Triops-induced morphological defences in *Daphnia magna*:

Combining large scale-, micro- and ultrastructural defences

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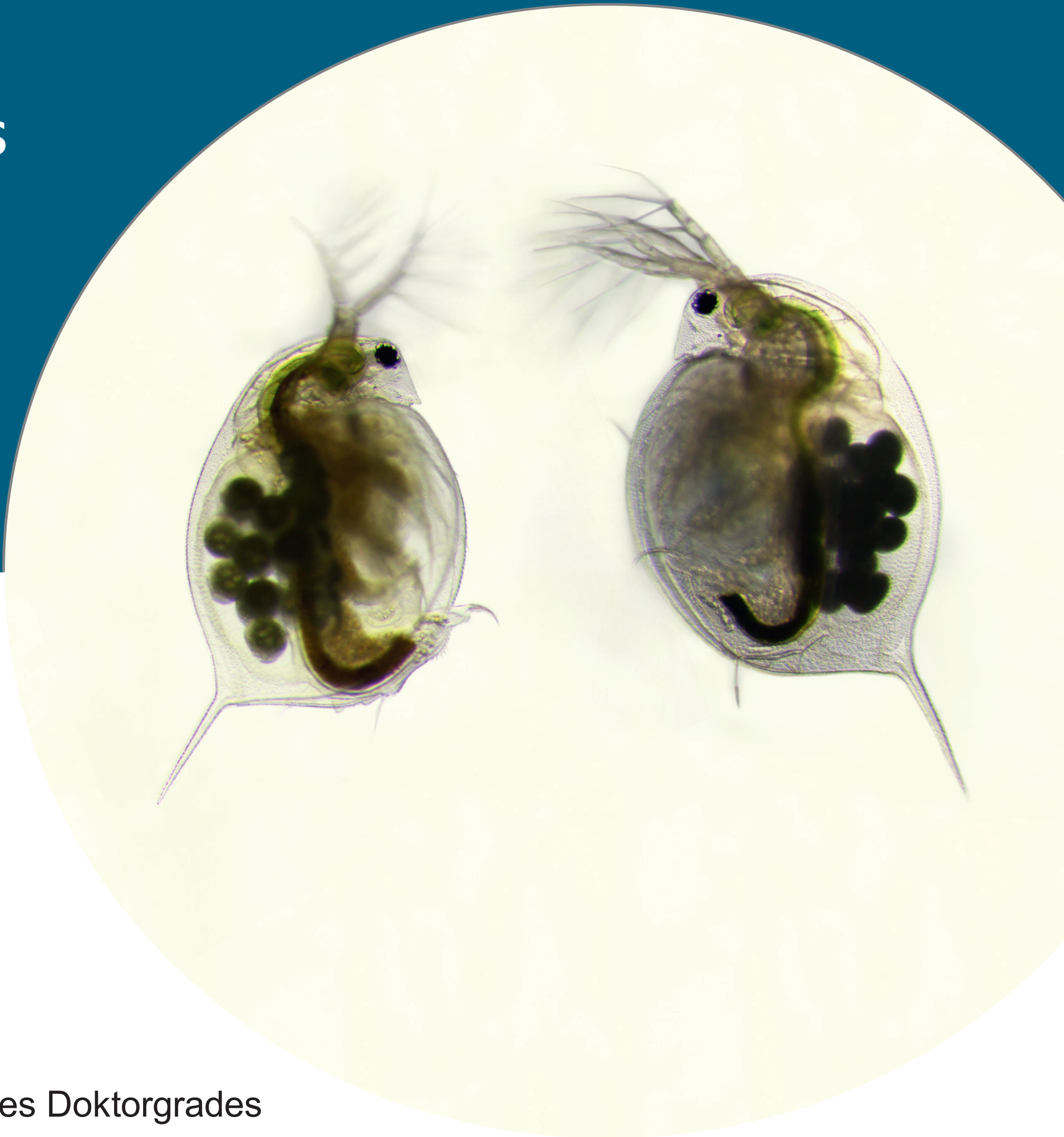

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Summary

In nature, predation plays an important role as one of the major drivers of natural selection. To cope with this stressor, a huge variety of anti-predator defences has evolved in prey species. If predation pressure is unpredictable and temporally variable, the evolution of inducible defences is favoured. Such phenotypic plasticity in defensive traits, which includes defences on the level of behaviour, life history and morphology, can be found in almost all taxa, ranging from bacteria to vertebrates. Inducible morphological defences typically alter the body shape and -size of the prey to impede handling by the predator, rendering the induced individuals less susceptible to being preyed upon. A textbook example for the study of inducible morphological defences is the cladoceran genus *Daphnia*.

In this thesis, I show for the first time that *Daphnia magna* responds to the presence of the predatory tadpole shrimp *Triops cancriformis* with the formation of a complete array of morphological defences. This is of special interest because *D. magna*, an important model organism in life sciences, was almost exclusively known for the expression of behavioural and life history defences rather than morphological responses. In *Triops*-exposed individuals, the most conspicuous change is the expression of a bulkier morphotype, a not yet described form of morphological defence in *Daphnia*. This bulkiness is based on a considerable increase in both, body length and -width, accompanied by a significant elongation of the tail spine and the development of a more pronounced shoulder shield. Although a clonal comparison reveals interclonal variation in the expression of the single defensive traits in *D. magna*, the *Triops*-induced bulkiness can be considered to be the general response to this particular predator. The results of a life cycle experiment further demonstrate that *D. magna* shows a gradual switch from easy-to-build start-up defences in juveniles, i.e. the longer tail spine, to the pronounced bulkiness in adults. This switch of the defensive traits enables *D. magna* to maintain an effective protection throughout its entire life span. The effectiveness of the induced defence, which is confirmed by predation trials, can be explained by the gape-limitation of *Triops*: Since the size of the midventral food groove, which is used to catch and transport the prey, and the opening width of the mandibles are restricted, the altered morphology of the induced daphnids should cause severe handling difficulties for the predator. Consequently the prey's chance to escape and survive the attack increases. The results of the predation trials are further supported by a long-term mesocosm study that shows a positive correlation between *Triops*-density and the intensity of the expression of the defensive traits. This indicates that *Triops* preferably preys upon less defended individuals, likely leading to a prevalence of clones with the ability to express very pronounced defences that enable them to coexist with the predator. Therefore, this result further supports the hypothesis that *Triops*, in its role as top predator and ecosystem engineer, acts as a strong agent in structuring pond communities.

Furthermore, I provide rare evidence for the existence of predator-induced small-scale and ultrastructural, “hidden” defences in Daphnia, which are expressed in addition to the prominent large-scale defences. Hence, my findings contribute to the growing awareness of these scarcely studied defences which are likely to play an important role in planktonic organisms. In *Triops*-exposed *D. magna*, both an approximately 50% increase in the length of the small spines, the so called spinules, along the dorsal ridge of the carapace, and a significant enlargement of the spinule bearing areas on the dorsal ridge and the ventral carapace margins could be observed. This increased spinescence of the induced individuals can be assumed to considerably impede the handling of the defended morph by interfering with the numerous fine cuticular structures in the feeding apparatus of *Triops*.

An interdisciplinary approach was used to assess the expression of ultrastructural defences in *D. magna*. Therefore, I combined classical microscopy techniques, i.e. optical microscopy and transmission electron microscopy, with atomic force microscopy, a state-of-the-art tool in material- and nanosciences, which I adapted to analyse the mechanical properties of the exoskeleton in *Daphnia*. Thereby, I reveal that *D. magna* expresses additional "hidden" defences in response to *Triops*. Compared to control individuals, the induced individuals show a two-fold increase in cuticle thickness and an approximate 10% increase in the diameter of pillars that connect the outer and inner cuticle layer of the carapace. The probably most striking change, however, is the five-fold increase in the elastic modulus, i.e. the hardness or rigidity, of the cuticle in predator-exposed *D. magna* which could be detected using atomic force microscopy based nanoindentation. Given the fact that *Triops* has to grasp and crush its prey prior to ingestion, these changes in the architecture of the daphnids' exoskeleton should provide considerable protection against being pierced and crushed by the predator's mouthparts. Together, the small-scale and ultrastructural defences can be assumed to synergistically add to the protective function of the increased bulkiness in *Triops*-exposed individuals.

With the help of this technique, carapace strength in Daphnia could be used as an indicator for the effects of global change, i.e. human driven climate warming and acid deposition, which are regarded as severe problems in aquatic ecosystems. As currently observed in many softwater lakes around the world, acidification alters biogeochemical cycles in lakes and ponds, i.e. it lowers calcium carbonate saturation states, and as a consequence the carbonate ion availability for calcifying aquatic organisms decreases. Since calcium is a major component of the crustacean zooplankton exoskeleton, decreasing calcium levels are supposed to have severe effects on crustacean zooplankton populations which may result in a major loss of biodiversity. Within the framework of a collaboration, I use atomic force microscopy to show that decreased calcium levels render specimen of *D. pulex* unable to express the fortification of the exoskeleton that usually accompanies the formation of neckteeth induced by larvae of the phantom midge *Chaoborus*. Together with the likewise impeded ability to express the large scale defences, i.e. neckteeth and an increased body size, the lack of structural defences increases the daphnids vulnerability to *Chaoborus*-predation and may thus explain the ongoing loss of *Daphnia* in habitats with declining calcium levels.

To conclude, my findings do not only provide new insights in the ecology of the model organism *D. magna* but also establish a new predator-prey-system based in temporary waters, the habitat *D. magna* and *Triops* share. In contrast to the well-studied lake ecosystems, the ecology of temporary ponds has just started to gain attention during the last few years. Due to the special characteristics of temporary waters and the resulting implications for their inhabitants, the study of this ecosystem may help to foster our understanding of several ecologically and evolutionary relevant processes such as rapid evolution and local adaptation. By introducing a novel and, above all, relatively accessible technique to test for the existence of ultrastructural defences in *Daphnia*, this thesis may stimulate further research on the distribution and the chemical and physiological basis of this presumably ubiquitous defence mechanism in crustacean zooplankton. As it becomes evident that several types of defensive morphological traits often act synergistically, the inclusion of small-scale and ultrastructural defences may help to reveal the exact mechanisms that underlie the effectiveness of the induced defences. And finally, the discovery of distinct, predator-induced morphological defences in *D. magna*, together with the current sequencing of its genome by the *Daphnia* Genomics Consortium, render the *D. magna - T. cancriformis* - system perfectly suitable for the study of the molecular mechanisms underlying the expression and evolution of phenotypic plasticity in defensive traits. Hence, my findings may contribute to a better understanding of the mechanisms governing the ecological interactions and evolutionary dynamics in predator-prey-systems.

Zusammenfassung

Als einer der bedeutendsten Selektionsfaktoren spielt Prädation in der Natur eine wichtige Rolle. Als Reaktion auf diese Bedrohung haben Beuteorganismen eine enorme Vielfalt an Verteidigungsmechanismen entwickelt. Ist die Bedrohung durch den Räuber dabei unvorhersehbar und zeitlich variabel, so werden induzierbare Verteidigungen ausgebildet. Diese stellen eine Form der phänotypischen Plastizität dar und finden sich von Bakterien bis hin zu Vertebraten bei fast allen Taxa und beinhalten Veränderungen auf Ebene des Verhaltens, der Life History und der Morphologie. Induzierbare morphologische Verteidigungen dienen typischerweise dazu, die Körperform bzw. –größe so zu verändern, dass die Handhabung durch den Räuber erschwert wird und somit die Überlebenswahrscheinlichkeit für die Beute gesteigert wird. Wasserflöhe der Gattung *Daphnia* stellen dabei ein Musterbeispiel für das Studium der induzierbaren Verteidigungen dar.

Im Rahmen dieser Arbeit zeige ich erstmals, dass *Daphnia magna*, ein wichtiger Modellorganismus in den Lebenswissenschaften, auf die Anwesenheit des räuberischen Kiemenfußkrebses *Triops cancriformis* mit der Ausbildung einer distinkten Reihe von morphologischen Verteidigungen reagiert. Dies ist von besonderem Interesse, da *D. magna* bisher fast ausschließlich dafür bekannt war, in Anwesenheit von Prädatoren Verteidigungen auf Verhaltens- und Life History-Ebene auszubilden, jedoch keine spezifischen morphologischen Verteidigungen. Bei *Triops*-exponierten Individuen stellt die Ausbildung einer sperrigeren Körperform, der sogenannten „Bulkiness“, die auffälligste Veränderung dar, wobei es sich um eine bei *Daphnia* bisher noch nicht beschriebene Form der morphologischen Verteidigung handelt. Diese “Bulkiness“ basiert auf einer deutlichen Zunahme von Körperlänge und –breite, die durch eine signifikante Verlängerung des Schanzstachels sowie einer stärkeren Ausprägung des Schulterschildes begleitet wird. Die Ausprägung der einzelnen Verteidigungsmerkmale kann dabei zwar zwischen Klonen von *D. magna* variieren, dennoch kann die *Triops*-induzierte „Bulkiness“ als allgemeine Reaktion auf diesen Räuber angesehen werden. Des Weiteren zeigen die Ergebnisse eines Lebenszyklus-Experiments, dass *Triops*-exponierte *D. magna* im Laufe ihres Lebens einen schrittweisen Wechsel in der Expression der Verteidigungsmerkmale vollziehen. Dabei wird die bei juvenilen Individuen besonders stark ausgeprägte Verlängerung des Schwanzstachels graduell durch die bei adulten Individuen immer stärker ausgeprägte „Bulkiness“ ersetzt. Dies ermöglicht den induzierten Daphnien während ihrer gesamten Lebensspanne eine effektive Verteidigung gegen *Triops* aufrechtzuerhalten, was mittels Prädationsexperimente bestätigt wird. Die Wirksamkeit dieser Verteidigung kann dabei durch die Größenlimitierung von *Triops* erklärt werden: Da sowohl die Größe der ventralen Nahrungsrinne als auch die Öffnungsweite der Mandibeln limitiert ist, sollte die veränderte Morphologie der induzierten *D. magna* die Handhabung durch den Räuber deutlich erschweren und damit die Wahrscheinlichkeit erhöhen, der Attacke zu entkommen und diese zu überleben. Die Ergebnisse der Prädationsexperimente werden außerdem durch ein Mesokosmos-Experiment unterstützt, welches eine positive Korrelation zwischen der Individuendichte von *Triops* und dem Ausprägungsgrad der Verteidigungsmerkmale bei *D. magna* zeigt. Diese Beobachtung lässt darauf schließen, dass *Triops* bevorzugt weniger stark verteidigte *D. magna* erbeutet und dadurch primär die Klone dominieren, die in der Lage sind eine stark ausgeprägte Verteidigung auszubilden. Dieses Ergebnis bestätigt somit auch die Hypothese, dass *Triops* als Ökosystemingenieur und Spitzenprädator einen starken Einfluss auf die Zusammensetzung und Struktur von Lebensgemeinschaften in temporären Tümpeln ausübt.

Des Weiteren zeige ich in meiner Arbeit die Existenz von zusätzlich zu den prominenten morphologischen Verteidigungen ausgebildeten Mikro-Verteidigungen und strukturellen, „versteckten“ Verteidigungen bei *D. magna*. Dadurch tragen meine Ergebnisse substantiell zu dem wachsenden

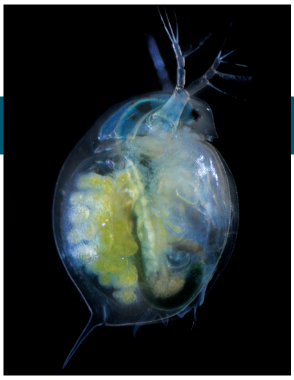
Bewusstsein für diese bisher kaum untersuchten Verteidigungen bei, die bei Planktonorganismen vermutlich eine wichtige Rolle spielen. So zeigen *Triops*-exponierte *D. magna* im Vergleich zu Kontrolltieren eine Längenzunahme der kleinen Stacheln entlang der Dorsalleiste des Carapax um etwa 50 Prozent. Zusätzlich findet eine signifikante Vergrößerung der Stachel-tragenden Bereiche auf der Dorsalleiste sowie den ventralen Rändern des Carapax statt. Die stärkere Bedornung der induzierten Individuen interferiert vermutlich mit den vielen feinen, kutikulären Strukturen im Freßapparat von *Triops* und sollte somit zusätzlich zum Schutz vor dem Prädator beitragen.

Die Ausbildung *Triops*-induzierter struktureller Verteidigungen bei *D. magna* wurde mit Hilfe eines interdisziplinären Ansatzes untersucht: Hierfür wurden klassische Mikroskopietechniken, d.h. Lichtmikroskopie und Transmissionselektronenmikroskopie, mit Rasterkraftmikroskopbasierter Nanoindentation kombiniert. Diese moderne Methode aus den Material- und Nanowissenschaften, wurde adaptiert um die mechanischen Eigenschaften des Exoskeletts von *Daphnia* zu analysieren. Die Ergebnisse dieser Studie zeigen, dass *D. magna* auf die Anwesenheit von *Triops* zusätzlich zur „Bulkiness" und der verstärkten Bedornung mit einer Verdopplung der Kutikuladicke sowie einer etwa zehnprozentigen Steigerung des Durchmessers der Stützsäulchen, die die beiden Carapaxschichten miteinander verbinden, reagiert. Die vermutlich gravierendste strukturelle Änderung ist jedoch die fünffache Zunahme des Elastizitätsmoduls, d.h. der Härte beziehungsweise Steifigkeit, der Kutikula, die mittels Rasterkraftmikroskopie festgestellt werden konnte. Nachdem *Triops* seine Beute zunächst greifen und zerkleinern muss, sollte die Verstärkung des Exoskeletts einen nennenswerten Schutz vor den Mundwerkzeugen des Räubers bieten. Dabei ist anzunehmen, dass die stärkere Bedornung und der verstärkte Carapax synergistisch zu der Schutzwirkung der gesteigerten „Bulkiness" der *Triops*-exponierten Individuen beitragen.

Ein weiteres Experiment zeigt zudem, dass der Einsatz des Rasterkraftmikroskops es ermöglicht, die Carapaxstärke von *Daphnia* beispielsweise als Indikator für die Auswirkungen des Klimawandels zu nutzen. Wie sich aktuell in vielen Weichwasserseen weltweit beobachten lässt, verändert die zunehmende Versauerung die biogeochemischen Kreisläufe der Gewässer was unter anderem in einer geringeren Verfügbarkeit von Calciumcarbonat resultiert. Da Calcium eine der wichtigsten Komponenten des Exoskeletts planktischer Crustaceen darstellt, steht zu erwarten, dass abnehmende Konzentrationen verfügbaren Calciums drastische Auswirkung auf deren Populationen haben was dann wiederum zu einem deutlichen Verlust an Biodiversität führen könnte. In Zusammenarbeit mit Kollegen zeige ich in diesem Kontext, dass die Verstärkung des Carapax bei *Chaoborus*-exponierten *D. pulex*, die üblicherweise die Ausbildung der sogenannten Nackenzähnchen bei induzierten Individuen begleitet, bei sinkende Calciumkonzentrationen nicht mehr möglich ist. Daneben fällt auch die Ausprägung prominenten morphologische Verteidigungen, die Nackenzähnchen sowie eine gesteigerte Körpergröße, als, deutlich vermindert aus. Dadurch steigt die Anfälligkeit gegenüber dem Prädator *Chaoborus*, was den fortschreitenden Rückgang der Daphnienfauna in Habitaten mit stetig abnehmenden Calciumkonzentrationen erklären könnte.

Insgesamt gewähren meine Ergebnisse neue Einblicke in die Ökologie des Modellorganismus *D. magna* und etablieren zusätzlich ein neues Räuber-Beute-System, dass in temporären Gewässern, dem gemeinsamen Habitat von *D. magna* und *T. cancriformis*, beheimatet ist. Im Gegensatz zu den sehr gut untersuchten permanenten Gewässern haben temporäre Gewässer und deren Ökologie erst im Laufe der letzten Jahre ein verstärktes Interesse erfahren. Aufgrund der speziellen Eigenschaften dieses Ökosystems und der daraus resultierenden Auswirkungen für seine Bewohner könnte ihre weitere

Erforschung unser Verständnis für einige ökologisch und evolutionär wichtiger Prozesse, beispielsweise „Rapid Evolution“ und lokale Adaptation, fördern. Die Vorstellung einer neuen und relativ gut verfügbaren Methodik zur Untersuchung struktureller Verteidigungen bei *Daphnia* könnte neue Impulse für die weitergehende Erforschung der Verbreitung sowie der chemischen und physiologischen Grundlagen dieser, bei planktonischen Crustaceen vermutlich weit verbreiteten, Verteidigung setzen. Da immer deutlicher wird, dass es Synergieeffekte der verschiedenen morphologischen Verteidigungsmerkmale gibt, könnte die Einbeziehung dieser strukturellen Verteidigungen helfen, die exakten Mechanismen, die der Wirksamkeit der induzierbaren Verteidigungen zugrunde liegen aufzudecken. Und schließlich bietet die Entdeckung der distinkten, induzierbaren morphologischen Verteidigungen in Kombination mit der aktuell erfolgenden Sequenzierung des Genoms von *D. magna* durch das *Daphnia* Genomics Consortium die vielversprechende Möglichkeit, die molekularen Mechanismen, die der Expression und Evolution der induzierbaren Verteidigungen zugrunde liegen, zu studieren. Insgesamt können die Ergebnisse dieser Arbeit somit zu einem besseren Verständnis der Mechanismen beitragen, die den evolutionären Prozessen und ökologischen Interaktionen in Räuber-Beute-Systemen zugrunde liegen.



Chapter 1:

Introduction

1

Phenotypic plasticity describes the ability of a genotype to express different phenotypes, depending on distinct environmental conditions (Pigliucci 2001, 2005, DeWitt & Scheiner 2004). Hence, phenotypic plasticity allows organisms to respond to changing environments, caused by abiotic (e.g. light, temperature, available nutrients) or biotic factors (e.g. competitors, pathogens, parasites or predators), in a way that ensures optimized fitness for the individual.

Inducible defences

Inducible defences represent a special kind of phenotypic plasticity. They are defined as phenotypic changes, triggered by cues associated with biotic agents (i.e. predators, parasites or pathogens), which can measurably decrease the effects of a subsequent attack by these agents (Tollrian & Harvell 1999). In contrast to constitutive defences, which are favoured if there is an unpredictable but permanent threat by a predator, parasite or pathogen, inducible defences are only expressed when the threat is actually present (Harvell & Tollrian 1999). There are four prerequisites for the evolution of inducible defences (Harvell & Tollrian 1999):

1. The presence of a temporary unpredictable, varying and sometimes strong biotic threat.
2. The existence of a reliable signal that indicates the presence and degree of the threat.
3. The expressed defence must offer an effective protection against the threat.
4. The defence should incur costs that outweigh its benefits in times when the threat is absent.

Inducible defences, which can be expressed on the levels of morphology, life history and behaviour (e.g. Tollrian & Harvell 1999, Kishida et al. 2010), are a widespread defensive mechanism and can be found in almost all taxa, including bacteria, protozoa, plants (here, they are usually referred to as resistance), crustaceans, insects, molluscs, amphibians and mammals.

Predator-induced changes in life history often comprise trade-offs between somatic growth and reproduction (Weiss et al. 2012). Thereby, the impact of predation can be lowered and the chance of successful reproduction increases (Lass & Spaak 2003). For instance, these defences allow the prey to sustain periods of high predation pressure by escaping the predator's prey spectrum by either producing more and smaller offspring at a decreased size at first reproduction (when threatened by a predator selecting for larger prey) or producing fewer but larger offspring at an increased size at first reproduction (when confronted with a size-limited predator). Additionally, life history defences include developmental shifts if only specific developmental stages are threatened by a predator. For instance, mayfly larvae of the species *Ephemerella invaria* emerge earlier and at a smaller size when fish are abundant (Dahl & Peckarsky 2003). Egg predation by leeches induces hatching in an earlier developmental stage in Pacific treefrogs and Cascade frogs (Chivers et al. 2001) and the presence of an odonate predator induces a decreased size at metamorphosis in juveniles of the American toad *Bufo americanus* (Skelly & Werner 1990). Another form of life history defences is the development of resting stages to escape temporary predation pressure. For example, some dinoflagellates can evade zooplankton gazers (Rengefors et al. 1998) or parasites (Toth et al. 2004) by the formation of temporary cysts.

Induced behavioural defences mainly act to prevent encounter, e.g. by hiding in a refugee, or ingestion, e.g. by an increased flight behaviour (Jeschke et al. 2008). Examples for this type of inducible defences include the predator induced diel vertical migration in marine and freshwater zooplankton (e.g.

Zaret & Suffern 1976, Stich & Lampert 1981) and juvenile fish (Scheuerell & Schindler 2003). Thereby, prey individuals typically avoid visually hunting predators by spending the day-time in deeper water layers with less available light. However, this bears two disadvantages: Firstly, those deeper water layers are usually colder than the surface water, resulting in slowed down developmental processes, and secondly, compared to the light flooded surface water, food, e.g. algae, is less abundant (Stich & Lampert 1981, Lampert 1989). Therefore, the prey organisms migrate to the shallow water layers at night-time to partially compensate for these disadvantages. However, also reverse diel vertical migration has been observed as a defence mechanism against invertebrate predators (Ohman et al. 1983). Diel horizontal migration into safer refugees, which can be found in freshwater zooplankton (e.g. Timms & Moss 1984, Lauridsen et al. 1996), is another predator-induced behavioural defence to avoid encounter with the predator. Besides changes in the migratory behaviour, predators can also induce aggregation to reduce predation risk, e.g. in cyanobacteria (Yang et al. 2006), green algae (Lurling & van Donk 1997, Lurling & Beekman 2009), mussels (Reimer & Tedengren 1997, Kobak & Kakareko 2011) and tadpoles (Watt et al. 1997). Predators can also induce reduced activity, which decreases the encounter rate between prey and predator. For instance, this has been shown in mosquito larvae (Uitregt et al. 2013), damselfly larvae (Strobbe et al. 2010) and tadpoles (Relyea 2001). In contrast to the latter defences, which act to prevent encounter, death feigning, i.e. a mimicked death posture, is a post-encounter defence, which increases the chance for a successful escape. This behaviour has been shown in insects (Honma et al. 2006, Ohno & Miyatake 2007), reptiles (Mutoh 1983) and birds (Sargeant & Eberhardt 1975).

Morphological defences are predominately post-encounter defences, which either prevent an attack due to the mere change in the prey's size or shape or cause handling difficulties for the predator and thus increase the prey's chance to escape and survive the attack. Thereby, it is a common defensive mechanism to express morphological features that interfere with the predator's feeding apparatus. Inducible morphological defences have been discovered in the rotifer *Brachionus calyciflorus*, which defends itself against the predator *Asplanchna* with the development of long spines (De Beauchamp 1952, Gilbert 1966). Following this discovery, numerous other morphological defences have been reported from a variety of taxa. For instance, grazing ciliates can induce a defensive change in the filament structure of some cyanobacteria, which prevents ingestion (Fiałkowska & Pajdak-Stós 1997). Browsing by herbivorous mammals induces the expression of longer spines in *Acacia* trees, which is an effective protection against defoliation (Young 1987, Rohner & Ward 1997). The ciliate *Euplotes daidaleos* responds to gape-limited predators by becoming larger and more round shaped (Kusch 1995, Kuhlmann et al. 1999) and marine bryozoans express long, chitinous spines when they are exposed to nudibranch predators (Harvell 1986). In arthropods it is known that the presence of fish induces the expression of longer abdominal spines in dragonfly larvae (Johansson & Samuelsson 1994, Mikolajewski & Johansson 2004) and an elongation of the caudal filaments in mayfly larvae of the species *Drunella coloradensis* (Dahl & Peckarsky 2002). Furthermore, it has been shown that barnacles can respond to predator cues by the expression of a bent shell, which renders them resistant to the predator (Lively et al. 2000). In vertebrates, tadpoles of many anuran species respond to the presence of predators with inducible morphological defences, e.g. bulkier morphotypes (against salamander) or the expression of a wider and heavier pigmented tail (against dragonfly larvae) (Relyea 2001, Kishida & Nishimura 2004, 2005). The predator-induced expression of deeper bodies is also known from fish, e.g. in the crucian carp *Carassius carassius* exposed to the piscivore northern pike *Esox lucius* (Brönmark & Miner 1992, Nilsson et al. 1995). A similar response has also been shown for other fish species, e.g. perch, roach and pumpkinseed sunfish, confronted with predatory fish (Eklöv & Jonsson 2007, Chivers et al. 2007, Robinson et al. 2007). In addition to morphological defences that alter the shape of the prey organism, there are also structural defences which protect the prey from being crushed by the predator. For instance, the sea urchin *Strongylocentrotus droebachiensis* has been shown to express a heavier and thicker exoskeleton when exposed to cues from predatory Jonah crabs (Selden et al. 2009). Similarly, many marine (Trussell 1996, Bourdeau 2011, 2012) and freshwater snails (Lakowitz et al. 2008, Auld & Relyea 2011, Brönmark et al. 2012) strengthen their shells when confronted with predatory crabs, fish or crayfish, respectively. Such structural defences can also be found in bivalves, e.g. the blue mussel *Mytilus edulis* increases shell thickness in response to predatory starfish (Reimer & Tedengren 1996) and crabs (Leonard et al. 1999). Likewise, the eastern oyster *Crassostrea virginica* shows an increased shell thickness as an effective inducible defence against a predatory gastropod, the oyster drill *Urosalpinx cinerea* (Lord & Whitlatch 2012). Apart from echinoderms and molluscs, inducible structural defences can also be found in arthropods. For example, the presence of fish induces a heavier exoskeleton in larvae of the mayfly *D. coloradensis* (Dahl & Peckarsky 2002) and an increased exocuticle thickness in dragonfly larvae of the species *Leucorrhinia dubia* (Flenner et al. 2009).

Inducible defences in *Daphnia*

Inducible defences are especially well studied in cladocerans of the genus *Daphnia* (Crustacea: Branchiopoda: Cladocera), a popular model organism in life sciences (Lampert 2011, Seda & Petrusek 2011). Members of the *Daphnia* family are particularly well suited as model organisms, e.g. to study the expression and evolution of inducible defences, for several reasons: As keystone grazers they play an important ecological role by linking primary production with higher trophic levels; under favourable conditions, daphnids reproduce by cyclic parthenogenesis, which means that distinct clonal lines can be established in the laboratory and therefore, genetic and environmental effects can be disentangled; due to their short generation time (approximately 10 days) they are perfectly suited for multigenerational studies; culturing and handling *Daphnia* in the laboratory is relatively easy; and, finally, the recent publication of the *Daphnia pulex* genome (Colbourne et al. 2011) will enable studies investigating the genetics underlying the expression of inducible defences in this genus.

In *Daphnia*, the expression of inducible defences is mainly mediated by info chemicals released by the predator, so called kairomones. These interspecific chemical messengers are defined by the fact that only the receiver, rather than the sender, benefits from them (Brown et al. 1970). For instance, the receiver can use kairomones to locate food or sense predators (Pohnert et al. 2007). Despite their ubiquity and considerable ecological role, the exact structure of almost all kairomones is either unidentified or only partially characterized (Laforsch & Tollrian 2009, Silberbush et al. 2010). One of the rare exceptions is the complete identification of aliphatic sulfates as *Daphnia*-kairomones, which induce colony formation in green algae (Yasumoto et al. 2006, 2008). Besides kairomones, other factors, e.g. alarm cues released by injured conspecifics (Pijanowska 1997, Stabell et al. 2003, Laforsch et al. 2006) or physical cues, such as predator born turbulence (Laforsch & Tollrian 2004a, Tollrian & Laforsch 2006) are also known to be involved in the induction of defences in *Daphnia*. Together, these factors can be used by *Daphnia* to fine-tune the expression of the defences to the actual type and degree of predation.

In response to predators, daphnids express a variety of life history defences. Thereby, size and age at maturity and subsequent resource allocation in adult growth are important traits, which can be affected by predators (e.g. Stibor & Lüning 1994). Theoretical models predict that invertebrate, gape limited predators should lead to a larger body size at maturity and investment into adult growth whereas vertebrate predators, selecting for larger prey, should cause a decreased size at maturity and minimal adult growth (Taylor & Gabriel 1993). Age at maturity should be delayed when invertebrate predators are present and never delayed in the presence of vertebrate predators (Taylor & Gabriel 1992). The predictions made by these models are supported by a vast number of empirical studies. When exposed to fish, many *Daphnia* species respond with a decreased size and age at first reproduction, e.g. *D. galeata* (Stibor 1992, Macháček 1995), *D. hyalina* (Stibor & Lüning 1994) and *D. magna* (e.g. Lampert 1993, Sakwińska 1998, Stibor & Navarra 2000). The opposite, i.e. an increased size and age at first reproduction, can be observed in many *Daphnia* species exposed to gape limited, invertebrate predators. For instance, exposure to kairomones released by larvae of the phantom midge *Chaoborus* induces these life history shifts in *D. hyalina* (Stibor & Lüning 1994) and *D. pulex* (e.g. Tollrian 1995, Boeing et al. 2006b). A predator-induced increase in size at first reproduction, although combined with an earlier maturation, has also been shown in cyclopoid-exposed *D. magna* (Pijanowska & Kowalczewski 1997a). A further, relatively common life history defence in *Daphnia* is a shift in the size and number of offspring. Concerning these traits, fish usually induce the production of smaller and sometimes more numerous offspring, amongst others reported for *D. pulex* (e.g. Dodson 1989), *D. hyalina* (e.g. Stibor 1992) and *D. magna* (e.g. Boersma et al. 1998), while invertebrate predators, such as *Chaoborus*, induce contrariwise reactions, e.g. in *D. pulex* (Black & Dodson 1990) and *D. hyalina* (Stibor & Lüning 1994). Another life history defence to sustain periods of high predation pressure is the fish-induced diapause observed in *D. magna* (Ślusarczyk 1995, 1999, Pijanowska & Stolpe 1996), a species which is particularly susceptible to fish predation due to its large size and often strong pigmentation. Hence, the production of tough ephippia, which even endure the gut passage of fish, represents an effective defence mechanism when fish are temporarily very abundant.

In addition to predator induced life history shifts, behavioural defences are very common in *Daphnia*. In contrast to induced life history or morphological shifts they can be expressed within a very short time span, and can thus be considered to serve as a first line defence. Probably the most striking example for behavioural defences in *Daphnia* is the diel vertical migration (DVM) (Lampert 1989). Usually triggered by fish-cues, the daphnids spend the daytime in the dark hypolimnion to evade predation by the visually hunting predators. At night-time they migrate to the epilimnion to benefit from the more abundant food and the higher temperatures. For instance, DVM has been documented in *D. hyalina* (Stich & Lampert 1981), *D. hyalina* x *galeata* hybrids (De Meester et al. 1995), *D. longispina* (Johnsen & Jakobsen 1987) and *D. magna* (Dawidowicz & Loose 1992). In contrast, invertebrate predators can also induce a reverse migration pattern, e.g. *Chaoborus* larvae induce an upward migration in *D. pulex* (Boeing et al. 2006a, Oram & Spitze 2013). A further predator-induced behavioural defence in *Daphnia*, which is closely related to the DVM, is a shift in the phototactic behaviour. It has been reported that the presence of fish induces a negative phototaxis in *D. ambigua* (Michels & De Meester 2004), *D. hyalina* (Ringelberg 1991) and *D. magna* (De Meester 1993, De Meester & Cousyn 1997). Especially in shallow lakes and ponds, diel horizontal migration is an effective predator avoidance behaviour. Thereby, the daphnids seek shelter in refugees where the predation pressure is less intense. For instance, in *D. longispina* (Kvam & Kleiven 1995) and *D. magna* (Van de Meutter et al. 2005), *Chaoborus* larvae induce a migration towards macrophytes in the littoral. Furthermore, it has been shown that fish predation triggers the same migration pattern in *D. magna* (Michels et al. 2007), whereas damselfly larvae, which are often associated with vegetation, induce migration away from the macrophytes towards the pelagial (Van de Meutter et al. 2005). Besides migration, predators can induce a variety of other behavioural defences. These include predator-induced swarming, which has been reported for *D. longispina* (Kvam & Kleiven 1995) and *D. magna* (Pijanowska & Kowalczewski 1997b), an increased alertness and more pronounced escape behaviour, e.g. in *D. pulicaria* (Brewer et al. 1999) and *D. magna* (Pijanowska & Kowalczewski 1997b, Boersma et al. 1998), and a decrease in the daphnids swimming speed, e.g. in *D. magna* (Pijanowska & Kowalczewski 1997b).

Finally, a great variety of morphological defences has been shown in *Daphnia*. Most of these defences are expressed in the presence of gape limited predators and incur morphological changes, which render the induced daphnids incompatible with the feeding apparatus of the predator ("anti-lock and key hypothesis"; Dodson 1974), imposing handling difficulties and increasing the chance to escape the attack. These defences are usually characterised by the expression of prominent morphological features. For instance, *Chaoborus* induces the formation a large helmet and an elongated tail spine in *D. cucullata*, which act as an effective protection (Laforsch & Tollrian 2004b). A *Chaoborus*-induced expression of defensive helmets has also been reported for *D. ambigua* (Hebert & Grewe 1985, Hanazato 1990), *D. longispina* (Brett 1992), *D. galeata* and *D. retrocurva* (Dodson 1988). When exposed to fish cues, *D. lumholtzi* expresses both an enormously large, pointy helmet and an elongated tail spine, making the induced individuals resemble a "swimming spike" (Tollrian 1994, Swaffar & OBrien 1996, Engel & Tollrian 2009) and offering an effective protection against juvenile fish (Dzialowski et al. 2003). A further morphological defence is the expression of large dorsal crests which can be observed in members of the *D. carinata* King complex exposed to predatory backswimmers of the genus *Notonecta* (Grant & Bayly 1981, Barry 2000). These large structures seem to help the daphnids to evade an attack and additionally prevent the predator from getting a firm grip on the prey and thus increase the likelihood for a successful escape (Grant & Bayly 1981). The so called "crown of thorns", a spine bearing, heart shaped lobe in the dorsal region of the head, which can be found in *D. atkinsoni* exposed to the predatory tadpole shrimp *Triops cancriformis,* represents another type of morphological defence in *Daphnia* (Petrusek et al. 2009). The protective effect of this defence is presumably based on the interference of the "crown of thorns" with the predator's feeding apparatus. One of the most common, and probably most universal, induced defensive traits is an elongated tail spine. This defence can already be expressed during embryogenesis without posing a risk to the other embryos in the brood pouch or consuming to much space, because it is folded and experiences post-hatching expansion and hardening (Laforsch & Tollrian 2004c). Hence, it can be considered to be a perfect start-up defence against gape-limited predators. Apart from the examples named above, an elongated tail spine can be found in *D. galeata* exposed to fish (Spaak & Boersma 1997), *D. hyalina* x *galeata* hybrids exposed to copepods (Caramujo & Boavida 2000), *D. middendorffiana* exposed to copepods, *D. pulex* exposed to fish (Boeing et al. 2006a), *Notonecta* (Dodson 1989) or *Chaoborus* (Black & Dodson 1990) and *Triops*-exposed *D. atkinsoni* (Petrusek et al. 2009). In contrast to those prominent large scale defences, also several less conspicuous, or even hidden, morphological defences can be found in *Daphnia*. Perhaps one of the best known examples for this type of defence is the expression of so called neckteeth, i.e. small, spiky protuberances in the neck region, in *D. pulex* (e.g. Krueger & Dodson 1981, Havel 1985, Riessen & Trevett-Smith 2009) exposed to *Chaoborus*. These neckteeth can also be found in members of the *D. curvirostris* complex (Kotov et al. 2006, Juračka et al. 2011). The adaptive value of this trait is presumably that it reduces the predator's handling efficiency by mechanically interfering with its mouthparts

(Havel & Dodson 1984, Parejko 1991). The more pronounced spinescence of the carapace margins in *Chaoborus*-exposed *D. ambigua* (Hebert & Grewe 1985) is a further example for the existence of small scale defences in *Daphnia*. Together with the enlarged helmet, these little spines are assumed to interfere with the fine cuticular appendages of the predator's feeding apparatus and thus increase the chance to escape the attack. The existence of "hidden" morphological defences in *Daphnia*, i.e. a fortification of the exoskeleton, which provides protection from being crushed or pierced by the predator, has often been discussed to accompany the more prominent defensive structures (e.g. Havel & Dodson 1984, Hebert & Grewe 1985, Parejko 1991). However, only few studies have actually revealed such defences so far, presumably because the detection of those defences can be relatively complicated and requires a specialized methodology. For instance, the predatory copepod *Heterocope septentrionalis* induces a thickening and strengthening (in terms of hardness) of the cuticle in *D. middendorffiana* (Dodson 1984). A study using ultrasound microscopy revealed that *Chaoborus* induces an increased hardness of the cuticle in *D. pulex* and *D. cucullata* (Laforsch et al. 2004). In *D. cucullata*, the increased carapace rigidity is additionally accompanied by an increase in cuticle thickness and in the diameter of the pillars which connect the outer and inner cuticle layer of the carapace.

The pond dwelling, large bodied species *D. magna* commonly inhabits temporary ponds (although it can sometimes also be found in permanent waters) in temperate regions of Europe, Asia, North America and Africa. It serves as an important model organism in a variety of research areas, including ecology, ecotoxicology and, since its genome is currently sequenced by the *Daphnia* Genomics Consortium (DGC; http://*Daphnia*.cgb.indiana.edu), also for evolutionary and ecological functional genomics. So far, studies on the expression of inducible defences in *D. magna* were primarily focused on the impacts of fish-kairomones. In this context, it has been documented, that *D. magna* responds to fish with a variety of behavioural (e.g. De Meester 1993, Van de Meutter et al. 2005), life history (e.g. Sakwińska 1998, Boersma et al. 1998, Ślusarczyk 1999) and sometimes also morphological defences, such as an elongation of the tail spine or the reduction in size of the compound eye to reduce visibility (e.g. Boersma et al. 1998). However, there is only sparse knowledge about the response to invertebrate predators. For instance, it has been shown that predatory cyclopoid copepods induce an earlier maturation combined with an increased size and the production of more offspring (Pijanowska & Kowalczewski 1997a), whereas *Chaoborus* induces a delayed maturation at an increased size (Coors & De Meester 2008). Although the induction of morphological defences in *D. magna* has rarely been reported, it was so far not known that this species can respond to a predator with an array of morphological defences, acting synergistically to form an effective protection.

Aims of this thesis

In this thesis, I analyse the morphological responses of *D. magna* to the predatory tadpole shrimp *T. cancriformis* and introduce this system as a novel model system to study the Evolutionary Ecology of inducible defences. Since it is the first predator-prey-model-system in temporary waters, it will even help to advance the understanding of pond dynamics.

Triops was chosen as the inducing agent for several reasons: First, it was recently revealed that it induces a distinct and novel morphological defence in the pond dwelling species *D. atkinsoni* (Petrusek et al. 2009); Second, *Triops* has not changed its morphology for approximately 220 million years (Kelber 1998) and thus it can be assumed that pond dwelling daphnids coexisted with this predator for a very long time; Third, and most important, due to its often very high abundance, its predatory impact and its function as an ecosystem engineer, *Triops* has been shown to play a major role in temporary waters (Boix et al. 2002, 2006, Yee et al. 2005, Waterkeyn et al. 2011). Thus, it seems very likely that several pond dwelling *Daphnia* species have adapted to this top predator by forming distinct defensive strategies. Based on the assumption that *D. magna* responds to *Triops*, I addressed the following issues:

1. I investigate whether *D. magna* responds to *T. cancriformis* with a distinct set of inducible morphological defences rendering them less susceptible to this gape limited predator. Since *Triops* threatens all size classes of *D. magna*, I analyse whether the expression of the individual morphological traits varies during the life span of *D. magna*, i.e. if there is a switch from easy-to-build start-up defences in juveniles to a more comprehensive defence in adults. Since the effectiveness of the defensive mechanism is one of the prerequisites for the evolution of an inducible defence, I test whether the *Triops*-induced defences offer an effective protection against this predator.

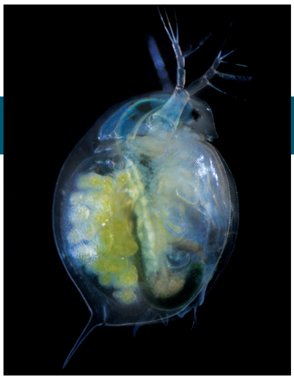
2. Since it has been shown that there is interclonal variation in the expression of inducible defences in *Daphnia*, I investigate if the same holds true for the *Triops*-induced morphological defences in *D. magna*. Since it can be assumed that different size classes of *D. magna* are usually confronted with *Triops* of different sizes, I studied the effectiveness, as well as the limitations, of the morphological defence by combining different size class combinations. Further, I analysed the direct, i.e. by predation, and indirect effects, i.e. by the induction of morphological defences, of *Triops* on *D. magna* populations.

3. I investigate, whether the *Triops*-induced large scale defences in *D. magna* are accompanied by less conspicuous small scale defences which interfere with the fine cuticular appendages *Triops* uses to catch its prey and to transport it towards the mouth. Namely, I analyse if the presence of *Triops* induces a change in the morphology of the spinules, i.e. the small spines located on the dorsal ridge and the ventral edges of the carapace. Further, I test whether the spinule bearing areas on the carapace are enlarged in response to *Triops*.

4. In some *Daphnia* species confronted with invertebrate predators, the existence of “hidden”, structural defences, such as a thickening and strengthening of the exoskeleton, has been shown. Since *Triops* is a predator that needs to crush its prey prior to ingestion, it seems very likely that such a response can also be found in *D. magna*. To achieve this aim, I use an interdisciplinary approach to assess induced changes in exoskeleton associated traits, namely cuticle hardness, cuticle thickness and the diameter of the pillars connecting the outer and the inner cuticular layer of the carapace. So far, the measurement of the elastic properties, i.e. hardness or rigidity, of the carapace in *Daphnia* required the application of very specialized, and thus not widely available methods. Therefore, I adapt and establish atomic force microscopy, a commonly used tool in material sciences and biophysics, as a very suitable, accessible and comparably fast method to perform such measurements. Cuticle thickness is analysed using light microscopy and transmission electron microscopy and the pillar diameter is analysed using fluorescence microscopy.

5. Atomic force microscopy based analysis of the mechanical properties is perfectly suited to answer a variety of ecological and morphological questions associated with changes in the exoskeleton in plankton organisms. For instance, it is known that changes in the water chemistry, either natural or driven by anthropogenic influence, can affect the expression of anti-predator defences which can lead to changes in the whole ecosystem. Within the framework of a collaboration, I use atomic force microscopy to analyse the impact of decreased calcium levels (as currently found in many softwater lakes in Scandinavia and eastern North America) on the ability of *D. pulex* to express a harder cuticle as part of the defensive response to *Chaoborus* larvae.

In the following, the journal articles and manuscripts will be given as separate chapters.



Chapter 2:

Growing large and bulky in the presence of the enemy: *Daphnia magna* gradually switches the mode of inducible morphological defences

Rabus M., Laforsch C. (2011)
Functional Ecology 25, 1137-1143

Abstract

1. Phenotypic plasticity in defensive traits has been proven to be effective in ecosystems with frequently changing predator regimes. However, if a single dominant predator exerts predation pressure on each ontogenetic stage, prey should adapt by developing defensive traits for each life stage within a cost-benefit framework. This may require a change of defensive mechanisms between juvenile and adult life stages.

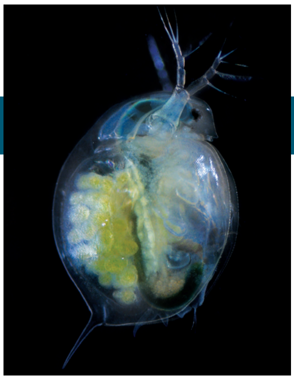
2. In this study, we examined the morphological defences of the cladoceran *Daphnia magna* Strauss induced by the tadpole shrimp *Triops cancriformis* Bosc. We tested for the induction of morphological defences by directly exposing daphnids to the predator and conducted a life span experiment to determine if the expression of the induced morphological defences varies throughout the life span of *D. magna*. In addition, we studied the adaptive value, i.e. the effectiveness, of the *Triops*-induced morphological defences in *D. magna* by conducting predation trials.

3. We found that, in *D. magna*, the expression of an array of inducible morphological defences, which act synergistically to provide effective protection, changes during the daphnids lifetime in response to the tadpole shrimp *T. cancriformis*. This gradual switch in the protective function of single traits between juvenile and adult stages represents a novel functionality and complexity of inducible defences. Both direct contact with the predator and chemical cues (kairomones) released by *T. cancriformis* induce an increased body length, body width and an elongation of the tail spine in *D. magna*. This study is the first to show that kairomones released by a predator can induce ‘bulkiness’ as a defensive mechanism in *Daphnia*. Finally, we demonstrate the effectiveness of the *Triops*-induced morphological defences (i.e. an elongated tail spine and increased bulkiness) by conducting predation trials.

4. Our study provides rare evidence for morphological defences in *D. magna*, and in addition shows that prey species gradually switch between plastic traits to maintain effective defences throughout their entire lifetime. Hence, our results help to shed light on the mechanisms governing phenotypic plasticity within natural populations.

Full-text article:

http://onlinelibrary.wiley.com/doi/10.1111/j.1365-2435.2011.01840.x/epdf



Chapter 3:

Interclonal variation, effectiveness and long-term implications of *Triops*-induced morphological defences in *Daphnia magna* Strauss

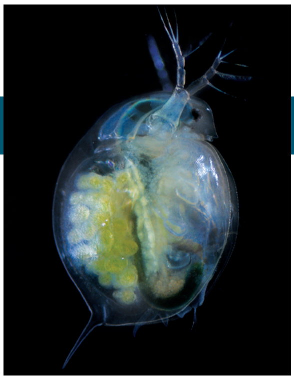
Rabus M., Waterkeyn A., Van Pottelbergh N.,
Brendonck L., Laforsch C. (2012)
Journal of Plankton Research 34, 152-160

Abstract

In response to the predatory tadpole shrimp *Triops cancriformis* Bosc. (Notostraca), the cladoceran *Daphina magna* develops a "bulky" morphotype as an inducible morphological defence. The aim of this study is to provide further insight in the *Triops*-induced defences by revealing interclonal variation of the induced traits, the effectiveness of the defence and their effects on the prey's population structure. In a clonal comparison experiment, we showed that clones of *D. magna* differed in their morphological response to *T. cancriformis*. By conducting predation trials with different combinations of *Daphnia* and *Triops* size classes, we could demonstrate that the morphological defences act as an effective protection throughout the entire life-span of *D. magna*. Finally, a long-term mesocosm study showed a significant, positive correlation between *Triops* density and the expression of the defensive traits. Thus, it confirmed that *T. cancriformis* is a strong agent in structuring *D. magna* populations through predation and induction of protective traits. Hence, this study provides further insight into this recently discovered predator–prey system, and might contribute to the knowledge of mechanisms of ecological interactions and evolutionary dynamics in aquatic communities.

Full-text article:

http://plankt.oxfordjournals.org/content/34/2/152.full.pdf+html



Chapter 4:

An inconspicuous fortification: The *Triops*-induced increase in spinescence as a small scale morphological defence in *Daphnia magna*

Rabus M., Böttcher A., Chaplinska M., Laforsch C.

(Manuscript to be submittet to *Journal of Plankton Research*)

4

An inconspicuous fortification: The *Triops*-induced increase in spinescence as a small scale morphological defence in *Daphnia magna*

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Keywords: phenotypic plasticity; inducible defences; predator-prey interactions; temporary waters; hidden defences

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Abstract

Phenotypic plasticity in defensive traits is a common response of prey organisms in ecosystems with variable predation regimes. In cladocerans of the genus *Daphnia*, inducible morphological defences are a widespread response to invertebrate predators. *Daphnia magna* has recently been shown to express an increased bulkiness, rendering the induced daphnids less susceptible to the gape limited predator *Triops cancriformis*. However, less prominent traits may contribute to the effectiveness of the defence. Here, we studied the expression of *Triops*-induced changes that may serve as small scale defences, namely changes in the morphology of the spinules, tiny spines located on the margins of the carapace, and the size of the spinule bearing areas. We found that the dorsal spinules grew almost 50% longer and had a broader base in induced individuals, whilst there was no predator-induced change in the ventral spinules. Moreover, we could show that *Triops* induces an increased size of the spinule bearing areas. Since these induced traits may interfere with the fine cuticular appendages of *Triops*, involved in catching and handling the prey, we anticipate that the observed expression of minute defensive structures may enhance the effectiveness of the more obvious protective traits.

Introduction

Predation is considered to be a major driver of natural selection (Kerfoot & Sih, 1987; Sih et al., 1998). To avoid predation, prey organisms have evolved a variety of phenotypically plastic defence mechanisms, so called inducible defences. This kind of anti-predator defence is especially favoured in environments with temporally changing predation pressure exerted by specific predators (Laforsch & Tollrian, 2009). Those defences can be found in almost all taxa, ranging from bacteria to vertebrates (Tollrian and Harvell, 1999; Kishida, et al. 2010), and especially in aquatic ecosystems, many organisms have been shown to express a variety of inducible defences (Lass & Spaak, 2003; Lampert, 2011a).

Particularly cladocerans of the genus *Daphnia*, important model organisms in life science research (Lampert, 2011b; Seda & Petrusek, 2011), are textbook examples for the study of inducible defences since they show predator-induced changes in life history, behaviour and morphology. Induced shifts in life history traits include changes in size and age at first reproduction (Weider & Pijanowska, 1993; Boersma, et al. 1998) as well as an altered clutch size (Spitze, 1992; Riessen, 1999). Inducible defences on the behavioural level include swarming (Kvam & Kleiven, 1995; Pijanowska & Kowalczewski, 1997a), a more pronounced alertness (Pijanowska & Kowalczewski, 1997a; Brewer, 1999) and diel vertical migration (Lampert, 1989; De Meester et al., 1999).

Finally, many *Daphnia* species express remarkable morphological defences in the presence of predators (Lampert, 2011a). Morphological defences are predominantly expressed in response to gape-limited predators because these changes are assumed to render the prey incompatible to the predator's feeding apparatus, q.v. Dodson's "anti-lock and key" hypothesis (Dodson, 1974), and thus increase the chance to escape and survive an attack. Thereby, a variety of morphological traits can be altered in different *Daphnia* species. For instance, individuals of *D. carinata* and *D. longicephala* express huge dorsal crests in the presence of notonectid predators (Barry, 2000), whereas *D. galeata*, *D. retrocurva* (Dodson, 1988) and *D. cucullata* (Laforsch and Tollrian, 2004b) build large helmets and tail spines in response to *Chaoborus* larvae. In *D. lumholtzi*, fish kairomones induce a massive elongation of the helmet and the tail spine (Tollrian, 1994; Dzialowski et al., 2003). Moreover, the expression of the so called "crown of thorns", a heart shaped, spine bearing dorsal lobe, could be shown in the *D. atkinsoni* species complex exposed to the predatory tadpole shrimp *Triops cancriformis* (Petrusek et al., 2009). Besides these large and prominent defensive structures, also smaller and less conspicuous defences have been reported for some *Daphnia* species, for instance the *Chaoborus*-induced expression of small neckteeth in *D. pulex* (Krueger & Dodson, 1981; Riessen & Trevett-Smith, 2009) and in some members of the *D. curvirostris* complex (Juračka et al. 2011). Moreover, Laforsch et al. (2004) showed *Chaoborus*-induced "hidden" defences in *D. pulex* and *D. cucullata*, namely an increased thickness and stability of the carapace and an increased diameter of the pillars connecting the carapace layers.

In *D. magna* an increased bulkiness as effective inducible defence against *T. cancriformis*, which can be considered a top predator and ecosystem engineer in ephemeral ponds (Yee et al., 2005; Boix et al., 2006; Waterkeyn et al., 2011), was revealed recently (Rabus & Laforsch, 2011; Rabus et al., 2012). Their enlarged body length and width, an elongated tail spine and a broader shoulder shield, protect the induced individuals against *Triops*. Since this predator can be assumed to be gape-limited due to its morphology and feeding mode, it is consequently impeded in the handling of induced *D. magna* (Rabus et al., 2012). Besides such noticeable traits it has been shown that *D. magna* increases the rigidity of its exoskeleton in response to *Triops* (Rabus et al. 2013) and it is possible that *D. magna* additionally expresses other less conspicuous morphological traits as part of the entire defensive mechanism.

In particular, we investigated two questions: (i) Do individuals of *D. magna* enlarge the small spinules along the dorsal ridge and the ventral margins of the carapace when exposed to *T. cancriformis*? (ii) Since not the entire ridge carries these spinules, does *Triops* induce an increased size of the spinule bearing areas?

We discovered that the spinules located on the dorsal ridge were increased in length and width in the *Triops*-exposed daphnids. Moreover, we could show that the spinule bearing areas were extended in *Triops*-exposed *D. magna*. However, we observed neither an elongation nor a broadening of spinules along the ventral margin of the carapace.

Method

Scanning electron microscopy (SEM) analysis of the spinule morphology

In this experiment, a single clone of *D. magna* was exposed to the predatory tadpole shrimp *T. cancriformis* to test whether this predator induces altered spinule morphology. The *D. magna* clone ($K_3$4J) used in this experiment originates from a former fishpond near Munich, Germany, and is known to respond to *Triops*-kairomones by an increased bulkiness (Rabus & Laforsch, 2011). As predator, we used a laboratory cultured strain of *T. cancriformis* provided by Dr. E. Eder, Zoological Institute, University of Vienna. All experiments were conducted in a climate chamber at a constant temperature of 20 ± 0.5°C under fluorescent light with a day-night-cycle of 15:9 h, including half an hour artificial dusk and dawn.

The experiment was carried out in 1.5 L glass beakers, filled with 1.2 L of a semi-artificial medium based on ultrapure water, phosphate buffer and trace elements (Rabus & Laforsch, 2011). Ten randomly picked, age synchronized neonate daphnids were placed into each beaker. Additionally, a net cage, made of an acrylic glass cylinder (height: 135 mm; diameter: 75 mm) with three openings at the side (65 x 50 mm) and the bottom covered with gauze (230 µm mesh width), was placed into each of the beakers to prevent direct contact between predator and prey but allowing chemical cues to pass through. For the induction treatment, one *Triops* of 3 - 4 cm body length, measured from the anterior end of the carapace to the end of the abdomen, was placed in the net cages whilst the cages remained empty in the control treatment. Each treatment was replicated three times.

The daphnids were fed daily ad libitum (approximately 2 mg C/L) with the green algae Scenedesmus obliquus. The *Triops* were fed every other day with 10 freshly killed *D. magna*, to take alarm cues into account (Stabell et al., 2003; Laforsch et al., 2006), and 10 pellets of fish-food (Grana Discus, JBL GmbH& Co. KG, Neuhofen, Germany), which has been tested to be ineffective in inducing morphological changes in *Daphnia* in preliminary experiments. The daphnids used for feeding were killed using carbonated water. The same amount of fish-food was also added to the net cages in the control treatment. Food remnants, faeces and exuviae were removed daily.

The *Daphnia* were checked on a daily basis for offspring and after being released from the brood pouch, ten randomly chosen neonates per beaker were transferred to a beaker containing fresh medium. Primiparous individuals of the fifth generation were preserved in 70% ethanol for the SEM analysis. The preserved daphnids were rinsed in a 1:1ethanol-acetone mix (both 70% p.a.) for 10 minutes. The liquid was removed and daphnids were desiccated in a graded acetone series (10 minutes once in 70%, 80%, 90% and 96% acetone p.a. and twice in 99% and 100% acetone p.a. dried over $CaCl_2$, respectively). After each step, except the last one, the liquid was not completely removed to avoid shrinking artefacts in the daphnids. Finally, the *Daphnia* were rinsed twice in hexamethyldisilazane (HDMS). Subsequently, the samples were transferred immediately into a desiccator and vacuum was applied. For detailed information on this method see Laforsch & Tollrian (2000). After 24 hours, the daphnids were mounted on aluminium stubs (Plano, GmbH, Wetzlar, Germany) using adhesive carbon tabs (Plano GmbH, Wetzlar, Germany) and stored dry in a desiccator until analysis.

Samples were sputter coated with a thin layer of gold for 120 sec using the SEM coating System (SC510, BIO-RAD Microscience Division, Hertfordshire, England). The microstructure of the specimen was characterised by a scanning electron microscope (LEO 1450VP, LEO Electron Microscopy, Oberkochen, Germany) operating at 15 kV.

Morphological traits were analyzed using the digital image analysis software Cell^P (Olympus, Hamburg, Germany) for Windows. For the measurement of the spinules, two regions of interest (ROI) were chosen on the ventral carapace margin and the dorsal ridge, respectively (Fig. 1). Then spinule width, defined as the distance between the lowest indentations on both sides of the spinule, and spinule length, defined as the distance between the spinule base and the tip of the spinule, of five randomly chosen spinules per ROI were measured (Fig. 2).

Comparison of the spinule bearing areas (SBAs)

This experiment was conducted to test for a *Triops*-induced increase in the range of the SBAs on the ventral carapace margin and the dorsal ridge in *D. magna*. Therefore, three clones of *D. magna* ($K_3$4M, $K_3$4J and Max4) were exposed to *T. cancriformis*. The clones used for this experiment originated from different countries and environments: a temporary pond in the nature reserve of the Tour du Valat research station, Camargue, France (Max4), where *D. magna* and *T. cancriformis* still coexist, and a former fishpond near Munich, Germany ($K_3$4M, $K_3$4J), an area where *Triops* went extinct approximately 40 years ago. We used those three clones because we recently showed that all of them express *Triops*-induced large scale defences (Rabus et al., 2012) and hence we wanted to test whether the same holds true for the small scale defences.

The experiment was carried out in 12 L glass aquaria that were kept in a climate chamber at

20 ± 1°C under fluorescent light (constant photoperiod 15 h light: 9 h dark). The aquaria contained 10 L of artificial medium and a thin layer of sterilized sand at the bottom. At the beginning of the experiment, 50 third-clutch neonates (F0-generation) from age synchronized mothers were placed into each aquarium. Control and induction treatments were replicated five times for each clone. The control treatment contained only daphnids and no predator, whereas in the induction treatment three *T. cancriformis* with a body length of 10 ± 3 mm were placed into each aquarium. This setup allowed both physical and chemical contact with the predator. Thus, the daphnids were not only exposed to the *Triops*-kairomones, but also to alarm cues released by injured conspecifics (Pijanowska & Kowalczewski, 1997b; Stabell et al., 2003; Laforsch et al., 2006), and physical stimuli such as turbulences produced by the predator (Laforsch & Tollrian, 2004b) to guarantee maximum induction of all morphological traits.

The daphnids were fed daily with S. obliquus at a concentration of 0.042 mM C L^{-1}. Five pellets of commercial fish food (JBL Grana Discus, JBL GmbH & Co. KG, Neuhofen, Germany) were added daily into the induction aquaria as a food source for *T. cancriformis*. The fish food was also added to the control treatment to guarantee identical experimental conditions. Dead *Triops* or animals grown bigger than 15 mm were replaced. Remnants of fish food, faeces and *Triops*-exuviae were removed on a daily basis. Every four days, 40% of the medium was exchanged in all aquaria. Once the daphnids released their neonates, the adults were removed from the experiment and only 50 randomly chosen neonates were kept in each aquarium to avoid crowding (Goser & Ratte, 1994) and keep the *Daphnia*-density constant. When the F3-generation reached maturity, the daphnids were preserved in 70% ethanol (Black & Dodson, 2003).

For the analysis, 15 randomly chosen individuals per replicate were measured using a digital image-analysis system Cell^P (Olympus, Hamburg, Germany) at 12x (body length) and 24x (SBA) magnification, respectively. Thereby, body length, defined as the distance between the upper edge of the compound eye and the base of the tail spine; dorsal SBA, defined as the line of spinules located on the dorsal ridge of the daphnids, starting from the first distinctly seen spinule and ending at the base of the tail spine; ventral SBA, defined as the line of spinules located on the ventral part of the body, starting with the first distinctly seen spinule and ending at the base of the tail spine, were measured.

Statistical analysis

Statistical data analysis was conducted using the software package PASW Statistics 18 (SPSS Inc., Chicago, USA). For the analysis of the SEM data, we pooled the data from all five spines measured per ROI and calculated a mean value per individual and ROI. For normally distributed and homogeneous data we used a nested ANOVA with treatment as fixed factor and replicates as random factors and for the data that were not normally distributed and homogeneous, the Mann-Whitney U test was used for analysis. The data obtained from the measurement of the SBAs were first tested for normality and homogeneity of variance. Then, a nested ANCOVA was applied for analysis, using body length as covariate to control for size dependent effects.

Results

The SEM analysis of the spinule morphology showed that the length of the spinules located on the dorsal ridge was significantly increased in the induced individuals (anterior ROI: Mann-Whitney U test; $Z = -3.995$; $P < 0.001$; posterior ROI: Mann-Whitney U test; $Z = -5.320$; $P < 0.001$; Fig. 3a).

Moreover, the base width of the spinules was significantly increased in induced compared to control *Daphnia* (anterior ROI: Mann-Whitney U test; Z = -3.832; P < 0.001; posterior ROI: Mann-Whitney U test; Z = -3.157; P = 0.002; Fig. 3b). In contrast, neither the width (anterior ROI: nested ANOVA; $F_{1, 4.389}$ = 5.316; P = 0.077; posterior ROI: nested ANOVA; $F_{1, 5.718}$ = 7.162; P = 0.139) nor the length (anterior ROI: nested ANOVA; $F_{1, 4.158}$ = 2.542; P = 0.183; posterior ROI: nested ANOVA; $F_{1, 4.487}$ = 1.246; P = 0.321) of the spinules on the ventral carapace margin differed between *Triops*-induced and non-induced *D. magna*.

For the comparison of the ventral and dorsal SBAs, three different clones of *D. magna* ($K_3$4J, $K_3$4M, Max4) were directly exposed to the predator *T. cancriformis* to compare the SBAs at the dorsal and ventral ridges of induced and control individuals. Compared to control daphnids, induced individuals of the German clone $K_3$4J showed a significantly greater length of the dorsal SBA after controlling for the effect of body length (nested ANCOVA; $F_{1, 28}$ = 12.98; P = 0.001; Fig. 4a) whereas there was no difference in the length of the ventral SBA (nested ANCOVA; $F_{1, 7}$ = 1.54; P = 0.258; Fig. 4a) . In the other German clone, $K_3$4M the dorsal (nested ANCOVA; $F_{1, 7}$ = 29.05; P = 0.001; Fig. 4b) and ventral SBA (nested ANCOVA; $F_{1, 7}$ = 12.93; P = 0.009; Fig. 4b) was significantly increased in the induced individuals. The results obtained from the French clone, Max4, were similar to those of the German clones. Induced individuals possessed a significantly greater length of the dorsal (nested ANCOVA; $F_{1, 9}$ = 24.00; P = 0.001; Fig. 4c) and the ventral SBA (nested ANCOVA; $F_{1, 8}$ = 11.81; P = 0.008; Fig. 4c).

Discussion

Only few studies have indicated that prominent morphological defences in *Daphnia* may sometimes be accompanied by more subtle but nevertheless effective “hidden” defences (Dodson, 1984; Laforsch et al., 2004, Rabus et al. 2013). With respect to this, we investigated whether *T. cancriformis* induces changes in length and width of the small spinules at the carapace margins as well as an altered range of the SBAs.

We could show that the spinules located on the dorsal ridge grew almost 50% longer and also significantly wider in predator exposed *D. magna*. In contrast, neither the length nor the width of the spinules along the ventral carapace margin differed between induced and non-induced daphnids. Moreover, we could show for all studied clones that the dorsal SBAs, and except from clone K34J also the ventral SBAs, are significantly larger in the induced individuals. Since we used body length as a covariate in our analysis to compensate for size dependent differences, we conclude that the observed differences are caused by the exposure to the predator, i.e. they are inducible traits.

The enlargement of the SBAs and the increased length and width of the dorsal spinules might therefore serve as a defensive trait in addition to the induced bulkiness. Since *Triops* catches its prey with the anterior legs and then transports it towards its mouth through a mid-ventral food groove (Fryer, 1988; Gruner, 1993) it seems likely that the handling of induced *D. magna* is impeded not only by the increased bulkiness, that has been shown to increase the survival rate of induced individuals in predation trials (Rabus & Laforsch, 2011; Rabus et al., 2012), but also by the more pronounced spination. The defensive mechanism underlying the enlargement of the spine bearing areas seems to be the interference of the spinules with the predator´s limb appendages involved in capturing and transporting the prey towards the mandibles. When *Triops* catches a prey item, it is enclosed by a cage built by the numerous appendages of the endites and is then transported towards the mouth by the stout denticles of the gnathobases and the so called sweeping spines (Fryer, 1988). Given the morphology of *Triops*' feeding apparatus, it seems possible that the increased size of the SBAs and the longer dorsal spinules in induced *D. magna* interact with the predator's spines and thus impede handling of the prey. An example for the effectiveness of small spines is, amongst others, the *Chaoborus*-induced expression of tiny neckteeth in *D. pulex* (e.g. Krueger & Dodson, 1981; Havel, 1985). In this system it has been supposed, that the small neckteeth interfere with the feeding apparatus of the predator and increase the chance to escape the attack (Havel & Dodson, 1984; Tollrian, 1995). Moreover, the "crown of thorns" in *D. atkinsoni* has been shown to offer an effective protection against *Triops* (Petrusek et al., 2009). This defensive structure consists of a heart-shaped lobe covering large parts of the head. Additionally, the edges of these lobes are bearing long spinules. Thus, the effectiveness of this defensive trait is presumably based on the incompatibility of the feeding apparatus of *Triops* and the spinule bearing lobes in the neck region of *D. atkinsoni*. Hence, it is likely that the enlarged SBAs in *Triops*-exposed *D. magna* serve as an additional defensive trait, acting synergistically to the yet discovered bulkiness. In addition, these traits may be accompanied by the fortification of the exoskeleton as shown in *D. pulex* and *D. cucullata* (Laforsch et al., 2004; Riessen et al., 2012) and just recently in *D. magna* (Rabus et al., 2013). Given that these arthropods frequently moult cuticle fortification including the development of small spinules may act as a first-line defence since morphometric defences such as helmets cannot be developed within a single moult. Furthermore, costs involved in forming these defensive traits can be saved within a very short timeframe, i.e. in the subsequent moult, if predation impact decreases.

To conclude, we found that *D. magna* enlarges the spinule bearing areas along the dorsal ridge and, except one clone, the ventral carapace margin as an additional morphological defence against the predatory tadpole shrimp *T. cancriformis*. Moreover, we could observe a predator-induced elongation of the spinules located on the dorsal ridge. The effectiveness of these spinules is thereby presumably based on the interference with the delicate cuticular structures of the predator's food groove. Since the feeding apparatus of many invertebrate predators partly consist of fine cuticular structures, it seems likely that a more pronounced spination, as a less prominent but nevertheless effective defensive trait, may be expressed in other *Daphnia* species as well.

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Figure legends

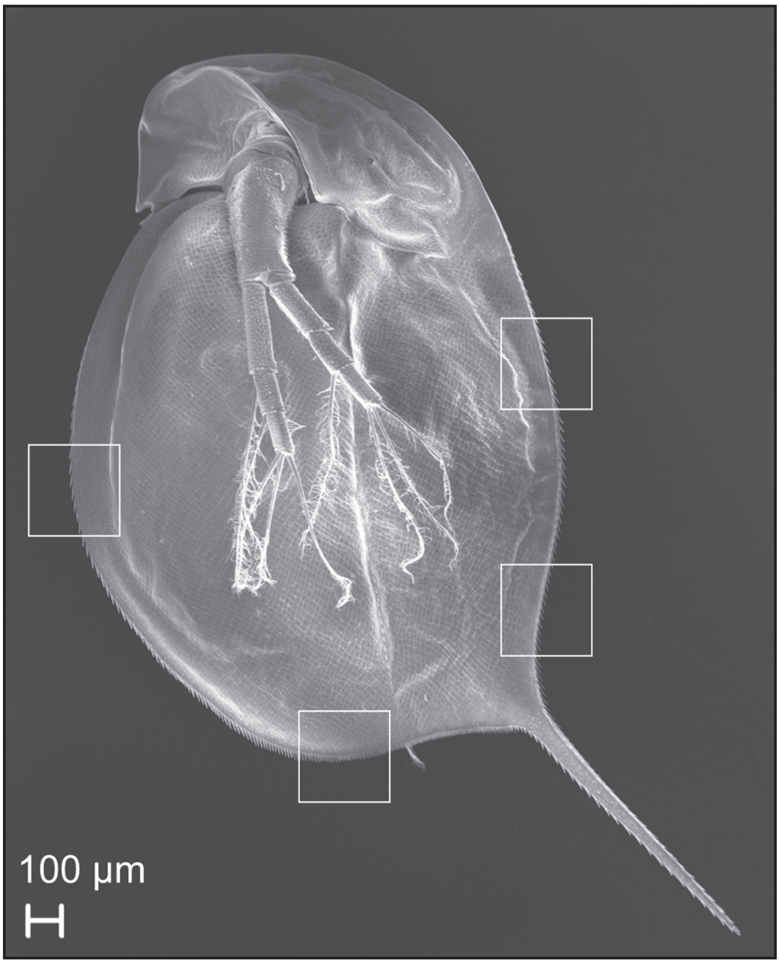
Figure 1: Scanning electron micrograph of a *Triops*-induced primiparous *D. magna*. The white boxes indicate the regions of interest (ROI) from which the measurements of spinule length and -width were taken.

Figure 2: Scanning electron micrograph of the spinules located on the dorsal ridge. The two micrographs show the differences in spinule morphology of a) an induced and b) a non-induced *D. magna*.

Figure 3: Comparison of a) the dorsal spinule length and b) -width between induced and non-induced *D. magna*. The error bars indicate the standard error of mean (SE), the asterisks indicate significant differences between induced and non-induced daphnids (** = $P < 0.01$; *** = $P < 0.001$).

Figure 4: Comparison of the relative spinule bearing areas (SBAs) of induced and non-induced *D. magna*. The graphs show both ventral and dorsal SBAs of three different clones: a) $K_3$4J, b) $K_3$4M and c) Max4. The error bars indicate the standard error of mean (SE), the asterisks indicate significant differences between induced and non-induced daphnids (** = $P < 0.01$).

Figure 1:



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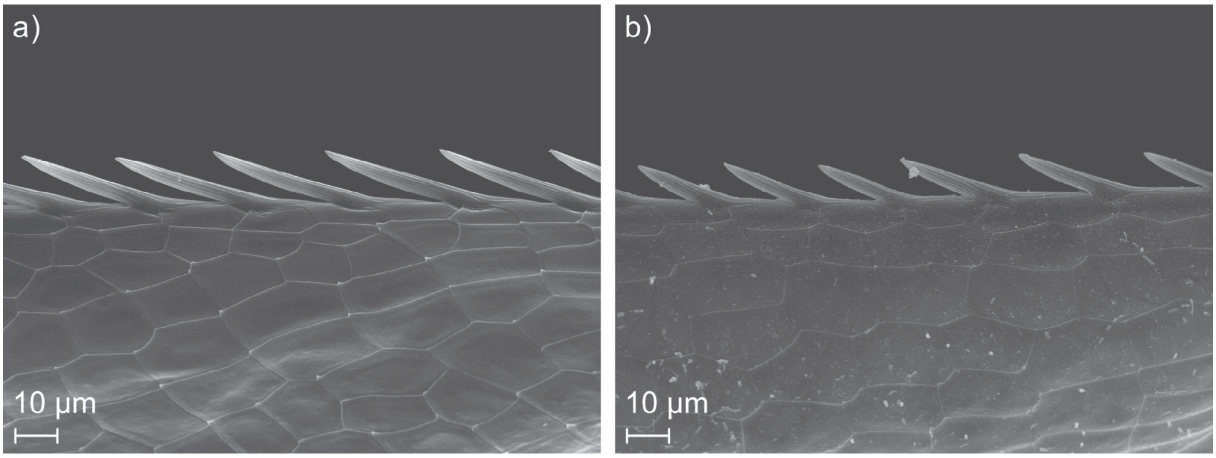

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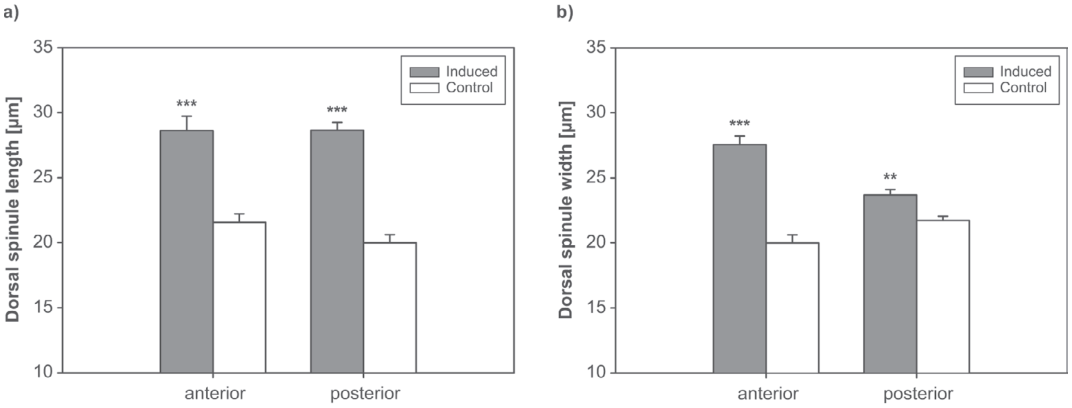

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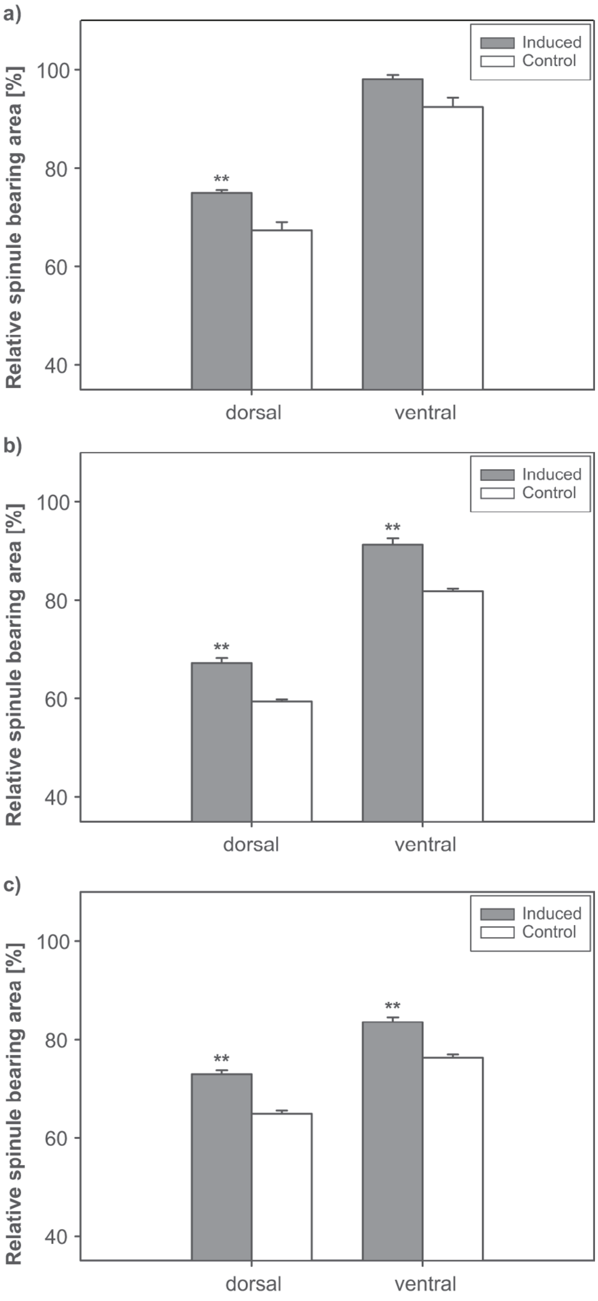

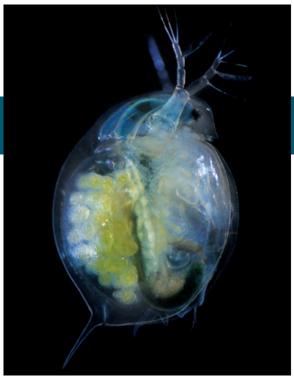

Chapter 5:

Uncovering Ultrastructural Defences in *Daphnia magna* – An Interdisciplinary Approach to Assess the Predator-Induced Fortification of the Carapace

Rabus M., Söllradl T., Clausen-Schaumann H.,
Laforsch C. (2013)
PloS ONE 8

Abstract

The development of structural defences, such as the fortification of shells or exoskeletons, is a widespread strategy to reduce predator attack efficiency. In unpredictable environments these defences may be more pronounced in the presence of a predator. The cladoceran *Daphnia magna* (Crustacea: Branchiopoda: Cladocera) has been shown to develop a bulky morphotype as an effective inducible morphological defence against the predatory tadpole shrimp *Triops cancriformis* (Crustacea: Branchiopoda: Notostraca). Mediated by kairomones, the daphnids express an increased body length, width and an elongated tail spine. Here we examined whether these large scale morphological defences are accompanied by additional ultrastructural defences, i.e. a fortification of the exoskeleton. We employed atomic force microscopy (AFM) based nanoindentation experiments to assess the cuticle hardness along with tapping mode AFM imaging to visualise the surface morphology for predator exposed and non-predator exposed daphnids. We used semi-thin sections of the carapace to measure the cuticle thickness, and finally, we used fluorescence microscopy to analyse the diameter of the pillars connecting the two carapace layers. We found that *D. magna* indeed expresses ultrastructural defences against *Triops* predation. The cuticle in predator exposed individuals is approximately five times harder and two times thicker than in control daphnids. Moreover, the pillar diameter is significantly increased in predator exposed daphnids. These predator-cue induced changes in the carapace architecture should provide effective protection against being crushed by the predator's mouthparts and may add to the protective effect of bulkiness. This study highlights the potential of interdisciplinary studies to uncover new and relevant aspects even in extensively studied fields of research.

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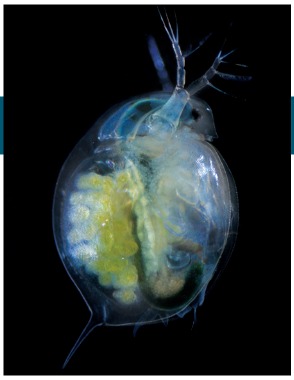

Chapter 6:

Changes in water chemistry can disable plankton prey defenses

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Abstract

The effectiveness of antipredator defenses is greatly influenced by the environment in which an organism lives. In aquatic ecosystems, the chemical composition of the water itself may play an important role in the outcome of predator-prey interactions by altering the ability of prey to detect predators or to implement defensive responses once the predator's presence is perceived. Here, we demonstrate that low calcium concentrations (<1.5 mg/L) that are found in many softwater lakes and ponds disable the ability of the water flea, *Daphnia pulex* to respond effectively to its predator, larvae of the phantom midge, *Chaoborus americanus*. This low-calcium environment prevents development of the prey's normal array of induced defenses, which include an increase in body size, formation of neck spines, and strengthening of the carapace. We estimate that this inability to access these otherwise effective defenses results in a 50–186% increase in the vulnerability of the smaller juvenile instars of *Daphnia*, the stages most susceptible to *Chaoborus* predation. Such a change likely contributes to the observed lack of success of daphniids in most low-calcium freshwater environments, and will speed the loss of these important zooplankton in lakes where calcium levels are in decline.

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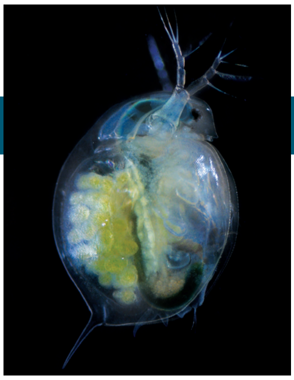

Chapter 7:

Discussion

7

Discussion

Temporary waters have long been considered to be a habitat type where predation plays a minor role because key predators such as fish and most dragonfly larvae cannot colonise them due to their ability to sustain extended drought periods. There is, however, growing evidence that invertebrate predation indeed plays an import role in structuring the communities in temporary ponds (e.g. Blaustein 1998; Brendonck et al. 2002; Urban 2007). The cladoceran *D. magna*, the focal species of this thesis, primarily inhabits such temporary waters and thus often co-occurs with the predatory tadpole shrimp *T. cancriformis*, one of the top predators in this habitat (Waterkeyn et al. 2011). Given the facts that *Triops* temporarily reaches very high densities of up to 300 individuals per square metre (Boix et al. 2002) and has a strong predatory impact on small crustaceans such as cladocerans (Yee et al. 2005, Boix et al. 2006), it seems very likely that *Triops* temporarily imposes strong predation impact upon *D. magna*, favouring the evolution of inducible defence mechanisms against this predator.

The predator-induction experiments revealed that the presence of *Triops* induces a distinct array of morphological defences in *D. magna*, a species that was so far not known to respond to predators with a whole set of morphological defences. Thereby, the most noticeable morphological change is the expression of a “bulky” morphotype due to the predator-induced increase in body length, body width and shoulder-shield width, which is accompanied by a considerable elongation of the tail spine. The increased bulkiness in *D. magna* thus resembles the typical response to gape-limited predators, i.e. the expression of morphological changes that render the prey less compatible to the predator’s feeding apparatus (Dodson 1974). Due to its morphology, namely the size of its food groove and the opening width of its mandibles, *Triops* can be considered to be a negatively size-selective, gape-limited predator and should thus prefer smaller over larger prey. This assumption is confirmed by prey-choice experiments with closely related notostracans with an almost identical morphology (Walton et al. 1991; Christoffersen 2001).

It is known that *Daphnia* can restrict the expression of inducible defences to the life stages, which are actually threatened by a specific predator. For instance, the expression of neckteeth in *D. pulex* is limited to the second and third juvenile instars which are susceptible to *Chaoborus*-larvae predation (Krueger & Dodson 1981; Riessen & Trevett-Smith 2009), whereas *D. cucullata*, where all size-classes are threatened by this predator, maintains its morphological defences throughout its entire lifespan (Laforsch & Tollrian 2004). Concerning the *D. magna* - *T. cancriformis* system, I was able to show that the induced defence is expressed in all life stages although the expression of the single morphological traits (except shoulder-shield width which was not recorded in this particular experiment) varies over time and life stages. In juvenile, *Triops*-exposed *D. magna* the elongated tail spine is the most prominent defensive structure, although the increase in body length and -width already emerges early in the juvenile stage. However, the difference in bulkiness caused by body length and -width is most pronounced in adults. Based on these results, it can be assumed that the tail spine, which is already enlarged in the offspring of *Triops*-exposed individuals, serves as an easy-to-build and cost-effective start up defence which helps to boost the overall length of the daphnids. In adult *D. magna*, however, the length of the tail spine is reduced soon after reaching maturity, which indicates that its importance as an anti-predator defence is decreasing concurrently. This conclusion is further supported by the observation that the tail spine length shows a negative allometric growth in relation to body length, beginning with the first juvenile moult. In contrast, the expression of an increased bulkiness likely needs more time and resources and is thus gradually expressed in juveniles before it becomes the dominant defensive structure in adults. By shifting the expression of the single defensive traits, from an easy to build start-up defence to a more complex defence in adults, *D. magna* ensures to maintain an effective protection throughout its entire life span. This adaptation might is of particular importance since *Triops* of different sizes usually co-occur (Boix et al. 2002), resulting in a threat for all *Daphnia* size-classes.

A further experiment revealed that *D. magna* is not only able to switch the expression of the defensive traits according to its own life stage but also seems to be capable to adjust the expression of the defence to the actual predation risk exerted by *Triops*. Thereby, I compared the expression of the defensive traits in *D. magna* that were exposed to three different treatments: 1) control treatment, i.e. no predator-related cues present; 2) kairomone treatment, i.e. exposure to kairomones; and 3) direct contact treatment, i.e. exposure to physical contact with the predator, predator-generated microturbulence and kairomones. Compared to the control, the kairomone-exposed individuals showed a significant increase in the observed morphological parameters, i.e. body length, -width, and tail spine length (Fig. 1). In *D. magna* that were directly exposed to *Triops*, however, body length and tail spine length were significantly enlarged compared to both, control and the kairomone-exposed individuals and body width was significantly increased compared to control individuals (Fig. 1). Thus, these findings are in accordance with previous studies, which show that the expression of inducible defences in *Daphnia* is triggered by a variety of cues. Each of these cues alone, i.e. kairomones (e.g. Dodson 1989), alarm cues (e.g. Pijanowska 1997) and turbulence (Hrbáček 1959; Laforsch et al. 2004), have been shown to induce morphological changes. However, it has also been shown that they act synergistically. Hence, a combination of cues leads to a different expression of the defence compared to the effect of a single cue. For instance, it has been shown that alarm cues from macerated conspecifics trigger morphological changes in several *Daphnia* species while exposure to the kairomones of feeding predators causes a considerably stronger response (Laforsch et al. 2006). This study further revealed that *D. cucullata* reacts more sensitive to both, macerated conspecifics and predators feeding on conspecifics, than to macerated *D. magna* or predators feeding on alternative prey, respectively. Furthermore, it has been shown that artificial turbulence and predator kairomones act synergistically, triggering the expression of a significantly larger helmet than each factor alone (Tollrian & Laforsch 2006). Hence, both the results of the previous studies and my results clearly confirm the idea that *Daphnia* is not only able to precisely adjust the expression of defensive traits to the type of predation (e.g. Riessen & Trevett-Smith 2009; Herzog & Laforsch 2013) but also to the actual degree of predation pressure.

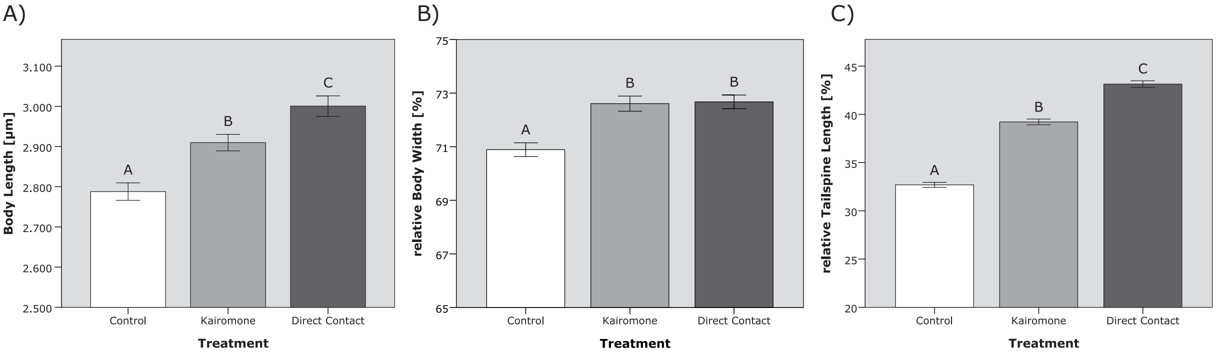

Figure 1: Expression of the defensive morphological traits in *D. magna* exposed to different predator-borne cues. A) Body length, B) relative body width and C) relative tail spine length of primiparous *D. magna*, which were either exposed to no predator cues (control), kairomones released by *Triops* (kairomone) or direct contact with *Triops*, i.e. physical contact, turbulence and kairomones, (direct contact). Error bars represent 1 SE. Treatments that do not have a letter in common are significantly different from one another ($P < 0.05$) based on a one-way ANOVA followed by pairwise comparisons using the Tukey HSD post-hoc test.

To test for interclonal variation in the expression of the defensive traits mentioned above, a comparison of several clones of *D. magna*, originating from different locations across Europe, was conducted. It revealed that the *Triops*-induced expression of an increased bulkiness seems to be the general response to the presence of this predator. However, I also revealed that there are exceptions, i.e. one of the clones responded to *Triops* in a different way. Namely, induced individuals of this clone reduced body length and did not change body width but increased shoulder-shield width and tail spine length, a response which closely resembles a morphological response to fish (e.g. Boersma et al. 1998). The origin of this clone, a former fishpond near Munich, Germany, further suggests that this clone is adapted to fish rather than *Triops*. The expression of the defensive traits could have been triggered by alarm cues released by conspecifics that have been crushed by *Triops*, indicating an unspecific predation threat. This assumption is supported by several studies reporting the relevance of alarm cues as an agent for the induction of defence mechanisms (e.g. Pijanowska 1997; Stabell et al. 2003; Schoeppner & Relyea 2005). In the context of morphological defences in *Daphnia*, Laforsch et al. (2006) were able to show that the morphological response to alarm cues alone is clearly less pronounced as to kairomones released by feeding predators. Nevertheless, compared to its undefended conspecifics, induced individuals of this clone should still be better defended against *Triops*-predation due to the elongated tail spine and the increased width of the shoulder shield. Interestingly, this clone originates from the same pond as the clone used for most of the experiments presented in this thesis ($K_3$4J), which expresses very pronounced morphological defences against *Triops*. The observed interclonal variation in the expression of the *Triops*-induced morphological defences, even in clones originating from the same habitat, is in accordance with previous studies focussing on this issue. For instance, interclonal variance in the expression of induced defences could be observed in *D. pulex* (Havel 1985; Boeing et al. 2006), *D. hyalina* (Stibor & Lampert 2000) and *D. magna* (Boersma et al. 1998), indicating that this phenomenon is relatively common in *Daphnia*.

In addition to the very obvious large scale defences that result in an increased bulkiness of the induced individuals, *D. magna* also expresses a number of less conspicuous small scale or even “hidden” defences as a response to *Triops*. *D. magna*, as well as most *Daphnia* species, is bearing a large number of small spines, so called spinules, on the dorsal ridge and the ventral edges of the carapace (Fryer 1991). Although it has been assumed that these spinules fulfil defensive function, there have only been few studies addressing this issue. By using SEM and light microscopy, I revealed that the presence of *Triops* induces considerable changes in both the morphology of the spinules and the size of the spinule bearing areas (SBAs). Thereby, the length of the dorsal spinules is increased by approximately 50% and the width of their base is also significantly increased. Contrarily, no changes were observed in the ventral spinules. Concerning the size of the SBAs, this study revealed that the dorsal SBAs were significantly enlarged in all analysed clones, whereas one clone did not enlarge the size of the ventral SBA, indicating that interclonal variance also plays a role in the expression of small scale defences. The defensive function of these predator-induced traits is presumably based on an interference with the predator’s feeding apparatus, which includes numerous small spines. A comparable morphological defence has already been shown in *Chaoborus*-exposed *D. ambigua*, where an increased size of the helmet is accompanied by an elongation of the spinules located on the dorsal and ventral carapace margin (Hebert & Grewe 1985). Further examples for the defensive value of relatively small spines are the neckteeth expressed in *D. pulex* (e.g. Krueger & Dodson 1981; Havel & Dodson 1984) and *D. curvirostris* (Juračka et al. 2011). Based on these findings and the fact that most *Daphnia* species possess the spinules located on the carapace margins, I would anticipate that induced changes in spinule morphology and/or distribution may be a common response to invertebrate predators that bear fine cuticular structures within their feeding apparatus that are likely to interfere with the spinules.

The existence of “hidden”, ultrastructural defences in *Daphnia* has often been discussed (e.g. Havel & Dodson 1984; Parejko 1991), however, there have been only few studies actually proving their existence (Dodson 1984; Laforsch et al. 2004). This discrepancy might be due to the fact that the analysis of ultrastructural defensive traits, such as an increased rigidity of the exoskeleton, require specialised techniques not commonly used in ecological research. By applying an interdisciplinary approach, I was able to reveal that both the large and small scale defences in *D. magna* are accompanied by hidden defences that considerably increase the stability of the carapace in induced individuals and should therefore provide additional protection against *Triops*. Bright field-, fluorescence- and transmission electron microscopy based studies revealed that the thickness of outer cuticle layer of the carapace is approximately doubled in induced *D. magna* and the diameter of the pillars which connect the outer and the inner cuticle layer is increased by roughly 10%. The probably most striking ultrastructural change, an approximately five-fold increase in cuticle hardness, was detected by the use of atomic force microscopy (AFM). This considerable increase is presumably caused by changes in the ultrastructure, e.g. a modified architecture of the chitin fibres, and/or the chemical structure of the cuticle, such as a stronger calcification (Alstad et al. 1999) or a higher melanin content, which acts as a strengthening agent in the arthropod cuticle (Hepburn & Roberts 1975). Due to the very small indentation depth of approximately 50nm and the modified Hertz model used to extract the elastic properties, the observed increase in rigidity can be considered to be independent from the simultaneous changes in cuticle thickness and pillar strength. Hence, *D. magna* responds to the presence of *Triops* with an, at least partly, independent set of ultrastructural changes. AFM-imaging further revealed that the cuticle associated changes are accompanied by changes in the nanostructure of the cuticle surface, which becomes more compact in induced individuals. The observed increase in cuticle thickness in induced *D. magna*, which is mainly caused by an amplification of the procuticle, is in accordance with studies that have shown the predator-induced increase in cuticle thickness in *D. middendorffiana* (Dodson 1984), *D. cucullata* and *D. pulex* (Laforsch et al. 2004). Alike, an increase in the diameter of the pillars that contribute to the overall stability of the exoskeleton has been shown in *Chaoborus*-induced *D. cucullata* (Laforsch et al. 2004). Together, the predator-induced ultrastructural changes in *D. magna* lead to a considerable fortification of the carapace which should be of special importance when confronted with *Triops*, which has to crush its prey prior to ingestion. The fortification of the carapace is thereby realized in a sort of lightweight architecture that maximises stability while keeping the costs, i.e. material and energy, minimized. Keeping the expenditures low is especially important since these ultrastructural defences have to be rebuilt at each moult. On the other hand, the fact that they are built at each moult implies that such ultrastructural defences may be rapidly expressed to serve as “first-line-defences” which offer protection while the much slower expression of the more prominent morphological defences takes place. In addition, these defences can also be assumed to be rapidly reduced to save costs when the predation pressure declines. Due to the anticipated costs of inducible defences, which can be saved in the absence of the inducing predator, the reversibility of induced defences should play an important role in predator-prey interactions (e.g. Gabriel 1999; Tollrian & Harvell 1999; Relyea 2003). However, this aspect is still not very well studied and thus remains mostly unknown. In contrast to other morphological defences that are irreversible or only partially reversible (Gabriel et al. 2005), the ultrastructural defences in

Daphnia bear the potential for rapid changes within single moults. Furthermore, it can be assumed that they are incurred with measurable costs, such as an increased energy expenditure for locomotion because induced daphnids may be heavier than their undefended conspecifics. Therefore, the study of these defences may help to understand and explain the mechanisms of reversibility in phenotypically plastic traits.

In order to comply with the definition of inducible defences (Harvell & Tollrian 1999), the *Triops*-induced morphological changes in *D. magna* should provide an efficient protection against this predator. Therefore, predation trials were conducted to quantify the advantages of the induced changes. In an initial step, the effectiveness of the defences was tested by respectively confronting both morphs of juvenile and adult *D. magna* with medium sized *Triops*. These trials revealed that a significantly larger number of induced individuals survived and that induced individuals of both life stages were equally well defended against *Triops*. A second set of predation trials, using different *Daphnia* and *Triops* size-class combinations, was then conducted to test for possible limitations of the defence. For instance, it seems probable that the defence offers only little protection when a very small, juvenile *D. magna* is confronted with a very large *Triops*. Overall, this experiment proved that the predator-induced defences offer an effective protection for all life stages of *D. magna* and that it is effective against *Triops* of different size classes. This is of particular importance since a simultaneous occurrence of different *Triops* size-classes has been shown to be common in natural habitats (Boix et al. 2002). On the other hand, the experiment also revealed some limitations of the defence: While the defence of small *D. magna* significantly increased survival when exposed to small- or medium-sized predators, no such effect was observed against large predators. The observation that there was no difference between the two morphs in medium-sized daphnids exposed to small *Triops* and large-sized daphnids exposed to small and large *Triops* can be explained by the fact that the daphnids were too large to be properly handled by the predators, regardless of their morphotype. This assumption is further supported by the low total number of eaten daphnids in the respective predation trials.

The effectiveness of the morphological defences can be explained by the morphology of the predator's feeding apparatus and its mode of feeding. When *Triops* catches a prey item, it is first encaged by the spines of the endites of the anterior limbs and it is then entered in the midventral food groove, build by the heavily armed gnathobases (Fryer 1988). While the abduction-adduction movements of the gnathobases are used to tear or crush softer prey items, the denticles of the gnathobases and the longer "curved-" and "sweeping spines" are used to transport larger, more robust prey towards the mandibles where it is crushed and ingested. The *Triops*-induced bulkiness in *D. magna* should therefore impede the handling of the prey by posing several difficulties to the predator. Firstly, encaging larger prey items should be more difficult since the size of the "cage" built by the spines of the endites is limited. Further, the wider shoulder shield and the elongated tail spine likely interfere with these spines and therefore increase the daphnids chances to escape the attack. Secondly, if the catch was successful, the uptake of daphnids with the bulky morphotype into the food groove should be more difficult since its opening width is limited. And thirdly, it seems plausible that getting a firm grip on the prey, to hold it in the food groove and to transport it towards the mouth, is further impeded by the more round shaped and larger body of the induced morph. Due to the fact that numerous small spines are involved in handling and transporting the prey, it seems very likely that the predator-induced enlargement of the pointy shoulder shield, the tail spine, the spinules on the carapace margins and the spinule bearing areas act synergistically to the bulkiness and thus contribute to the effectiveness of the whole array of defences. The mechanism underlying the protective effect of the increased spinescence may thereby be based on an interference of the daphnids' spinules with the small spines in the feeding apparatus of *Triops*, which should considerably impede or even prevent the handling of the induced morphotype. Additionally, the spinules may hurt or even injure the predator. As a consequence, induced *D. magna* are more likely to escape the predator during transport in the food groove. This assumption is further supported by observations made during the predation trials because it was often observed that induced *D. magna*, which have already been taken to the food groove, were suddenly repelled by the predator. Following the repulsion, the *Triops* usually showed a strong behavioural reaction, i.e. they displayed very fast swimming with frequent changes in direction, which was repeatedly interrupted by a bending of the body. Although no behavioural data were recorded during the predation trials, this behaviour was almost exclusively observed when *Triops* handled induced *D. magna*. Finally, the measured fortification of the carapace should provide further protection against *Triops*-predation. The harder and more rigid exoskeleton should provide an efficient protection against being pierced or crushed by the heavily armed gnatobases during transport through the food groove and also the resistance against the sharp mandibles should be increased. Moreover, the ultrastructural defences may also decrease the ability of the predator to get a firm grip on the prey. Instead of being indented, a more rigid carapace should favour that the daphnid simply slips out of the predator's grip and consequently escapes the attack. To conclude, the proven effectiveness of the *Triops*-induced morphological defences in *D. magna* is presumably based on the synergistic action of the large- and small scale defences as well as the hidden ultrastructural defences, which should severely impede the handling of the prey.

A long-term mesocosm study further confirmed the effectiveness of the morphological defences and the negative size selection of *Triops*. Thereby, it was revealed that *D. magna* is indeed able to coexist with *Triops*, without sustaining periods of increased predation pressure by the production of resting eggs, and further, that the expression of the induced traits is positively correlated with the predator's density. This positive correlation indicates that *Triops* is a powerful agent in structuring populations of *D. magna* and other pond-dwelling macroinvertebrates by preferably preying upon smaller and less defended individuals. In turn, this leads to an increasing prevalence of clones that possess the ability to express pronounced morphological defences. Hence, this result supports previous findings, which show that predation plays a major role in structuring prey populations (Brett 1992; Pijanowska et al. 1993; Brendonck & De Meester 2003).

The study on *Triops*-induced structural defences in *D. magna* revealed the potential of AFM as a powerful tool to assess the mechanical properties of the exoskeleton in planktonic crustaceans. As it is an accessible and comparably time-efficient method, AFM-based force spectroscopy facilitates the study of further ecologically relevant questions related with exoskeleton rigidity, such as the effects of global change on calcifying crustacean zooplankton. For instance, I used this method within the framework of a study on the effects of changes in water chemistry, specifically low calcium levels, on the expression of *Chaoborus*-induced morphological defences in *D. pulex*. The phenomenon of decreasing calcium levels, which is often caused by human activity, such as forest harvesting and atmospheric acid deposition (Watmough et al. 2003), can be found in many softwater lakes and ponds around the world (Cairns & Yan 2009). In our study, we revealed that low calcium levels had a negative effect on the predator-induced increase in body length and the formation of neckteeth. To test whether the *Chaoborus*-induced increase in cuticle hardness is also negatively affected by low calcium concentrations, I adjusted and applied the AFM-method to probe induced and non-induced juvenile *D. pulex* raised under different calcium levels. These AFM measurements indeed confirmed that decreased calcium levels render *D. pulex* incapable to strengthen their carapace in response to *Chaoborus*-larvae. In contrast, predator-exposed daphnids that were raised under a normal calcium level almost doubled the hardness of their cuticle. No difference in cuticle hardness was detected between the respective control treatments, indicating that the calcium level per se did not influence the elastic properties of the cuticle but that low calcium levels impede the expression of a harder cuticle. These results suggest that the vulnerability to *Chaoborus*-predation may be considerably increased under low calcium conditions. Together with the already known consequences of declining calcium levels, such as a reduced fecundity and an increased susceptibility to food limitation and temperature stress (Ashforth & Yan 2008), my results suggest that the on-going calcium depletion poses severe threats to the *Daphnia* fauna in many softwater lakes. Due to their intermediate position in the food web, i.e. as a major grazer of phytoplankton and prey for both invertebrate and vertebrate predators, the decline of the *Daphnia* populations can be assumed to have an extensive impact on the whole lake ecosystem (Jeziorski et al. 2013).

To conclude, my studies are the first to show that the cladoceran *D. magna* expresses a complete array of predator-induced morphological defences. Hence, my findings provide novel insights in the ecology of *D. magna*, an important model organism in ecological, evolutionary and environmental research, and may give way to further research on phenotypic plasticity in this species and in *Daphnia* in general. Thereby, I also establish a new predator-prey model system, which may help to foster our understanding of the ecology of temporary ponds as it is the first model system to be located in this habitat type. Furthermore, my studies confirm the idea that ultrastructural defences, i.e. a strengthening of the exoskeleton, may be a ubiquitous defence mechanism in *Daphnia* species confronted with predators that have to crush their prey prior to ingestion. Based on my results, the next logical step to gain further insight into those “hidden” defences would be to investigate the underlying ultrastructural- and chemical changes, e.g. by the application of AFM, TEM and energy dispersive X-ray spectroscopy (EDX). Furthermore, the role of the increased stability of the carapace, which accompanies the large-scale defences, should be taken into consideration when explaining the adaptive value of morphological defences in *Daphnia*. An appropriate way to analyse for the effects of the ultrastructural defences may be the application of the finite element method (FEM), a technique commonly used to predict the effects of mechanical stress on given structures. By combining different microscopy methods and FEM with a detailed analysis of the predation event, it may be possible to finally elucidate the exact mechanisms that cause the efficiency of morphological defences in *Daphnia*. The complete assessment of the induced traits and their contributions to the defence may also allow a better understanding of the cost-benefit-framework associated with the expression of inducible defences, since especially costs of plasticity in defensive traits have rarely been found. As demonstrated in this thesis, analyses of exoskeleton rigidity in *Daphnia* are facilitated by the use of atomic force microscopy, which is an accessible method with the advantage of being relatively time-efficient. Therefore, this technique seems to be perfectly suitable to study the effects of changing environments, e.g. acidification, on zooplankton by using carapace rigidity as a proxy. The probably most promising aim for the future, however, is to reveal the molecular background of inducible morphological defences in *Daphnia*. The discovery of the distinct predator-induced morphological defences in *D. magna* and the almost completed sequencing of its genome by the *Daphnia* Genomics Consortium (DGC; http://*Daphnia*.cgb.indiana.edu), render this species a very suitable model organism to study the molecular mechanisms and pathways that lead to the expression and evolution of inducible morphological defences.

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M. Rabus designed and performed research, analysed the data and wrote the paper. C. Laforsch designed research and wrote the paper.

Rabus et al. 2012

M. Rabus designed and performed research (clonal comparison and predation trials), analysed the data and wrote the paper. A. Waterkeyn designed and performed research (mesocosm study), analysed data and wrote the manuscript. N. Van Pottelbergh performed research (mesocosm study) and analysed the data. L. Brendonck designed research (mesocosm study) and wrote the manuscript. C. Laforsch designed research (clonal comparison and predation trials) and wrote the paper.

Rabus et al. (Manuscript)

M. Rabus designed and performed research, analysed the data and drafted the manuscript. A. Böttcher performed research (SEM study), analysed the data and drafted the manuscript. M. Chaplinska performed measurements (measurement of the SBAs), analysed the data and drafted the manuscript. C. Laforsch designed research and drafted the manuscript.

Rabus et al. 2013

MR designed and performed research, analysed the data and wrote the paper. T. Söllradl designed and performed research (AFM-measurements) and wrote the paper. H. Clausen-Schaumann designed research (AFM-measurements), contributed analytic tools and wrote the paper. C. Laforsch designed research and wrote the paper.

Riessen at al. 2012:

H.P. Riessen designed and performed research, analysed the data and wrote the paper. R.D. Linley designed and performed research, and analysed the data. I. Altshuler performed research. M. Rabus designed and performed research (AFM measurements), analysed the data and wrote the paper. T.S. analysed the data (AFM-measurements) and wrote the paper. H. Clausen-Schaumann contributed analytic tools (AFM-measurements) and wrote the paper. C. Laforsch designed and performed research (AFM-measurements), and wrote the paper. N.D. Yan designed research and wrote the paper.

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