the specific modality (that is, the qualitative aspect of natural selection caused by predation) also plays an important role. Modality describes where natural selection is leading in terms of direction and magnitude. Differences in this modality can result from a variety of entangled ecological factors, such as prey-preference, feeding mechanism, predation strategy, habitat use, dangerousness and the mode of perception of the predator [14]. In contrast to predation intensity, measuring, characterizing and comparing modality is difficult, even more so without clear categories and definitions. Additionally, variation in intensity and modality are non-exclusive changes, which can occur both on a spatial and a temporal scale, further complicating an assessment. Since most studies concentrate on single predator systems, modality differences have been largely neglected. However, as Sih *et al.* [15] pointed out, almost all prey organisms have to face multiple predators. Under these circumstances, modality matters. Indeed, many studies on amphibians [7,8,16,17], mollusks [11,18-20], insects [21], rotifers [22,23] and crustaceans [6,24] have demonstrated predator-specific responses, emphasizing the importance of modality.

Daphnia, a group of model organisms in ecology, evolution and biomedical research [25,26], provide a classical example for the role of modality. The predators they are facing are commonly categorized as invertebrate and vertebrate predators [27]. While vertebrate predators are considered to be primarily visual hunters and prefer larger prey, invertebrates are generally regarded as size-limited and mostly tactile predators. Corresponding to these different modalities, the well-known responses of daphnids exposed to fish are to reproduce earlier at a smaller size [28,29], to release more but smaller offspring [28] and to migrate into darker and deeper water layers during the day [30,31]. In contrast, when encountering invertebrate predators, such as *Chaoborus* larvae, daphnids mature later at larger size and produce fewer but larger offspring [28,29,32]. These above mentioned changes are, however, restricted to life history and behavioral defenses, with especially the latter considered to adapt fast and reversibly [33,34]. Yet, more prominent features of the genus *Daphnia* are numerous plastic morphological responses, such as helmets [35], crests [36], neckteeth [37,38], elongated tail-spines [13,39] and a crown of thorns [40]. Except in one species (*Daphnia lumholtzi* [39]), these defenses are solely built against invertebrate predators. While in one case they indeed have been shown to be caused by and act against multiple invertebrate predators [41], in most cases they seem to be predator specific [36,37,39,40,42]. Although this clearly questions the grouping of 'invertebrate predators' together as a single functional group, the potential differences in their modality have not been the focus of research so far.

In this context, we investigated if differences in the modality of invertebrate predators are relevant for the expression of inducible defenses. We used two contrasting predators with distinct differences in their morphology and ecology (that is, predation strategy): *Triops cancriformis* (Notostraca) and *Notonecta glauca* (Hemiptera). In addition, both predators are known to induce morphological defenses in *Daphnia* [13,36,40,43]. As the prey organism, we used a clone of *Daphnia barbata*, an African pond and lake dwelling species [44], which shares distribution and habitats with predators of both genera [45-47]. As a first step, we exposed *D. barbata* to the chemical cues released from both predators separately and analyzed morphological responses among all experimental groups. As a second step, we used direct predation trials to assess the adaptive value of each morphotype. We show that two invertebrate predators can induce different morphological defensive traits in *D. barbata*, which are based on the same structures, but built in different shapes. This is not only the first record of inducible defenses in *D. barbata*, but a unique case of defensive specialization across a wide range of taxa. Surprisingly, the defense against one predator also offered protection against the other predator, in one case even matching the specialized defense. To explain why the prey shows nonetheless not one general but two distinctively defended morphotypes, a theoretical framework is needed. Therefore, we introduce a 'concept of modality', which categorizes multipredator-prey systems into three major groups (functionally equivalent, functionally inverse and functionally diverse) and describes optimal responses in environments where predators co-occur

or alternate. This concept is in line with the existing literature, but provides a general framework. It offers an explanation for the evolution of the different induced morphotypes of *D. barbata*, generates a basis to assess and compare the importance of modality in different multi-predator-prey systems and emphasizes the importance of a differentiation between predator co-occurrence and predator succession.

Results

Morphological parameters

Significant changes in the morphology of *D. barbata* (Figure 1) between the treatments and within all measured parameters were observed (Kruskal-Wallis one-way analysis of variance, all *P* <0.001). Relative helmet length was significantly different in all three treatments (all pair wise comparisons *P* ≤0.001; Table 1). The control (non-predator exposed) daphnids had the smallest helmets. Larger helmets were found in the *Triops*-induced treatment and the longest helmets overall were from *Notonecta*-exposed daphnids (Table 1, Figure 1). The shape of the helmet varied as well. Daphnids exposed to *T. cancriformis* built a backwards bending helmet which differs significantly in its angle relative to the body axis from both the control (*P* <0.001; Table 1) and *Notonecta*-induced daphnids (*P* <0.001).

Furthermore, the length of the tail-spine increased significantly with exposure to *Notonecta* as compared to both the control (*P* <0.001; Table 1) and *Triops*-induced daphnids (*P* <0.001; Table 1). *D. barbata* exposed to *Triops* did not increase tail-spine length compared to the control, but the morphology of the tail-spine was altered.

Specifically, the tail-spine was bent backwards (lower spine angle) and had significantly more curvature as compared to the two other treatments (*P* <0.001; Table 1).

Triops-induced *D. barbata* showed an increase in microspine density at the cranial dorsal ridge (distance between 1st and 10th microspine; Table 1), a widening of the dorsal ridge, longer microspines and a sideways orientation of the 5th microspine (all *P* <0.001 compared to control; Table 1). *D. barbata* exposed to chemical cues released by *Notonecta* on the other hand showed a much smaller decrease in the distance between 1st and 10th microspine (*P* = 0.001; Table 1) and no changes in the dorsal ridge width (*P* = 1; Table 1). Additionally, they possessed longer microspines than *Triops*-induced daphnids (*P* = 0.043; Table 1) and compared to the control showed only a minor increase in the angle of the fifth microspine relative to the dorsal ridge (*P* <0.001; Table 1).

Predation trials

Predation trials using *Notonecta* revealed that the *Notonecta*-induced morphotype is better protected, having an 80% higher survivorship compared to the control (Wilcoxon signed-rank test, *P* = 0.012, Figure 2). The *Triops*-induced morphotype also held an advantage, having a 52% higher survivorship compared to the control (Wilcoxon signed-rank test, *P* = 0.028). However, the defenses proved to be less effective against notonectids in direct comparison with the *Notonecta*-induced morphotype (Wilcoxon signed-rank test, *P* = 0.017). In contrast, when *T. cancriformis* was the predator, both morphs showed higher survival rates compared to the control
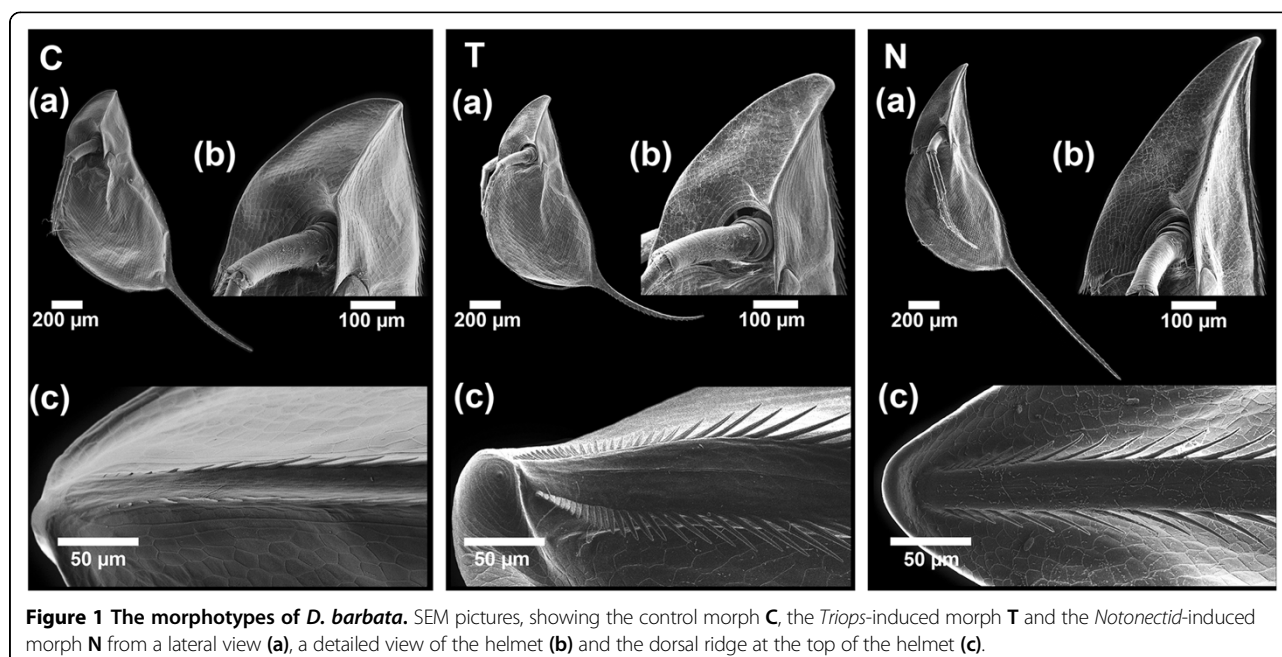
Figure 1 The morphotypes of *D. barbata*. SEM pictures, showing the control morph **C**, the *Triops*-induced morph **T** and the *Notonectid*-induced morph **N** from a lateral view **(a)**, a detailed view of the helmet **(b)** and the dorsal ridge at the top of the helmet **(c)**.

Table 1 Measured morphological parameters

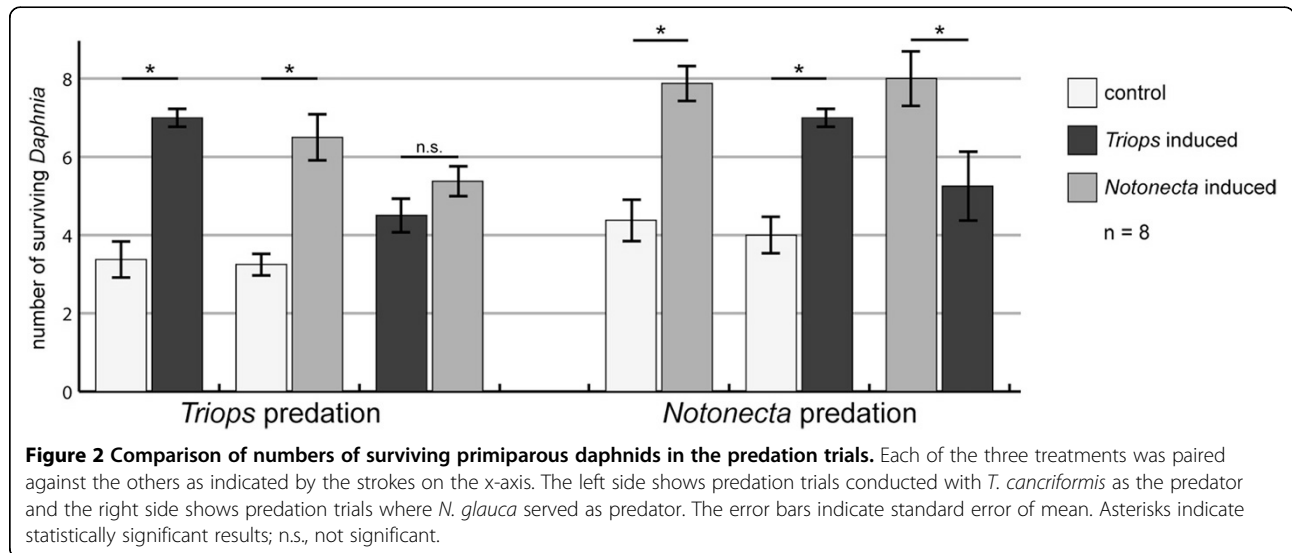| Parameters	Group	Mean	SEM	Kruskal-Wallis main test		H	*P*-value
					Kruskal-Wallis pairwise comparison		
Helmet							
Relative helmet length (helmet length/body length)	**C** (n = 17)	0.260	0.004	df = 2	**C - N**	−37.765	<0.001
	N (n = 17)	0.384	0.008	H = 47.235	**C - T**	−19.190	0.001
	T (n = 21)	0.322	0.004	*P* = 0.001	**N - T**	18.574	0.001
Helmet angle [°]	**C** (n = 17)	110.320	0.897	df = 2	**C - N**	−11.706	0.099
	N (n = 17)	115.463	0.955	H = 38.662	**C - T**	20.106	<0.001
	T (n = 21)	103.573	0.559	*P* = 0.001	**N - T**	31.812	<0.001
Tail-spine							
Relative tail-spine length (tail-spine length/body length)	**C** (n = 17)	0.583	0.014	df = 2	**C - N**	−26.529	<0.001
	N (n = 17)	0.859	0.019	H = 34.720	**C - T**	1.756	1
	T (n = 21)	0.581	0.008	*P* = 0.001	**N - T**	28.286	<0.001
Spine angle (°)	**C** (n = 17)	160.518	1.264	df = 2	**C - N**	−3.471	1
	N (n = 17)	162.494	0.811	H = 38.222	**C - T**	25.61	<0.001
	T (n = 21)	143.596	0.844	*P* = 0.001	**N - T**	29.081	<0.001
Curvature (absolute/effective spine length)	**C** (n = 17)	1.005	0.001	df = 2	**C - N**	5.529	0.943
	N (n = 17)	1.003	0.000	H = 34.493	**C - T**	−22.964	<0.001
	T (n = 21)	1.018	0.001	*P* = 0.001	**N - T**	−28.493	<0.001
Dorsal ridge							
Dorsal ridge width (µm)	**C** (n = 17)	30.391	0.554	df = 2	**C - N**	0.294	1
	N (n = 17)	29.857	0.676	H = 37.094	**C - T**	−26.853	<0.001
	T (n = 21)	50.289	0.905	*P* = 0.001	**N - T**	−27.147	<0.001
Dist. 1. to 10. microspine (µm)	**C** (n = 17)	192.558	3.070	df = 2	**C - N**	17.000	0.005
	N (n = 17)	134.432	3.981	H = 47.016	**C - T**	35.500	<0.001
	T (n = 21)	47.235	1.293	*P* = 0.001	**N - T**	18.500	0.001
Max. microspine length (µm)	**C** (n = 17)	39.181	1.690	df = 2	**C - N**	−33.706	<0.001
	N (n = 17)	61.260	1.283	H = 39.885	**C - T**	−20.982	<0.001
	T (n = 21)	54.249	1.004	*P* = 0.001	**N - T**	12.724	0.043
Microspine angle (°)	**C** (n = 17)	19.533	0.906	df = 2	**C - N**	−13.941	0.029
	N (n = 17)	28.490	1.243	H = 43.776	**C - T**	−33.971	<0.001
	T (n = 21)	78.571	1.307	*P* = 0.001	**N - T**	−20.029	<0.001

C, non-induced daphnids (control); N, *Notonecta*-induced daphnids; T, *Triops*-induced daphnids; SEM, standard error of mean; H, test statistics.

(107% increase for the *Triops*-induced morphotype, Wilcoxon signed-rank test, *P* = 0.017; 100% increase for the *Notonecta*-induced morphotype, Wilcoxon signed-rank test, *P* = 0.018). Between the two induced morphs, no significant differences in the number of surviving *Daphnia* were found (*P* = 0.230).

Discussion

Our findings are the first records of inducible defenses in *D. barbata*. Furthermore, we show that *D. barbata* responds to two different invertebrate predators (*Notonecta* and *Triops*) with distinctive morphological responses, rather than displaying a general defense. Unlike in previous records of predator-specific morphological responses across wide taxonomical groups, they consist of neither a gradual extension of the same trait (that is, an intermediate response against one predator and a stronger response against the other predator for example, [24,36]), nor of opposing traits (that is, when a trait increases against one predator and decreases against the other predator compared to the non-induced morph for example, [11,48,49]) or the addition of a new trait (for example, a high-tail against one predator and a high tail and a bulgy head against another [7]). Instead, the defenses are based on the same structures, but formed in a different way. This makes it impossible to order the morphotypes of *D. barbata* by

Figure 2 Comparison of numbers of surviving primiparous daphnids in the predation trials. Each of the three treatments was paired against the others as indicated by the strokes on the x-axis. The left side shows predation trials conducted with *T. cancriformis* as the predator and the right side shows predation trials where *N. glauca* served as predator. The error bars indicate standard error of mean. Asterisks indicate statistically significant results; n.s., not significant.
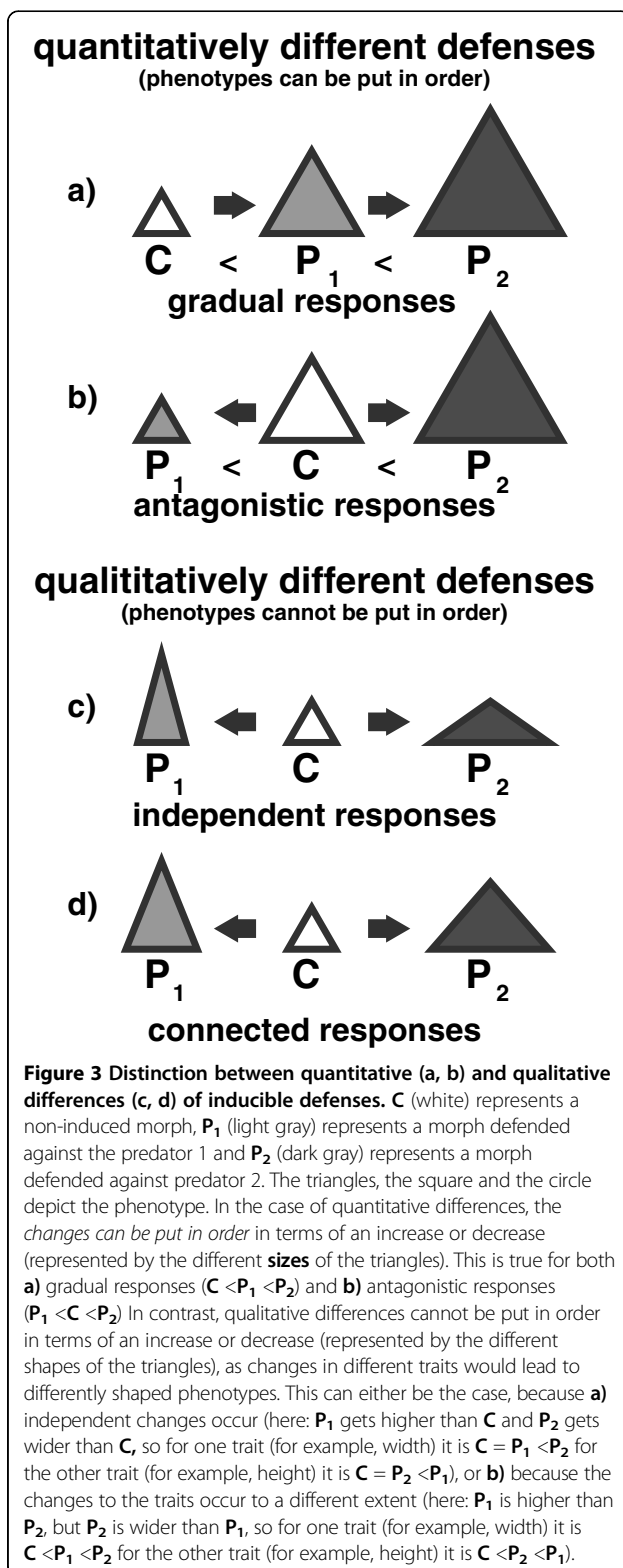
the magnitude of expression of their traits (that is, quantitative differences, see Figure 3). Rather, the differences represent distinctive shapes, providing a rare example of qualitative predator specific defenses (see Figure 3, in accordance with Bourdeau [20]).

Regarding the adaptive value of these differing traits, the morphs exposed to chemical cues released by *Triops* had a clear disadvantage under predation by *Notonecta* compared to the morphs exposed to *Notonecta* cues. Still, compared to non-induced daphnids, they showed a limited defensive value. Surprisingly, both defended morphotypes performed equally well against *T. cancriformis*. At first glance, it seems contradictory that a mismatching defense works just as good as the specific adaptation. Even so, as two distinctive morphotypes have evolved instead of a single general defense, either the benefits or the costs (or both) have to differ in favor of the specific defense. Although the predation trials showed no direct benefits (increased survivorship), indirect benefits might exist. Such could be an increase in handling time or in predator mortality (the saw-like orientated microspines along the dorsal ridge may be able to cause injuries within *Triops'* food groove). Differences in costs are more difficult to assess, as they are often manifold [50] and depend on both abiotic and biotic factors. As such, they differ in multi-predator environments from single predator environments [48]. Depending on whether predators co-occur or occur subsequently, the costs may change even further. Therefore, it is insufficient to assess the costs of defenses by simple comparisons of predator-exposed and non-predator-exposed individuals. Predator-related environmental costs, like 'survival trade-offs' [48,49], can possibly surpass 'costs of plasticity' (that is, the costs for the ability to be plastic, for a review see [51]) by far. Costs may also be reduced under certain circumstances; for instance,

when a defense against one predator simultaneously offers protection against another predator (as here in the case of *D. barbata*). Consequently, it is crucial to understand the modalities of the predators in a given system to evaluate the costs of inducible defenses. To this end, it is helpful to visualize modality as an Euclidean vector, showing both the direction and limit of natural selection caused by a predator. Based on that, we developed a novel concept on the influence of modality in multi-predator regimes (Figure 4). In a system with one prey and two predators, three different scenarios are possible: The predators can be *functionally equivalent* (type I, Figure 4), with both vectors pointing in the same direction, *functionally inverse* (type II), with both vectors pointing in opposite directions or *functionally diverse* (type III), with both vectors pointing in different directions. Depending on the conditions, predator-specific inducible defenses can be found within each of the three categories.
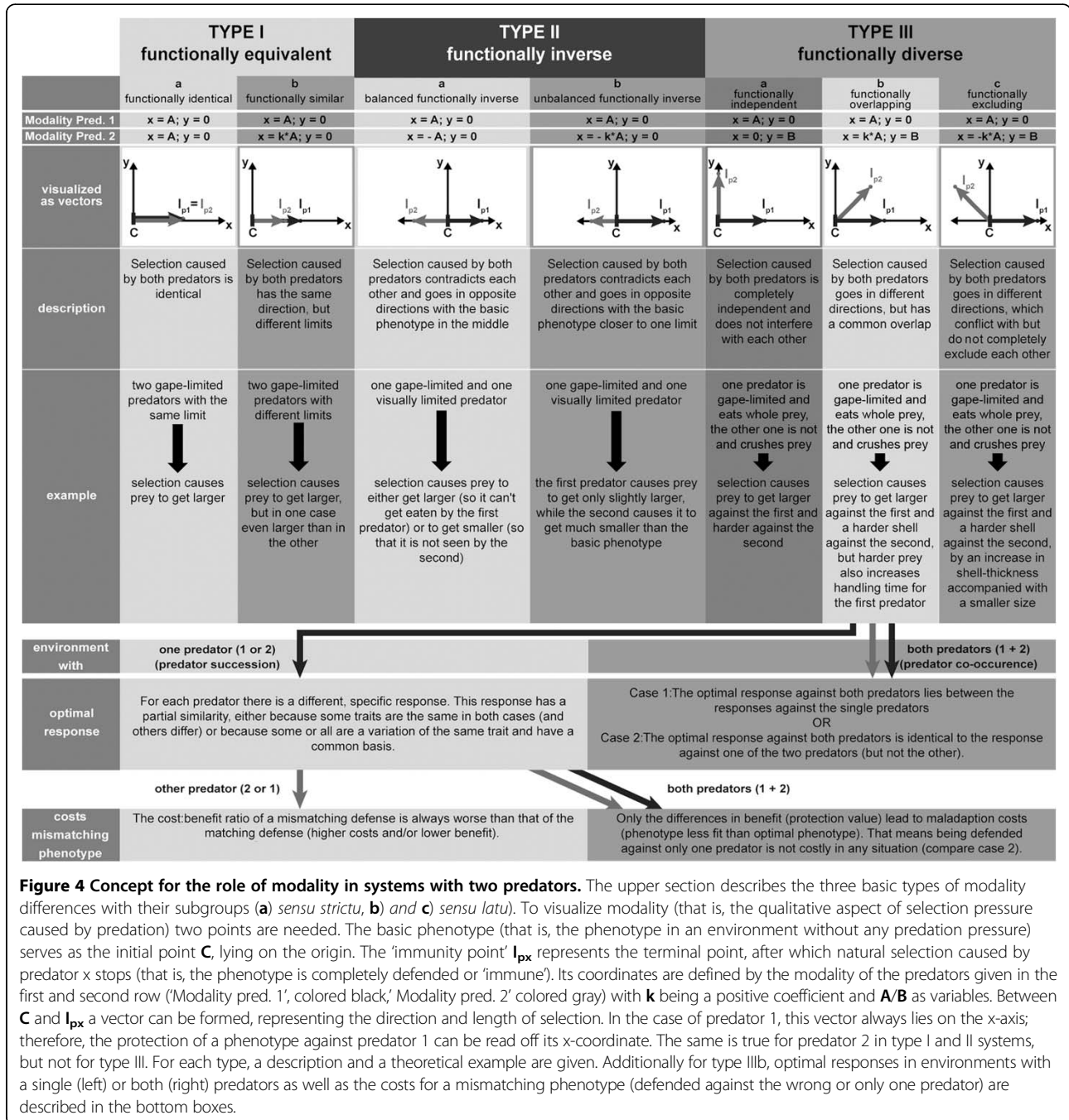
Previous reports of predator specific-defenses cover either type I [3,24,36,41] or type II [48,49,52,53] but rarely type III [7,20]. Yet, systems with two predators should be most realistically described with two dimensions (type III, Figure 4). In this case, the x-axis shows phenotypic characteristics relevant for the risk caused by the first predator, while changes in the y-axis only influence the predation risk from the second. A reason for the predominance of types I and II may be a simplification by observation, which can happen if only one or a small number of related traits are observed. Then it is likely that a second predator causes selection to go in the same or the opposite direction (type I and II, respectively, Figure 4). Vice versa, with more observed traits, the chance increases to find changes relevant to one predator only (y-axis, type III, see Figure 4). Additionally, natural selection can also lead to a simplification when

quantitatively different defenses
(phenotypes can be put in order)

a)

C < P₁ < P₂

gradual responses

b)

P₁ < C < P₂

antagonistic responses

qualititatively different defenses
(phenotypes cannot be put in order)

c)

P₁   C   P₂

independent responses

d)

P₁   C   P₂

connected responses

Figure 3 Distinction between quantitative (a, b) and qualitative differences (c, d) of inducible defenses. C (white) represents a non-induced morph, **P₁** (light gray) represents a morph defended against the predator 1 and **P₂** (dark gray) represents a morph defended against predator 2. The triangles, the square and the circle depict the phenotype. In the case of quantitative differences, the *changes can be put in order* in terms of an increase or decrease (represented by the different **sizes** of the triangles). This is true for both **a)** gradual responses (**C** <**P₁** <**P₂**) and **b)** antagonistic responses (**P₁** <**C** <**P₂**) In contrast, qualitative differences cannot be put in order in terms of an increase or decrease (represented by the different shapes of the triangles), as changes in different traits would lead to differently shaped phenotypes. This can either be the case, because **a)** independent changes occur (here: **P₁** gets higher than **C** and **P₂** gets wider than **C,** so for one trait (for example, width) it is **C** = **P₁** <**P₂** for the other trait (for example, height) it is **C** = **P₂** <**P₁**), or **b)** because the changes to the traits occur to a different extent (here: **P₁** is higher than **P₂**, but **P₂** is wider than **P₁**, so for one trait (for example, width) it is **C** <**P₁** <**P₂** for the other trait (for example, height) it is **C** <**P₂** <**P₁**).

fitness trade-offs do not exist and predators always co-occur. Selection should then favor that type IIIa and b systems convert into type Ib, and thus display only one general defense (compare also case 2 for type IIIb,

Figure 4). The same should happen if the cost of differentiating between predators is higher than the benefit of a predator-specific response. Since *D. barbata* does not display a general defense, acting against both predators, this suggests that *Triops* and *Notonecta* have a different spatial or temporal occurrence. *D. barbata* is known to inhabit both small temporary ponds and larger lakes in Africa [44,54] as does *Notonecta* [45,55,56], while *Triops* lives in temporary freshwaters as pioneer species [46,57]. Therefore, habitats might exist with only one of these two predators. An alternative explanation is that the different plastic defenses are an adaptation to a common succession pattern. When a dry pond gets filled with water, daphnids and *Triops* hatch from resting eggs. Thus, while there is an immediate threat caused by *Triops*, *Notonecta* have to migrate to the pond [55] and lay their eggs. Adult *Notonecta* occur in smaller numbers, have a reduced feeding rate (1/8 to 1/4 of earlier instars), consume more surface prey than juveniles [58,59] and, therefore, exert less predation impact on pelagic organisms such as *Daphnia*. As soon as juvenile *Notonecta* hatch they are in high numbers and represent an immediate and strong threat to *D. barbata*. By then, the daphnids should already possess their defenses (from reacting to the chemical cues of the adult notonectids), being now well adapted.

Further experiments are needed to analyze the response of *D. barbata* exposed to both predators simultaneously. Previous studies (for a review see [60]) showed that responses to two different predators usually result in an intermediate response or a response identical to the 'more risky' predator. However, it is just as important to acquire field data about the predator-regimes that *D. barbata* faces. Especially, as it is a condition for the two specialized defenses to evolve that the composition of the predator-regimes changes. For that predator succession seems to be the most plausible explanation. That predator succession influences the expression of inducible defenses is already known for frogs [61], but not for any daphnid species so far. The importance of predator succession might even apply to many other prey organisms as well, not only in temporary habitats, but also due to seasonal changes in temperate zones. According to our concept (see details for case IIIb, Figure 4 and Additional file 1: Figure S1), these frequently changing environments would allow for the persistence of type III systems. However, even then it is a basic condition for type III, that the predators show qualitative differences in their selection pressure. If the predators belong to different main types (true predators, grazers, parasites, parasitoids [62]) these differences might be more likely, but this is not the case for *Triops* and *Notonecta*. Thus, whether or not predators exert different selection pressures on their prey can only be answered by looking directly and in detail at the species in question.

	TYPE I functionally equivalent		TYPE II functionally inverse		TYPE III functionally diverse		
	a functionally identical	b functionally similar	a balanced functionally inverse	b unbalanced functionally inverse	a functionally independent	b functionally overlapping	c functionally excluding
Modality Pred. 1	x = A; y = 0	x = A; y = 0	x = A; y = 0	x = A; y = 0	x = A; y = 0	x = A; y = 0	x = A; y = 0
Modality Pred. 2	x = A; y = 0	x = k*A; y = 0	x = - A; y = 0	x = - k*A; y = 0	x = 0; y = B	x = k*A; y = B	x = -k*A; y = B
visualized as vectors	(vector diagram $I_{p1}=I_{p2}$)	(vector diagram I_{p2} I_{p1})	(vector diagram I_{p2} I_{p1})	(vector diagram I_{p2} I_{p1})	(vector diagram I_{p2} I_{p1})	(vector diagram I_{p2} I_{p1})	(vector diagram I_{p2} I_{p1})
description	Selection caused by both predators is identical	Selection caused by both predators has the same direction, but different limits	Selection caused by both predators contradicts each other and goes in opposite directions with the basic phenotype in the middle	Selection caused by both predators contradicts each other and goes in opposite directions with the basic phenotype closer to one limit	Selection caused by both predators is completely independent and does not interfere with each other	Selection caused by both predators goes in different directions, but has a common overlap	Selection caused by both predators goes in different directions, which conflict with but do not completely exclude each other
example	two gape-limited predators with the same limit → selection causes prey to get larger	two gape-limited predators with different limits → selection causes prey to get larger, but in one case even larger than in the other	one gape-limited and one visually limited predator → selection causes prey to either get larger (so it can't get eaten by the first predator) or to get smaller (so that it is not seen by the second)	one gape-limited and one visually limited predator → the first predator causes prey to get only slightly larger, while the second causes it to get much smaller than the basic phenotype	one predator is gape-limited and eats whole prey, the other one is not and crushes prey → selection causes prey to get larger against the first and harder against the second	one predator is gape-limited and eats whole prey, the other one is not and crushes prey → selection causes prey to get larger against the first and a harder shell against the second, but harder prey also increases handling time for the first predator	one predator is gape-limited and eats whole prey, the other one is not and crushes prey → selection causes prey to get larger against the first and a harder shell against the second, by an increase in shell-thickness accompanied with a smaller size

environment with	one predator (1 or 2) (predator succession)	both predators (1 + 2) (predator co-occurence)
optimal response	For each predator there is a different, specific response. This response has a partial similarity, either because some traits are the same in both cases (and others differ) or because some or all are a variation of the same trait and have a common basis.	Case 1: The optimal response against both predators lies between the responses against the single predators OR Case 2: The optimal response against both predators is identical to the response against one of the two predators (but not the other).
	other predator (2 or 1)	both predators (1 + 2)
costs mismatching phenotype	The cost:benefit ratio of a mismatching defense is always worse than that of the matching defense (higher costs and/or lower benefit).	Only the differences in benefit (protection value) lead to maladaption costs (phenotype less fit than optimal phenotype). That means being defended against only one predator is not costly in any situation (compare case 2).

Figure 4 Concept for the role of modality in systems with two predators. The upper section describes the three basic types of modality differences with their subgroups (**a**) *sensu strictu*, **b**) *and* **c**) *sensu latu*). To visualize modality (that is, the qualitative aspect of selection pressure caused by predation) two points are needed. The basic phenotype (that is, the phenotype in an environment without any predation pressure) serves as the initial point **C**, lying on the origin. The 'immunity point' I_{px} represents the terminal point, after which natural selection caused by predator x stops (that is, the phenotype is completely defended or 'immune'). Its coordinates are defined by the modality of the predators given in the first and second row ('Modality pred. 1', colored black,' Modality pred. 2' colored gray) with **k** being a positive coefficient and **A/B** as variables. Between **C** and I_{px} a vector can be formed, representing the direction and length of selection. In the case of predator 1, this vector always lies on the x-axis; therefore, the protection of a phenotype against predator 1 can be read off its x-coordinate. The same is true for predator 2 in type I and II systems, but not for type III. For each type, a description and a theoretical example are given. Additionally for type IIIb, optimal responses in environments with a single (left) or both (right) predators as well as the costs for a mismatching phenotype (defended against the wrong or only one predator) are described in the bottom boxes.

Conclusion

In the case of *D. barbata*, it is evident that even the modality differences of two invertebrate predators matter. This led to the ability to react to *Triops* and *Notonecta* with a wide array of distinctive and specific morphological defenses, making *D. barbata* the morphologically most plastic daphnid based on current knowledge. With all the advantages that have established *Daphnia* as model organisms, including a sophisticated genetic background [63], we hope that this study provides an experimental basis for future research and further insight into the ultimate causes for the evolution of inducible defenses. From a theoretical perspective, we hope our concept proves to be a useful extension of the four prerequisites for the evolution of inducible defenses, outlined by Tollrian and Harvell [9]. Furthermore, our concept can be easily adapted to any number of predators by using combinations of the three categories, their subgroups and, if necessary, by the addition of more dimensions. In conclusion, our study highlights the need to include predator modality in

research regarding inducible defenses and predator-prey interactions in general.

Methods

General procedure

We used an Ethiopian clone (Eth 1) of *D. barbata*, provided by Joachim Mergeay. Of the predators used, *T. cancriformis* derived from a clonal line provided by the University of Vienna (Dr. E. Eder), while adult *N. glauca* were caught in the field and treated against bacteria and fungi (Tetra-Medica General Tonic, Tetra GmbH, Melle, Germany) prior to the experiments. Juvenile notonectids were obtained by hatching the adults' eggs. Three stable laboratory cultures of *D. barbata* (beaker-set A) for all three treatments were established, starting with 13 adult, pre-induced (*Triops* or *Notonecta*) or control daphnids, which were each put in a 1.5 L beaker containing semi-artificial medium [64]. In each beaker, a 125 μm mesh net-cage was placed, which was either empty (control), or contained a single predator (*Triops* or *Notonecta*). The daphnids were fed daily with 1 mg C/l of green algae (*Scenedesmus obliquus*) and 50% of the medium was exchanged every five days. Each predator was fed 5 to 10 adult *D. barbata* and 3 live chironomid larvae per day, which were also placed in the control treatment. Impurities and feces were removed every other day. After obtaining a stable population of more than 100 daphnids in each beaker, a batch of juveniles was randomly removed once a week and put into fresh beakers (beaker-set B), which were treated in the same way as the corresponding beaker-set A and considered as biological replicates. All beakers (set A, set B and the predation trials) were kept in a climate-controlled chamber at 20 ± 0.5°C under a constant period of fluorescent light (15 h day:9 h night). Beaker-set B was checked daily for primiparous daphnids, which were then removed and counted. If a beaker contained at least 11 primiparous daphnids, 10 randomly chosen (or decimal multiples) were used in the predation trials and the rest were preserved in 70% EtOH (p.a.) for later measurements of morphological traits. If a beaker did not contain at least 11 primiparous daphnids or if not enough daphnids from another treatment were available (as each predation trial consisted of 20 daphnids, 10 from one, 10 from another morphotype), then the replicate could not be used in the predation trials and was excluded from analysis. This resulted in a total number of 21 *Triops*-induced (131 measured daphnids) and 17 control and *Notonecta*-induced replicates (control 110 and *Notonecta*-induced 95 measured daphnids).

Measurements

Using a digital image analysis system (cell^P software and Altra 20 Camera, Olympus, Hamburg, Germany) mounted on a stereo microscope (Olympus SZX12), the following parameters were measured from a lateral view:

– body length, defined as the distance between the tail-spine base and the upper edge of the compound eye;
– helmet length, defined as the distance between the edge of the compound eye and the tip of the helmet;
– helmet angle, defined as the angle enclosed between tail-spine base, center of the compound eye and tip of the helmet;
– absolute spine length, defined as the ventral edge of the tail-spine, measured from the base to the tip using a polygon line with at least five points;
– effective spine length, defined as the straight distance between base and tip of the tail-spine;
– spine angle, defined as the angle enclosed by the tip of the tail-spine, the base of the tail-spine and the center of the compound eye.

Four additional parameters were measured from a dorsal view of the head:

– distance between the 1st and the 10th dorsal spine, as a measurement of microspine density;
– maximum dorsal spine length;
– maximum dorsal ridge width;
– angle of the fifth dorsal spine relative to the dorsal ridge.

From the ratio between absolute and effective tail-spine length, another parameter, "curvature", was calculated. To exclude body-size effects, relative values of helmet length, body width and tail-spine length were calculated. For each replicate the arithmetic mean of each trait was calculated from the single measurements and then analyzed statistically. Since the assumptions for parametric tests were not met (normal distribution and/or homogeneity of variance), Kruskal-Wallis one-way analysis of variance was performed using IBM SPSS 20.0 (IBM, Armonk, New York, USA).

Predation experiment

Predation trials were conducted under fluorescent light in a climate chamber at 20+/−0.5°C. Each morph was tested against the others (*Notonecta* induced/control, *Triops* induced/control, *Notonecta* induced/*Triops* induced) with either *Notonecta* or *Triops* as the predator. Ten female primiparous daphnids of both respective morphs were placed into an 800 ml beaker, containing 200 ml medium. The trial started when the predator/s (one *Triops*, sized 20 to 30 mm, or three 2nd to 3rd instar *Notonecta*s, 3 to 5 mm) were placed into the beaker and ended after 90 minutes (*Triops*) or 3 hours (*Notonecta*), or when half of

the daphnids were eaten. Numbers of surviving daphnids were subsequently counted using a stereo microscope (Leica MS5, Leica Microsystems, Wetzlar, Germany, 6.3× magnification). All combinations of treatments and predators was replicated eight times and analyzed with a Wilcoxon signed-rank test using IBM SPSS 20.0 (IBM, Armonk, New York, USA).

Additional file

Additional file 1: Figure S1. Full concept for the role of modality in systems with two predators. For detailed description see Figure 4. In addition to Figure 4, optimal responses and maladaption costs of mismatching phenotypes in environments with predator succession and predator co-occurrence are given for each subgroup.

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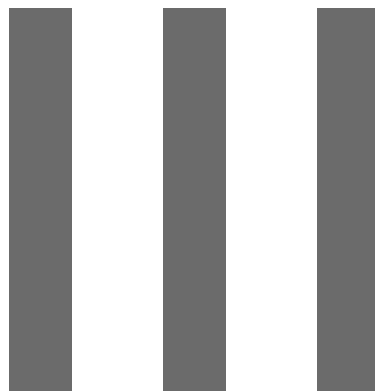www.biomedcentral.com/submit

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CHAPTER III: ARTICLE

Inducible Defenses with a "Twist": *Daphnia barbata* Abandons Bilateral Symmetry in Response to an Ancient Predator

Herzog, Q., Rabus, M., Wolfschoon Ribeiro, B. and C. Laforsch. 2016,

CHAPTER

III

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RESEARCH ARTICLE

Inducible Defenses with a "Twist": *Daphnia barbata* Abandons Bilateral Symmetry in Response to an Ancient Predator

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Abstract

Predation is one of the most important drivers of natural selection. In consequence a huge variety of anti-predator defenses have evolved in prey species. Under unpredictable and temporally variable predation pressure, the evolution of phenotypically plastic defensive traits is favored. These "inducible defenses", range from changes in behavior, life history, physiology to morphology and can be found in almost all taxa from bacteria to vertebrates. An important group of model organisms in ecological, evolutionary and environmental research, water fleas of the genus *Daphnia* (Crustacea: Cladocera), are well known for their ability to respond to predators with an enormous variety of inducible morphological defenses. Here we report on the "twist", a body torsion, as a so far unrecognized inducible morphological defense in *Daphnia*, expressed by *Daphnia barbata* exposed to the predatory tadpole shrimp *Triops cancriformis*. This defense is realized by a twisted carapace with the helmet and the tail spine deviating from the body axis into opposing directions, resulting in a complete abolishment of bilateral symmetry. The twisted morphotype should considerably interfere with the feeding apparatus of the predator, contributing to the effectiveness of the array of defensive traits in *D. barbata*. As such this study does not only describe a completely novel inducible defense in the genus *Daphnia* but also presents the first report of a free living Bilateria to flexibly respond to predation risk by abandoning bilateral symmetry.

Introduction

Phenotypically plastic defensive traits in prey organisms typically evolve in environments characterized by strong variation in predation risk. These traits, termed inducible defenses, are known to affect organismic interactions and population dynamics and are therefore crucial for the understanding of ecosystem functioning and evolutionary processes (e.g. [1,2]). The prerequisites for inducible defenses to evolve include, next to the variable and unpredictable predation risk, the existence of a cue that reliably indicates the presence of the predator, the

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effectiveness of the defense and finally defense-associated costs, which exceed the benefits in the absence of the threat [3]. Inducible defenses can be expressed on the level of behavior, life history, physiology and morphology and are found in almost all taxa ranging from bacteria and unicellular organisms to vertebrates (e.g. [4,5]).

Water fleas of the genus *Daphnia* (Crustacea: Cladocera) are important model organisms in ecological, evolutionary and environmental research. They are well known for their ability to respond to predators with an enormous variety of inducible morphological defenses, which are thought to function by impeding handling and ingestion by the predator [6]. So far primarily helmet-like (e.g. enlarged and pointy helmets, dorsal crests), spine-like (e.g. elongated tail spines, neckteeth) or structural (i.e. fortification of the carapace) defenses have been reported from this genus [7]. With the exception of the development of a spiky helmet and a longer tail spine in *D. lumholtzi* exposed to fish [8], most of these inducible morphological defenses are expressed in response to predatory insects, e.g. phantom midge larvae and back swimmers, and pelagic carnivorous crustaceans, e.g. cyclopoid copepods and *Leptodora*. Over the last years, another crustacean predator, the pond dwelling tadpole shrimp *T. cancriformis*, and responses of its prey received increasing attention. Being extant for 220 million years, this most ancient animal species acts as strong selective force on coexisting *Daphnia* species. Intriguing morphological defenses such as a "crown of thorns" in the *D. atkinsoni* species complex [9] and the "bulkiness" in *D. magna* [10] are attributed as effective means against *Triops* predation. Recently, it has been shown that the African species *D. barbata*, which coexists with *Triops* in temporary freshwater ponds and lakes, responds to *T. cancriformis* and the backswimmer *Notonecta glauca* with specialized defenses which are based on the same structures (e.g. helmet, tail spine, dorsal ridge), but built in a different shape [11]. Both induced defenses have been shown to enhance survival when the daphnids are exposed to the respective predator. Based on the latter study, we here report on a unique inducible morphological defense in *D. barbata*, the body torsion.

Results and Discussion

Triops-exposed *D. barbata* alter their body symmetry in response to this predator (Fig 1). In detail, this change is characterized by a torsion of the whole body that leads to an S-shaped dorsal ridge. As both tail-spine and helmet are bent backwards the torsion further results in both structures to point into opposite directions as they laterally deviate from the body axis. Interestingly, the orientation of this change is apparently not random, but genetically fixed: All measured specimens had their helmets pointed to the right and the tail spine to the left from a dorsal view. As a consequence, the bilateral axis, which normally aligns along the dorsal ridge in *D. barbata*, is abolished. The body torsion, here quantified as the sum of helmet- and tail spine deviation from the body axis (defined as the line connecting the base of the tail spine and the middle between the fornices of the shoulder shield), was significantly increased in predator-exposed daphnids compared to the control morph not exposed to predator released cues (control: 63.47μm ± 25.97 SD; predator-exposed: 342,87μm ± 49,51 SD; F-Test, $F(1, 22) = 264,09$, $P < 0.001$; Fig 2). Therefore, the twisted body can be considered to be predator-induced.

The body torsion likely contributes considerably to the increased survival rate of *Triops*-induced *D. barbata*, as it should act synergistically with or additively to the previously described induced traits, i.e. the elongated, dorsally bent helmet, the curved and dorsally bent tail spine, and more and larger spinules on the dorsal ridge [11]. The functioning of the body torsion may be explained by the way *Triops* is handling its prey [12]: the prey is caught by encaging it with the numerous legs and placed into the narrow food groove, a symmetrical, conveyor-like structure built by the gnathobases of the trunk limbs. Then it is transported

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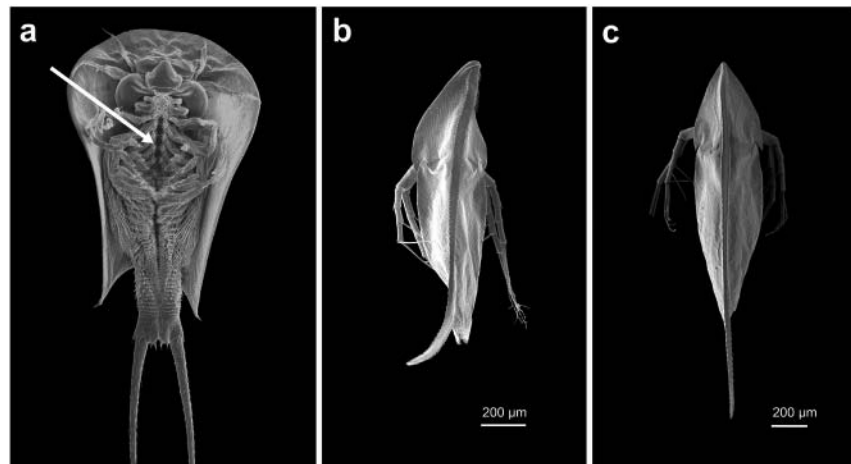
Fig 1. Scanning electron microscope images of the experimental animals. a) *Triops cancriformis*, ventral view with the arrow pinpointing to the narrow food groove. The ancient predator feeds on *Daphnia*, which are caught, subsequently placed into the food groove and transported towards the mandibles; b) Dorsal view of a *Triops*-exposed morph of *Daphnia barbata* showing the "twisted" appearance. The tips of helmet and tail spine deviate from the body axis in opposite directions, leading to an S-shaped dorsal ridge and thus abolishing bilateral symmetry of the individual. The twisted morphotype can be assumed to severely impede the transport through the food groove as it should cause the daphnid to wedge within the food groove of the predator. c) Dorsal view of *D. barbata* not exposed to the predator. The dorsal ridge aligns with the bilateral body axis, the tips of helmet and tail spine do not deviate from the body axis.

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towards the mandibles through movements of the limbs. When *Triops* catches a *Daphnia*, it is almost exclusively placed in the food groove in a way that the dorsal side of the prey faces the predator while the head of the daphnid points towards the mandibles (Rabus, unpublished observation). Given this mode of feeding, we expect the body torsion to effectively interrupt
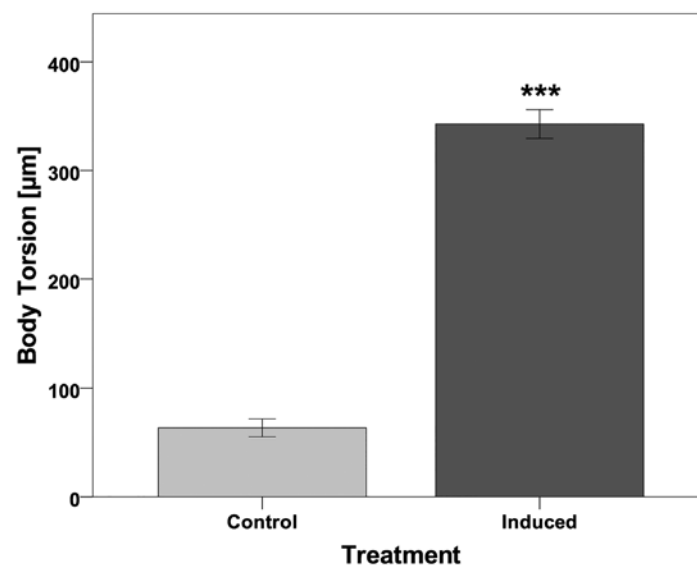
Fig 2. Comparison of the body torsion in non-predator-exposed (Control) and predator-exposed (Induced) primiparous *Daphnia barbata*. Body torsion is here defined as the sum of helmet and tail spine deviation from the body axis. The error bars show the standard error of Mean (SE), the asterisks indicate the significance level (*** $P < 0.001$) based on a F-Test ($F(1, 22) = 264.09$).

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the transport within the food groove. The sidewards bent helmet and the sidewards curved tail spine may each thread into opposing small gaps between the gnathobases, causing the daphnid to become wedged within the food groove. This would in turn cause a complete blockage of the transport of the daphnid towards the mandibles, which should require *Triops* to release its prey from the food groove in order to rearrange its position, giving the daphnid the chance to escape.

In contrast to the classical defensive traits in *Daphnia*, e.g. spines or helmets, the observed body torsion in *D. barbata* causes a massive change in the morphology of the whole body since not only the helmet and the tail spine deviate from the body axis, but also the carapace is twisted. This morphological alteration is so far unique since no other free living animal has been shown to completely abandon its bilateral symmetry as an induced response to predation. So far, only few ontogenetically determined deviations from bilateral symmetry have been described [13]. Only two cases of predator-induced asymmetry are known at this point: a one-sided enlargement of a single spine in the rotifer *Keratella tropica* exposed to the predatory rotifer *Asplanchna* [14]; and the sessile barnacle *Chthamalus anisopoma*, which changes its shell shape from the typical conical morph to an atypical "bent-over" morph when exposed to the carnivorous gastropod *Acanthina angelica*, resulting in a shift of the bilateral axis [15]. In *D. barbata* however, the bilateral axis is not changed to another plane, but abolished completely, leaving the animal without a symmetrical axis. This tremendous change in morphology should considerably alter the hydrodynamic properties of the induced individuals. This may negatively affect locomotion leading to ecological costs (e.g. escape behavior against other predators) and physiological costs, respectively. Additionally, the twisted carapace may also affect feeding efficiency, i.e. by impairing the suction-and-pressure pump built by the thoracic limbs and the carapace [16], and possibly also reproduction, i.e. by limiting the available space in the brood pouch. Finally, the formation of the body torsion itself likely incurs developmental costs. In sum, this suggests high costs associated with the body torsion. Since it is an evolutionary prerequisite that an inducible defense provides a net benefit under predation [17], this is an indication that the body torsion plays an essential role in the defense against the predator *Triops*. It is therefore likely that the previously described increase in survival rate [11] is to a great extent caused by this trait.

To conclude, we report on the "twist", a torsion of the whole body, as an intriguing novel inducible defense in the extensively studied model genus *Daphnia*. Hence, our finding further adds to the emerging awareness of the complexity of inducible morphological defenses in *Daphnia*, which often include a full array of morphological alterations, ranging from prominent structures (e.g. helmets and spines) to minute (e.g. the tiny spinules along the dorsal ridge) or even "hidden" defenses (e.g. a fortified exoskeleton). Therefore, it also shows the need for further studies to reveal and entangle the mechanisms underlying the effectiveness of these defenses. Moreover, body torsion presumably requires a complex developmental pattern for its formation. Since the symmetry of adult Bilateria is usually established during the cleavage period [18], the deviation from this symmetry in later life stages must apparently be triggered by well-defined interactions of genes with the environment. Given that *Daphnia* has emerged as important model organism for biomedical research and environmental genomics [19,20], the body torsion in *D. barbata* might be an extraordinary model system for understanding the developmental mechanisms underlying phenotypic variations.

Material and Methods

The specimens of *D. barbata* analyzed in this study derive from the induction experiment described in detail in Herzog & Laforsch [11]. In this study, we used a single clone of *D.*

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barbata (Eth 1), originating from Ethiopia and a laboratory cultured clonal line of *T. cancriformis* as predator. The induction experiment was conducted in a temperature controlled climate chamber at 20 ± 0.5°C under fluorescent light with a constant photoperiod (15h light: 9h dark). As starting point for the induction experiment, two stable cultures (control and *Triops*-induced) were established starting with 20 adult, brood bearing *D. barbata* in 1.5L glass beakers containing semi artificial medium based on ultrapure water, well water, phosphate buffer and trace elements. A net cage (mesh width: 125µm) was placed in each beaker and was either empty (control) or stocked with a single *T. cancriformis* (*Triops*-induced). The daphnids were fed daily with 1 mg C/L of the green alga *Scenedesmus obliquus*, *Triops* were fed daily with 5 to 10 live *D. barbata* and 3 live red chironomid larvae. Every 5 days half of the medium was exchanged. Every week, randomly sampled juveniles, less than 2 days old, were transferred from the starting cultures into new beakers, each representing a biological replicate, which were treated as described above. As soon as the daphnids in the new beakers reached primiparity, they were preserved in 70% ethanol and stored until further analysis.

To quantify the body torsion, we measured the deviation of the tip of the helmet and the tip of the tail spine from the body axis. Since the body axis becomes asymmetric in predator exposed animals, it is here defined as the line connecting the base of the tail spine and the middle between the fornices of the shoulder shield. Then the torsion was calculated as the sum of helmet and tail spine deviation from the body axis. Mean torsion was calculated for each replicate (control N = 10; induced N = 14), as several individuals (on average 4) from each replicate were measured (S1 Dataset). Then the data was tested for normality and homogeneity of variance and a F-Test was conducted to test for treatment-dependent differences in body torsion.

Supporting Information

S1 Dataset. Body Torsion in *D. barbata*, including replicate means.
(XLSX)

Acknowledgments

Author Contributions

Conceived and designed the experiments: QH CL. Performed the experiments: QH. Analyzed the data: MR. Contributed reagents/materials/analysis tools: CL. Wrote the paper: QH MR BWR CL. Performed morphological measurements: MR BWR.

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CHAPTER IV:
ARTICLE

Predator specific reversibility of morphological defenses in *Daphnia barbata*

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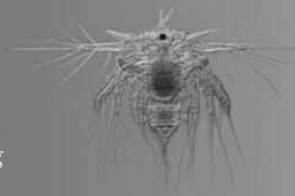
CHAPTER

IV

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),

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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 ± 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 $1 \, \text{mg} \, \text{C} \, \text{L}^{-1}$ 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: $n_{+3\text{days}} = 26$, $n_{+3\text{days}} = 22$, $n_{+3\text{days}} = 15$; T_{ind}: $n_{+3\text{days}} = 20$, $n_{+3\text{days}} = 18$, $n_{+3\text{days}} = 16$; T_{rem}: $n_{+3\text{days}} = 17$, $n_{+3\text{days}} = 18$, $n_{+3\text{days}} = 16$; N_{ind}: $n_{+3\text{days}} = 21$, $n_{+3\text{days}} = 16$, $n_{+3\text{days}} = 9$ and N_{rem}: $n_{+3\text{days}} = 18$, $n_{+3\text{days}} = 13$, $n_{+3\text{days}} = 8$.

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 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).

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.

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, $P = 0.353$).

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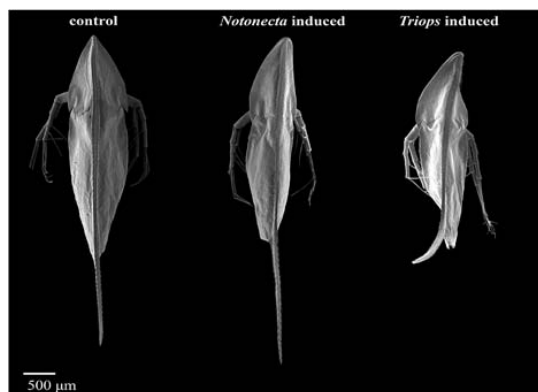
Fig. 2. Scanning electron microscope images of adult *D. barbata* (dorsal). The body torsion of *Triops*-induced daphnids compared to either control or *Notonecta*-induced daphnids is shown.
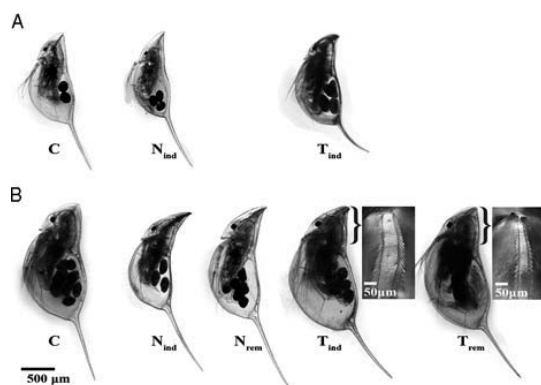
Fig. 3. Lateral view of *D. barbata* through the experiment, sorted by treatment control (C), *Notonecta* induced (N_{ind}), *Notonecta* removed (N_{rem}), *Triops* induced (T_{ind}), *Triops* removed (T_{rem}) at the end of phase I (**A**) and at the end of the experiment/phase II (**B**), respectively. 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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Chapter V:
Article

Plasticity of defensive traits in adult *Daphnia magna* in response to *Triops cancriformis*

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CHAPTER
V

Plasticity of defensive traits in adult *Daphnia magna* in response to *Triops cancriformis*

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Abstract

Daphnia are well known to possess phenotypically plastic adaptations in order to cope with a heterogeneous predation risk. So far, most research has focused on the expression of these 'inducible defenses' in juvenile and primiparous daphnids. Here we show that even adult *D. magna* are able to flexibly change morphology and life-history according to the presence or absence of the tadpole shrimp *Triops cancriformis*. Previously non-induced daphnids increased clutch size and relative tail-spine length to the same level as continuously induced specimen, whereas previously induced *D. magna* reduced both traits to non-induced levels. This induction and reversion respectively of morphologic and life-history defenses happened within a few molts after the introduction or removal of predator cues. A similar switch could be observed for size and relative tail-spine length of neonates produced in the new environment. Consequently, our findings provide evidence that *D. magna* largely retain their phenotypic plasticity as adults.

Introduction

Daphnia are known for their ability to flexibly cope with a large number of environmental changes through their eco-responsive genome (Colbourne et al. 2011). Prominent and well-studied examples (Lampert 2011) are 'inducible defenses', phenotypically plastic responses to predation (Harvell and Tollrian 1999). This includes the ability of *Daphnia* to change their behavior, e.g. diel vertical migration (Stich and Lampert 1981; De Meester and Cousyn 1997); induce life history shifts, e.g. changes in age and size at maturity (Stibor and Luning 1994; Riessen 1999); or to alter their morphology, for instance the expression of helmet- or spine-like structures (Dodson 1989; Kolar and Wahl 1998; Laforsch and Tollrian 2004a), all in accordance to the experienced predation regime. This flexibility to react to changing environments is further enhanced through a fast parthenogenetic reproduction, that enables *Daphnia* to produce genetically identical offspring, which then develops correspondingly to the predation. It has been shown, that the induction of defenses is often influenced by factors throughout early development, for instance maternal effects during embryogenesis (Agrawal et al. 1999). However, this also applies to the influence of kairomones, infochemicals released by the predator, which can start to affect the induction during the embryogenesis (Laforsch and Tollrian 2004b; Naraki et al. 2013) and seems to have the most effect in the first instars (Mikulski et al. 2004, 2005). In consequence, it is common practice to 'pre-induce' mothers for a maximum induction of the offspring. Furthermore, some inducible defenses, like neckteeth in *Daphnia pulex* are only expressed during early instars, which are susceptible to attacks from the predator (Riessen and Trevett-Smith 2009). Therefore, it could be inferred that the decision to induce (but not necessarily to maintain) morphological defenses is already reached in juvenile *Daphnia*. Possible explanations can range from developmental windows to the mentioned fast parthenogenetic reproduction in *Daphnia*: genetically identical offspring of a maladapted adult daphnid could develop into a well-adapted adult (~ one to two weeks) faster than the adult itself could change its phenotype. However, since the vast majority of studies have concentrated on the

induction of morphological defenses up until primiparity, with only a few studies providing clear evidence, that these defenses are maintained or continue to grow in adults (Rabus and Laforsch 2011; Herzog et al. subm.), the question, whether and how adult daphnids react to sudden changes in predation risk, is still largely unanswered. Behavioral changes, e.g. diel vertical migration, which are generally considered to be very flexible (Gabriel et al. 2005) seem to remain plastic in adults (Beklioglu et al. 2008) but for morphological and life-history defenses the situation remains unclear. Only recently, a study found plasticity of morphological defenses in adult *D. barbata* (Herzog et al. subm.). The sudden absence of predator cues from *Triops cancriformis* caused the adult induced daphnids to revert parts of their defenses, with first changes within a week. Nevertheless, this showed only adult plasticity in one direction.

In the present study, we followed the question of predator associated phenotypic plasticity of *Daphnia magna* in both directions, i.e. induction and reversion of defenses. For this, we used *T. cancriformis* as predator and *D. magna* as prey species. Adult *D. magna* are known to remain defended against *T. cancriformis* with continuous exposition to the predator (Rabus and Laforsch 2011). However, so far, it was unknown whether adult non-induced *D. magna*, could express induced morphological and life history defenses or adult induced *D. magna* could reverse these defenses if they each experience the respective other environment. Therefore, we tested (i) how adult, non-induced *D. magna* react to the sudden appearance and (ii) how adult, induced *D. magna* react to the sudden disappearance of *Triops*-kairomones.

Methods

A single clone of *D. magna*, K34J, originating from a former fish pond near Munich, Germany, was used in this experiment. This clone has already been shown to respond to *Triops* with the expression of prominent morphological defenses (Rabus and Laforsch 2011). As predator, we used a laboratory-cultured clonal line of *T. cancriformis*. The experiment was conducted in a climate chamber at 20±0.5°C under a constant period of fluorescent light (15h day:9h night). We started with age synchronized adult *D. magna*, which were reared in semi artificial SSS-medium (Jeschke and Tollrian 2000) until they released their third clutch. They were then randomly assigned to two groups (control and induction) and individually transferred to 160 mL glass beakers, either containing 100 mL pure SSS-medium (control) or 100 mL SSS-medium preconditioned with *Triops*-kairomones (induction) to ensure a pre-induction. As food source for the daphnids 54 µM C L^{-1} of the green algae *Scenedesmus obliquus* were added daily to the media. This concentration lies above the limit for producing offspring, but below ad libitum (Glazier 1992) and at a level found in German lakes during summer (Lampert 1978; Müller-Navarra and Lampert 1996). We chose to conduct the experiment with limited food resources, since a typical 'ad-libitum'-food supply might counteract with the immediate need to change by overshadowing energetic maladaptation costs. The kairomone conditioned medium was prepared by keeping large (> 2 cm) *Triops* at a density of one individual per liter in 10 L glass aquaria filled with SSS-medium (Jeschke and Tollrian 2000). Each *Triops* was being fed three large live chironomid larvae and ten live *D. magna*. After 24h, the

medium was filtered through a 11 µm cellulose filter prior to experimental use. After that, the aquaria were cleaned and restocked with *Triops*, medium and food, in order to prepare kairomone conditioned medium for the next day. Control SSS-medium was prepared in the same fashion without predators. Until they released their forth clutch, which was then used in the main experiment, the daphnids were daily transferred into new beakers containing the respective (fresh) medium. Within 12h after their birth, for both treatments (control and kairomone) 140 randomly sampled neonates were distributed separately into fresh 160 mL glass beakers containing 100 mL of their respective medium (control or kairomone conditioned) and 0.65 mg C L^{-1} of *S. obliquus*. The 140 neonates of each treatment were then randomly designated to seven groups of 20 daphnids each. Every day, daphnids were transferred into fresh beakers containing fresh medium and algae. With the release of the first clutch, one of the seven groups of each treatment was removed and its adult daphnids were frozen at -80°C, while the neonates were preserved in 80% ethanol for later analysis. The daphnids of three of the remaining six groups from each treatment were then transferred into medium of the other treatment. This resulted in four experimental treatments with 60 replicates each (or 3 groups of 20 replicates): continuously kairomone exposed daphnids (TT), *Triops*-induced daphnids in control medium (TC), non-induced daphnids in kairomone medium (CT) and non-induced daphnids in control medium (CC). After each molt, all neonates and one group of adults per treatment were removed and preserved as described above. The experiment stopped with the removal of the last groups, three molts after the medium switch. All adult daphnids were measured (body length, body width, tail-spine length, clutch size) in accordance to previous studies (Rabus and Laforsch 2011; Rabus et al. 2012). For comparison of morphological traits in the offspring, the neonates of the last group, which was maintained until the end of the experiment (i.e. had three clutches), were used. For each clutch and replicate, three neonates were randomly chosen and their morphology measured (body length and tail-spine length). Relative tail-spine length was used as a proxy for the expression of defenses in neonates as it has been shown to be the start-up defense against *Triops* (Rabus and Laforsch 2011).

To compensate for size-dependent differences, relative body width and relative tail-spine length were calculated by dividing trait length through body length. For each replicate, mean values for body length, relative body width and relative tail-spine length were calculated.

Data analysis was performed using IBM SPSS Statistics Version 21 (IBM Corporation, Armonk, NY, USA). Data was then tested for normality and homoscedasticity. Measured parameters of adult daphnids were compared with one-way ANOVA and Tukey-HSD post hoc-tests. For heteroscedastic data, Welch-corrected ANOVA with Tamhane T2-post-hoc tests were conducted. Since clutch size was not normally distributed, Kruskal-Wallis test was used for a comparison. Relative tail-spine length in neonates was analyzed using a repeated measures ANOVA followed by pairwise comparisons using the Tamhane T2 post hoc test.

Results

After the release of the first clutch, *Triops*-induced daphnids were significantly larger than control daphnids and showed a larger relative tail-spine length (table 1). A significantly larger relative body width of induced daphnids, as shown in previous studies with this clone (Rabus and Laforsch 2011), could only be observed at the end of the experiment (table 1). *Triops* induced daphnids also produced a significantly larger number of eggs (table 1). Although the expression of the morphological defense is less pronounced, it can be considered to be in accordance with previous induction experiments with this clone and *T. cancriformis* (Rabus and Laforsch 2011).

The difference in body length between control daphnids (CC) and induced daphnids (TT) of ~300 µm was maintained throughout the experiment (table 1 and figure 1A). In the switch treatments, this difference remained for two molts (table 1 and figure 1A). However, by the end of the experiment daphnids of the CT treatment were still found to be larger than the control but smaller than continuously induced daphnids, whereas TC daphnids were smaller than continuously induced daphnids and larger than the control (table 1 and figure 1A). In consequence, both switch treatments had on average a similar and intermediate body length. In general, tail-spine length decreased with each molt for all treatments, with one exception (table 1 and figure 1B): CT daphnids grew larger tail-spines in their fourth adult molt (table 1 and figure 1B). Correspondingly, TC daphnids showed a faster decrease in tail-spine length than TT daphnids (table 1 and figure 1B). Proportional to their body size (i.e. relative tail-spine length), a full reversion/induction to the levels of continuously exposed/control daphnids was achieved (table 1 and figure 1C). Absolute body width showed response similar to body length, but by the time of the fourth adult molt, relative body width of TT and TC daphnids was larger compared to both CC and CT daphnids (table 1 and figure 1D). Number of offspring in the TC treatment compared to the TT treatment was already decreased in the first brood that was produced after the switch (table 1 and figure 1E). One clutch later, CT daphnids showed an increasing number of eggs compared to CC daphnids and another clutch later, both switch treatments produced on average approximately the same number as the treatments they were switched to (table 1 and figure 1E).

In neonates, relative tail-spine length differed significantly between treatments (repeated measures ANOVA, test for between subject effects; $F_{3, 63}$ = 107.642; $P < 0.001$). In neonates from the CC treatment, relative tail-spine length was significantly greater than in TC neonates (Tamhane's T2; P = 0.042). CT neonates showed a significantly greater relative tail-spine length than neonates from the CC (Tamhane's T2; $P < 0.001$) and the TC treatment (Tamhane's T2; $P < 0.001$) and TT neonates showed a significantly greater relative tail spine length than neonates from the CC (Tamhane's T2; $P < 0.001$), CT (Tamhane's T2; P = 0.048) and TC treatment (Tamhane's T2; $P < 0.001$). The time-treatment interaction was not significant (repeated measures ANOVA, F(5.781) = 1.959; P = 0.079), indicating no treatment effect over time.

Discussion

Our findings show that even adult *D. magna* can respond phenotypically plastic to the sudden absence or presence of predator cues. After releasing their first clutch, *Triops*-induced daphnids were larger, wider, had elongated tail-spines and a larger clutch size than their non-induced counterparts. Within three molts (~ seven to nine days) after the switch into medium without predator cues, the relative tail-spine length and clutch size of daphnids switched from kairomone to control medium (TC) did not differ from control daphnids (CC) anymore. Correspondingly and within the same timeframe, previously non-induced daphnids, which were exposed to predator cues, increased relative tail-spine length and clutch size to the level of continuously induced daphnids. Still, while clutch size as life-history defense showed full reversibility, the morphological defense of larger tail-spines was only partially reverted. Absolute tail-spine length did not completely induce or revert to the level of the respective continuous treatment. In accordance to earlier findings (Rabus and Laforsch 2011) adult *D. magna* reduced their tail-spine length with age, regardless of the treatment. Interestingly, the partial induction in the CT treatment posed an exception. From the third to the fourth adult molt, an increase in absolute tail-spine length was observed that could only be explained by an active growth of the structure. The physiological ability to reverse, not only halt the reduction of the tail-spine, shows that this trait remains plastic in both directions, indicating no developmental window. Changes in body length occurred in the switch treatments by the end of the experiment, although a full reversion could not be observed within this timeframe. Interestingly, the 'bulkiness' seemed to be the only parameter predetermined by the original environment, although it could only be found at the end of the experiment in the TT and TC treatments. Regarding the offspring, relative tail-spine length seemed to correspond to the environment in which the eggs and embryos developed: Relative tail-spine length in the offspring of TT and CT animals, which were exposed to *Triops*-kairomones in the brood pouch of their mothers, was significantly larger than in both the offspring of CC and TC animals, which lacked the exposure to predator cues. The finding that relative tail-spine length was significantly larger in TT neonates compared to CT neonates seems to indicate that maternal effects may add to the induction of this defense in neonate *D. magna*. Similar maternal effects have been described in *D. cucculata* (Agrawal et al. 1999): mothers that had previously experienced an environment with *Chaoborus*-larvae produced neonates with larger helmets compared to mothers stemming from a predator-free environment. In contradiction to these findings, we also observed that even TC neonates had a significantly smaller relative tail-spine length than CC neonates. At first glance, this rather indicates against the existence of maternal cues. However, it is also possible that the disappearance of *T. cancriformis* leads to contrasting maternal effects. In the case that the sudden absence of kairomones is a safe information not only that the predator is not any longer present, but also that it will not return, the best way to prepare the offspring is to reduce investment in defensive traits as much as possible. This is in line with the finding that *D. barbata* reverses its induced defenses against *T. cancriformis*, but not against the mobile predator *Notonecta glauca*, which presumably has a high chance of repopulating a habitat. Furthermore, a mother that has never experienced predator cues cannot be sure that a predator might suddenly appear, explaining

a longer tail-spine.

In conclusion, our findings hint towards a retained phenotypic flexibility of defensive traits in adult *D. magna* and against the existence of developmental windows. Nevertheless, stronger responses to *Triops* are found, if *D. magna* is directly exposed to the predator (Herzog et al., in prep.) and the limits of a late induction or reversion of inducible defenses remain to be tested. Together with another study on *D. barbata* (Herzog et al., in prep), this study presents some of the first evidence for adult reversibility and induction of inducible defenses in *Daphnia*.

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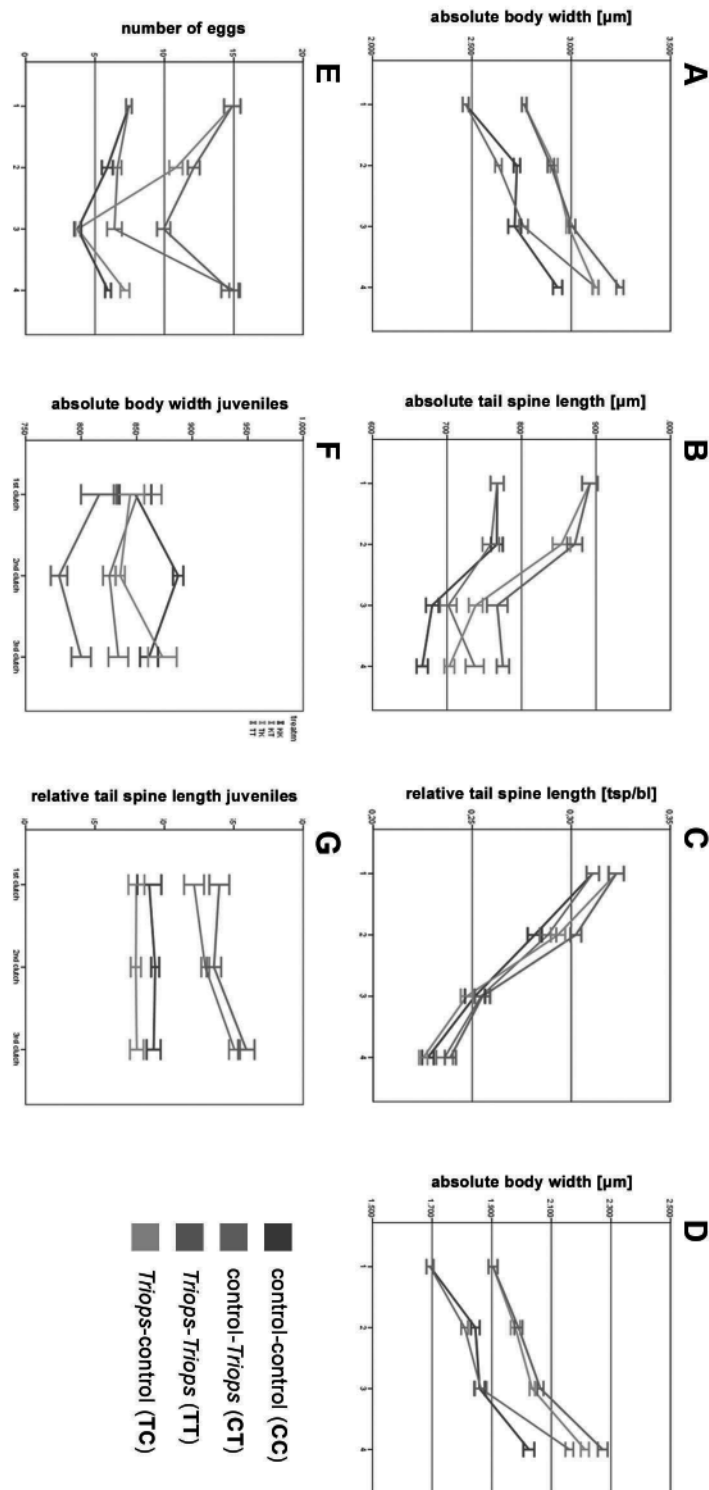
Figure 1. Development of reversible traits. The graphs show mean trait values during the four stages of the experiment (after first, second, third and fourth adult molt), including: body length (A), absolute tail spine length (B), relative tail spine length (C), absolute body width (D), number of eggs (E), body width of the neonate offspring (F) and relative tail spine length of neonate offspring (G). The error bars indicate +/- one standard error of mean.

Table 1. Statistical comparisons of morphological and life-history parameters.

body length		1. adult molt		one-Way ANOVA	dF	F	P
					2.0000		
	N	mean	sem	CC	CT	TC	TT
CC	18	2468.33	15.24	-			
CT					-		
TC						-	
TT	18	2763.21	11.59				

		2. adult molt		ANOVA (T-HSD)	dF	F	P
					3.0000	62.1997	> 0.0001
		mean	sem	CC	CT	TC	TT
CC	18	2726.73	16.09	-	0.0017	< 0.0001	< 0.0001
CT	18	2633.50	17.11	0.0017	-	< 0.0001	< 0.0001
TC	19	2913.33	18.78	< 0.0001	< 0.0001	-	0.9120
TT	18	2897.39	16.51	< 0.0001	< 0.0001	0.9120	-

		3. adult molt		Welch (T-T2)	dF	dF2	P
					3.0000	36.6469	> 0.0001
		mean	sem	CC	CT	TC	TT
CC	20	2715.18	32.63	-	0.8405	< 0.0001	< 0.0001
CT	17	2760.07	22.18	0.8405	-	< 0.0001	< 0.0001
TC	20	2986.14	10.86	< 0.0001	< 0.0001	-	0.9112
TT	18	3004.84	15.56	< 0.0001	< 0.0001	0.9112	-

		4. adult molt		ANOVA (T-HSD)	dF	F	P
					3.0000	51.9749	> 0.0001
		mean	sem	CC	CT	TC	TT
CC		2931.42	23.07	-	< 0.0001	< 0.0001	< 0.0001
CT		3123.88	14.52	< 0.0001	-	0.9995	0.0001
TC		3120.98	14.58	< 0.0001	0.9995	-	< 0.0001
TT		3244.73	18.10	< 0.0001	0.0001	> 0.0001	-

absolute tail spine length		1. adult molt		one-Way ANOVA	dF	F	P
					2.0000		
	N	mean	sem	CC	CT	TC	TT
CC	18	767.20	9.01	-			
CT					-		
TC						-	
TT	18	891.65	10.46				-

		2. adult molt		ANOVA (T-HSD)	dF	F	P
					3.0000	29.9250	> 0.0001
		mean	sem	CC	CT	TC	TT
CC	17	766.89	7.94	-	0.9468	< 0.0001	< 0.0001
CT	18	758.85	11.25	0.9468	-	< 0.0001	< 0.0001
TC	18	853.49	11.89	< 0.0001	< 0.0001	-	0.6408
TT	15	871.42	10.13	< 0.0001	< 0.0001	0.6408	-

		3. adult molt		Welch (T-T2)	dF	dF2	P
					3.0000	38.2360	> 0.0001
		mean	sem	CC	CT	TC	TT
CC	20	680.14	8.44	-	0.5646	0.0002	< 0.0001
CT	17	701.80	11.06	0.5646	-	0.0929	0.0048
TC	20	738.46	9.30	0.0002	0.0929	-	0.4434
TT	18	767.33	13.80	< 0.0001	0.0048	0.4434	-

		4. adult molt		ANOVA (T-HSD)	dF	F	P
					3.0000	28.7118	> 0.0001
		mean	sem	CC	CT	TC	TT
CC	18	666.68	7.59	-	< 0.0001	0.0165	< 0.0001
CT	16	737.07	12.18	< 0.0001	-	0.0366	0.0188
TC	20	703.12	6.73	0.0165	0.0366	-	< 0.0001
TT	18	775.00	8.31	< 0.0001	0.0188	< 0.0001	-

relative tail spine length		1. adult molt		one-Way ANOVA	dF	F	P
					2.0000		
	N	mean	sem	CC	CT	TC	TT
CC	18	0.31	0.00	-			
CT					-		
TC						-	
TT	18	0.32	0.00				-

		2. adult molt		ANOVA (T-HSD)	dF	F	P
					3.0000	5.3834	0.0023
		mean	sem	CC	CT	TC	TT
CC	17	0.28	0.00	-	0.0051	0.0051	0.0053
CT	18	0.29	0.00	0.0051	-	0.0050	0.0053
TC	18	0.29	0.00	0.0051	0.0050	-	0.0053
TT	15	0.30	0.00	0.0053	0.0053	0.0053	-

		3. adult molt		ANOVA (T-HSD)	dF	F	P
					3.0000	0.7478	0.5272
		mean	sem	CC	CT	TC	TT
CC	20	0.25	0.01	-	-	-	-
CT	17	0.25	0.00	-	-	-	-
TC	20	0.25	0.00	-	-	-	-
TT	18	0.26	0.00	-	-	-	-

		4. adult molt	ANOVA (T-HSD)	dF	F	P
				3.0000	4.9251	0.0037
		mean	sem	CC	CT	TC	TT
CC	18	0.23	0.00	-	0.0043	0.0041	0.0042
CT	16	0.24	0.00	0.0043	-	0.0042	0.0043
TC	20	0.23	0.00	0.0041	0.0042	-	0.0041
TT	18	0.24	0.00	0.0042	0.0043	0.0041	-

absolute body width		1. adult molt	one-Way ANOVA	dF	F	P
				2.0000		
	N	mean	sem	CC	CT	TC	TT
CC	18	1692.80	12.06	-			
CT					-		
TC						-	
TT	18	1904.38	15.39				-

		2. adult molt	ANOVA (T-HSD)	dF	F	P
				3.0000	43.8194	< 0.0001
		mean	sem	CC	CT	TC	TT
CC	18	1845.02	14.73	-	0.2693	< 0.0001	< 0.0001
CT	18	1808.80	11.42	0.2693	-	< 0.0001	< 0.0001
TC	19	1979.27	15.59	< 0.0001	< 0.0001	-	0.9529
TT	18	1989.53	13.33	< 0.0001	< 0.0001	0.9529	-

		3. adult molt	ANOVA (T-HSD)	dF	F	P
				3.0000	36.2232	0.2474
		mean	sem	CC	CT	TC	TT
CC	20	1859.24	17.43	-	0.9969	< 0.0001	< 0.0001
CT	17	1863.48	19.34	0.9969	-	< 0.0001	< 0.0001
TC	20	2035.71	8.92	< 0.0001	< 0.0001	-	0.5380
TT	18	2063.19	10.50	< 0.0001	< 0.0001	0.5380	-

		4. adult molt	ANOVA (T-HSD)	dF	F	P
				3.0000	45.9914	> 0.0001
		mean	sem	CC	CT	TC	TT
CC	18	2024.54	18.28	-	< 0.0001	< 0.0001	< 0.0001
CT	16	2160.45	13.14	< 0.0001	-	0.0988	< 0.0001
TC	20	2212.53	13.69	< 0.0001	0.0988	-	0.0350
TT	18	2272.33	16.58	< 0.0001	< 0.0001	0.0350	-

relative body width		1. adult molt	one-Way ANOVA	dF	F	P
				2.0000		
	N	mean	sem	CC	CT	TC	TT
CC	18	0.69	0.00	-			
CT					-		
TC						-	
TT	18	0.69	0.00				-

		2. adult molt		ANOVA (T-HSD)	dF	F	P
					3.0000	1.6930	0.1766
		mean	sem	CC	CT	TC	TT
CC	18	0.68	0.00	-	-	-	-
CT	18	0.69	0.01	-	-	-	-
TC	19	0.68	0.00	-	-	-	-
TT	18	0.69	0.00	-	-	-	-

		3. adult molt		Welch (T-T2)	dF	dF2	P
					3.0000	36.2232	0.2474
		mean	sem	CC	CT	TC	TT
CC	20	0.69	0.01	-	-	-	-
CT	17	0.68	0.00	-	-	-	-
TC	20	0.68	0.00	-	-	-	-
TT	18	0.69	0.00	-	-	-	-

		4. adult molt		Welch (T-T2)	dF	dF2	P
					3.0000	38.3213	> 0.0001
		mean	sem	CC	CT	TC	TT
CC	18	0.69	0.00	-	0.0047	0.0054	0.0063
CT	16	0.69	0.00	0.0047	-	0.0048	0.0057
TC	20	0.71	0.00	0.0054	0.0048	-	0.0063
TT	18	0.70	0.01	0.0063	0.0057	0.0063	-

CHAPTER VI:
ARTICLE

Effects of inducible defenses in *Daphnia magna* on sinking and swimming efficiency

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CHAPTER

VI

Effects of inducible defenses in *Daphnia magna* on sinking and swimming efficiency

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Abstract

Originally cyclomorphosis was speculated to act as buoyancy adjustment to temperature related changes in water viscosity. While it has been shown that the phenomenon of cyclomorphosis in *Daphnia* is an adaption to predation termed inducible defenses, the morphologic changes still might affect the hydrodynamics and thus cause costs. Here we show through sinking experiments, that drag is largely increased in *Triops*-induced *D. magna* and that this difference also found in juvenile daphnids and adult daphnids without brood. The cause is most likely not a change in surface hydrodynamics, but rather attributed to an increase in density through carapace fortification. We estimate the metabolic costs inflicted by the increase in drag to only lie around less than one per mill of the total energy consumption of a daphnid. Furthermore, no differences could be observed in swimming parameters, such as speed, stroke frequency and distance covered by each stroke. Consequently, no swimming impairment was found, while induced *D. magna* seem to be able to compensate for the large differences in drag. Therefore, movement related costs of inducible defences do not seem to explain the plasticity of morphological defenses against *Triops* in *D. magna*.

Introduction

Waterfleas of the genus Daphnia have long been known for a phenomenon called cyclomorphosis (Wesenberg-Lund 1900; Coker and Addlestone 1938). Typically during the summer months many species exhibit helmets and elongated tailspines. These seasonal changes in the morphology have originally been speculated to be related to buoyancy (for a review of those ideas see (Coker and Addlestone 1938). Since a higher water temperature leads to a lower viscosity, a parachute effect was discussed (Woltereck 1913). However, this would result in a lower sinking speed of helmeted 'summer' morphs and so far only the opposite could be observed (Jacobs 1967; Dodson 1984). In contrast, a connection between the morphological changes and predation became more and more apparent (Brooks and Dodson 1965; Jacobs 1967; Dodson 1974). In the last decades, numerous studies have confirmed this relationship and showed that these reactions are form of phenotypic plasticity, termed inducible defenses (Hebert and Sciences 1978; Hanazato and Dodson 1993; Barry 2000; Laforsch and Tollrian 2004b; Rabus and Laforsch 2011). Typically through chemical signals, such as alarm cues, produced by wounded or killed prey organisms (Laforsch et al. 2006), or kairomones, which are produced by the predator itself (for a review see (Lass and Spaak 2003), or other cues, such as turbulences (Laforsch and Tollrian 2004a) daphnids are able to detect predators. The list of species of *Daphnia* exhibiting inducible defenses is still constantly growing (Rabus et al. 2011; Herzog and Laforsch 2013), leading to the identification of even more defensive traits. Apart from life-history (Black 1993; Riessen 1999; Carter et al. 2008) and behavioral defenses (Lampert 1989; Brewer et al. 1999), this also includes previously unknown morphological defenses, such as microspines (Petrusek et al. 2009; Herzog and Laforsch 2013) Rabus in prep.), tail-spine curvature and body torsion (Herzog et al. 2016) or the 'hidden' defense of carapace fortification (Laforsch et al. 2004; Rabus et al. 2013).

While the number of known inducible defensive traits and species featuring them is increasing, many open questions remain, such as the nature and quantity of costs associated with them. These costs could be saved in times with no or low predation, thus offsetting the benefit of the defense. Consequently, they have been stated as a basic condition for the evolution of inducible defenses (Harvell and Tollrian 1999). Without them, one would expect the defense to be permanent. Still, not much is known about the costs of inducible defenses in *Daphnia*. An obvious way of calculating costs by measuring the quantity and quality of offspring is impaired by the fact that such changes are common Life-History defenses to predation (Tollrian 1995). For example it is known that *Daphnia* can increase their clutch size in response to predators (Lüning 1992; Barry 2000). On the other hand, some species decrease the number, but increase the size of the offspring (Dodson 1984; Lüning 1992). Consequently, a clear differentiation, which change reflects an adaptation and which change reflects a cost is often not possible.

In contrast to the measurement of direct fitness costs through lifetime reproductive success (Hammill et al. 2008), another way is to look for the possible causes of costs and to quantify them. Since cyclomorphosis in *Daphnia* turned out to be regularly occurring inducible defenses, studies have focused on the relation between predators and prey morphology. However up to this point, temperature seemed to hold the greatest influence on the occurrence of cyclomorphosis (Coker and Addlestone 1938) and even more recently direct induction could be observed through temperature change (Yurista 2000). However, since temperature is not the ultimate cause, it might only sometimes act as proximate cue, but actually be a condition for inducible defenses to occur. Indeed, studies have found that lower temperatures decrease the degree of development (Hanazato 1991; Laforsch and Tollrian 2004a). Temperature dependent costs are a likely explanation for these observations. For this reason, we designed an experiment in which we induced swimming behavior in induced and non-induced *Daphnia magna* in a temperature of 5° and 25° Celsius to determine swimming efficiency. Afterwards the daphnids were preserved in order to conduct sinking experiments. In order to rule out methodical errors, we repeated a sinking experiment with life, anesthetized daphnids.. Furthermore, in this second experiment, we controlled for the influence of body length and differences in clutch size by using daphnids of the same size, as well as juveniles and adults with removed brood. In addition to the first clone originating from Germany, we used a second clone from Kenia to broaden the approach.

Method

First experiment:

Induction

We used a clone of *D. magna*, K34J, which was known through several laboratory studies to induce defenses in response to chemical cues released by the predator *Triops cancriformis* (Rabus and Laforsch 2011; Rabus et al. 2013; Otte et al. 2014)Rabus et. al in prep). This clone was originally isolated from a former fish pond near Munich, Germany. The predator *T. cancriformis* was cultured in the laboratory and stemmed from a clonal line from the University of Vienna. The induction of the daphnids was conducted within a climate chamber at 20°C (+/- 0.5°C) under constant fluorescent

light (15 h day:9 h night) using glass aquaria (30 x 20 x 20 cm) filled with 9 L of semi-artificial SSS-medium (Jeschke and Tollrian 2000). The bottom of the aquaria was covered with white aquarium sand (White Sun, grain size 0.9-1.2 mm, Colorstone, Germany). The experiment was started using fifty neonate daphnids, which were born within 12h, randomly chosen and equally distributed among four aquaria. One live *Triops* of 1 cm total length was put into each of two of the aquaria and was being fed 10 pellets of crushed fishfood (Grana Discus, JBL, Germany) daily. The same amount was also given into the control aquaria. Old pellets as well as feces were removed every other day. Upon death or reaching a body length of over 2 cm predators were replaced with new *Triops* larger than 1 cm. Daily, the daphnids were being fed 1 mg C L^{-1} of the green algae *Scenedesmus obliquus* and half of the medium was changed every four days. Sinking experiments were conducted after a large stable population of approximately 100 adult daphnids was established within each aquarium. The populations were then kept at this size in order to avoid crowding effects caused by high daphnid densities (Burns 2000; Tollrian et al. 2015).

Swimming efficiency

Thin PMMA cuvettes with internal dimensions of 108 x 144 x 9 mm (www.antstore.de, Berlin, Germany) were used as experimental vessels. A fitting PMMA septum was used to divide the cuvette into two equal partitions. The cuvette was then filled with 20°+/-0.5°C SSS-medium containing 0.5 mg C L^{-1} of *S. obliquus* and a daphnid from the *Triops* treatment randomly placed in one partition and daphnid from the control treatment of approximately similar size in the other. The cuvette was then taken into a climate chamber where it remained for 90 minutes at a temperature of 5+/-0.5°C and 40 minutes at 25+/-0.5°C respectively for acclimatization. Preliminary experiments showed that this time period was sufficient for both the water temperature to change to 5+/-0.5°C and 25+/-0.5°C respectively, as well as for the daphnids to habituate to the new temperature. Temperature was measured before and after the experiment to control for changes caused by the experimental procedure and did not change for more than 0.5°C. In order to induce vertical swimming behavior in the daphnids, the experiment was set up utilizing their positive phototaxis (see figure 1). The cuvette was placed on top of a transparent PMMA board with a light source (KL 1500 electronic, Schott, Germany) coming from below. Between board and cuvette, a white sheet of paper was used to diffuse the light and a black cardboard aperture was used to either let the light transmit into the cuvette or shut it off completely. On top of the cuvette, a cover made of dark cardboard with two slits (see figure 1) ensured that light coming from the top light source (Olympus ILP-1, Olympus Deutschland GmbH, Hamburg, Germany) was not reflected in the PMMA walls of the cuvette but only transmitted into the water filling it. This way, we avoided somersaulting/looping behavior, which preliminary tests showed to be caused by reflections.

At the beginning of each swimming trial, the bottom light source ('gathering light') was turned on and the aperture was removed (figure 1A). The daphnids then swam to the bottom and remained there, until the aperture was used to block the 'gathering light', while the top light ('swimming light') was turned on (figure 1B). After 5 seconds the intensity of the 'swimming light' was reduced by 50% and after the daphnids reached the water surface, the 'swimming light' was turned off.

This procedure was repeated for 30 minutes or until a maximum of four valid stroke series could be observed. Swimming behavior was filmed using a highspeed camera (i-SPEED 3, Olympus Deutschland GmbH, Hamburg, Germany) at 100 frames per second and a resolution of 1280 x 1024 pixel with a Sigma 50mm 2.8 EX DG Macro lens(Sigma Corporation, Kawasaki, Japan) at F8 from a distance of 32 cm, covering the whole cuvette on video. Only continuous series of four strokes in a vertical direction without pauses (no second antennae movement for more than 50ms) were used for analysis (see figure y). The first stroke was disregarded. For the each of the remaining three strokes body length (in pixels) at the beginning and the end, distance covered per stroke and swimming velocity of were measured in pixel and pixel per second respectively using a video analysis software (iSpeed Suite 3.0.2.9, Olympus, Germany). Strokes per second were calculated from the measured time of the series of three strokes. After the swimming experiment, each daphnid was frozen and preserved at -62°C for the sinking experiment and detailed morphological measurements. Using the microscopic measurements of body length, pixel values were then converted into metric values for each individual.

Sinking experiment

The sinking experiment was conducted using a 50 cm glas tube with an inner width of 6 mm and markings 10 cm from each end, defining a 30 cm sector within the tube. The preserved daphnids from the swimming experiments were carefully defrosted using ice-water and then brought to the specific temperature at which they were tested (5 and 25°C). Each single daphnid was then put into the tube, filled with SSS-medium of the corresponding temperature and the tube was then closed without remaining air. After the daphnid sunk to the bottom, the tube was turned 180° and hung completely vertical onto a hanger. Using a stopwatch, the time the daphnid took to pass through the sector of 30 cm was measured. This was replicated for each daphnid. The 10 cm before each measurement ensured that the daphnids reached their final sinking position and speed before the measurement was conducted.

Morphological measurements

Directly after conducting the sinking experiments, the daphnids were measured using a Leica MS 5 stereo-microscope (Leica Microsystems GmbH, Germany) connected to a digital camera (Altra 20, Olympus Soft Imaging Solution GmbH, Münster, Germany) in combination with the software Cell^P (Olympus Soft Imaging Solution GmbH, Münster, Germany). Measured parameters included body length (the top of the compound eye to the base of the tail spine), tail-spine length and body width (greatest dorso-ventral expansion).

Second experiment

Induction

The induction of the daphnids was in accordance to the first experiment, but with two clones. In addition to K34J, we used a second clone of *D. magna*, MAKE21, which originated from Kenia. Other changes compared to the first experiment were a daily feeding of 0.5 mg C L^{-1} of *S. obliquus*, the use of six aquaria for each clone (three with *Triops* and three without) and the use of three

predators per aquarium with a size larger than 10 mm but below 20 mm. *Triops* were being fed daily with larvae of non-biting midge (Chironomidae). The larvae were also put into the control aquaria and regularly exchanged.

Sinking experiment

The second sinking experiment was carried out at constant temperature conditions (20°C +/- 0.5°C), using 6 glas tubes with a length of 500mm and inner width of 10mm, filled with SSS-medium. In accordance to the first experiment, markings 100mm from each end defined a sinking distance of 300mm. The tubes were arranged within an apparatus, which made it possible to turn all six tubes at the same time along the same axis (see figure 2). Prior to the sinking experiments, the daphnids were anesthetized for 15min in 7% ethanol (p.a.), then 10min in 20% ethanol (p.a.). In the case the second antennae were retracted during that procedure, they were put back gently into the natural sinking position (uplifted) using a fine cactus needle. The anesthetized daphnids were then transferred into the glas tubes for the sinking experiments. After the daphnids reached the ground, the glas tubes were closed without remaining air. A video camera (Sony HDR-CX 550VE, Sony Corporation, Minato, Japan) was started to film at 50 fps in a resolution of 1280x720p and the apparatus was then turned 180° to start the experiment. Upon the daphnids reaching the ground, the apparatus was again turned 180° for four more times. Only daphnids that sank along their longitudinal axis with fully expanded second antennae throughout the whole distance and that stayed anesthetized during the experiment were taken into account for later analysis. If these daphnids carried brood, they were transferred into SSS-Medium containing 6-Well plates after the sinking experiment and brood chambers were flushed out using a syringe filled with SSS-medium. After the daphnids recovered, they were anesthetized again and the sinking experiment was repeated. At the end of the experiment, all daphnids were fixed in 70% ethanol (Black and Dodson 2003) and stored in Eppendorf tubes for morphological measurement in accordance to the first experiment and previous induction studies with *D. magna*. It is known that induced *D. magna* are larger than non-induced individuals (Rabus and Laforsch 2011; Rabus et al. 2011) and that larger daphnids sink faster than smaller ones. Therefore, in order to exclude this factor, induced and non-induced daphnids of the same size were used for analysis. Furthermore, three size classes were defined for K34J (1: smaller than 2150 µm; 2: smaller than 2650 µm; 3: larger than 2650 µm) and MAKE21 (1: smaller than 2100 µm, 2: smaller than 2600 and 3: larger than 2600 µm).

The recorded sequences were analyzed frame by frame using the software Avidemux 2.5.4 (MEAN, www.avidemux.org) to calculate the time each daphnid needed to cover the distance of 30 cm. The times of all valid sinking experiments were averaged for each daphnid. For those daphnids, that had their brood removed two separate averages were calculated (with and without brood).

Statistical analysis

The software PASW statistics 18.0 for Windows (SPSS Inc., USA) was used for statistical analysis of our data. A one-way ANOVA was performed, when data was normal distributed and homoscedastic, for all other cases Mann-Whitney-U-Tests were conducted.

Results

First experiment

Induction

The morphology showed an induction in accordance to the literature (Rabus and Laforsch 2011; Rabus et al. 2011), with elongated tail-spines of induced daphnids (19.95 +/- 0.008 % relative length) which were significantly longer than tail-spines of control daphnids (4.92 +/- 0.005 % relative length, ANOVA, N=87, $F_{1,85}$ = 345.116; p < 0,001). Additionally, body width was significantly larger in induced daphnids compared to control daphnids (Mann-Whitney U-Test, N=101, Z=2.507, p = 0.012, 68.33 +/- 0,40 % and 67.35 +/- 0.40 % respectively). As daphnids of approximately same size were chosen for this experiment, no significant differences were detected between the two treatment (Mann-Whitney U-test, Z=1.209 p = 0.303), although *Triops* induction is known to cause a larger body length in *D. magna* and controls were with 3134.78 +/- 40.43 µm on average slightly smaller than induced daphnids with 3203.55 +/- 46.06 µm.

Swimming experiment

Neither the stroke frequency at 5°C nor the stroke frequency at 25° C showed any significant differences between the two treatments (ANOVA, $F_{1,50}$ = 2.072; p = 0.156 and $F_{1,37}$ = 1.230; p = 0.274 respectively, table 1). Furthermore, the distance covered per stroke relative to body size showed no difference both at 5 and 25°C either (ANOVA, $F_{1,50}$ = 0.238; p = 0.628 and $F_{1,37}$ = 0.052; p = 0.820 respectively, table 1). In consequence, there were also no differences in the swimming speed relative to body size of induced and non-induced daphnids (ANOVA, $F_{1,50}$ < 0.001; p = 0.991 and F1,37 = 0.542; p = 0.466 respectively, table 1).

Sinking experiment

On average it took a control daphnid 58.46 +/-2.53 seconds and induced daphnids with 48.73 +/- 2.17 seconds approximately 16.6% less time to fall 300 mm at 5°C (figure 2). This difference was highly significant (ANOVA: $F_{1,49}$ = 8.577; p = 0.005). At 25° C, the induced daphnids fell with 34.94 +/- 0.87 again significantly faster than the control with 42.86 +/- 1.68 seconds (ANOVA: $F_{1,42}$ = 17.523; p < 0.001) (figure 2). The difference was slightly larger than at 5° C with the induced daphnids falling approximately 18.5% faster than the control (figure 2).

Second experiment

Induction and general parameters

Since pairs of same-sized control and induced daphnids were used in order to exclude size as a factor, differences in body size between the treatments were all non-significant (all p > 0.7). Daphnids of the clone K34J had a body length of 1818.30 +/- 53.27 µm (control)/1823.79 +/- 51.51 µm (induced) in size class 1, 2308.12 +/- 22.13 µm (control)/2303.40 +/- 21.97 µm (induced) in size class 2 and 2987.85 +/- 55.12 µm (control)/2977 +/- 55.58 µm (induced) in size class 3. Daphnids of the clone MAKE21 had a body length of 1888.09 +/- 31.68 µm (control)/1897.95 +/- 31.60 µm (induced) in size class 1, 2337.28 +/- 36.02 µm (control)/2341.63 +/- 36.35 µm (induced) in size class 2 and 2808.90 +/- 32.55 µm (control)/2804.58 +/- 35.28 µm (induced) in size class 3. In K34J only daphnids of the third size class (>2650 µm) possessed brood, whereas in

MAKE21 the second and third size class had eggs or larvae in their brood chamber. In all these cases, induced daphnids had a significantly larger number of eggs or larvae than control daphnids (table 2). In accordance to previously published induction experiments with K34J, body width of induced daphnids was significantly larger in induced compared to control daphnids (table 2). Induced daphnids of the clone MAKE21 only showed a significantly larger body width in the second size class, whereas in size class 1 induced daphnids were even significantly more narrow (table 2). The tail-spines were much more pronounced than in the first experiment with 58 - 73% of their body length in K34J, but especially in MAKE21, where induced daphnids reached relative tail-spine lengths of 72 - 91% of their body length (table 2), which is the largest tail-spine induction in *D. magna* so far. As reported in previous studies, differences in tail-spine length between induced and non-induced daphnids were highly significant in all size classes of both clones and relative length decreased with age (table 2).

Sinking experiment

In all three size classes of K34J, induced daphnids fell significantly faster than control daphnids (see table 2 and figure 3). With the exception of the first size class, the same was true for MAKE21 (see table 2 and figure 3). After the removal of the brood of daphnids from the third size classes of both clones, these differences between induced and non-induced daphnids remained both in quantity and quality (see table 2).

Discussion

In the first experiment, induced daphnids fell significantly and much faster than control daphnids, whereas no differences in the swimming efficiency could be detected. Neither at 5°C nor at 25°C did stroke distance, stroke frequency or swimming speed differ between the treatments. On the other hand, induced daphnids fell significantly faster than control daphnids at both temperatures. The difference was with 18.5% even larger at 25°C compared to 16.6% at 5°C and induced daphnids seemed to compensate for the sinking differences at both temperatures. This is in contrast to inducible defenses in *Eubosmina*, where the difference was larger at lower temperatures and affected swimming behavior (Lagergren et al. 2000). However, the found large difference in drag between induced and non-induced daphnids is in accordance to findings in *D. middendorfiana* and *D. pulex* (Dodson 1984). The faster sinking rate of induced daphnids can have two possible explanations. The first is, that induced daphnids have a lower water resistance and therefore sink faster, in other words the opposite of the 'parachute effect' (Woltereck 1913). The second is that induced daphnids have a higher density than non-induced daphnids, leading to a higher drag and thus faster sinking. A lower water resistance, for example coming from a more stream-lined body, could help daphnids by swimming faster in order to escape predators. Still, this would have an impact on the swimming efficiency, which could be either observed by a longer distance covered by each swimming stroke or a lower stroke frequency, without losing speed.

Interestingly, no difference in any parameter could be detected during the swimming experiment. It is well known, that kairomones can have an impact on the behavior in daphnids (Weber and Noordwijk 2002), especially in relation to phototaxis (Gool and Ringelberg 1998, 2002). However,

our experiment was designed in order to compare swimming efficiency, not natural swimming behavior. As such, the requirement was to induce a continuous series of swimming strokes in a completely vertical orientation. With the presented method, both induced and non-induced daphnids showed valid behavior. Consequently, the same stroke frequency, but a different swimming speed or vice-versa would have provided evidence for differences in swimming efficiency between the groups, e.g. through differences in hydrodynamic properties of the shape of the two morphotypes. On the other hand, mixed differences in both stroke frequency and swimming speed could have been more difficult to interpret. However, neither could be observed in the swimming experiment. The logical conclusion is that the difference in drag in *D. magna* is the result of an increased body density. A candidate for this increase is the carapace. It has been shown that induced *D. magna* fortify their carapace (Rabus et al. 2013), as do other species of *Daphnia* (Dodson 1984; Laforsch et al. 2004). This is achieved through thicker epidermal layers and more massive small pillars connecting them (Laforsch et al. 2004; Rabus et al. 2013). A calcification of the carapace is known in *Daphnia* (Porcella et al. 1969) and the lack of Ca++ seems to affect the fortification of the carapace in *D. magna* (Riessen et al. 2012). This would suggest that the fortification is at least partly achieved through calcification of the cuticule, which would have a strong impact on the density (Amato et al. 2008). Other factors, such as clutch size do not seem to be the primary cause of this difference or even contribute much. The results of the second experiment, which could show that the large difference in sinking speed even occurs in juvenile daphnids and for adult daphnids the difference is maintained after the removal of all eggs or larvae. Body width also does not seem to be directly causing the sinking speed differences, since the wider induced daphnids of size class1/MAKE21 showed no significant difference compared to control daphnids in sinking speed, whereas a clear difference was detected in size class 3 of the same clone, where induced daphnids did not show a significantly wider carapace. Although not tested within this experiment, tail-spine length also seems to be irrelevant for the sinking speed (Vega and Clausse 1998). Even without the results of the swimming experiment, it is clear that daphnids that do not somehow compensate for a higher sinking speed will not be able to maintain their position within the water column and sink to the ground. Therefore, it can be assumed, that these differences hold a relevancy in the field. However, Dodson estimated the resulting energetic costs for *D. middendorfiana* to be insignificantly low at around 0.1 % of the metabolic rate of a daphnid (Dodson 1984). To describe the energetic effort for a planktonic crustacean to maintain a vertical position, the weight in water is multiplied with the terminal sinking velocity (Spaargaren 1980). In our case, the sinking speed of an adult control daphnid (~3mm) is 0.006 m s^{-1}, whereas the weight in water has to be estimated. The most amount of material with a higher density than water in a daphnid should be composed of chitinous structures. The density of chitin ranges from 1.39 (pure α-chitin) to 2.34 g/cm^3 (100% calcificated chitin) (Amato et al. 2008). The amount of calcification in *D. magna* under non-limiting control condition is only 4% (Alstad et al. 1999), thus the density should be close to the lower limit around 1.43 g/cm³. This means for 1.43 g the weight in water is ~4.3 mN. In consequence for a *D. magna* with a size of around 3 mm and a dry weight of approximately 160 µg (Hessen et al. 2000; Bäumer et al. 2002), the weight in water should amount to approximately 481 nN, resulting in an

energetic effort of ~2.89 nW (P = 481 nN x 0.006 m s^{-1}) to stay in vertical position. Regular used values for the muscle efficiency in crustaceans are 20-25 % (Spaargaren 1980; Morris et al. 1985) so the total energy consumption for a 3mm *D. magna* should lie between 11.5 and 14.4 nW. The oxygen consumption rate of similar sized *D. magna* lies around 40 nmol/h (Bohrer and Lampert 1988; Bäumer et al. 2002), which converts (Elliott and Davison 1975) into ~5228 nW. In consequence, for non-induced *D. magna*, the costs for maintaining a vertical position lie between 2.2 and 2.75 ‰ of the total metabolism. The result of up to a maximum of 32% higher sinking rates in *Triops*-induced daphnids therefore equals only an increase of less than 1 ‰ in energy consumption. This is an extremely small amount of costs, even considering, that this does not include any horizontal movement. While the reason for the increase drag is likely an increase in density and thus body mass due to the fortification of the carapace, meaning additional costs should be inflicted by horizontalmovement as well, these costs should lie in the same order of magnitude or less. Thus, we deduce that the increased energetic costs of swimming caused by inducible defenses in *D. magna* only attribute to less than a few tenths of a per mill of the general metabolism. This is even one order of magnitude less than Dodsons estimation for *D. middendorfiana* (Dodson 1984). Thus, it seems unlikely that movement related costs are the reason for *D. magna* to exhibit inducible rather than constitutive defenses against *T. cancriformis*. Similarly, we expect movement-related costs of inducible defenses like body fortification to only attribute to a very small amount of energetic costs. However, since it is likely that the carapace fortification is achieved through calcification it seems more than possible that costs related to an upregulation, which is known to lead to physiological trade-offs (Findlay et al. 2009), can outweigh these movement related costs. Especially in environments with low-calcium concentrations, which negatively affect calcification (Alstad et al. 1999; Hessen et al. 2000) and carapace fortification (Riessen et al. 2012) these costs might be severe. Even more so, as only around 10 % of Ca can be regained during the molting process (Alstad et al. 1999) calcification related costs would persistently impact induced daphnids. Therefore, we conclude that further research should focus on direct developmental costs of the induction of defenses rather than their hydrodynamic properties in order to explain their plasticity.

Acknowledgements

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Tables

Table 1. Comparison of swimming parameters measured in experiment I.

Treatment	temperature	n	strokes/s	distance per stroke [μm]	velocity [μm/s]
control	5°C	27	5.045 ± 0.091	47.976 ± 2.571	245.949 ± 16.757
induced	5°C	25	5.257 ±0.117	46.283 ± 2.305	246.209 ± 15.509
control	25°C	19	9.075 ± 0.189	72.998 ± 1.559	664.395 ± 23.492
induced	25°C	20	9.580 ± 0.406	72.405 ± 2.040	700.215 ± 41.838

Table 2. Comparison of morphological traits and sinking times in experiment II. With the exception of clutch size in size class 2 of MAKE21, all traits were testes using Mann-Whitney-U-tests.

| trait	size class	treatment	n	value (K34J)	Z (K34J)	p (K34J)	n	value (MAKE21)	Z (MAKE21)	p (MAKE21)
relative body width	1	control	20	0.67 ± 0.007	Z = 2.705	0.006	20	0.66 ± 0.004	Z = -2.056	0.040
		induced	20	0.71 ± 0.12			20	0.62 ± 0.01		
	2	control	19	0.67 ± 0.009	Z = 4.219	0.000	19	0.66 ± 0.005	Z = 2.350	0.018
		induced	19	0.73 ± 0.006			19	0.67 ± 0.006		
	3	control	24	0.67 ± 0.005	Z = 5.815	0.000	20	0.66 ± 0.003	Z = 1.136	0.265
		induced	24	0.73 ± 0.005			20	0.67 ± 0.008		
relative tail-spine length	1	control	20	0.39 ± 0.013	Z = 5.410	0.000	20	0.37 ± 0.008	Z = 5.410	0.000
		induced	20	0.73 ± 0.012			20	0.91 ± 0.044		
	2	control	19	0.30 ± 0.009	Z = 5.270	0.000	19	0.30 ± 0.01	Z = 5.270	0.000
		induced	19	0.68 ± 0.01			19	0.77 ± 0.017		
	3	control	24	0.20 ± 0.008	Z = 5.588	0.000	20	0.22 ± 0.01	Z = 5.410	0.000
		induced	24	0.58 ± 0.012			20	0.72 ± 0.025		
clutch size	2	control					19	0.53 ± 0.31	$F_{1,36}$=16.124	0.000
		induced					19	4.21 ± 0.86		
	3	control	24	3.25 ± 0.42	Z = 4.839	0.000	20	4.70 ± 0.63	Z = 4.543	0.000
		induced	24	16.88 ± 1.7			20	10.35 ± 0.73		
sinking time	1	control	20	69.13 ± 4.02	Z = -3.273	0.001	20	66.69 ± 1.85	Z = -1.434	0.157
		induced	20	53.19 ± 3.19			20	64.85 ± 3.70		
	2	control	19	53.33 ± 2.61	Z = -3.547	0.000	19	62.04 ± 3.67	Z = -4.335	0.000
		induced	19	43.39 ± 1.49			19	43.45 ± 1.69		
	3	control	24	50.00 ± 0.93	Z = 4.660	0.000	20	49.37 ± 1.64	Z = -3.787	0.000
		induced	24	34.22 ± 2,47			20	40.11 ± 1.84		
sinking time, removed brood	3	control	10	51.90 ± 3.99	Z = -2.419	0.015	11	59.35 ± 2.90	Z = -3.273	0.001
		induced	10	38.53 ± 2.64			13	41.41 ± 2.80		

Figures
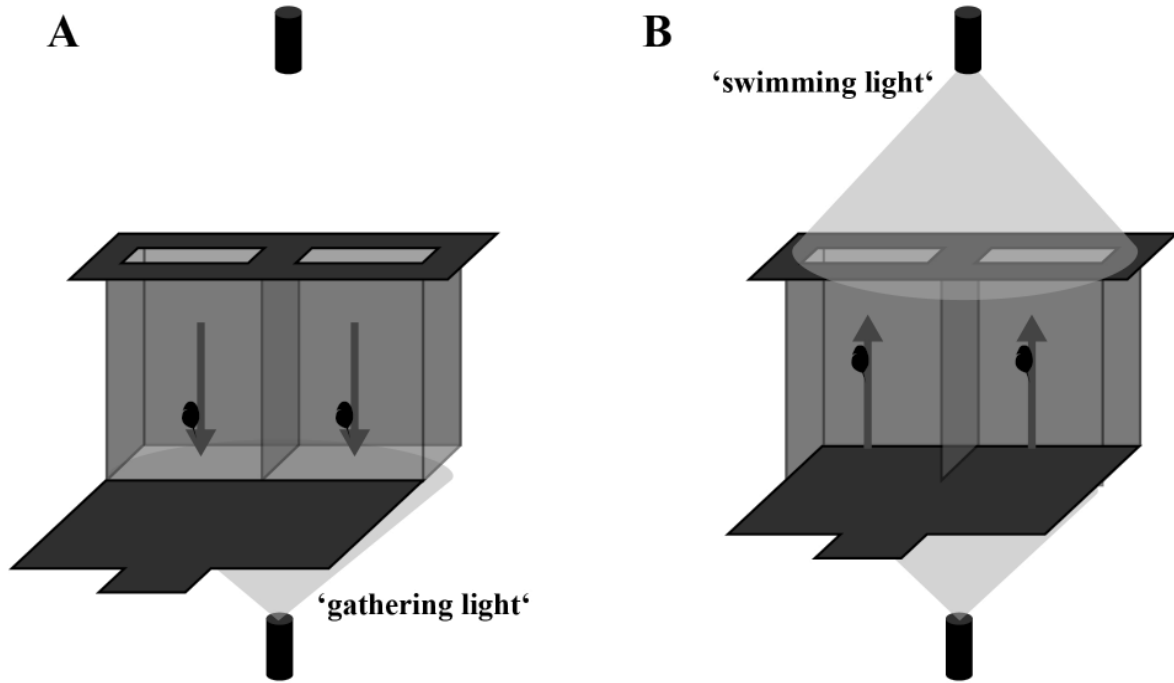
Figure 1. Apparatus used for the swimming experiment. A shows the gathering phase, in which the 'swimming light' was turned off, wheras the aperture was removed to allow the transmission of the 'gathering light'. In consequence, daphnids moved to and stayed on the bottom of the cuvette until settings were changed to B: The aperture was closed and the 'swimming light' turned on and after 5 seconds, its light intensity was reduced by half. Daphnids now immediately swam in straight lines to the top of the cuvette.
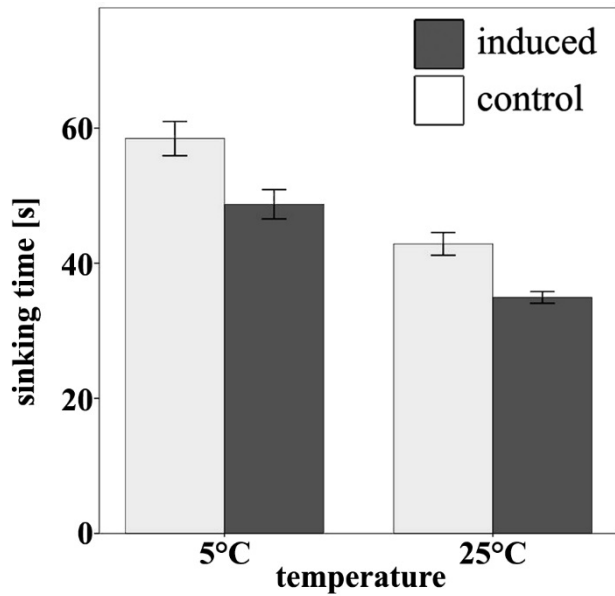
Figure 2. Comparison of mean sinking times between induced and non-induced *D. magna* at 5° C and 25° C. Error bars indicate +/- one standard error of mean.
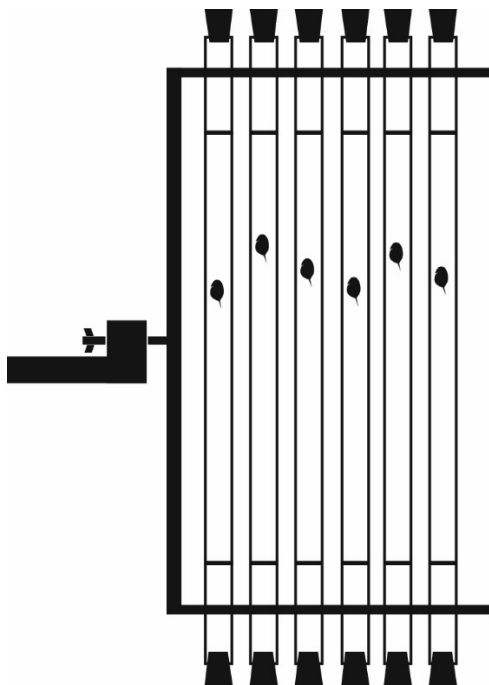
Figure 3. Sinking apparatus used in experiment 2. An array of six glass tubes was fixed into a mount, with which all tubes could be filmed and rotated along the same axis at the same time, allowing parallel and consecutive measurements of sinking times.
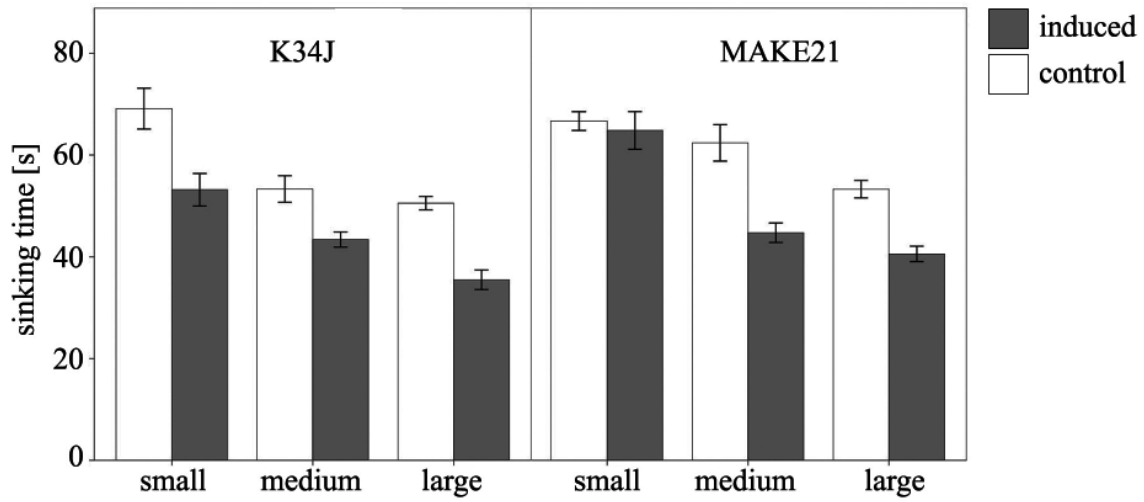
Figure 4. Comparison of mean sinking times between induced and non-induced *D. magna* of the clones K34J (left) and MAKE21 (right) for all three size classes. Error bars indicate +/- one standard error of mean.

CHAPTER VII:
DISCUSSION

CHAPTER
VII

Despite being supposedly a widespread phenomenon (Utz *et al.* 2014), reversibility of inducible defenses is still widely a neglected area of research. This is even more so the case within the genus *Daphnia*, in contrast to the numerous described inducible defenses. Consequently, the scope of this thesis was to identify reversibility and irreversibility of inducible defenses within this group of model organisms and to increase the understanding of factors contributing or inhibiting reversibility. This includes information about energetic and maladaptation costs as well as an assessment of the roles of ecological and physiological reasons for reversibility or its absence. Moreover, a detailed new framework for inducible defenses in multi-predator regimes and a novel study-system for predator related phenotypic plasticity in relation to physiological and ecological factors were introduced. The latter also includes the description of previously unknown defensive traits, most notably the 'twist', an abolishment of bilateral symmetry, and its reversibility in *D. barbata*.

7.1 Costs of inducible defenses

Costs of defensive traits in environments without the respective predator are essential for the evolution of both inducible defenses and their reversibility (Gabriel 1999; Gabriel *et al.* 2005; Utz *et al.* 2014). However, reversibility is promoted by costs that burden the prey organism after predation has decreased or changed (Utz *et al.* 2014), e.g. running or maladaptation costs. The higher these costs are, the likelier does reversibility occur. Such costs could potentially be energetic costs that have to be paid continuously in order to maintain a defense. Since cyclomorphosis has originally been thought to affect the hydrodynamic properties of daphnids (Wesenberg-Lund 1900), such running costs could potentially be caused by inducible defenses affecting the swimming ability and efficiency respectively. However, as shown in chapter VI, while large differences in sinking rates were indeed found between non-induced *D. magna* and induced specimens, the resulting energetic costs only lie in the order of less than a per mill of the whole energy consumption rate of a daphnid. This is in accordance to similar estimations in *D. middendorfiana* and *D. pulex* (Dodson 1984) and consistent with other studies finding either no or extremely low costs for inducible defenses in *Daphnia* (Dawidowicz & Loose 1992; Tollrian 1995; Hammill, Rogers & Beckerman 2008). Moreover, induced *D. magna* fully compensated for the differences in sinking rates and showed no decrease in swimming efficiency. Therefore, an impairment of the movement was not observed in *D. magna*. Based on these findings, it seems improbable that running costs related to swimming exert a strong selection pressure for the reversion of inducible defenses, at least in *D. magna*. It seems more plausible that other factors would have to contribute to evolution and sustenance of reversibility. Since a fortified carapace or an elongated tail-spine are built anew with each molt (Porcella, Rixford & Slater 1969; Halcrow 1976), buildup and growth related costs are to be expected, but have not been measured so far. Alternatives or additions to these physiological running costs are ecological maladaptation costs like survival trade-offs, which can be severe in systems with multiple and changing predators (Benard 2006; Hoverman & Relyea 2009). The

induction experiment in chapter II revealed that *D. barbata* possesses different defenses against two predators. Nevertheless, survival trade-offs could not be found. Instead, both defenses offered protection against both predators. For *Triops*-induced daphnids this protection was less efficient compared to the matching defense, when exposed to *Notonecta*-predation. In contrast, both, *Notonecta*- and Triops-induced morphotypes showed no differences in their efficiency against *Triops* predation. This means that maladaptation costs exist for *Triops*-induced morphs, when exposed to *Notonecta*, but not the other way around. Consequently, higher costs for the establishment and maintenance of the *Notonecta*-induced defense have to exist, else there would only be one general defense. Comparisons of lifetime reproductive success (the cumulative number of offspring produced within the lifetime of an organism (Hammill *et al.* 2008)) in combination with the survival rate could to allow for a better assessment of the cost/benefit relationship of the two defensive morphotypes in the future. However, without field data on the composition, characteristics and changes of the predation regimes *D. barbata* faces under natural conditions, any assessment of actually experienced costs will be constrained. So, while costs could be identified and estimated, further investigations are needed for a complete picture.

7.2 Reversibility in *Daphnia*

Reversibility of inducible defenses in *Daphnia* is still a largely neglected topic. So far, only a small transfer experiment suggests the reversibility of neckteeth in juvenile *D. pulex* (Vuorinen I. *et al.* 1989). Apart from this record, there is only the more substantial evidence of reversibility of clutch size alterations in response to fish predation in *D. magna* (Mikulski *et al.* 2005) and the generally reversible diel vertical migration, a migration to deeper water layers that is reversed daily (Lampert 1989; Beklioglu *et al.* 2008). Consequently, the findings within this thesis add to the spare records of reversibility in this genus and provide the first records of reversibility of morphological defenses for adult *Daphnia* in general. Despite the lack of apparent high running or maladaptation costs in either *D. magna* or *D. barbata*, it could be revealed, that both species show highly reversible traits. In the case of the morphological defenses of *D. barbata* many parameters, such as tail spine curvature, body torsion and dorsal ridge width showed reversibility, whereas helmet related traits could be considered mostly stable. In *D. magna* both morphological and life history defenses were either reduced after the removal of predator cues (body growth, absolute tail spine length) or even changed to control levels (clutch size, relative tail spine length). Furthermore, the changes in both species occurred extremely fast within only a few molts. Hence, the reversion of inducible defenses occurs in a similar timeframe compared to the parthenogenetic production of adult offspring, adapted to the new environment (~ six days for *D. magna* and ~7-9 days for *D. barbata*). This shows that reversibility holds importance even for organisms with fast clonal reproduction. Additionally, the results from *D. barbata* suggest that the predator modality matters for the reversibility as much as for the induction of defenses. Using the multi-predator-prey system of

Notonecta-Triops-D. barbata, it was possible to bring reversibility or the lack thereof in direct connection with either physiological (trait related) or ecological constraints (predator related). The predator specific responses in the reversibility experiment with *D. barbata* have revealed surprising results. The defense against *Notonecta* showed no reversion of induced traits and even continued helmet and tail spine growth after the predators were removed. On the other hand, the defenses against *Triops* showed high reversibility for most traits. Only helmet length did not revert, but did reduce its further growth. This hints at physiological limitations regarding a reversion of helmet structures. However, apart from this exception, it seems that ecological factors (predator species) hold a higher relevancy for reversibility in *D. barbata* than physiological factors. As mentioned, the results from chapter II show that the *Notonecta*-defense offers best protection against both predators. This indicates that this defense is costlier than the more specialized defense against *Triops*, otherwise the latter would provide no benefit over a general defense. However, if the *Notonecta*-induced defense was costlier, the benefit by reverting it could also be higher. Nevertheless, none of the traits showed reversibility, not even the elongated microspines along the dorsal ridge, which reverted in the *Triops* morph. This emphasizes that neither costs or physiological limitations are sufficient to explain reversibility. In accordance to the conditions for inducible defenses to evolve (Harvell & Tollrian 1999), information quality and heterogeneity of predation risk is essential. The lack of finding reversibility in the *Notonecta*-induced morph is likely attributed to one of the two or even both. It is possible, that the disappearance of *Notonecta* cues is not a reliable sign for an end of the threat, since *Notonecta* can fly and recolonize the habitat (Ward & Blaustein 1994; Wilcox 2001). Another possibility is that the *Notonecta*-induced morph is protected against many predators, similar to the also helmet bearing *D. cucullata* (Laforsch & Tollrian 2004b), and the chances are high that other predators fill the gap once *Notonecta* leaves. However, another explanation could be, that the disappearance of *Notonecta* is an event, that simply does not occur under usual natural conditions, unless the habitat itself (and with it all daphnids) disappears. Since two induced morphotypes of one clone already show extreme differences in reversibility, a general conclusion about reversibility in *Daphnia* seems far-fetched. However, it could be shown that the reversibility of morphological traits related to the dorsal ridge (which only *Ctenodaphnia* possess) and tail-spine is physiologically possible, just not necessarily advantageous, while limitations seem to exist for helmet-like structures. As both studied species, *D. magna* and *D. barbata*, reversed defenses against *Triops*, it seems likely, that other species, which induce dorsal ridge or tail-spine related defenses in response to this predator, for instance *D. atkinsoni* (Petrusek *et al.* 2009), also possess the ability to at least partially revert their induced traits.

7.3 The importance of multi-predator regimes and the introduction of a new model system

Most studies regarding inducible defenses focus on only one predator. In contrast, most prey organisms are exposed to multiple predators (Lima 1992), which can co-occur, but also differ in their timing of arrival and change in densities (Olito & Fukami 2009; Stier *et al.* 2013). To understand inducible defenses, including their reversibility, it is necessary to understand these complex relationships between prey organisms and varying predation regimes. Every predator causes a selection pressure on their prey, but the direction in which it influences evolution can differ. Even one and the same predator can change the quantity of its predation pressure throughout its ontogeny (Murdoch & Scott 1984; Murdoch, Scott & Ebsworth 1984; Scharf, Juanes & Rountree 2000) or the quality by changing foraging modes (Hirsch, Cayon & Svanbäck 2014). Predators are not simply present or not, they change and with them all the related qualitative differences in selection pressure. This 'modality' has often been left out of the focus or has only been discussed within the specific studied system. Thus, a general concept and clear classification for a broader context was developed within this thesis. The implications modality has for the evolution of prey organisms have been pointed out within this new framework introduced in chapter II. Through the vectorized visualization of the selection pressures cause by predation, these differences become apparent. Depending on the qualitative differences in the modality of predators, three categories have been defined: functionally equivalent (selection pressure in the same direction), functionally inverse (selection pressure in opposing directions) and functionally diverse (selection pressure in different directions). Whether a multi-predator-system falls into one or another category and whether the predators co-exist or follow subsequently, costs, benefits and optimal responses of prey organisms differ. The assessment of predator related cost/benefit-relationships of plasticity should be simplified and improved following the novel concept. This conceptualization also allows for a better comparability of different multi-predator-prey systems across different taxa or ecosystems.

Results from two studies on *D. barbata* (chapter II and IV) have emphasized this importance of modality, even without high maladaptation costs like survival trade-offs (Benard 2006; Hoverman & Relyea 2009). *D. barbata* with its predators *Notonecta* and *Triops* provides a rare case of a functionally overlapping system (a subcategory of functionally diverse). Since *D. barbata* possesses predator specific inducible defenses that are based on the same structures, but modified in a different shape - or to a different extent - it became possible to differentiate between ecological and physiological factors influencing the plasticity in chapter V. Furthermore, it has pointed out, that using larger numbers of traits shows benefits regarding the analysis of costs of plasticity (Relyea 2002). Consequently, research regarding the evolution on inducible defenses benefits from the large number of inducible traits found in *D. barbata*. Some of them are also found in other species of *Daphnia*, i.e. the formation of helmets (e.g. (Brooks 1946, 1965; Dodson 1988;

Laforsch & Tollrian 2004a) or elongated tail-spines (e.g. (Dodson 1984; Swaffar & O'Brien 1996; Kolar & Wahl 1998; Rabus & Laforsch 2011; Engel *et al.* 2014). However, many others of the described traits add up to the already large list of morphologic defenses within this genus. This includes a widening of the dorsal ridge lined with elongated microspines, somewhat similar to the crown-of-thorns in *D. atkinsoni* (Petrusek *et al.* 2009), but without the extension of lobes. It further spans to the change in orientation (angle) of helmet and tail-spine and even to a curvature of the latter. However, from all these previously undescribed induced traits, the most noteworthy is the body torsion, a completely new type of an inducible defense, previously unknown within *Daphnia*. This plastic response, which we termed the 'twist', is characterized by an abolishment of bilateral symmetry and expressed during exposition to chemical cues from the predator *Triops* (see chapter III and IV), but are reverted, when predator cues vanish (chapter IV). A loss of body symmetry is generally a rare phenomenon in bilateria, but known for example in gastropods (Pennington & Chia 1985), flatfish (Brewster 1987), scale-eating cichlid fish (Hori 1993; Kusche, Lee & Meyer 2012) and fiddler crabs (Morgan 1923). As plastic response to predation body asymmetry has only been found in the sessile barnacles (Lively *et al.* 2000) and rotifers (Gilbert 2011) but *D. barbata* shows a previously unknown complete loss of a body axis. Furthermore, the ability to reverse this drastic change in morphology shows a flexibility to an even larger degree. Interestingly, the body asymmetry in *D. barbata* as well as the complete *Triops*-induced morphotype has never been described through field research, while records exist for the control morph (Korinek 1984) and the *Notonecta*-induced morph (Benzie 2005) records. This means, that with the exception of the hidden defense of carapace fortification (Laforsch & Tollrian 2004a), this is the first time morphological defenses in *Daphnia* have been discovered in the laboratory.

Finally, in consideration of the high number of easily measurable defenses, the predator specific responses in induction and reversibility, and the trait specific flexibility, I highly recommend this species as a model system for the evolution of reversible and irreversible inducible morphologic defenses.

7.4   Outlook

In order to gain a picture, whether reversibility is as common a phenomenon as suggested by theoretical studies (Utz *et al.* 2014) and to test the hypothesis that other species of *Daphnia* coexisting with *Triops*, like *D. atkinsoni* (Petrusek *et al.* 2009) or *D. brooksi* (own observation) exhibit the ability to reverse their defenses, more species have to be tested. Additionally, reversibility in relation to other known daphnid-predators, such as different species of *Chaoborus* (Riessen & Trevett-Smith 2009), fish (Kolar & Wahl 1998; Weber 2003; Engel *et al.* 2014), *Leptodora*, *Cyclops* (Laforsch & Tollrian 2004b) or others is needed to be looked into further.

Regarding a general understanding of the evolution of inducible defenses and their reversibility, *D. barbata* could act as model system. As it was shown in chapter IV, by being able to study questions

related to inducible morphologic defenses in dependency of both trait characteristics and predator characteristics within one species and even within one genotype, some questions can be better addressed than by comparison of different species. This offsets the apparent disadvantage of a low number of offspring in *D. barbata*, especially compared to larger species like *D. magna*. Still, further steps are needed for a concrete establishment of this system. Especially field studies to fill the lack of knowledge about the habitats, predator regime composition and transitions etc. are needed. The paradoxon of the possession of two specific defensive morphotypes instead of a general defense is still not completely solved. Predation trials suggested the *Notonecta*-induced morphotype as optimally defended against both tested predators. On the other hand, the same defense remained stable, even with the removal of predator cues. If it was costlier, the opposite would have been expected. While this could be attributed to differences in information quality and the heterogeneity of the specific predation risk, conventional predation trials are inherently artificial and should consequently not be used as the sole basis for the assessment of the adaptive value of a defense. A clarification of the concrete defensive mechanisms against each predator is needed. Unfortunately, the mechanisms of morphological defenses of *Daphnia* in general are still mostly speculated. In order to allow for an observation and analysis of the feeding mechanism of *Triops*, I developed a new, minimal-invasive method together with Stefan Bindereif (Bindereif 2014). This method could not only be used to identify the mechanism of the different defenses, but also allow for a better differentiation in the efficiency of both defensive morphotypes.

In addition to a better understanding of the functioning of the defenses, the most important step is an expansion of the research to the field. So far there is a lack of clear information about the predation regimes *D. barbata* is exposed to under natural conditions. A characterization of the predation-regimes *D. barbata* encounters, is essential for the understanding of both induction and reversion of defenses. Spatial and temporal distribution, abundance and composition of predators are likely to show large variations between different habitat-types. For example, *Triops* populates temporary ponds (Takahashi 1977; Turki & Turki 2010) and not larger permanent water bodies, such as dam lakes, where *D. barbata* also occurs and is described with a morphotype matching the *Notonecta*-defense (Benzie 2005). This incorporation of concrete field data about spatial and temporal predation regime heterogeneity generally holds a large importance, also for other inducible defense systems. Furthermore, since clonal differences in morphological defenses are known for other species of daphnids (Rabus *et al.* 2011), an investigation of clonal differences in induction and reversibility of defenses in dependency to the encountered predator regimes might provide interesting insights. The resulting implications would not only allow for a better description of the system itself, but lead to a detailed understanding of the mechanisms underlying the evolution of phenotypic plasticity in general. So, while the reversibility of morphologic defenses in the genus *Daphnia* could finally be described and foundations have been laid out, an increased attention in this field of study (especially studies in the field) is needed to understand its prevalence and ultimate causes.

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Author contributions

Q. Herzog and C. Laforsch, 2013

Q. HERZOG and C. LAFORSCH designed the experiment. Q. HERZOG conducted the experiment, analyzed data and developed the concept. C. LAFORSCH provided methods and materials. Q. HERZOG wrote the first draft of the manuscript and C. LAFORSCH contributed substantially to revisions. Both authors read and approved the final manuscript.

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Q. HERZOG and C. LAFORSCH conceived and designed the experiments. Q. HERZOG performed the experiments. M. RABUS analyzed the data. C. LAFORSCH contributed reagents/materials/analysis tools. M. RABUS, Q. HERZOG, B. WOLFSCHOON-RIBEIRO and C. LAFORSCH wrote the paper. M. RABUS and B. WOLFSCHOON-RIBEIRO performed morphological measurements.

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Q. HERZOG, M. RABUS and C. LAFORSCH conceived and designed the experiments. Q. HERZOG performed the experiments and measurements. Q. HERZOG and M. RABUS analyzed the data. C. LAFORSCH contributed reagents/materials/analysis tools. Q. HERZOG, M. RABUS and C. LAFORSCH wrote the paper.

Q. Herzog, R. Immler, M. Sternhardt and C. Laforsch (manuscript to be submitted to Journal of Plankton Research)

Q. HERZOG, R. IMMLER, M. STERNHARDT and C. LAFORSCH designed the experiment. M. STERNHARDT conducted experiment I, R. IMMLER conducted experiment II. Q. HERZOG conducted the video measurements of swimming efficiency in experiment I. Q. HERZOG analyzed data and C. LAFORSCH provided materials. Q. HERZOG wrote the first draft of the manuscript, C. LAFORSCH and R. IMMLER contributed substantially to revisions. All authors read and approved the final manuscript.

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QUIRIN HERZOG

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Eidesstattliche Erklärung

Ich versichere hiermit an Eides statt, dass die vorgelegte Dissertation von mir selbständig und ohne unerlaubte Hilfe angefertigt ist.

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<center>(Unterschrift)</center>

Erklärung

Hiermit erkläre ich, ∗

X   dass die Dissertation nicht ganz oder in wesentlichen Teilen einer anderen Prüfungskommission vorgelegt worden ist.

X   dass ich mich anderweitig einer Doktorprüfung ohne Erfolg **nicht** unterzogen habe.

☐   ~~dass ich mich mit Erfolg der Doktorprüfung im Hauptfach~~ ....

~~und in den Nebenfächern~~ ....

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~~unterzogen habe.~~

☐   ~~dass ich ohne Erfolg versucht habe, eine Dissertation einzureichen oder mich~~ ~~der Doktorprüfung zu unterziehen.~~

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∗) Nichtzutreffendes streichen