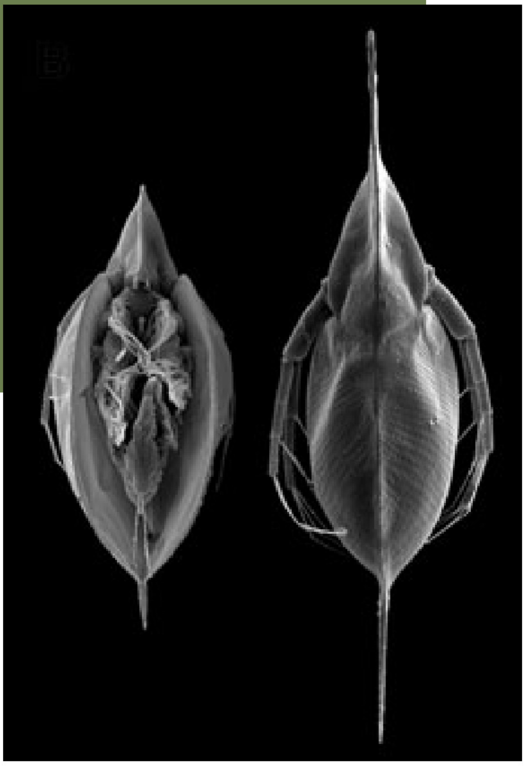
Morphologische Plastizität:

Die Zyklomorphose bei *Daphnia cucullata*

Christian Laforsch



Dissertation
zur Erlangung des Doktorgrades
der Fakultät für Biologie
der Ludwig-Maximilians-Universität München
September 2003

Erstgutachter: PD Dr. Ralph Tollrian

Zeitgutachter: Prof. Dr. Sebastian Diehl

Tag der mündlichen Prüfung: 08.12.2004

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Zusammenfassung

Die vorliegende Arbeit beschäftigt sich mit der morphologischen Plastizität bei *Daphnia cucullata*, die eine stark ausgeprägte Saisonalvariation, die so genannte Zyklomorphose zeigt. Die Ergebnisse dieser Studie haben gezeigt, dass sowohl von Räubern abgegebene chemische Substanzen (Kairomone), als auch von den Daphnien abgegebene Warnsignale eine induktive Wirkung auf die plastischen Merkmale von *Daphnia cucullata* haben, wobei die Induktion durch Warnsignale im Vergleich zur Induktion der prädator-exponierten Tiere gering ist. Ebenso sind im Labor erzeugte Mikroturbulenzen, die möglicherweise den Turbulenzen ähnlich sind, die andere Organismen durch Ihre Fortbewegungsweise im Wasser verursachen, als proximater Faktor der Zyklomorphose identifiziert worden. Als ultimater Faktor der Zyklomorphose konnte ein eindeutiger Schutzeffekt der plastischen Merkmale bei der Prädation verschiedenster invertebrater Räuber nachgewiesen werden. Diesbezüglich konnte durch eine neuartige hochfrequente Ultraschallmikroskopiertechnik eine „versteckte“ morphologische Plastizität, die Verstärkung des Panzers der Daphnien nachgewiesen werden. Zudem konnte durch eine selbst entwickelte neue rasterelektronenmikroskopische Präparationsmethode anhand der Ontogenie von prädatorexponierten und nicht-räuberexponierten Daphnien gezeigt werden, dass *Daphnia cucullata* ihre Verteidigung bereits im letzten Embryonalstadium ausbildet. Außerdem haben transgenerationale Kairomoninduktionsexperimente gezeigt, dass bereits verteidigte Mütter Nachkommen bilden, deren Ausprägung der Verteidigung stärker ist, als bei Nachkommen unverteidigter Mütter. Weiterhin sind die Daphnien in der Lage das aktuelle Prädationsrisiko anhand der Räuber-, als auch der Beutedichte abzuschätzen und nivellieren den Grad der Verteidigung anhand dieser Information. Dadurch werden mögliche, mit einer bestimmten Verteidigung verbundene Kosten gespart. Die Ergebnisse dieser Studie zeigen, dass sich die Zyklomorphose von *Daphnia cucullata* als eine Adaptation an eine variable Umwelt evolviert hat.

Abstract

The aim of this study was to reveal factors affiliated with morphological plasticity in *Daphnia cucullata.* This planktonic crustacean shows a seasonal variation in body shape that is commonly known as cyclomorphosis. Here I show that both predator-released chemicals and water-soluble “alarm” signals from wounded *Daphnia* can induce certain morphological features in *Daphnia cucullata.* Specifically, the inductive effect of the “alarm” signals is minute compared to the predator-exposed animals. Moreover, laboratory-generated small-scale turbulence was identified to be a proximate factor in cyclomorphosis. This special kind of turbulence is compared to turbulence evoked by the movement of other organisms.

A distinct protective effect of the morphological traits against invertebrate predation was revealed as the likely ultimate factor of cyclomorphosis. Furthermore, a unique method of ultrasonic microscopy reveals “hidden” morphological plasticity in daphnids. The armor of predator-exposed animals is much stronger than that of those not exposed to predators. I developed a new SEM-preparation method to get detailed insight in ontogenesis in *Daphnia*. I detected morphological defenses in predator-chemical-induced *Daphnia cucullata* already in the last embryonic stage.

Induction experiments on transgenerational effects in *Daphnia cucullata* show that defended mothers release offspring that are better defended than the descendants of unthreatened parents. Furthermore, daphnids could asses their actual predation risk by taking both predator and prey density into account. Thereafter the degree of a defense is adjusted to the individual hazard, thus saving unnecessary costs associated with the development of a particular defense.

The combined results of this study indicate that cyclomorphosis in *Daphnia cucullata* has evolved as an adaptation to variable environments.

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Danksagung

Lebenslauf

Einleitung

Induzierbare Verteidigung

Der Begriff phänotypische Plastizität beschreibt die Fähigkeit von Organismen als Reaktion auf variable Umweltbedingungen unterschiedliche Ausprägungen desselben Genotyps auszubilden (Stearns 1989, Pigliuggi 2001). Induzierbare Verteidigungen sind phänotypische Veränderungen, welche durch Reize ausgelöst werden die im Zusammenhang mit einer Gefahr aus der belebten Umwelt für diesen Organismus stehen. Die veränderte plastische Eigenschaft dient meist messbar dem Schutz des Individuums vor dieser Bedrohung (Tollrian & Harvell 1999).

Der Mechanismus der induzierbaren Verteidigung ist sowohl im Tier-, als auch im Pflanzenreich weit verbreitet, wobei in der Botanik oftmals die Terminologie Resistenz anstelle von Verteidigung gewählt wird. Im Pflanzenreich induzieren Reize von Pathogenen oder Herbivoren eine veränderte morphologische Ausprägung oder eine unterschiedliche Zusammensetzung der chemischen Inhaltsstoffe (Agrawal & Karban 1999).

Im Tierreich zeigen vertebrate und invertebrate Organismen plastisch veränderliche Morphologie, Lebenszyklusparameter und Verhaltensstrategien, ausgelöst durch die Wahrnehmung einer akuten Bedrohung. Bei den Wirbeltieren stellt das Immunsystem, das selektiv pathogene Keime durch Anschalten eines komplexen Mechanismus bekämpft, wohl eines der faszinierendsten Beispiele induzierbarer Verteidigungen dar (Frost 1999). Gleichfalls können Insekten durch die Produktion von antibakteriellen Substanzen, induziert durch eingedrungene Pathogene, auf diese reagieren.

Verhaltensanpassungen ausgelöst durch die Anwesenheit von Fressfeinden umfassen beispielsweise die Aktivität oder eine erhöhte Wachsamkeit des Beutetiers, um entweder die Begegnungswahrscheinlichkeit zu verringern oder die Fluchtchancen bei bereits erfolgter Attacke zu erhöhen.

Induzierbare Veränderungen der „Life History“ – Parameter, wie beispielsweise die Körpergröße bei Geschlechtsreife oder die Größe und Anzahl der Nachkommen, ermöglichen es der Beute aus dem Größenspektrum des Räubers zu entkommen. Ebenso dient die induzierbare morphologische Veränderung, welche die Entwicklung von sperrigen Verteidigungsstrukturen, eine Variabilität der Schalendicke oder eine unterschiedliche Körperform- oder Färbung beinhalten kann, dem Schutz vor Prädation (Tollrian & Harvell 1999).

Induzierbare Verteidigungen können sich nur unter spezifischen ökologischen Rahmenbedingungen evolviert haben, da sonst angenommen werden kann, dass die jeweilig spezifische Verteidigung im Laufe der Entwicklung genetisch und phänotypisch fixiert worden wäre. Hierbei werden vier Faktoren diskutiert, welche die Evolution dieses Spezialfalls phänotypischer Plastizität bedingen.

1. Der Selektionsdruck des jeweiligen Räubers sollte variabel sein, da bei permanentem Raubdruck eine konstitutive Verteidigung zu erwarten wäre. Jedoch sollte aber zeitweise ein starker Prädationsdruck von dem Räuber ausgehen.
2. Ein zuverlässiger Hinweis auf die Anwesenheit des Prädators muss existieren um die spezifische Verteidigung aktivieren zu können.
3. Die Verteidigung muss einen Vorteil bedingen.
4. Die Verteidigung sollte aber auch einen Nachteil haben, der in Situationen, in welchen der Vorteil nicht existent ist, überwiegt. Ansonsten wäre eine permanente Verteidigung zu erwarten.

Zur Untersuchung des Phänomens der phänotypischen Plastizität sind Daphnien („Wasserflöhe“), kleine planktonische Krebse als Studienobjekt hervorragend geeignet. Zum einen bedingt ihre parthenogenetische Fortpflanzungsweise das Arbeiten mit genetisch identischen Individuen, wodurch einerseits in der Interpretation der Ergebnisse die genetische Variabilität ausgeschlossen werden kann, anderseits für evolutionäre Studien unterschiedliche Klone verglichen werden können. Zum anderen vereinfachen die kurzen Generationszeiten die Versuchsbedingungen. Weiterhin spielen Daphnien in limnischen Ökosystemen eine tragende Rolle, da sie die wichtigsten Konsumenten der Primärproduktion darstellen (McCauley et al.1999) und demzufolge eine wichtige Funktion in der Verbindung der trophischen Interaktion zwischen invertebraten und vertebraten Prädatoren einnehmen. Aufgrund der bedeutenden ökologischen Relevanz waren Daphnien schon sehr bald im Blickpunkt des wissenschaftlichen Interesses.

Induzierbare Verteidigung und morphologische Plastizität bei *Daphnia*: Die Zyklomorphose

Bereits Anfang des letzten Jahrhunderts wurde ein saisonaler Gestaltwechsel bei planktonischen Organismen des Süßwassers beschrieben. Da für viele Arten eine Abfolge ähnlicher Arten oder die Selektion auf Morphen unterschiedlicher Gestaltausprägung ausgeschlossen werden konnte, war der beobachtete Formenwechsel auf phänotypische Plastizität der Organismen zurückzuführen. Diese in der Natur während des Jahresverlaufs auftretende Veränderung der Morphologie in einer Abfolge von genetisch identischen Generationen, wird als Zyklomorphose bezeichnet (Brooks 1946).

Die Zyklomorphose ist vor allem in der Familie der Daphnien beschrieben, obgleich diese Temporalvariation auch bei diversen Cladoceren, wie Bosminen, deren erste Antennen saisonal variieren (Brock 1980) und Rotatorien (Gilbert 1966; Gilbert & Stemberger 1984), die abspreizbare Dornen ausbilden können, zu beobachten ist. Dabei zeigen Daphnien eine besondere Vielfalt morphologischer Plastizität. Es kann generell der Gesamthabitus, zum Beispiel hin zu einer schmaleren Körperform (Jacobs 1967; Havel & Dodson 1985), oder die Gesamtgröße einzelner Entwicklungsstadien saisonal variieren (Dodson 1974; Culver 1980). Weiterhin konnte eine Veränderung der Größe des Komplexauges, des Filterapparates (Jacobs 1966; Lee 1984), der Antennenborsten (Jacobs 1967) und der Spina (Dodson 1989) festgestellt werden. Die auffälligsten zyklomorphen Veränderungen sind jedoch im Kopfbereich einiger Daphnienarten zu finden. So zeigen einige Daphnien in bestimmten Entwicklungsstadien im Dorsalbereich so genannte Nackenzähne oder bilden je nach Art Helme unterschiedlicher Form aus (review: Tollrian & Dodson 1999).

Seit der Entdeckung des Phänomens der Zyklomorphose wurde sowohl nach den proximaten als auch den ultimaten Faktoren, welche diese morphologische Plastizität bedingen, geforscht. Dabei wurden in den frühen Untersuchungen hauptsächlich abiotische Faktoren wie Temperatur, Wasserturbulenzen oder Photoperiode als Ursache des temporalen Formenwechsels diskutiert (Brooks 1947; Hrbácek 1959; Jacobs 1962; Hazelwood 1966). Obwohl diese abiotischen Reize im Labor nur eine geringe Induktionswirkung zeigten, gab es verschiede Hypothesen zur Funktion der plastischen Merkmale. Anfänglich wurden die verlängerten Körperfortsätze als Schwebehilfen interpretiert. Dabei sollten diese als „Fallschirm“ fungieren, um die im Sommer durch hohe Wassertemperaturen auftretende Dichteverringerung des

Mediums zu kompensieren. Woltereck (1913; 1921) schrieb sowohl den Helmen, als auch der Spina eine balance- und richtungsweisende Funktion zu, die es dem Tier ermöglichen sollte, in horizontaler Schwimmlage, die von ihm postulierten, nahrungsreichen Schichten effektiv zu nutzen.

Erst die Einbeziehung biotischer Faktoren, brachte weiterführende Erkenntnisse in der Untersuchung der Zyklomorphose. Ausschlaggebend hierfür war eine Untersuchung von Gilbert (1966) der zeigte, dass chemische Substanzen, abgegeben von räuberischen Rotatorien, Dornen bei dem Rädertier *Brachionus calyciflorus* induzieren und dass diese Dornen einen Schutzeffekt bezüglich der Prädation von *Asplanchna* aufweisen. Diese Gruppe chemischer Signalstoffe die zwischen Individuen verschiedener Arten wirken und einen ökologischen Vorteil für den Empfänger bedingen werden als Kairomone definiert.

Seit dieser Zeit wurde in zahlreichen Untersuchungen die induktive Wirkung von Prädatorkairomonen auf Daphnien nachgewiesen. Diese wasserlöslichen Signalstoffe, von deren chemischer Natur bisher wenig bekannt ist, beeinflussen zum Beispiel „Life-history“ - Parameter und das Verhalten von Daphnien (review: Tollrian & Harvell 1999). Das diesbezüglich wohl bekannteste Beispiel ist die tagesperiodische Vertikalwanderung von Daphnien in Anwesenheit von planktivoren, visuell jagenden Fischen im Gewässer. Die Daphnien halten sich tagsüber in dunkleren und kälteren Wasserschichten auf und wandern erst nachts in die oberen nährstoffreichen Wasserschichten, um dem Raubdruck der Fische zu entkommen (Lampert 1989; Loose 1993). Aber auch in der zyklomorphen Gestaltausprägung spielen Kairomone eine bedeutende Rolle. Chemische Signalstoffe der räuberischen Büschelmücke induzieren bei *Daphnia pulex* und *Daphnia hyalina* Nackenzähne in den ersten Juvenilstadien, wobei aber klonale Unterschiede in der Merkmalsausprägung festgestellt wurden (Krueger & Dodson 1981; Spitze & Sadler 1996). Ebenso konnten die „Pickelhauben“ von *Daphnia ambigua* durch Chaoborusstoffe induziert werden (Hebert & Grewe 1985; Hanazato 1992, 1995). Die australische Art *Daphnia longicephala* entwickelt große dorsale Kopfhauben bei Anwesenheit von Notonectiden (Grant & Bayly 1981). Ebenso reagieren *Daphnia galeata mendotae* und *Daphnia retrocurva* mit der Bildung eines Helmes (Dodson 1988) auf Kairomone spezifischer invertebrater Prädatoren. Eine sehr stark ausgeprägte Formvariation zeigt *Daphnia lumholtzi,* die auf Fischstoffe mit der

Bildung von langen Dornen an Kopf und Spina reagiert (Tollrian 1994; Kolar & Wahl 1998).
Nachdem Kairomone in zahlreichen Beispielen als auslösender Faktor der Formvariabilität nachgewiesen wurde, sind neue Hypothesen für den Adaptivwert der zyklomorphen Ausprägungen aufgestellt worden, in denen vor allem Räuber – Beute Interaktionen in Vordergrund stehen. So vermutete Jacobs (1967) dass schmälere, behelmte Morphen bessere Schwimmeigenschaften besitzen und somit Prädatoren besser entkommen können. Ebenso deutete Dodson (1974) die Zyklomorphose als Schutz vor Räubern. Er erstellte die „Anti-Schlüssel-Schloss“ Hypothese, die besagt, dass der Fangapparat invertebrater Prädatoren an die Normalform der Beute angepasst ist. Durch Veränderung der Körperform werden Beutetiere sperrig und können demzufolge schlechter gefangen werden. Aufbauend auf den gewonnenen Ergebnissen entwickelte Zaret (1980) die Theorie, dass die Temporalvariation dem Schutz vor größenselektiven, invertebraten Prädatoren diene. Durch Verlängerung oder Vergrößerung bestimmter Körperteile fallen die Beutetiere aus dem gefährdeten Größenspektrum invertebrater Prädatoren, da diese durch Ihre Fangapparate größenlimitiert sind. Die Ausbildung von Verteidigungsstrukturen und deren Vorteil bei der Prädation wurde vor allem im System *Chaoborus* - *Daphnia pulex* gezeigt (Krueger & Dodson 1981; Parejko & Dodson 1991; Tollrian 1995). Aber auch in anderen Räuber - Beute Interaktionen konnte ein deutlicher Nutzen der phänotypischen Plastizität nachgewiesen werden, wie zum Beispiel für die Kopfhauben von *Daphnia longicephala* oder der Spina bei *Daphnia middendorffiana* (O’Brien & Vinyard 1978; Balseiro & Vega 1994). Demzufolge wird in der heutigen Literatur vor allem eine Schutzfunktion der zyklomorphen Formvariation bei Daphnien als ultimater Faktor diskutiert.

Die heimische Art *Daphnia cucullata* zeigt eine stark ausgeprägte morphologische Plastizität. Sie ist eine in Europa weit verbreitete pelagisch lebende Cladocerenart, die vorwiegend im Epilimnion eutropher Seen vorkommt. Der Körper ist äußerst hyalin und weist einen zart gefelderten Carapax auf. Das Komplexauge ist klein und ein Naupliusauge ist nicht vorhanden (Abb. 1).

Abb 1: Lichtmikroskopische Aufnahme einer rundköpfigen und einer behelmten Morphe des gleichen Klons von *Daphnia cucullata*.

Ihr augenfälligstes Charakteristikum ist die Fähigkeit zur Zyklomorphose. Die Temporalvariation äußert sich bei dieser Art durch die Ausbildung von seitlich abgeflachten Helmen (Abb. 2). Im Frühjahr entwickeln sich aus rundköpfigen Müttern, im Laufe der Zeit immer stärker behelmte Tiere. Die längsten Helme werden in der Regel in den Monaten Juli und August gebildet. Die nachfolgenden Generationen bauen diesen Helm bis zum Erreichen des Winters wieder kontinuierlich ab (Lampert & Wolf 1986). Obgleich *Daphnia cucullata* als exemplarischer Modellorganismus zur Erklärung der Zyklomorphose angeführt wird (Lampert & Sommer 1993, Herder 1994), sind bisher erstaunlicherweise erst wenige Untersuchungen über die Mechanismen dieses Phänomens durchgeführt worden.
Die vorliegende Arbeit behandelt sowohl biotische, als auch abiotische Faktoren die in kausalem Zusammenhang mit der zyklomorphen Gestaltvariation bei *Daphnia cucullata* stehen:

1. Da *Daphnia cucullata* in der Natur Beute verschiedener invertebrater Räuber ist, teste ich die Induktion der zyklomorphen Gestaltvariation durch Kairomone von verschiedenen Invertebraten Prädatoren und den damit verbundenen Schutzeffekt als Adaption an ein ständig wechselndes Prädatorenregime.

2. Weil nicht ausgeschlossen werden kann, dass Daphnien und nicht die Prädatoren die aktiven chemischen Signale abgeben, untersuche ich die Rolle möglicher innerartlicher Warnsignale.
3. Weiterhin teste ich die Transgenerationseffekte der Kairomoninduktion, da aufgrund einer Verzögerungsphase der Induktion, bereits verteidigte Mütter besser verteidigte Nachkommen in ihre Umwelt entlassen sollten.
4. Ich untersuche die Abschätzung des aktuellen Prädationsrisiko anhand der Räuber- und der Beutedichte, da unnötige Kosten durch Anpassung der Verteidigung an den jeweiligen Raubdruck vermieden werden können.
5. Aufgrund alter Literaturangaben, die eine leichte Induktion durch Turbulenzen beschreiben, teste ich die Induktion der plastischen Merkmale von *Daphnia cucullata* durch Mikroturbulenzen. Hierbei wird die Rolle von möglichem Windstress und die, durch andere Organismen erzeugte Mikroturbulenz, bezüglich der Zyklomorphose diskutiert.
6. Anhand einer selbst entwickelten, neuartigen Präparationsmethode analysiere ich durch rasterelektronenmikroskopische Technik die Embryonalentwicklung von kairomonexponierten Daphnien und Kontrolltieren, um morphologische Unterschiede in der Ontogenie der beiden Morphen zu untersuchen.
7. Ich verwende eine neuartige hochfrequente Ultraschallmikroskopietechnik zur Untersuchung der Stärke des Panzers, um eine möglicherweise versteckte morphologische Plastizität aufzudecken. Weiterführend habe ich den Carapax von *Daphnia cucullata* mittels Semidünnschnitten und digitaler Bildanalyse analysiert, da bei einem verstärktem Panzer Unterschiede in der Carapaxbeschaffenheit von induzierten und nicht induzierten Tieren bestehen könnten.

Im Anschluss sind die Veröffentlichungen und Manuskripte als einzelne Kapitel aufgeführt. Ergebnisse, die nicht in die Manuskripte aufgenommen wurden, werden in der Synopsis der einzelnen Kapitel wiedergegeben.

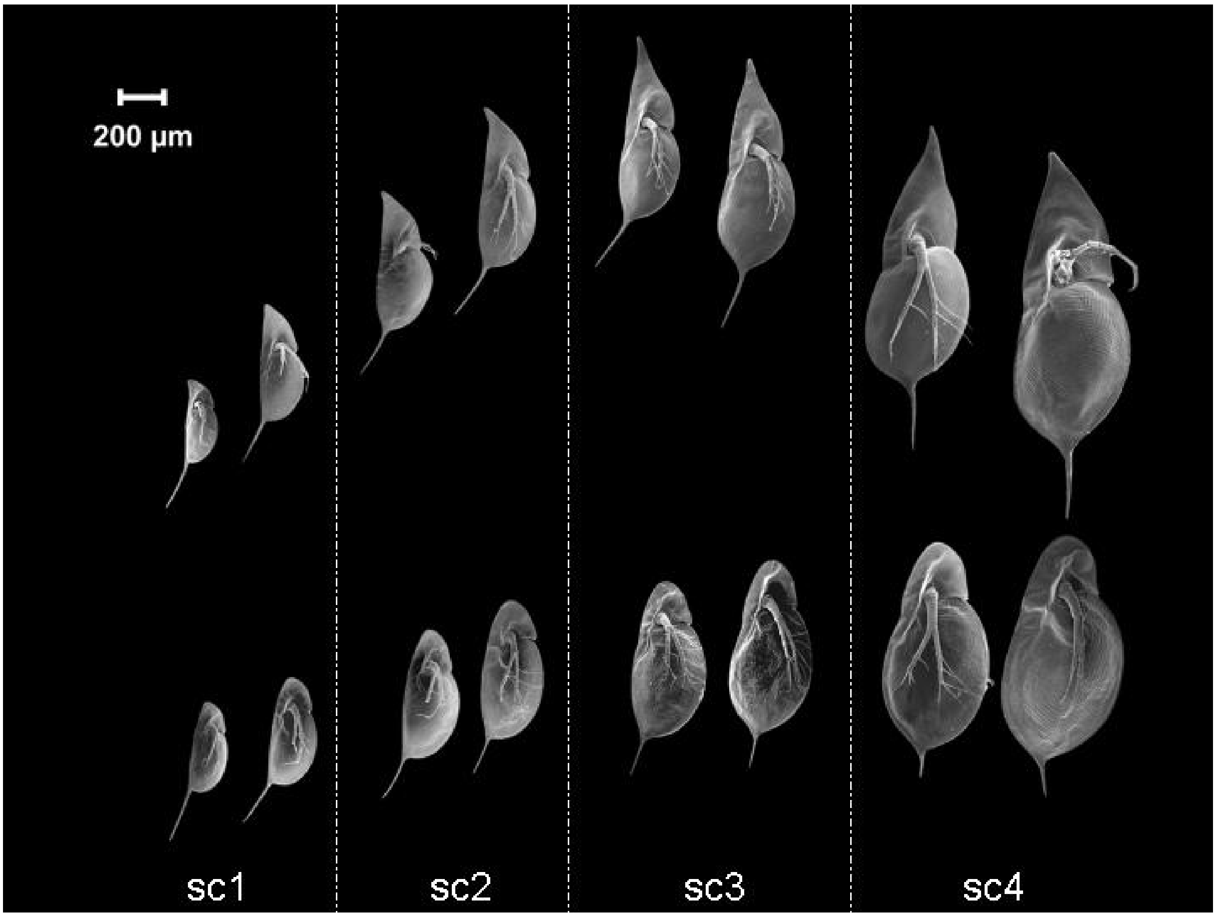

Abb. 2 Vergleichende rasterelektronenmikroskopische Aufnahme aller Entwicklungsstadien von induzierten (oben) und nicht induzierten (unten) Individuen von *Daphnia cucullata*.

Zusammenfassung der Artikel

1.

INDUCIBLE DEFENSES IN MULTIPREDATOR ENVIRONMENTS: CYCLOMORPHOSIS IN *DAPHNIA*

(Laforsch und Tollrian, *Ecology*, im Druck)

Die Prädation als Selektionsfaktor stellt eine Schlüsselfunktion für die Evolution von Beuteorganismen dar. In natürlichen Habitaten sind die meisten Beuteorganismen einer Vielzahl von unterschiedlichen Prädatoren ausgesetzt. Diese können sich erheblich, sowohl in ihrem Suchschema, als auch in Ihrem Beutegreifmechanismus unterscheiden. Planktonorganismen sind dabei sowohl Beute für vertebrate als auch für invertebrate Räuber, welche sowohl in ihrer Abundanz als auch temporär variieren. Da im Pelagial jegliche Versteckmöglichkeit fehlt, besteht der Selektionsdruck diverse Verteidigungsstrategien zu evolvieren. Die Morphologische Plastizität von Verteidigungsstrukturen scheint dabei ein ausgezeichneter Mechanismus zu sein, um auf eine saisonal variierende Bedrohung eines ständig wechselnden Räuberregimes zu reagieren. Um den Adaptivwert einer Verteidigungsstrategie zu verstehen ist es notwendig den Effekt der Verteidigung im Zusammenhang mit einer Vielzahl von potentiellen Räubern zu untersuchen, anstatt sich auf ein Räuber-Beute System zu konzentrieren. Hierbei erlauben induzierbare Verteidigungen den Schutzeffekt einer spezifischen Verteidigung einzuschätzen, da dieser anhand genetisch identischer Individuen zwischen der verteidigten und der nicht verteidigten Form verglichen werden kann.

Ich habe am Modellsystem *Daphnia cucullata* die Induktion und die damit im Zusammenhang stehende Schutzwirkung der Helm- und Spinabildung untersucht.

Daphnia cucullata koexistiert im Epilimnion mit Fischen und ist als Anpassung an diese optisch orientierten Räuber eine sehr kleine und transparente Daphnienart mit geringer Gelegegröße (Hrbácek 1962). Allerdings haben auch viele invertebrate Prädatoren, obwohl diese zu den großen Organismen des Zooplanktons zählen, diverse Verteidigungsstrategien gegen Fischprädation entwickelt. Als prominentestes Beispiel für eine derartige Anpassung, ist die tagesperiodische Vertikalwanderung von Chaoboruslarven unter Anwesenheit von Fischen zu erwähnen (Dawidowicz 1993). Ebenso wie die Büschelmückenlarve zeichnet sich auch die räuberische Cladocere *Leptodora* durch einen extrem durchsichtigen Körper aus. Aus diesem

Grund ist *Daphnia cucullata* eine potentielle Beute einer Vielzahl von invertebraten Prädatoren und sollte die Fähigkeit besitzen flexibel auf ein wechselndes Prädatorenregime zu reagieren.

Daher wurde in diesem Kapitel die Hypothese getestet, ob die plastischen morphologischen Eigenschaften von *Daphnia cucullata* durch verschiedene Räuberstoffe induziert werden können. Weiterhin wurde untersucht ob behelmte Individuen einen Vorteil bei der Prädation gegenüber den typischen rundköpfigen Morphen besitzen und welcher funktionelle Mechanismus diesen Vorteil bedingt.

Meine Laboruntersuchungen haben gezeigt, dass Kairomone abgegeben von drei invertebraten Prädatoren (*Leptodora kindtii*, *Chaoborus flavicans*, *Cyclops*) die alle im Freiland mit *Daphnia cucullata* koexistieren, signifikant längere Helme und Spinae bei dieser Art induzieren, wobei die Laborinduktion nicht die im Freiland beobachteten Ausprägung der Merkmale erzielt hat.

Meine Prädationsversuche mit *Cyclops*, *Leptodora* und *Chaoborus* haben ergeben, dass die plastischen Merkmale Helm und Spina, eine eindeutige Schutzfunktion bei *Daphnia cucullata* gegenüber den rundköpfigen Morphen darstellen.

Interessanterweise waren hierbei Unterschiede zwischen den verschiedenen Prädatoren, sowohl bezüglich der geschützten Größenklasse der Daphnien, als auch im Schutzmechanismus zu finden. Helm und Spina ist demnach von Vorteil bei der Prädation verschiedenster Räuber auf *Daphnia cucullata*, die sich alle in Ihrer Jagdstrategie unterscheiden. Dabei geht das Spektrum von Lauerjägern (*Chaoborus*), über aktiv suchende Räuber (*Cyclops*), bis hin zu Streifgängern (*Leptodora*).

Diese Ergebnisse deuten an, dass im natürlichen Habitat chemische Stoffe die momentane Abundanz und Relevanz des jeweiligen Räubers anzeigen und die Zyklomorphose im Freiland initiieren. Die Kombination von verlängertem Helm und Spina scheint ein perfekter Mechanismus zu sein sich gegen eine Vielzahl unterschiedlicher Räuber zu schützen. Der Schutz einer einzelnen Verteidigung gegen unterschiedliche Prädatoren kann möglicherweise das Ergebnis einer „diffusen“ Coevolution sein. Der additive Vorteil könnte den Adaptivwert dieser allgemeinen Verteidigung erhöhen und somit evolutiv die Manifestierung dieser Verteidigung fördern.

2.

INDUCIBLE DEFENSES: THE RELEVANCE OF ALARM SIGNALS IN DAPHNIDS

(Laforsch und Tollrian, *Limnology and Oceanography*, Akzeptiert in Abhängigkeit der Revision)

Der Informationsaustausch über chemische Signalstoffe spielt eine tragende Rolle sowohl in der innerartlichen, als auch in der zwischenartlichen Kommunikation. Hierbei können Informationen zum Beispiel über Nahrungsqualität, die Dichte möglicher Sexualpartner oder das Prädationsrisiko empfangen werden (Atema et al. 1988). Viele aquatische Organismen verwenden chemische Signale (Kairomone) um Prädatoren zu detektieren und um eine spezifische Verteidigung zu induzieren. Chemische Alarmsignale können mit Warnrufen von Säugern und Wirbeltieren verglichen werden (z. B. Staton 1978; Klump & Shalter 1984) und vermitteln somit keinerlei Information über die Quelle der Bedrohung. Sowohl chemische Signale abgegeben von Prädatoren, als auch Alarmsignale, die von der Beute ausgesendet werden, können wichtige Informationen über den aktuellen Bedrohungsgrad aufzeigen.

In terrestrischen und aquatischen Lebensräumen aktivieren die durch Verletzung hervorgerufenen chemischen Warnstoffe spezifische Verteidigungen in den Beuteorganismen. Hierbei sind zahlreiche Untersuchungen über Verhaltensänderungen der Beute, ausgelöst durch chemische Alarmsignale durchgeführt worden (z.B. Brown & Godin 1997; Wisenden et al. 2001). Im Gegensatz dazu sind wenig Arbeiten über den Einfluss von chemischen Alarmstoffen auf die Induktion morphologischer Verteidigungen veröffentlicht worden (siehe review Chivers & Smith 1998). Ebenso konzentrierten sich bei der Untersuchung der phänotypischen Plastizität bei Daphnien die meisten Arbeiten auf Räuberkairomone, da in ersten Studien bei *Daphnia pulex* (Walls & Ketola 1989; Parejko & Dodson 1990) und *Daphnia galeata mendotae* (Stirling 1995) kein Effekt von homogenisierten Artgenossen auf die Morphologie der Daphnien nachgewiesen wurde.

Allerdings werden viele Planktonorganismen während einer Attacke verletzt, da zum Beispiel Fischlarven erst lernen müssen wie sie die Beute fressen können (Ibrahim & Huntingford 1992) und einige invertebrate Prädatoren ihre Beute zerkleinern bevor sie diese fressen (Branstrator 1998). Aus diesem Grund könnten auch chemische Alarmstoffe für Daphnien einen zuverlässigen Reiz in der Abschätzung des

Prädationsrisiko darstellen. Zwei Studien an *Daphnia magna* zeigen, dass sowohl Lebenszyklusparameter als auch das Verhalten durch Stoffe homogenisierter Artgenossen beeinflusst werden (Pijanowska 1997; Pijanowska & Kowalczewski 1997).

Daher war in diesem Kapitel das Ziel meiner Arbeit die Relevanz von chemischen Alarmsignalen im Vergleich zu Prädatorkairomonen auf die morphologische Plastizität von Daphnien zu untersuchen. Die Definition von chemischen Alarmsignalen war hierbei relativ weit gefasst, da angenommen werden konnte, dass diese Signale nicht in speziellen Drüsen produziert werden, sondern dass es sich hierbei um Zellinhaltsstoffe handelt, welche durch eine Verletzung in das Medium gelangen. In dieser Arbeit wurden drei *Daphnia* Arten untersucht, die alle eine induzierbare morphologische Verteidigung aufweisen. *Daphnia cucullata* bildet Helme (Tollrian 1990), *Daphnia longicephala* entwickelt hohe Nackenkämme (Grant & Bayly 1981) und *Daphnia lumholtzi* zeigt große Schwanz- und Kopfstacheln (Tollrian 1994).

Die Versuche haben ergeben, dass chemische Substanzen abgegeben von mazerierten Artgenossen die morphologischen Merkmale aller drei untersuchten Arten beeinflussen. Jedoch war die Induktion mit Räuberkairomonen in allen Fällen deutlich stärker als die Induktion mit Alarmsignalen. Experimente mit *Daphnia cucullata* haben weiterhin gezeigt, dass die Helmlänge höher ist wenn der Räuber mit Artgenossen gefüttert wird, anstatt mit Alternativbeute. „Cross-species“ Experimente weisen auf eine höhere Sensitivität von *Daphnia cucullata* auf das Homogenisat von Artgenossen, als auf das von *Daphnia magna* hin. Weiterhin war ein akkumulativer Effekt der Alarmsignale, sowie eine alarmstoffkonzentrationsabhängige Reaktion bei *Daphnia cucullata* bezüglich der Helminduktion festzustellen.

Chemische Alarmstoffe können Informationen über das momentane Prädationsrisiko liefern, geben dabei aber keinen Hinweis auf den Räuber. Ein potentieller Adaptivwert könnte darin liegen, dass die Daphnien durch die Warnsignale in einen Alarmzustand versetzt werden. Weiterhin könnten möglicherweise bei einer Vielzahl von koexistierenden Prädatoren, die alle in geringer Dichte vorkommen, die spezifischen Räuberstoffe nicht ausreichen, um einen hohen Raubdruck anzuzeigen. Hierbei könnte die Kombination von Alarmsignalen und Kairomonen ein signifikantes Prädationsrisiko signalisieren. Es besteht daher entweder ein synergistischer Effekt zwischen Kairomon und Alarmstoffen, oder der Räuber produziert einen Signalstoff mit höherer Effektivität für die jeweilige Daphnienart wenn er sich von deren Artgenossen ernährt. Meine Ergebnisse deuten an, dass Alarmstoffe nur ein Bestandteil einer Mixtur verschiedener Komponenten sind, die vornehmlich aus Räuberstoffen bestehen, und gemeinsam induzierbare Verteidigungen in der Natur aktivieren.

3.

TRANSGENERATIONAL INDUCTION OF DEFENSES IN ANIMALS AND PLANTS

(Agrawal, Laforsch und Tollrian, *Nature*, 401:60-63,1999)

In experimentellen Studien wurde gezeigt, dass die Einführung eines Prädators in ein System eine rasche Evolution von morphologischer oder verhaltensorientierter Verteidigung im Tierreich (z. B. Reznick et al. 1997, Thompson 1998) oder chemischer Verteidigung bei Pflanzen hervorrufen kann (Berenbaum et al. 1986, Thompson & Burdon 1992). Eine derartige Verteidigung kann konstitutiv, also phänotypisch fixiert ausgebildet werden oder induziert werden. Induzierbare Verteidigungen sind eine adaptive Verteidigungsstrategie, in der nicht letale Reize eines Räubers oder Parasiten, einen zuverlässigen Indikator über das zukünftige Prädationsrisiko darstellen. Ein Nachteil einiger Systeme ist jedoch eine Verzögerungsphase zwischen der Induktion und der Ausprägung der Verteidigung.

Falls die Umwelt der Eltern bereits eine Aussage über die Umwelt der Nachkommen zulässt, könnten die Eltern ihren Netto-Reproduktionserfolg steigern, indem diese den Phänotyp Ihrer Nachkommen auf die jeweilige Bedrohung hin anpassen. Derartige maternale Effekte sind bereits im Pflanzen- und Tierreich beobachtet worden (Roach & Wulff 1987, Mousseau & Fox 1998). Demnach setzen adaptive maternale Effekte voraus, dass die Mutter auf einen Reiz der Umwelt reagiert indem sie den Phänotyp ihrer Nachkommen so verändert, dass dieser in der jeweiligen Umwelt eine erhöhte Fitness aufweist.

In diesem Kapitel wurde der Effekt der Räuberexposition auf den Phänotyp der Nachkommen von *Daphnia cucullata* getestet.

Da sowohl die Induktion von *Daphnia cucullata* mit Kairomonen verschiedener Prädatoren beschrieben und der Schutzeffekt von Helm und Spina nachgewiesen wurde, gehe ich in diesem Kapitel nur auf die transgenerationale Induktion der Verteidigungsstrukturen ein.

Die Experimente haben gezeigt, dass signifikante Unterschiede in der Helmlänge von *Daphnia cucullata* bestehen die in derselben Umwelt aufgewachsen sind (räuberfrei oder mit Räuberstoffen), aber deren Mütter unterschiedlichen Treatments ausgesetzt waren. Ebenso zeigten die nachfolgenden Bruten das gleiche Muster. Dies verdeutlicht den Einfluss der maternalen Umwelt auf die transgenerationale Induktion der Verteidigungsmechanismen. Ebenso stellte sich heraus, dass die Umwelt im frühen Embryonalstadium einen Einfluss auf die Helmlänge von *Daphnia cucullata* hatte. Dieser war allerdings deutlich geringer als der Einfluss der maternalen Umgebung.

Weiterhin haben die Ergebnisse aufgezeigt, dass auch die Umwelt in der die F_0 Generation aufgewachsen ist, noch den Phänotyp der Neonaten der F_2 Generation nachhaltig beeinflusst.

Der Adaptivwert von transgenerationalen Effekten liegt zum Teil darin begründet, dass ontogenetische Grenzen in kritischen Lebensphasen abgepuffert werden können. Zum Beispiel scheint ausgehend von einem rundköpfigen Individuum die spätere Ausbildung der maximalen Helmlänge von *Daphnia cucullata* nicht möglich. Demgegenüber könnten transgenerationale Effekte beeinflusst durch die maternale Umgebung, die maximale Induktion der Verteidigung ermöglichen. Ein großer Nachteil induzierbarer Verteidigungen ist, dass die Verteidigung nicht sofort präsent ist, wenn sie benötigt wird, da eine lag-Phase zwischen Induktion und Ausprägung der Verteidigung besteht. Diesbezüglich können Nachkommen mit bereits maternal induzierter Verteidigung diesen Nachteil nivellieren. Da die Prädation einen erheblichen Selektionsfaktor darstellt, scheint die transgenerationale Induktion von Verteidigungen eine logische Erweiterung der fitnessfördernden Maßnahmen von Beuteorganismen zu sein, zumal dieser Effekt sowohl im Pflanzen-, als auch im Tierreich zu beobachten ist.

4.

EXACT PREDATION-RISK ASSESSMENT AND DEFENCE-LEVEL ADJUSTMENT TO PREY DENSITY (Tollrian, Duggen, Laforsch und Kopp, *Nature*, eingereicht)

Sehr viele Organismen sind darauf angewiesen den Aufwand für eine Verteidigung dem aktuellen Prädationsrisiko anzupassen, da sich die meisten Verteidigungen in einer Kosten-Nutzen Beziehung evolviert haben (Tollrian & Harvell 1999). Dies bedeutet, dass Kosten mit dem Grad der Verteidigung steigen. Die Selektion bewirkt, dass sich diejenigen Organismen durchsetzen, die den optimalen Grad an Verteidigung unter den jeweils spezifischen Umweltbedingungen ausgebildet haben und damit unnötige Kosten vermeiden. Es ist aus diesem Grund für ein Beutetier von ausschlaggebender Bedeutung das exakte Prädationsrisiko dem es ausgesetzt ist abzuschätzen, um die Verteidigung optimal anpassen zu können.

In bisher durchgeführten Studien wurde nur die Dichte der Prädatoren zur Abschätzung des Prädationsrisiko verwendet (Lima & Dill 1990, Kats & Dill 1998). Jedoch sollte das Prädationsrisiko des einzelnen Individuums ebenso mit steigender Anzahl der Beutetiere geringer werden. Demzufolge sollte eine exakte Abschätzung des Prädationsrisiko sowohl die Räuber, als auch die Beutedichte berücksichtigen.

Diese Hypothese wurde in diesem Kapitel anhand von sechs Räuber-Beute-Systemen getestet, bei der die Beuteorganismen jeweils eine induzierbare morphologische Verteidigung aufweisen.

In allen untersuchten Systemen, die von *Protozoa*, über *Rotatoria*, bis hin zu *Daphnia* reichen, konnte gezeigt werden, dass die Ausbildung der Stärke der Verteidigung (z.B. in dem von mir untersuchten System: die Helmlänge von *Daphnia cucullata*) mit steigender Räuberdichte zunahm, aber ebenso mit steigender Beutedichte abnahm.

Weiterhin wurde am System *Daphnia lumholtzi* gezeigt, dass ein chemischer und kein mechanischer Reiz für die Erkennung der Beutedichte verantwortlich ist, wobei es sich aber um kein artspezifisches Signal handelt.

Die Ergebnisse dieser Studie lassen aufgrund des breiten taxonomischen Ansatzes vermuten, dass eine exakte Abschätzung des Prädationsrisiko sowohl auf der Räuberdichte, als auch auf der Beutedichte basierend, ein weit verbreiteter Mechanismus in induzierbaren Verteidigungssystemen ist. Die genaue Abschätzung des Prädationsrisikos könnte zur Vermeidung von Kosten beitragen, indem keine Energie in unnötige Verteidigungen investiert wird. Dies spielt vor allem in einer

Umwelt eine bedeutende Rolle in der eine starke intra- und interspezifische Konkurrenzsituation vorliegt. Ebenso zeigt diese Untersuchung eine Erklärungsmöglichkeit für Unterschiede in Versuchen zur induzierbaren Verteidigung auf, da die Dichte der Artgenossen die Ausbildung der Verteidigung beeinflusst. Daher sollte in zukünftigen Studien die Dichte der Versuchsorganismen genauestens kontrolliert werden.

Weiterführend ist das Ergebnis dieser Untersuchung auch relevant für das Verständnis der Evolution von Verteidigungsmechanismen per se, und nicht ausschließlich für induzierbare Verteidigungen. Denn auch bei permanenten Verteidigungen bildet das individuelle Prädationsrisiko den Rahmen für die Ausbildung einer Verteidigung, auf die selektiert wird und spielt somit eine zentrale Rolle für lokale Adaptationen.

5.

EXTREME HELMET FORMATION IN *DAPHNIA CUCULLATA* CAUSED BY SMALL-SCALE TURBULENCES

(Laforsch und Tollrian, *Journal of Plankton Research*, Akzeptiert in Abhängigkeit von der Revision)

Die Saisonalvariation ist bei verschiedenen Daphnien bereits Anfang des letzten Jahrhunderts beschrieben worden (review: Jacobs 1987). Als Lehrbeispiel für die Beschreibung der Zyklomorphose wird meist die Helmbildung von *Daphnia cucullata* angeführt. Jedoch sind bei dieser Art bisher nur die Induktion der Helmbildung durch Kairomone der räuberischen Büschelmücke *Chaoborus* nachgewiesen worden (Tollrian 1990). Die Beobachtung, dass in zwei polnischen Seen die höchsten Helme von *Daphnia cucullata* dann zu finden waren, wenn die Häufigkeit der invertebraten Prädatoren am höchsten war, unterstrich die Relevanz von chemischen Signalen bezüglich der Helminduktion (Pijanowska 1991), ebenso wie die von mir gewonnenen Daten der Multiprädatorexposition. Allerdings war die in allen Laborversuchen induzierte Helmbildung geringer als die im Freiland gemessenen Helmlängen.

Eine ältere Studie, in der nur die Rolle abiotischer Faktoren auf die zyklomorphe Gestaltausprägung getestet wurde, deutet an, dass die Helme von *Daphnia cucullata* auch mittels Wasserturbulenzen induziert werden können (Hrbácek 1959). Jedoch konnte Hrbácek mittels einer rotierenden Trommel in der die Versuchsgläser montiert waren nur eine äußerst geringe Induktionswirkung erzielen.

In diesem Kapitel habe ich die Rolle von Mikroturbulenzen bezüglich der Induktionswirkung auf *Daphnia cucullata* getestet. Die Turbulenzen wurden mit zwei unterschiedlichen Methoden erzeugt. Vergleichend wurde die Zyklomorphose von *Daphnia cucullata* in zwei Seen der Eggstätt-Helmhofer-Seenplatte mit ähnlicher Morphometrie untersucht. Weiterhin wurde im Labor getestet, ob klonale Unterschiede zwischen beiden Seen bezüglich der Turbulenzinduktion zu beobachten sind. Ebenso wurden Räuberstoffe und Mikroturbulenzen als Induktionsfaktoren der morphologischen Plastizität von *Daphnia cucullata* verglichen. Abschließend wurde die Induktion der plastischen Merkmale mittels Mikroturbulenzen unter Kältebedingungen getestet, da Coker und Addlestone (1938) eine Schwellentemperatur von 11°C bei der Ausbildung der zyklomorphen Strukturen von *Daphnia longispina* festgestellt haben.

Die Versuche haben gezeigt, dass Mikroturbulenzen die maximale Merkmalsausprägung bei *Daphnia cucullata* induzieren können. Die Helme erreichen im Labor die gleichen maximalen Längen, die auch im Freiland gefunden wurden. Die verschiedenen Methoden der Turbulenzerzeugung ergaben unterschiedliche Ergebnisse in den plastischen Merkmalen. Jedoch scheinen windinduzierte Mikroturbulenzen nicht der alleinige Grund für die Zyklomorphose im Freiland zu sein, da Klone aus zwei Seen mit ähnlicher Morphometrie, die sich im Freiland stark in Ihrer Helmbildung unterscheiden, keinerlei Unterschiede im Labor im Hinblick auf die Induktion der plastischen Merkmale aufweisen. Weiterhin sprechen die niedrigen Helme im Frühjahr und Herbst gegen eine induktive Wirkung von Turbulenzen verursacht durch Windexposition. Obwohl die Helminduktion durch Kairomone niedriger war als die Induktion durch Turbulenzen, könnten synergistische Effekte die extreme Zyklomorphose von *Daphnia cucullata* im Freiland erklären, da Jungfische (0+) und invertebrate Prädatoren ähnliche Mikroturbulenzen erzeugen könnten, wie meine Versuchsapparatur (siehe Synopsis Abb.7). Zumal nicht ausgeschlossen werden kann, dass die Helme nicht nur als Schutz gegenüber invertebraten Prädatoren, sondern auch gegenüber Jungfischen dienen.

Ferner könnten die Helme eine energiesparende Funktion bezüglich der Fortbewegungsweise in warmer turbulenter Umgebung aufweisen, wobei diese im kalten viskoseren Medium die Manövrierbarkeit wieder einschränken und demzufolge in kaltem Wasser nicht mehr induziert werden.

Die Induktion der zyklomorphen Merkmale von *Daphnia cucullata* sowohl mit abiotischen, als auch biotischen Faktoren deutet darauf hin, dass synergistische Effekte von verschiedenen Parametern die Merkmalsausprägung bedingen, wobei die Helme multifunktional in einer sich ständig verändernden Umwelt eingesetzt werden.

6.

A NEW PREPARATION TECHNIQUE OF DAPHNIDS FOR SCANNING ELECTRON MICROSCOPY USING HEXAMETHYLDISILAZANE

(Laforsch und Tollrian, *Archiv für Hydrobiologie,* 149: 587-596, 2000)

Die Rasterelektronenmikroskopie (REM) ist ein wichtiges Hilfsmittel um die Oberfläche von biologischen Proben darzustellen, wobei neben der sehr hohen Vergrößerung, vor allem die hohe Tiefenschärfe, welche einen dreidimensionalen Eindruck des Objekts vermittelt, von Vorteil ist. Für die Analyse von biologischen Präparaten werden spezielle Präparationsmethoden benötigt, um Artefakte in der Oberflächendarstellung zu vermeiden. Dabei ist die Entwässerung der Präparate der kritischste Schritt der Präparation. Die am häufigsten verwendete Methode zur Dehydrierung der Präparate ist dabei die „critical point“ (CPD) Trocknung. In Untersuchungen zur Ultrastruktur von Daphnien wurden bisher immer nur Teile des Organismus verwendet, wie zum Beispiel der Filtrierapparat (z.B. Brendelberger 1991) oder die Ephippien (z. B. Du & Li 1990). Präparate des Gesamthabitus zeigen im CPD Verfahren starke Schrumpfungsartefakte.

Da Hexamethyldisilazan (HMDS) bereits erfolgreich zur Dehydrierung verschiedener Gewebe eingesetzt wurde habe ich in diesem Kapitel die Oberflächenstruktur von verschiedenen *Daphnia* Arten verglichen, die jeweils entweder mittels CPD oder HMDS entwässert wurden.

Die Analyse der Oberflächenstruktur der sehr kleinen und zerbrechlichen Daphnien ergab, dass die HMDS Methode zur Dehydrierung besser geeignet ist als das herkömmlich verwendete CPD Verfahren. Die Präparate die „critical point“ getrocknet wurden, zeigten vorwiegend im Nacken und am Carapax im Bereich des Brutraumes Schrumpfungsartefakte, wohingegen HMDS dehydrierte Präparate eine exzellente Oberflächenstruktur aufwiesen. Sensitive und fragile Strukturen scheinen durch HMDS besser erhalten zu bleiben, wobei dieser Effekt durch die extrem langsame Evaporation über Nacht unter absolut wasserfreien Bedingungen noch verstärkt wird.

Die sehr guten Resultate und die äußerst günstige und einfache Methode sprechen für eine HMDS Dehydrierung zur Präparation von Daphnien für rasterelektronenmikroskopische Untersuchungen, wobei neue Erkenntnisse in der Verbindung zwischen Funktionsmorphologie und Ökologie gewonnen werden können. Die exakte REM Präparation ermöglichte in dieser Arbeit wichtige Fragestellungen sowohl zur

Embryonalentwicklung der Daphnien (siehe Kapitel 7), als auch zur Detektion der Carapaxstärke (siehe Kapitel 8) zu klären.

7.

EMBRYOLOGICAL ASPECTS OF INDUCIBLE MORPHOLOGICAL DEFENSES IN DAPHNIA (Laforsch und Tollrian, *Journal of Morphology*, eingereicht)

Bei einer Vielzahl von pelagischen Cladocera ist bereits eine chemisch induzierte Verteidigung beschrieben, die sowohl Änderungen der Lebenszyklusstrategie, des Verhaltens, als auch der Morphologie beinhalten kann. Daphnien zeigen die auffälligsten morphologischen Veränderungen in Anwesenheit eines Prädators (Tollrian and Harvell 1999). Jedoch ist bisher wenig über die Embryonalentwicklung dieser Verteidigungsstrukturen bekannt.

Der Verteidigungsmechanismus verschiedener Daphnienarten kann sich unterscheiden, obwohl sie vom gleichen Prädator bedroht werden. Zum Beispiel entwickelt *Daphnia pulex* nur Nackenzähne im zweiten Juvenilstadium (Krueger & Dodson 1981), wohingegen *Daphnia cucullata* in allen Lebensphasen einen Helm bei Anwesenheit von *Chaoborus* ausbildet (Tollrian 1990). Ebenfalls wurde gezeigt, dass sich Daphnienarten in der sensitiven Phase der Kairomoninduktion unterscheiden. *Daphnia ambigua* scheint nur in den ersten Juvenilstadien sensitiv für die Kairomondetektion zu sein (Hanazato 1990), wohingegen die Nackenzahnbildung bei *Daphnia pulex* bereits beim Embryo induziert werden kann (Parejko 1992).

Die Ausbildung großer Verteidigungsstrukturen stellt die Daphnien in der Embryonalphase vor ein logistisches Problem, aufgrund des limitierten Platzes im Brutraum der Mutter. Einerseits sollten die morphologischen Verteidigungen bereits vor der Geburt entwickelt werden, falls die Daphnie direkt nach der Geburt bereits ins Beutespektrum des jeweiligen Prädators fällt. Anderseits sollten die oft spitzen Verteidigungsstrukturen im Brutraum der Mutter „entschärft“ sein um eine mechanische Verletzungsgefahr der Geschwister zu vermeiden.

Da in früheren Studien bisher nur die Induktion bei Neonaten mittels Lichtmikroskopie untersucht wurde, habe ich in diesem Kapitel die Unterschiede der verschiedenen Embryonalstadien von kairomoninduzierten Tieren und Kontrolltieren von fünf verschiedenen Daphnienarten mit der in Kapitel 6 beschriebenen REM-Präparationsmethode verglichen.

Die Ergebnisse meiner Arbeit haben gezeigt, dass sich die Embryonalentwicklung bei den verschiedenen Daphnienarten in Grundzügen nicht unterscheidet. Der Konflikt des limitierten Raumes wird durch einen komprimierten weichen Körper gelöst, der sich kurz nach der Geburt entfaltet und einen harten Panzer bildet.

Weiterhin konnte ich nachweisen, dass sowohl bei *Daphnia cucullata* als auch bei *Daphnia pulex* bereits im letzten Embryonalstadium Veränderungen der Morphologie zwischen räuberexponierten Daphnien und Kontrolltieren feststellbar sind. *Daphnia cucullata* zeigt bereits im Brutraum der Mutter einen stark ausgeprägten Helm. Da diese Art bereits ab dem Zeitpunkt der Geburt von invertebraten Räubern bedroht ist (Kapitel 1), ist es für sie besonders wichtig bereits bei der Geburt vollständig verteidigt zu sein. Zudem kann der Helm nicht innerhalb eines Häutungstadiums gebildet werden und somit scheint eine Induktion der Verteidigungsstruktur bereits im Embryonalstadium adaptiv.

Demgegenüber ist *Daphnia pulex* erst im zweiten Embryonalstadium bedroht und Verteidigunkstrukturen wie Nackenzähne können innerhalb eines Häutungsstadiums gebildet werden. Daher ist es nicht verwunderlich, dass Nackenzähne bei prädatorexponierten Embryonen noch nicht ausgebildet werden. Zudem würden die kantigen Nackenzähne ein potentielles Verletzungsrisiko für die Geschwister darstellen. Jedoch wird bei dieser Art die Basis für die Verteidigungsstruktur, ein Nackenkamm bereits während der Embryonalphase induziert.

Bei *Daphnia ambigua*, *Daphnia longicephala* und *Daphnia lumholtzi* konnten keine sichtbaren Unterschiede im Entwicklungsverlauf von kairomoninduzierten und nicht kairomoninduzierten Tieren festgestellt werden. Allerdings verteidigt sich *Daphnia lumholtzi* morphologisch gegen Fische und zeigt daher auch bereits kurz nach dem Schlüpfen sehr spitze Dornen. In der Embryonalphase wird dieses Problem durch eine scheinbar sehr weiche und elastische Cuticula und dem Einklappen von Fornices, Spina und Helm gelöst. Nach der Geburt dauert es nur einige Sekunden bis diese Strukturen vollständig entfaltet sind. Hierbei konnte ich bei *Daphnia lumholtzi* die Häutung aus einer vierten Embryonalmembran dokumentieren. Diese Membran reißt unmittelbar bei der Geburt und kann die rapide Veränderung der Morphologie vom letzten Embryonalstadium zum Neonatenstadium erklären. Diese Veränderung die offensichtlich einer Häutung entspricht, wurde bisher als „pseudomolt“ deklariert (Parejko 1992). Weiterhin konnte ich die Resultate von Kotov (1997) bestätigen der ebenfalls die Häutung aus vier Membranen postuliert, wobei sich die zweite und dritte Embryonalmembran fast simultan ablöst. Die Häutung aus den Membranen könnte auch mit der Sensitivität gegenüber dem Kairomon in

Zusammenhang stehen, da die erste derbwandige Eimembran höchstwahrscheinlich undurchlässig für diese chemische Substanzen ist. Nach dem Ablösen der dritten Eimembran sind bereits Chemosensillen an der Basis der ersten Antennen ausgebildet. Ab diesem Zeitpunkt beginnt bei *Daphnia pulex* die Sensitivität gegenüber dem Kairomon (Tollrian, unveröffentlichte Daten).

Die verschiedenen Ausprägungen der morphologischen Verteidigungen (z.B. Helme, Nackenzähne) der in dieser Studie untersuchten Daphnienarten, haben sich aufgrund des Selektionsdrucks ausgehend von unterschiedlichen Prädatoren und Umwelten evolviert. Meine Studie zeigt, dass Daphnia bei der Embryonalentwicklung der Verteidigungsstrukturen einen Kompromiss zwischen ontogenetischen Zwängen und den Anforderungen der Umwelt einen Schutz zu bilden, gefunden hat.

8.

BEYOND THE TIP OF THE ICEBERG – A UNIQUE ACOUSTIC MICROSCOPY TECHNIQUE REVEALS HIDDEN MORPHOLOGICAL DEFENCES

(Laforsch, Ngwa, Grill und Tollrian, *Nature*, eingereicht)

Induzierbare Verteidigungen sind in zahlreichen Lebensgemeinschaften dokumentiert und ermöglichen den Organismen eine bestimmte Verteidigung nur dann auszubilden, wenn ein zuverlässiges Signal über eine zukünftige Bedrohung existiert (Harvell 1984, Brönmark & Miner 1992, Van Buskirk et al. 1997, Leonard et al.1999). Hierbei können die Organismen mögliche Kosten einsparen, die mit einer spezifischen Verteidigung verbunden sind, falls der Schutz nicht benötigt wird.

In limnischen Habitaten initiieren vom Räuber abgegebene chemische Substanzen diese Verteidigung. Daphnien zeigen als Reaktion auf Räuberstoffe oft beachtliche morphologische Veränderungen (Grant & Bayly 1981, Spitze & Sadler 1996, Tollrian & Dodson 1999). Jedoch haben auch kleine induzierte morphologische Veränderungen einen Schutzeffekt gegen invertebrate Prädatoren gezeigt. Der genaue Verteidigungsmechanismus konnte hierbei nie aufgezeigt werden, da er nicht offensichtlich erklärbar war. Da viele invertebrate Räuber spezialisierte Mundwerkzeuge aufweisen, wurde die „Anti-Schlüssel-Schloss“ Hypothese aufgestellt (Dodson 1974), die postuliert dass die Beute durch die induzierten morphologischen Eigenschaften nicht mehr mit den Mundwerkzeugen der Räuber kompatibel ist und somit nur erschwert gefressen werden kann. Jedoch wurde auch vermutet, dass die sichtbaren morphologischen Veränderungen nur die „Spitze des Eisberges“ darstellen und versteckte Verteidigungen den Schutzeffekt bedingen (Tollrian 1993).

In bisher durchgeführten Studien zur morphologischen Plastizität wurde der Panzer der Daphnien, der Carapax nur selten berücksichtigt (Dodson 1984).

Ich habe die Stärke des Panzer von *Daphnia pulex* und *Daphnia cucullata* mittels einer neuartigen hochfrequenten Ultraschallmikroskopiertechnik mit Vectorkontrast, bezüglich eines Unterschieds von kairomoninduzierten und nicht kairomoninduzierten Tieren untersucht.

Das Ergebnis der Ultraschallmikroskopie hat in beiden Fällen einen hoch signifikanten Unterschied in der Reflektion von räuberexponierten Tieren und Kontrolldaphnien ergeben. Eine stärkere Reflektion ist gleichbedeutend mit einer höheren akustischen Impedanz des Carapax, welche wiederum ein Indikator für die Härte eines Materials ist. Die Härte eines Materials charakterisiert die Fähigkeit dem

Eindringen eines Fremdkörpers zu widerstehen, die in diesen Fall die Mandibel des Prädators wären. Die Reflektion des Schalls war bei induzierten *Daphnia pulex* 3,5 mal so hoch wie bei den Kontrolltieren, bei *Daphnia cucullata* doppelt so hoch. Diese Verstärkung des Panzers kann die hohen Fluchtraten auch nach bereits erfolgten Fang durch eine Chaoboruslarve erklären (Spitze 1992), da für den Räuber die Zerstörung des Panzers erheblich erschwert oder sogar verhindert wird und somit der Daphnie die Möglichkeit eröffnet wird sich aus dem Fangapparat zu befreien.

Eine ultrastrukturelle Untersuchung anhand von Semidünnschnitten des Carapax von *Daphnia cucullata* ergab, dass der Panzer von räuberexponierten Tieren dicker ist als der der Kontrolltiere. Ebenso ist bei *Daphnia cucullata* der Durchmesser der Säulchen, die die beiden epidermalen Schichten des Carapax verbindet, erheblich größer bei kairomoninduzierten Tieren. Die Vergrößerung der Säulchen, die wiederum aus feinen Fasern bestehen, festigt die mechanische Stabilität des Panzers, ähnlich der Leichtbauweise bei architektonischen Konstruktionen.

Die hier gewonnenen Ergebnisse zeigen, dass kairomoninduzierte Daphnien durch einen mechanisch verstärkten Panzer vor Attacken der Prädatoren besser gewappnet sind. Diese „versteckten“ morphologischen Schutzfunktionen könnten das Paradoxon erklären, dass augenscheinlich „unverteidigte“, aber den Räubersignalen ausgesetzte Daphnien trotzdem ihre Mortalitätsrate bei Räuberkontakt verringern konnten. Dies wurde bisher mit noch unaufgedeckten Verhaltensänderungen, die den Verteidigungseffekt bedingen sollten, erklärt.

Obwohl in dieser Studie nur zwei Daphnienarten untersucht wurden, scheint es nicht unwahrscheinlich dass dieser Verteidigungsmechanismus sich in vielen Cladoceren evolviert hat. Der evolutionäre Rüstungswettlauf fördert die Aufrecherhaltung von effektiven aber Kosten sparenden Verteidigungsstrukturen. Daher scheint die Leichtbauweise des Panzers mit verstärkter Oberfläche einen deutlichen Vorteil zu haben. Es ermöglicht einen zuverlässigen Schutz, bei minimalem Materialaufwand.

Synopsis

Die Fähigkeit eines Genotyps, sich in variablen Umwelten auf verschiedene Weise zu exprimieren wird als phänotypische Plastizität definiert.

Daphnia cucullata eine pelagisch lebende Cladocerenart, zeigt im Jahresverlauf eine deutliche morphologische Plastizität, indem sich Individuen mit einem hohen Helm und einer verlängerten Spina entwickeln. Aufgrund dieser sogenannten Zyklomorphose eignet sich *Daphnia cucullata* hervorragend als Studienobjekt zur Erforschung der evolutiven Mechanismen phänotypischer Plastizität.

Meine Ergebnisse haben gezeigt, dass sowohl biotische als auch abiotische Umwelteinflüsse als proximate Faktoren dieser Temporalvariation fungieren. Chemische Signalstoffe, sogenannte Kairomone, abgegeben von verschiedenen invertebraten Prädatoren induzieren die Helmbildung bei *Daphnia cucullata*.

Neben den im Manuskript aufgeführten Prädatoren *Leptodora kindtii*, *Chaoborus flavicans* und *Cyclops*, zeigten auch chemische Signale abgegeben von Süßwassermilben und den räuberischen Rotatorien *Asplanchna* eine signifikant induktive Wirkung auf die plastische Morphologie von *Daphnia cucullata* (Abb. 3).

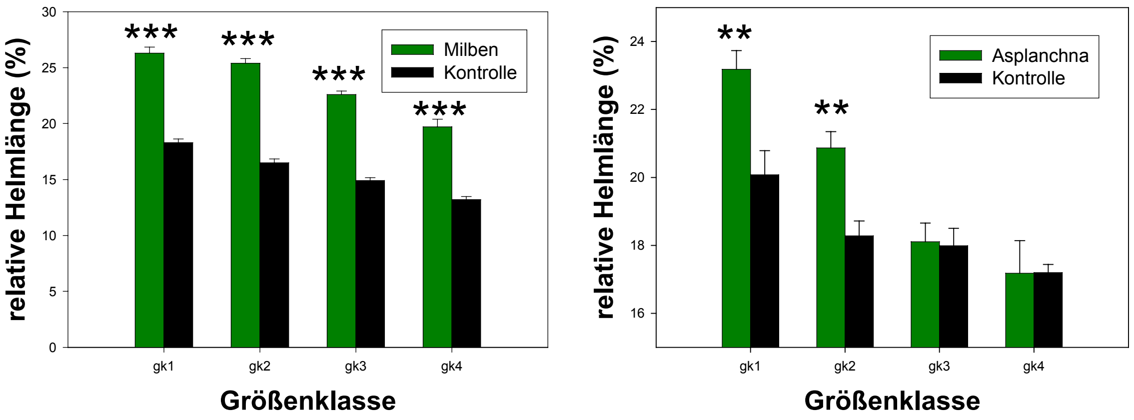

Abb. 3 Vergleich der relativen Helmlänge von *Daphnia cucullata* bei der Induktion mit Räuberstoffen von *Hydrachnella* (a,) und *Asplanchna* (b,) in den verschiedenen Körperlängen-Größenklassen (gk 1 < 600 µm; gk 2 = 600 – 800 µm; gk 3 = 800 – 1000 µm; gk 4 > 1000 µm). Die Sternchen geben das Signifikanzniveau wieder (*** = p < 0,001; ** = p < 0,01). Das hierfür verwendete Versuchsdesign entspricht der im Manuskript 1 beschriebenen Methode zur Induktion.

Die in den Zusatzexperimenten erzielte Induktionswirkung entspricht in dem Milbenversuch, den Ergebnissen der in der Veröffentlichung verwendeten Prädatoren. Im Induktionsversuch mit den räuberischen Rädertieren, zeigen nur die

ersten beiden Juvenilstadien von *Daphnia cucullata* einen längeren Helm. Hierbei kann spekuliert werden, dass die sehr kleinen Räuber nur für Neonate und Individuen im ersten Juvenilstadium eine Gefahr für *Daphnia cucullata* darstellen, da die Daphnien im Laufe ihrer Entwicklung aus dem gefährdeten Größenspektrum fallen. Demzufolge ist eine Helmbildung in älteren Entwicklungsstadien bei ausschließlicher Anwesenheit des Prädators *Asplanchna* nicht mehr relevant.
Da die meisten invertebraten Prädatoren ihre Beute nach dem Fang erst einmal zerkleinern, könnte nicht ausgeschlossen werden, dass nicht Kairomone, sondern Zellinhaltsstoffe der Daphnien als chemische Warnsignale fungieren und die Induktion der Helmbildung bewirken. Meine Versuche haben jedoch ergeben, dass Alarmstoffe homogenisierter Daphnien zwar eine leichte Helmbildung induzieren, die induktive Wirkung der Kairomone aber deutlich stärker ist. Da bei *Daphnia cucullata* die Helmbildung zusätzlich verstärkt wurde, wenn der Räuber *Chaoborus* mit Artgenossen gefüttert wurde, scheinen synergistische Effekte bei der Induktion eine Rolle zu spielen oder die Qualität des Kairomons wird durch die Nahrung des Prädators beeinflusst. Kairomone sind wasserlöslich, bakteriell abbaubar, haben ein geringes Molekulargewicht (< 500 Dalton) und weisen Hydroxyl- und Carboxylgruppen in ihrer Struktur auf (Hebert & Grewe 1985; Parejko & Dodson 1990; Tollrian and van Elert 1994). Da über die chemische Zusammensetzung der Kairomone weiterhin noch wenig bekannt ist, könnte es durchaus möglich sein, dass die aktive Substanz aus mehreren Komponenten besteht. Diese Komponenten scheinen von der Daphnie nach der Rezeption miteinander verrechnet zu werden, um das aktuelle Prädationsrisiko abzuschätzen, damit die der momentanen Situation angepasste bestmögliche Verteidigung initiiert werden kann. Hierfür spricht auch das Ergebnis des dichteabhängigen Induktionsexperiments. Daphnien in höherer Dichte zeigen signifikant niedrigere Helme bei der Induktion mit Kairomon von *Chaoborus flavicans*. Die Merkmalsausprägung folgt somit dem Prädationsrisiko, das für das einzelne Individuum bei hoher Beutedichte sinkt. *Daphnia cucullata* scheint sowohl die eigene Populationsdichte, als auch die Prädatorkairomone im chemischen Milieu getrennt voneinander auflösen zu können.

Obwohl ich in meiner Arbeit eine eindeutige Induktion durch chemische Botenstoffe nachweisen konnte, ist im Freiland eine stärkere Merkmalsausprägung im Jahresverlauf zu beobachten.

Die Ergebnisse meiner Arbeit haben gezeigt, dass die Helmbildung bei *Daphnia cucullata* auch mittels Turbulenzen induzierbar ist. Dabei spielt die Art der Turbulenzen eine wesentliche Rolle. Die erste hierfür entwickelte Apparatur generierte Turbulenzen (Turbulenz 1), die ebenso große Helme induzierte, wie die im Freiland beobachteten Werte. Die zweite Apparatur erzeugte ein wenig moderatere Turbulenzen (Turbulenz 2), wobei die plastischen Merkmale von *Daphnia cucullata* ähnlich stark induziert wurden, wie mit Prädatorkairomonen (Abb. 4).

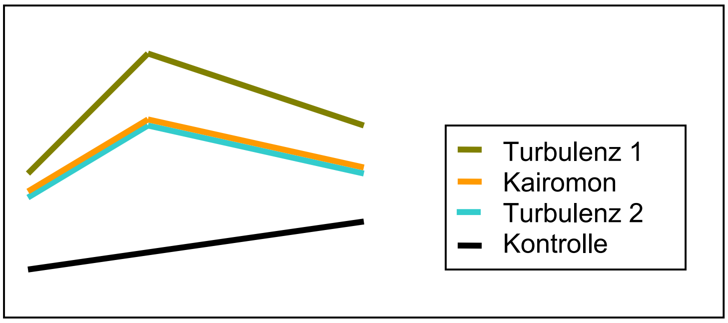

Abb. 4 Schematische Darstellung der relativen Helmlänge von *Daphnia cucullata* vom Neonaten bis zum Adultstadium bei unterschiedlicher Induktionsmethode (Turbulenz 1, 2 und Räuberkairomone) im Vergleich zur Entwicklung der relativen Helmlänge der rundköpfigen Morphen (Kontrolle) während des Lebenszyklus.

Weiterhin haben meine „Turbulenzversuche“ im Labor ergeben, dass kein Unterschied in der Merkmalsausprägung bei verschiedenen Klonen von *Daphnia cucullata* aus zwei benachbarten Seen besteht. Jedoch sind im Bansee während des Jahres nur rundköpfige Morphen anzutreffen. *Daphnia cucullata* zeigt demgegenüber im Thalersee die klassische Zyklomorphose. Aufgrund der regionalen Nähe und einer ähnlichen Morphometrie, scheint Windstress nicht der ausschlaggebende Faktor für die Induktion von *Daphnia cucullata* zu sein. Ebenso spricht der Verlauf der Zyklomorphose gegen eine starke Rolle der durch Wind erzeugten Turbulenzen, da ansonsten während der Herbst- und Frühjahreszirkulation in dimiktischen Seen die Ausbildung von hohen Helmen zu erwarten wäre. Die Zyklomorphose von *Daphnia cucullata* zeigt allerdings in unseren Breitengraden eine genau gegenläufige Entwicklung (Abb. 5). Die höchsten Helme werden während der Sommerstagnation gebildet, in einer Phase in der sowohl die Abundanz als auch die Aktivität von vertebraten und invertebraten Räubern am stärksten ist.

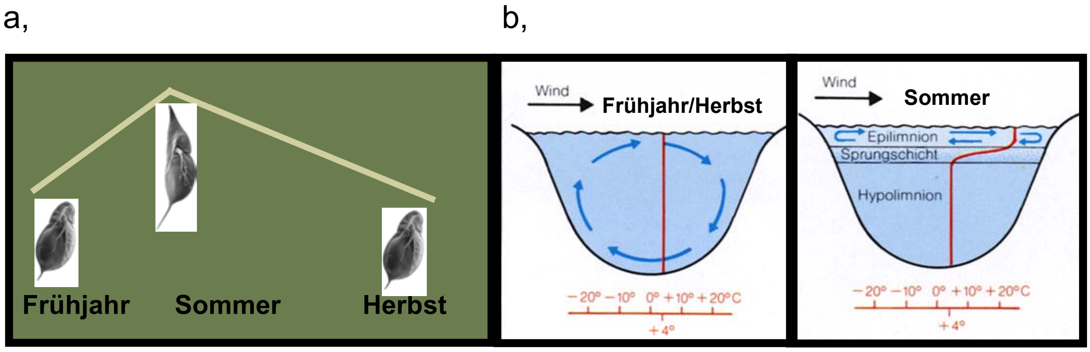

Abb.5 a, Schematische Darstellung der Zyklomorphose von Daphnia cucullata im Jahresverlauf. b, Darstellung der durch Windstress verursachten Seenschichtung im Jahresverlauf. Herbst- und Frühjahresstürme verursachen eine vollständige Durchmischung des Sees.

Da sowohl Fische als auch invertebrate Prädatoren bei Ihrer Fortbewegung Mikroturbulenzen erzeugen, könnte es möglich sein, dass diese Art von Turbulenzen ähnlich den von meiner Apparatur erzeugten Turbulenzen ist. Diese Turbulenzen könnten als weiterer Indikator für das Auftreten einer Gefahr fungieren und somit die induktive Wirkung erklären.

Meine Versuche haben gezeigt, dass *Daphnia cucullata* die gleichzeitig sowohl Mikroturbulenzen (Turbulenz 2) als auch Räuberkairomonen ausgesetzt war ebensogroße Helme entwickelt, wie diese im Freiland beobachtet werden können oder mittels Turbulenz 1 im Labor induziert werden konnten. Hierbei konnte ein signifikanter synergistischer Effekt zwischen Kairomon und Mikroturbulenzen nachgewiesen werden (Abb. 6).

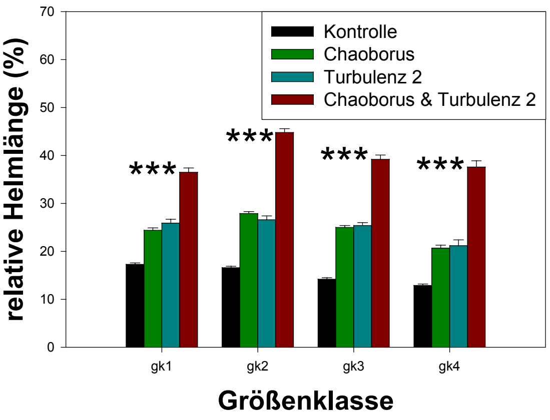

Abb. 6 Vergleich der relativen Helmlänge von *Daphnia cucullata* bei der Induktion mit Räuberstoffen von *Chaoborus*, der Induktion mit Mikroturbulenzen (Turbulenz 2) und einem additiven Versuchsansatz (*Chaoborus* und Turbulenz 2) in den verschiedenen Körperlängen-Größenklassen (gk 1 < 600 µm; gk 2 = 600 – 800 µm; gk 3 = 800 – 1000 µm; gk 4 > 1000 µm). Die Sternchen geben das

Signifikanzniveau wieder (*** = p < 0,001). Das hierfür verwendete Versuchsdesign entspricht der im Manuskript 4 beschriebenen Methode zur Induktion. Im additiven Treatment wurde ein Acrylkäfig (ø3cm; h 10cm) der den Stoffaustausch der Kairomone gewährleistet (10 Chaoboruslarven/L) dem Turbulenz 2 Versuchsaufbau hinzugefügt.

Weiterhin konnte ich einen stärkeren Induktionseffekt bei direktem Kontakt der Räuber mit *Daphnia cucullata*, als bei reiner Kairomoninduktion im Labor nachweisen. Die hierbei erzielten Ergebnisse, exemplarisch aufgezeigt an der Direktkontaktinduktion mit *Leptodora kindtii* (Abb. 7), geben einen weiteren Hinweis darauf, dass die plastischen Merkmale von *Daphnia cucullata* sowohl durch chemische Botenstoffe als auch durch mechanische Reize induzierbar sind und dass beide Reize eine additiv-induktive Wirkung aufweisen. Dieses Ergebnis bietet eine plausible Erklärungsmöglichkeit für die maximal im Freiland beobachtete Helmbildung.

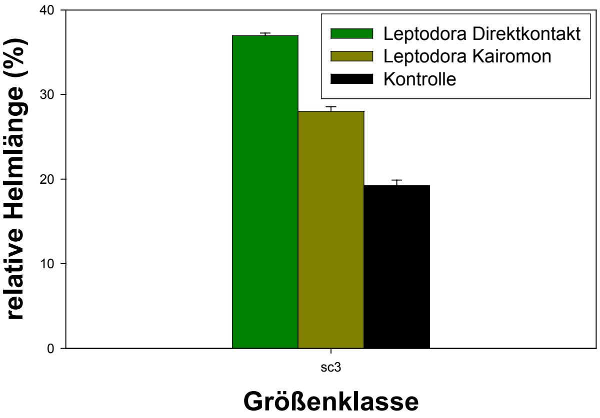

Abb. 7 Vergleich der relativen Helmlänge von *Daphnia cucullata* bei der Induktion mit Räuberstoffen von *Leptodora* und der additiven Induktion mit Mikroturbulenzen erzeugt durch den Prädator und dessen abgegeben Kairomone in der dritten Körperlängen-Größenklassen (gk 3 = 800 – 1000 µm). Das Signifikanzniveau zwischen den einzelnen Treatments beträgt p < 0,001 (ANOVA; Tamhane Post Hoc Test). Das verwendete Versuchsdesign für die Kairomoninduktion entspricht der im Manuskript 1 beschriebenen Methode. Der Direktkontaktversuch wurde in 10L Aquarien mit einer Konzentration von 20 Leptodora/L durchgeführt.

Da die induzierten plastischen Merkmale einen eindeutigen Vorteil bei der Prädation durch unterschiedliche invertebrate Prädatoren bedingen, die sich in ihrer Jagdstrategie unterscheiden, konnte ich einen eindeutigen ultimaten Faktor der Zyklomorphose bei *Daphnia cucullata* nachweisen.

Diese Schutzfunktion der Helme unterschied sich bei den verschiedenen Räubern sowohl im Verteidigungsmechanismus, als auch zwischen den einzelnen Größenklassen der Daphnien. *Daphnia* cucullata ist eine sehr kleine Daphnienart und ist während des gesamten Lebenszyklus durch größenlimitierte Invertebrate Prädatoren bedroht. Dementsprechend sollte *Daphnia cucullata* bereits im Neonatenstadium die morphologischen Verteidigungsstrukturen ausbilden. Ich konnte mit meinen Experimenten nachweisen, dass die Induktion der Verteidigungsstrukturen über Generationen weitergegeben wurde. Die maternale Umgebung hatte somit einen Einfluss auf die Stärke der Induktion sowohl der F_1, als auch der F_2 Generation. Besser verteidigte Mütter verschaffen dabei ihren Nachkommen bereits ab der Geburt einen Vorteil gegenüber nicht verteidigten Individuen. Ebenso konnte ich mittels einer von mir entwickelten speziellen rasterelektronenmikroskopischen Präparationsmethode, einen Vergleich der Embryonalentwicklung von induzierten und nicht induzierten Individuen verschiedener Daphnienarten durchführen. Hierbei konnte ich zeigen, dass Daphnien die bereits ab den Neonatenstadium ihre Verteidigung benötigen, wie zum Beispiel *Daphnia cucullata*, diese bereits während der letzten Phase der Embryonalentwicklung ausbilden. Demgegenüber weisen Daphnien, die ihre Verteidigungsstrukturen in späteren Entwicklungsphasen benötigen keine sichtbaren morphologischen Unterschiede während der Embryonalentwicklung auf.

Als weiteren Vorteil bei der Prädation durch invertebrate Prädatoren hat sich ein verstärkter Carapax bei der Anwesenheit von Räuberstoffen im Wasser ergeben. Diese Materialverstärkung konnte mittels einer neuartigen hochfrequenten Ultraschallmikroskopietechnik detektiert werden. Ebenso variieren die Carapaxdicke und die Säulchenstärke innerhalb des Carapax, zwischen induzierten und nicht-induzierten Tieren von *Daphnia cucullata*. Da invertebrate Prädatoren ebenfalls filigrane Fangapparate aufweisen, scheint ein verstärkter Panzer ein guter Schutz bei bereits erfolgter Attacke zu sein.

Ich konnte in dieser Arbeit als proximate Faktoren der Zyklomorphose bei *Daphnia cucullata*, sowohl Mikroturbulenzen als auch chemische Botenstoffe nachweisen. Als ultimater Faktor steht ein eindeutiger Schutzeffekt der plastischen morphologischen Merkmale gegen eine Vielzahl von invertebraten Prädatoren und ermöglicht somit die Existenz von *Daphnia cucullata* unter ständig variierenden Umweltbedingungen.

Ausbilck

Während über ökologische Faktoren die zur Evolution phänotypischer Plastizität am Modellorganismus *Daphnia* geführt haben, ständig neue Erkenntnisse gewonnen werden, sind die entwicklungsbiologischen, zellulären Mechanismen und deren genetische Basis bisher noch weitgehend unerforschtes Gebiet. Polyploide Zellen, die in Regionen der plastisch veränderbaren Bereiche liegen werden als Grundlage der induzierbaren Verteidigung bei *Daphnia* diskutiert. Jedoch ist bisher nur die Anzahl der polyploiden Zellen mit der Größe der Verteidigungsstruktur bei adulten aus dem Freiland isolierten Daphnien verglichen worden (Beaton und Hebert,1997). Eigene Vorversuche konnten diesen Befund bestätigen und für weitere Charakteristika ausbauen (Abb. 8).

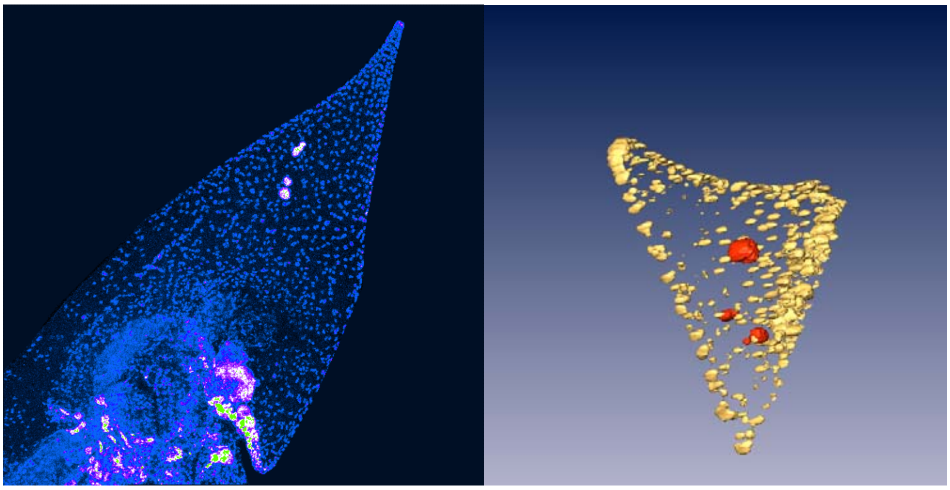

Abb. 8 a,Extended focus Aufnahme des Helmbereichs von Daphnia cucullata erstellt am konfokalen Laser scanning mikroskop (DNA Färbung mit Propidiumjodid). Im Helm sind deutlich größere Nucleoli der endopolyploiden Zellen im Gegensatz zu den diploiden Zellen des umgebenden Gewebes zu erkennen. b, Dreidimensionale Rekonstruktion der polyploiden Zellen im Helm von *Daphnia cucullata*.

Zum Beispiel finden sich zwei polyploide Zellen ab dem Adultstadium am Rande der Brutkammer. Dies könnte für die Weitergabe maternaler Informationen (siehe Kapitel 3) von ausschlaggebender Bedeutung sein. Die Rolle der polyploiden Zellen soll in weitergehen Untersuchungen, unter kontrollierten Bedingungen mittels Laser-ablation-setup, durch die nicht letal, eine Zelle aus dem lebenden Tier entfernt werden kann, geklärt werden. Anhand dieser Methode kann weiterführend der „pathway“ induzierbarer Verteidigungen entschlüsselt werden, indem Chemosensillen und nach deren Lokalisation, einzelne Neuronen aus dem Gewebe ablatiert werden (Abb. 8a). Durch eine wahrscheinlich große Menge an unterschiedlichen Genprodukten, die für die Ausbildung einer Verteidigungsstruktur notwendig sind, könnte zwischen kairomoninduzierten Daphnien und Kontrolltieren mittels Transkriptomanalyse oder Mikroarrays Einblicke in die genetischen Grundlagen der morphologischen Plastizität gewonnen werden.

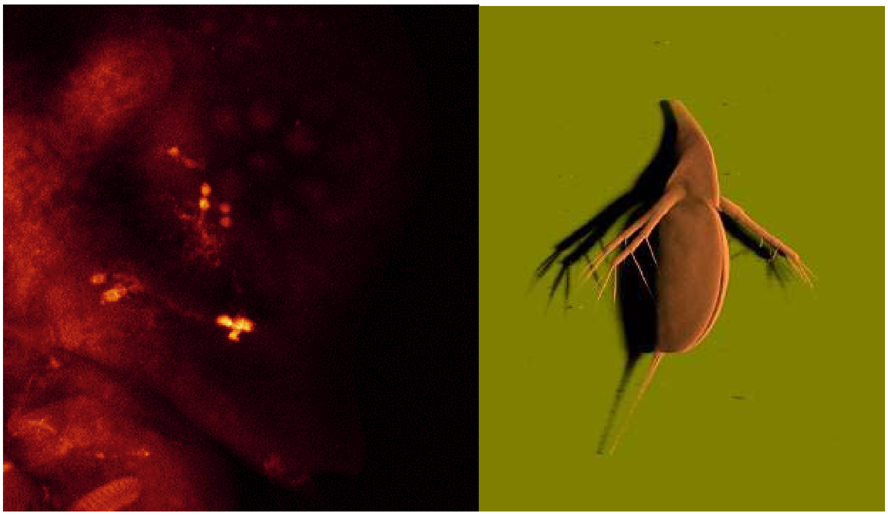

Abb. 8 a, Immunohistochemische Färbung der Nervenzellen im Kopfbereich von *Daphnia cucullata*. b, Dreidimensionale Rekonstruktion von *Daphnia cucullata*.

Allerdings bergen weiterhin auch autökologische Faktoren, die die Adaptation morphologischer Plastizität gefördert haben noch faszinierende Forschungsbereiche. Beispielsweise können hydrodynamische Unterschiede zwischen behelmten und rundköpfigen Daphnien sowohl als Vorteil, als auch als Nachteil, zum Beispiel bei unterschiedlicher Viskosität des Mediums, interpretiert werden. Hierbei können dreidimensionale Modelle (Abb.8b) mit finiter Elementstruktur und deren anschließende Untersuchung im digitalen Strömungskanal, Aufschluss bringen.

Die Untersuchung morphologischer Plastizität am Modellorganismus Daphnia ist demnach weiterhin ein spannendes Forschungsgebiet das verschiedenste Bereiche der Biologie vereint.

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1

INDUCIBLE DEFENSES IN MULTIPREDATOR ENVIRONMENTS: CYCLOMORPHOSIS IN *DAPHNIA CUCULLATA* (Laforsch und Tollrian, *Ecology*, im Druck)

Running Head: *DEFENSES IN MULTIPREDATOR ENVIRONMENTS*

INDUCIBLE DEFENSES IN MULTIPREDATOR ENVIRONMENTS: CYCLOMORPHOSIS IN *DAPHNIA CUCULLATA*

Christian Laforsch † and Ralph Tollrian *

†* Section of Evolutionary Ecology and GeoBioCenter, Department Biologie II,
Ludwig-Maximilians-Universität München, Karlstr. 23 – 25, 80333 München,
Germany

† Corresponding author: Tel:+49 89 5902253, Fax: +49 89 5902461,
laforsch@zi.biologie.uni-muenchen.de
* tollrian@lmu.de

ABSTRACT

Phenotypically-plastic defenses are strategies to reduce predation risk in variable environments. Predator-induced formations of protective devices in cladocerans are prominent examples. To understand the adaptive value of a defense it is necessary to investigate its effects in a multipredator context, instead of merely concentrating on a two-species interaction. Small cladocerans such as *Daphnia cucullata* are prey items for many aquatic invertebrate predators and thus comprise a perfect model system to investigate defenses and their effects. In this paper we tested the hypothesis that the helmets of *Daphnia cucullata* are inducible with chemical cues from different kinds of predators and that they act as a generalized defense offering protection against several predators, each using a different hunting strategy.

Results from our induction experiments show that chemical cues released from *Chaoborus flavicans*, *Leptodora kindtii*, and *Cyclops sp*. induce significantly longer helmets and tail spines and thus act as proximate factors for cyclomorphosis in *Daphnia cucullata.* Our predation experiments revealed that the induced morphological changes offered protection against each of the predators tested. Interestingly, the protective mechanisms and the prey size classes which were protected differed between predator systems.

Our results suggest that phenotypic plasticity in *Daphnia cucullata* evolved as a "diffuse" coevolution against different invertebrate predators which selectively feed on small prey items. The additive benefits may increase the adaptive value and thus facilitate the evolution and persistence of this generalized defense.

Keywords: phenotypic plasticity, multipredator environments, functional morphology, diffuse coevolution, inducible defenses, cyclomorphosis

INTRODUCTION

Predation is a key factor in the evolution of prey species and the dynamics of prey communities. Changes in the predator environment can have tremendous effects on prey species and the related communities (Sih, 1987; Lima & Dill 1990). In nature, most prey organisms are exposed to a variety of predators, which differ in their search and capture characteristics. Thus it is astonishing that most defense systems have been studied only in a two-species context. While defensive effects against multiple attackers have been studied in some plant systems (e.g., Rausher et al. 1993; Berenbaum & Zangerl 1999), corresponding work on animal prey in multipredator environments is sparse (e.g., Stemberger & Gilbert 1987, Kuhlmann et al. 1999, Iyengar & Harvell 2002) and mainly restricted to behavioral traits (Sih et al. 1998). Defenses may be especially advantageous if they protect against a variety of attackers, and thus function as a multipurpose tool against predators. Tollrian & Harvell (1999a) suggested that multipredator environments are important for the evolution of inducible defenses. These phenotypically-plastic responses have been observed in a variety of different taxa, including bryozoans (Harvell 1984) and barnacles (Lively 1986) in marine environments, and in algae (Van Donk et al. 1999), protozoans (Kuhlmann et al. 1999), rotifers (Gilbert 1999), cladocerans (Tollrian & Dodson 1999), amphibians (McCollum & Leimberger 1997), and fish (Brönmark et al. 1999) in freshwater environments.

A major difficulty inherent in devising methodology for estimating the protective effect of a specific defense is overcome with inducible defenses because the responses of predators to defended and undefended forms can be directly compared.

We used the predator-induced morphology in *Daphnia cucullata* to test the effectiveness of defenses in a multipredator context. The small water flea, *Daphnia cucullata* (Fig. 1), is a common species in the epilimnion of eutrophic lakes across Europe. As an adaptation against visually-hunting fish, which form a common selective force in large lakes, *Daphnia cucullata* is a relatively small species with a transparent body and a small clutch size (Hrbácek 1962). This renders *Daphnia cucullata* an inferior competitor against larger cladoceran species when fish are absent (Weider & Wolf 1991), but a superior competitor when fish are present. Thus, population growth in *Daphnia cucullata* is dependent on a moderate degree of fish predation (Hrbácek 1962). However, many invertebrate predators, while being large zooplankters, are adapted to fish predation and therefore coexist with fish. For example, *Chaoborus flavicans* avoids fish predation by an induced vertical migration (Dawidowicz 1993) and a transparent body. Similarly, the predatory cladoceran, *Leptodora kindtii*, is completely transparent, leaving the compound eye as the most visible part of the body. Hence, *Daphnia cucullata* is potential prey for many invertebrate predators and should possess the ability to react flexibly to a variable predation regime.

Daphnia cucullata shows a distinct cyclomorphosis with respect to helmet formation during the course of the year (Wesenberg-Lund 1908; Lampert & Wolf 1986). Although *Daphnia cucullata* is used as a textbook example for phenotypic plasticity (Jacobs 1987; Lampert & Sommer 1993), the conditions under which these changes arise and are beneficial remain poorly understood. In *Daphnia cucullata* only the induction of the helmet length by predatory larvae of the phantom midge *Chaoborus* have been shown (Tollrian 1990). However, an advantage of the helmets has not yet been proven.

Therefore, in this study we investigated the morphological structures of *Daphnia cucullata.* Specifically we tested whether they are inducible by chemical cues from various invertebrate predators. Additionally, we studied whether the induced traits of *Daphnia cucullata* act as defense against several invertebrate predators. Finally, we explored the functional mechanism by which these induced traits offer protection.

MATERIAL AND METHODS

Induction Experiments

A standard clone of *Daphnia cucullata* isolated from lake Thalersee, Germany was used for the experiments. All predators in our study coexist with *Daphnia cucullata* in lake Thalersee. However, for experiments we isolated them from adjacent lakes where they occurred in higher densities. Fourth instar larvae of *Chaoborus flavicans* were taken from lake Klostersee, *Leptodora kindtii* from lakes Klostersee and Eschenauersee. *Cyclops sp*. were isolated from lake Hartsee.

The experiments were conducted in the laboratory under constant conditions at 20° C and fluorescent light (14h:8h day/night rhythm) in 1.5 L glass beakers. The animals were reared in an artificial medium based on ultrapure water, trace elements and phosphate buffer at unlimited food conditions (S*cenedesmus obliquus*). The medium (1 L) was changed weekly. At the beginning of the experiment 20 age-synchronized non-helmeted animals with freshly deposited eggs were placed into each beaker. All beakers contained a cylindrical cage made of acryl (10 cm diameter and 12 cm length) and 100 µm nylon mesh that insured separation between predators and *Daphnia cucullata* while guaranteeing the exchange of chemical cues. The predators were placed into these cages and fed daily with adjusted amounts of prey according to their selectivity to ensure the release of kairomones. Ten *Chaoborus* per cage received a mixture of 30 juvenile *D. cucullata* and *D. pulex* each day. Three *Leptodora* per cage received daily 30-40 *D. cucullata*, *Bosmina* sp. and *Ceriodaphnia* sp. while in the copepod treatment 25 *Cyclops* sp. per cage were fed daily with 30 *D. cucullata* and *Bosmina* sp. Pupate *Chaoborus* larvae and dead predators were replaced and the cages were cleaned daily. Beakers without predators served as a control. Pilot experiments had shown that chemical cues released by the prey species without predators had no inductive effect on *Daphnia cucullata*. All treatments were replicated three times. The experiments with *Leptodora kindtii* were conducted in the dark because the animals tend to swim against the walls of the beaker under light conditions in the laboratory. All induction experiments lasted three to five weeks to include transgenerational induction effects (Agrawal et al. 1999). After this period approximately 100 randomly selected daphnids per beaker were measured under a dissecting microscope with a digital image-analysis system (Soft Imaging System, Analysis Pro, Münster, Germany). The morphological parameters recorded from *Daphnia cucullata* were helmet length (defined as the length between the tip of the helmet to the upper edge of the compound eye), body length (defined as the length between the upper edge of the compound eye to the base of the tail spine) and tail spine length (defined as the length between the base of the tail spine to the tip of the tail spine). For analysis, the daphnids were divided into four body length classes (sc1: <600 µm; sc2: 600 – 799 µm; sc3: 800 – 1000 µm; sc4: >1000 µm). To compensate for size-dependent changes in helmet and spine length within the classes, the relative helmet length (helmet length / body length *100) and the relative tail spine length (tail spine length / body length *100) were calculated. The relative values were arcsin-square-root transformed prior to analysis (Sokal & Rohlf 1995). Data were tested for normal distribution. A nested ANOVA was performed to analyze for treatment effects between induced and control animals with treatment as fixed factor and three replicates per treatment as random factor.

Predation Experiments

Three different predators, each showing a different hunting strategy, were used to test if the morphological changes of *Daphnia cucullata* act as a protection against invertebrate predation. The predators *Chaoborus flavicans*, *Leptodora kindtii* and *Cyclops sp*. were isolated as described above. For the predation trials, *Chaoborus* kairomone-induced and non-induced cultures of *Daphnia cucullata* were age-synchronized in order to reduce the variation within size classes and to ensure that induced and non-induced animals of the same size class were of equal age. Daphnids were grouped into three body-length size classes before the experiment (sc1: < 600 µm; sc2: 600 – 800 µm; sc3: > 800 µm) using the digital image-analysis system.

The predation trials with *Cyclops sp.* were conducted in 20 mL volume petri dishes filled with artificial medium. The average size of the copepods was 1.6 ± 0.1 mm (mean ± SE), measured from the tip of the head to the base of the furca. Only the first and second body length size classes of the daphnids were used in the experiment because the copepods did not succeed in grasping larger prey items. The predators were starved for 24 h before the start of the experiment. One *Cyclops* and either ten induced or non-induced *Daphnia cucullata* were exposed to the test conditions (20° C) for two hours in the dark. The trials were replicated 15 times for each size class. Three petri dishes without *Cyclops* served as controls to identify non-predator related mortality. The number of killed, completely eaten and surviving animals was recorded.

The predation treatment with *Leptodora kindtii* was also conducted in the dark in 200 mL artificial medium at 20° C. The body length of the predators was measured from the top of the head to the caudal part of the body (Manca & Comoli 1995). The average body length of *Leptodora* in the experiment was 8.4 ± 0.1 mm. The average trap basket length calculated according to Manca and Comoli (1995) and Branstrator (1998) was 1.4 mm. A single non-starved predator was placed into a glass beaker with both induced and non-induced daphnids (10 each) of the same size class. After 24 hours the number of killed and surviving daphnids was recorded. The trials were replicated 7 to 12 times in each *Daphnia* size class. Once again three beakers without predators served as controls.

We used five cm diameter petri dishes filled with 20 mL artificial medium for the predation experiments with the phantom midge, *Chaoborus flavicans*. A single, starved fourth-instar larva of *Chaoborus flavicans* was placed into each petri dish with either 20 induced or 20 non-induced *Daphnia cucullata*. We chose the high number of daphnids to increase the encounter rate between predators and prey. The larvae were directly observed under artificial light at 20° C for 30 minutes. Those *Chaoborus* larvae that failed to strike within 10 minutes were removed from the experiment. Eaten daphnids were replaced immediately. The predation trials were replicated 10 times in the smallest size class and 14 times in the other size classes.

To shed light on the protective mechanisms, we directly observed the *Chaoborus* larvae to clarify on which steps of the predation cycle (search; attack; capture or ingestion) the defense acts. We defined an attack as a jerky movement of the *Chaoborus* larvae directed towards a nearby prey, followed by a grasp. This could result in either an evasion or a capture, the latter of which we defined as the moment when the larvae had a hold on the *Daphnia*. Finally, ingestion was defined as the moment a *Daphnia* was swallowed into the pharynx of the *Chaoborus*. The number of attacks, contacts, evasions and captures which resulted in either escapes or ingestions was noted. From the data we calculated the following efficiencies:

(1) The attack efficiency, which is an inverse measure of the protective effect of the induced defenses, was defined as the ratio of the number of ingestions to the total number of attacks. We used the attack efficiency, instead of a prey-

oriented defense efficiency (= 1 – attack efficiency), because it is commonly used in functional response models (for review see Jeschke et al. 2002).

(2) The evasion efficiency, which is a measurement of the ability to escape without being caught, was defined as the ratio of the number of evasions to the total number of attacks.

(3) The escape efficiency, which is a measure of the ability to escape after capture, was defined as the ratio of the number of escapes to the total number of captures.

The effects of induced *Daphnia* phenotypes on predation were analyzed by using Mann-Whitney-U-tests for *Chaoborus* and *Cyclops* ($p < 0.05$). We used paired Wilcoxon tests for related samples for the *Leptodora* treatment ($p < 0.05$). Efficiency data in the *Chaoborus* treatment were arcsin-transformed prior to analysis (Sokal & Rohlf 1995).

RESULTS

Chemically Induced Traits

All predators induced longer absolute helmets and tail spines in the prey animals compared to the control animals in all size classes, with the only exception being the spine length in the first size class in the *Leptodora* treatment.

Similarly, the difference in the relative helmet length was highly significant between each of the predator kairomone treatments and controls in each of the four body length categories (Table 1). We always found the largest difference to be in the second size class.

The relative tail spine length of daphnids induced with *Chaoborus flavicans* or *Cyclops sp.* was in each size class significantly longer when compared to the control daphnids (Table 2). In the *Leptodora* treatment the relative tail spine length was not different in the smallest size category. However, as in the previous two predator treatments, the induced daphnids in each of the larger size categories had longer tail spines (Table 2).

Predation Experiments

We found protective effects of the induced traits against each of the predators tested. However, the magnitude of protection differed between predators and between prey size classes.

Direct observation of Chaoborus flavicans predation on Daphnia cucullata

The evaluation of the total number of attacks (Table 3), yielded a significantly higher attack rate on helmeted compared to non-helmeted animals in the second and third size category (Mann-Whitney-U; size class 2: $p = 0.039$; size class 3: $p < 0.001$). Only in juvenile instars with body length less than 600 µm did phantom midge larvae show no significant difference in total number of attacks (Mann-Whitney-U; size class 1: $p = 0.072$). None of the examined parameters, attack efficiency, escape efficiency and evasion efficiency, showed a significant difference in the smallest size class. However, there was a significant advantage of the induced morph in the remaining size categories (Table 4). In the second and third size class the attack efficiency, which is an inverse measure of the protective effect of the plastic traits, decreased. This decrease was caused by both an increased evasion efficiency, which is a measure for the ability to avoid captures, and by an increased escape efficiency, which is a measure for the ability to escape after capture, in daphnids of the helmeted morph (Table 4).

Predation experiment with Cyclops sp.

Only the juvenile instars of *Daphnia cucullata* are susceptible to predation by *Cyclops*. The helmeted morph had a higher survival rate and was less often eaten. We found significant differences between both morphs in the second size category of the surviving animals and in the first size class of the eaten animals (Mann-Whitney-U; survived animals: size class 1, $p = 0.205$; size class 2, $p < 0.001$; eaten animals: size class 1, $p = 0.004$; size class 2, $p = 0.086$). In the copepod experiment we frequently found daphnids which were killed but not completely eaten. Daphnids were killed significantly more often in the second size category of the non helmeted morph (Mann-Whitney-U; size class 1: $p = 0.256$; size class 2: $p < 0.001$).

Predation experiment with Leptodora kindtii

In the first size class, there was no significant difference between the two morphs of *Daphnia cucullata* in the number of prey items eaten by *Leptodora* (paired Wilcoxon test for related samples, $p = 0.546$; Fig. 2). Starting from a body length of 600 µm, there was a distinctly significant advantage for the induced daphnids compared to the non-induced ones (size class 2: $p = 0.018$; size class 3: $p = 0.009$).

DISCUSSION

Defenses of prey organisms are frequently studied in a simplified two-species context. While this approach provides important information about defensive mechanisms and protective effects, studies focusing on two-species interactions offer only limited insights into understanding the ecological relevance of defenses. The impact of multiple predators on prey is poorly understood, especially in systems where different predators vary temporally and spatially in their abundance and importance. In situations where predators with different prey selectivity or modes of predation vary in their impact or abundance, prey species should evolve phenotypic plasticity, given that trade-offs have to be assumed (Tollrian & Harvell 1999 b). In situations where a single kind of defense is effective against a variety of important predators (which may occur concurrently or alternately) a diffuse coevolution (sensu Janzen 1980) could be the consequence.

Our study revealed that helmet and tail spine formation in *Daphnia cucullata*, the textbook example of cyclomorphosis, offers protection against different types of invertebrate predators and is thus a likely example of diffuse coevolution against multiple predators. We showed that this particular prey defense is beneficial against predators with diverse foraging tactics, ranging from cruising predators (*Leptodora*), to ambush predators (phantom midge larvae) and active searching predators (copepods). In the natural habitat of *D. cucullata*, where predators vary in time and space, prey organisms must evolve defense strategies to respond flexibly to this changing multipredator environment. It may be speculated that in populations where the defense was originally induced by one predator, the individual benefit would increase with the abundance of other invertebrate predators. The additional defensive effect would further contribute to the total benefit of the inducible trait. It could thus evolutionary stabilize and establish as a predator-induced general defense.

Induction Experiments

Tollrian & Dodson (1999) pointed out that daphnids live in an “olfactory sea” and are able to discriminate between many chemical odors. Water-soluble substances released by predators provide reliable cues for prey species if they indicate the current predation pressure of a single predator. The so-called “kairomones” (Brown et al. 1970) are exclusively advantageous to the receiver in an interspecific information-transfer context (Dicke & Sabelis 1988). Kairomones have been shown to cause plastic responses in cladocerans, but most scientists have focused their studies on kairomones released from *Chaoborus*, *Notonecta* or fish (for review see: Havel 1987; Tollrian & Dodson 1999).

Our investigation reveals that *D. cucullata* have morphologically-plastic responses to cues from each of the predators tested. The result from our *Chaoborus* treatment concurs with the data published by Tollrian (1990). He showed that daphnids of intermediate and large size in the presence of the predator developed significantly longer helmets than control daphnids. We achieved an almost identical result in the induction experiments with other invertebrate predators, although extreme helmet formation as observed in nature could not be induced. Predatory copepods are reported to affect phenotypic plasticity in *Bosmina* and *Daphnia* (e.g., Dodson 1984; Post et al. 1995), but our study revealed for the first time that daphnids change their morphology in the presence of *Leptodora*.

Daphnia cucullata is a very small and transparent species and can be found in ponds with a high density of fish (Hrbácek 1958). Fish feed on larger prey items and the induced structures in *D. cucullata* should have no effect on fish predation. Hence, it is not surprising that helmets in *Daphnia cucullata* are not inducible with fish kairomones (Pawlowski 2000). Similarly, Dodson (1988) revealed that *Daphnia retrocurva* and *Daphnia galeata* developed helmets in the presence of *Chaoborus* and *Notonecta*, but not in the presence of fish. Field studies indicate that plastic responses in *Cladocera* are strongest when invertebrate predators are abundant (Mort 1989; Pijanowska 1991). Together with these observations, our results imply that cyclomorphosis in *Daphnia cucullata* in the wild is at least partly induced by predator kairomones.

Our three predator treatments differed in the magnitude of induction (see Tables 2, 3). This difference could be due to variation in kairomone concentration in our study. Such a dose-response relationship was demonstrated for neckteeth formation in *Daphnia pulex* (e.g. Havel 1985 a, Tollrian 1993). On the other hand, predator-specific kairomones could be the cause for the variable degree of response to the different predator environments. For example, Barry & Bayly (1985) demonstrated that *Daphnia carinata* is only inducible with notonectids. Laforsch & Tollrian (unpublished) revealed that *Leptodora* could induce helmet formation in *Daphnia cucullata* but not neckteeth development in *Daphnia pulex*. Accordingly, it seems likely that *Daphnia cucullata* can distinguish between different predators in a multipredator environment. However, these speculations are beyond the scope of our study because we controlled neither for equal amounts of predator biomass nor for equal amounts of prey consumed. Interestingly, the maximum induction of the traits in our study is found in the size range of *Daphnia* with the strongest threat of invertebrate predators (Swift & Fedorenko 1975; Pastorok 1981), indicating that selection has shaped the defense such that it is active during the most vulnerable life stages.

Predation Experiments

Our predation experiments, with three invertebrate predators that coexist with *Daphnia cucullata* in the wild, show for the first time a distinct advantage of the induced morph. So far only a few studies have shown protective effects of helmets in other species (Havel 1985 b; Mort 1986).

Spitze & Sadler (1996) showed that tail spines also act as a protection against *Chaoborus* predation. Similarly, an elongated tail spine in other species was reported to be protective against fish (e.g., Kolar & Wahl 1998) and copepods (e.g., Balseiro & Vega 1994). Invertebrate predators, which usually locate their prey with mechanoreceptors, are often restricted in their ability to catch, handle or ingest their prey items. The phantom midge larva is the best-documented example of a size-selective predator with a preference for smaller prey organisms (e.g., Tollrian 1995). This selectivity is based on a size-dependent interaction between the encounter probability and strike efficiency of the ambush predator (Pastorok 1981). Similarly, *Leptodora* are limited by the morphology of their feeding basket and consequently can only catch prey up to a certain size (e.g., Branstrator 1998). Size selectivity in copepods is not universally agreed upon. Williamson (1983) suggested that body size of prey items is not crucial to a successful strike, whereas Gliwicz & Umana (1994) demonstrated that smaller cladocerans such as *Daphnia cucullata* remained vulnerable to *Acanthocyclops* predation until maturation and larger animals were safe from copepod predation. The latter is in accordance with the result of our predation experiment, where only the first two size classes were susceptible to *Cyclops* predation. Copepods often attack their prey dorsally, and thus the tail spine is discussed as the predominant defense against this predator (Balseiro & Vega 1994). We show that the plastic traits act as protective devices against *Cyclops* predation, even in the smallest neonates. Similarly, *Leptodora* predation was significantly less successful on longer-helmeted and spined specimens in our trials (Fig. 2). Induced animals larger than 600 µm had a distinct advantage in both treatments.

In general, two different sorts of defense strategies can be distinguished (Tollrian & Dodson 1999). The first are pre-capture defenses which make prey less conspicuous or harder to detect, and the second are post-capture defenses which impede handling or ingestion of the prey. Induced morphological defenses belong predominantly to the second category as already suggested in Dodson’s (1974) “anti lock and key” hypothesis. For example, neckteeth in juvenile *Daphnia pulex* offer only a post-capture advantage (Havel & Dodson 1984). We showed that cyclomorphic features of *Daphnia cucullata* offer advantages at different steps of the *Chaoborus* predation cycle. We did not find an advantage before an attack, (i.e. a “stealth” mechanism which would allow the prey to pass a tactile predator without being attacked), since the attack rate on helmeted individuals was not lower. Induced individuals were actually attacked more often in size categories 2 and 3. However, these relatively high attack rates can be offset by the low attack efficiency for the induced morphs (Table 4). Induced daphnids escaped and the predator remained hungry and continued to attack. In our experiment, induced *Daphnia cucullata* of intermediate sizes had a significant advantage between the steps of attack and capture (Table 4). It was not possible to clarify whether the daphnids were harder to grasp or simply able to escape faster. Hebert (1978) suggested that helmets may supply more area for antenna muscle attachment which in turn would allow the animals to escape faster. However, in the case of *Chaoborus* this scenario seems unlikely. Since *Chaoborus* attacks take less than 1/100 second (Tollrian, unpublished data), certainly faster than *Daphnia* escape-swimming speed. Alternatively, helmets and tail spines could lead to a misjudgment of prey distance by the predator. Finally, our study revealed that induced daphnids of the largest size had an additional advantage between the steps of capture and ingestion. The induced traits render *Daphnia cucullata* difficult to swallow. Phantom midge larvae are gape-limited predators (Swift 1992; Pastorok 1981) and enlarged structures such as helmets and tail spines can interfere with their ability to ingest prey. Interestingly, the induced traits in *D. cucullata* acted during different life stages, from the smallest neonates which have been protected against copepods to the adults which escaped Chaoborus captures. This result may resolve the question of why some cladocerans form helmets and others neckteeth (Tollrian & Dodson 1999). Neckteeth have been shown to defend early instars of larger cladocerans against *Chaoborus* larvae (Tollrian 1995), while our study showed that smaller species require protection from several invertebrate predators and helmets act as general multipurpose defenses.

CONCLUSIONS

Phenotypically plastic organisms are adapted to heterogeneous environments. Multipredator regimes are common and the evolution of inducible defenses is a suitable agent to cope with the sometimes unpredictable hazard of a variety of predators, each foraging in a different way. *Daphnia cucullata*, a very small cladoceran, can coexist with fish and is threatened by several predaceous invertebrates. Chemical cues released by each of these predators may indicate their temporal impact and so induce cyclomorphosis in the field. Finally, after one century of research in this “classical” system, our study provides evidence that predator kairomones act as proximate cues and a defensive effect is an ultimate reason for cyclomorphosis in *Daphnia cucullata*. In freshwater habitats there is a strong pressure to adapt to a variety of predator’s foraging strategies and the combination of an elongated helmet and tail spine seems to be a perfect device to reduce the predation risk caused by multiple predators, especially since the same defensive traits can act at different stages of the predation cycle. The combined benefits of defenses against different predators may be the result of a “diffuse” coevolution and possibly the key factor for evolution and persistence of these inducible defenses in multipredator environments.

ACKNOWLEDGEMENTS

We thank W. Gabriel for encouragement and discussion; M. Kopp and J. M. Jeschke for comments; M. Kredler and E. Hochmuth for help during experiments; John Baines and Scott Stevens for linguistic improvements; and the editor John Havel and two anonymous reviewers for comments on the manuscript.

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Table 1

Mean relative helmet length (%) and standard error (SE) of *Daphnia cucullata* raised with (kairomone) and without (control) chemical cues released from different invertebrate predators. Only one control treatment was carried out, because all experiments were conducted at the same time. *Daphnia* were divided into four body length size classes (1: < 600 µm; 2 = 600 – 800 µm; 3 = 800 – 1000 µm; 4 > 1000 µm) for analysis. N indicates the number of individual *Daphnia* pooled from 3 replicate beakers per treatment. Size classes were compared for treatment effects using a nested ANOVA with 3 replicates per treatment as random factor (P value).

		control			kairomone			
Experiment	*Daphnia* size class	Mean	SE	N	Mean	SE	N	p
Chaoborus	1	15.12	0.41	62	30.19	0.47	94	< 0.001
	2	14.28	0.21	136	29.79	0.32	100	< 0.001
	3	13.03	0.25	62	25.84	0.34	74	< 0.001
	4	12.29	0.26	41	20.51	0.44	35	< 0.001
Cyclops	1	15.12	0.41	62	27.96	0.46	101	= 0.001
	2	14.28	0.21	136	27.70	0.41	75	< 0.001
	3	13.03	0.25	62	23.07	0.29	99	< 0.001
	4	12.29	0.26	41	20.10	0.44	25	< 0.001
Leptodora	1	15.12	0.41	62	30.62	1.97	10	< 0.001
	2	14.28	0.21	136	33.98	0.52	85	< 0.001
	3	13.03	0.25	62	28.02	0.54	72	= 0.001
	4	12.29	0.26	41	19.70	0.92	25	= 0.001

Table 2

Mean relative tail spine length (%) and standard error (SE) of *Daphnia cucullata* raised with (kairomone) and without (control) chemical cues released from different invertebrate predators. Only one control treatment was carried out, because all experiments were conducted at the same time. *Daphnia* were divided into four body length size classes (1 < 600 µm; 2 = 600 – 800 µm; 3 = 800 – 1000 µm; 4 > 1000 µm) for analysis. N indicates the number of individual *Daphnia* pooled from 3 replicate beakers per treatment. Size classes were compared for treatment effects using a nested ANOVA with 3 replicates per treatment as random factor (P value).

		control			kairomone			
Experiment	*Daphnia* size class	mean	SE	N	mean	SE	N	p
Chaoborus	1	38.40	0.86	62	54.05	1.01	94	= 0.001
	2	26.91	0.41	136	39.05	0.74	100	= 0.001
	3	22.51	0.35	62	29.21	0.43	74	= 0.001
	4	12.36	0.39	41	22.89	0.58	35	< 0.001
Cyclops	1	38.40	0.86	62	50.62	0.62	101	= 0.002
	2	26.91	0.41	136	38.20	0.59	75	= 0.001
	3	22.51	0.35	62	30.12	0.23	99	= 0.001
	4	12.36	0.39	41	23.45	0.72	25	< 0.001
Leptodora	1	38.40	0.86	62	44.07	2.01	10	n.s.
	2	26.91	0.41	136	33.11	0.49	85	= 0.045
	3	22.51	0.35	62	26.64	0.36	72	= 0.029
	4	12.36	0.39	41	19.40	1.15	25	< 0 .001

Table 3

Observed mean values and standard errors (±1 SE) of the predation of *Chaoborus flavicans* on two different morphs (morph) of *Daphnia cucullata* (h = helmeted; t = typical) in three size classes (body length: 1 < 600 µm; 2 = 600 – 800; 3 > 800 µm): attack = number of attacks; evasion: number of unsuccessful attacks; eaten: number of eaten animals; escape: number of escaped animals after capture.

Daphnia size class	morph	n	attack	evasion	eaten	escape
1	h	10	6.30 ± 0.79	1.30 ± 0.33	4.90 ± 0.67	0.10 ± 0.01
	t	10	8.50 ± 0.72	1.50 ± 0.27	6.70 ± 0.68	0.30 ± 0.15
2	h	14	8.71 ± 0.79	3.93 ± 0.54	2.57 ± 0.39	2.21 ± 0.79
	t	14	6.29 ± 0.67	1.57 ± 0.42	3.93 ± 0.45	0.79 ± 0.21
3	h	14	10.57 ± 1.24	6.43 ± 0.86	0.71 ± 0.22	3.43 ± 0.50
	t	14	4.71 ± 0.57	1.92 ± 0.48	1.86 ± 0.33	0.93 ± 0.25

Table 4

Direct observation of *Chaoborus flavicans* predation on *Daphnia cucullata.* Attack efficiency (number of eaten *Daphnia*/ total number of attacks x 100), evasion efficiency (number of evasions without capture / total number of attacks x 100) and escape efficiency (number of escaped daphnids after capture / total number of captures x 100) in the predation trials with *Chaoborus flavicans*. Data show means and standard errors (SE) of three size classes (body length: 1 < 600 µm; 2 = 600 – 800; 3 > 800 µm). Significance levels for comparisons between the helmeted and typical morphs were calculated using Mann – Whitney U – tests.

	Daphnia size class	typical			helmeted			
		N	Mean (%)	SE	N	Mean (%)	SE	p
Attack efficiency	1	10	78.60	3.47	10	75.97	4.92	= 0.543
	2	14	62.76	4.59	14	31.52	5.00	< 0.001
	3	14	47.02	8.87	14	7.10	2.21	< 0.001
Evasion efficiency	1	10	18.42	3.56	10	19.02	4.51	= 0.790
	2	14	20.73	4.72	14	46.23	5.27	= 0.002
	3	14	35.47	7.03	14	61.29	3.99	= 0.009
Escape efficiency	1	10	3.44	1.82	10	5.00	5.00	= 0.358
	2	14	19.65	5.01	14	36.45	8.79	= 0.239
	3	14	33.33	9.56	14	85.64	4.50	< 0.001

FIGURE LEGENDS

Figure 1

Comparative Scanning Electron micrographs (for method see Laforsch & Tollrian 2000) of the first eight instars of typical (bottom row) and helmeted (laboratory induced; top row) *Daphnia cucullata*. The grouping size classes in our study (sc1 – sc4) are separated by vertical lines. The arrangement of the daphnids follows the developmental pattern of the relative values of the plastic traits during these life stages. The morphological parameters recorded from *Daphnia cucullata* in our experiments were the helmet length (HL), the body length (BL) and the tail spine length (SL).

Figure 2

The number of eaten animals in the predation experiment with *Leptodora kindtii*. Given are means and the standard errors of two different morphs (helmeted; typical) of *Daphnia cucullata* in three size classes (body length: 1 < 600 µm; 2 = 600 – 800; 3 > 800 µm). Asterisks (*) indicate significant differences.

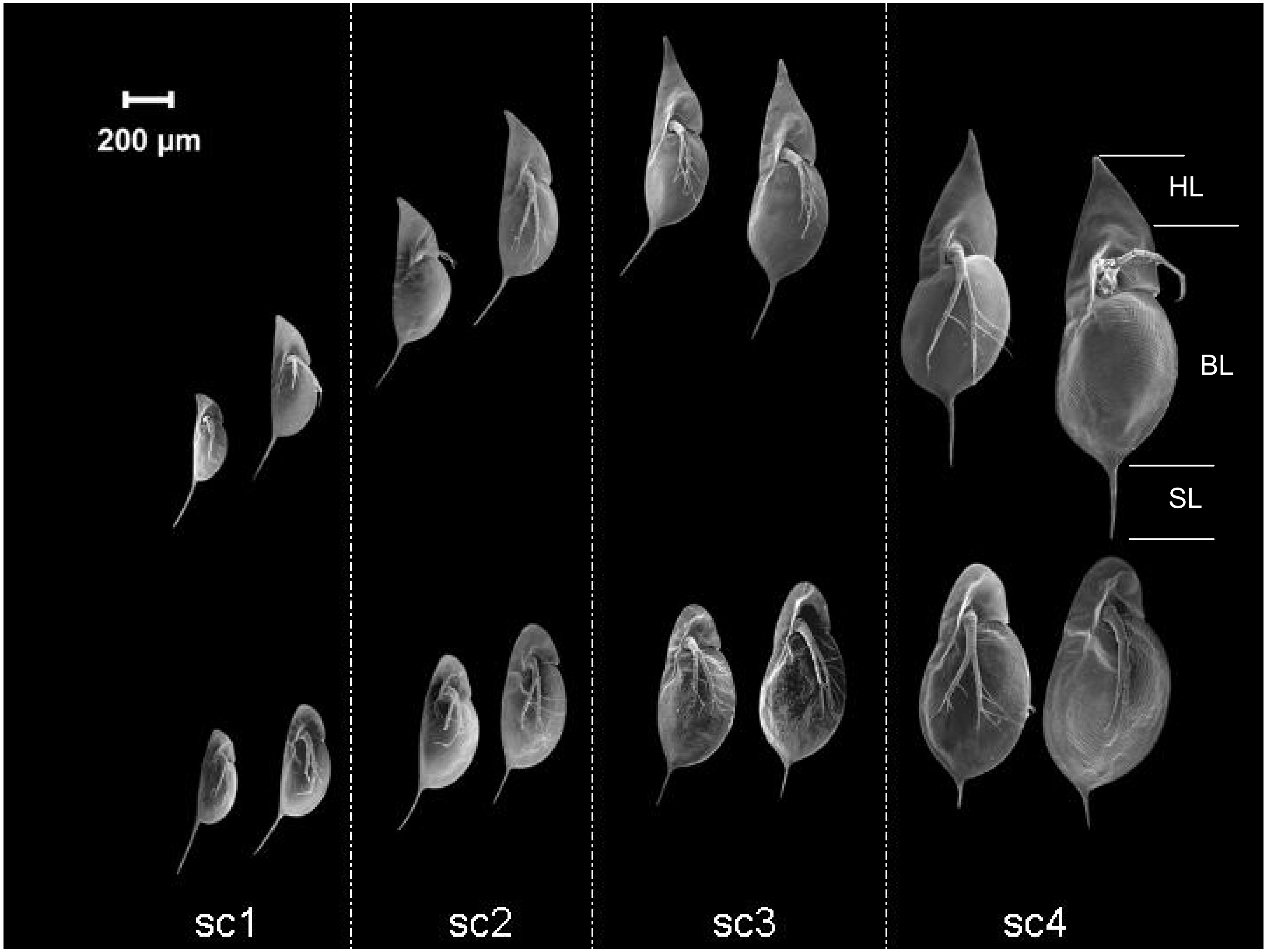
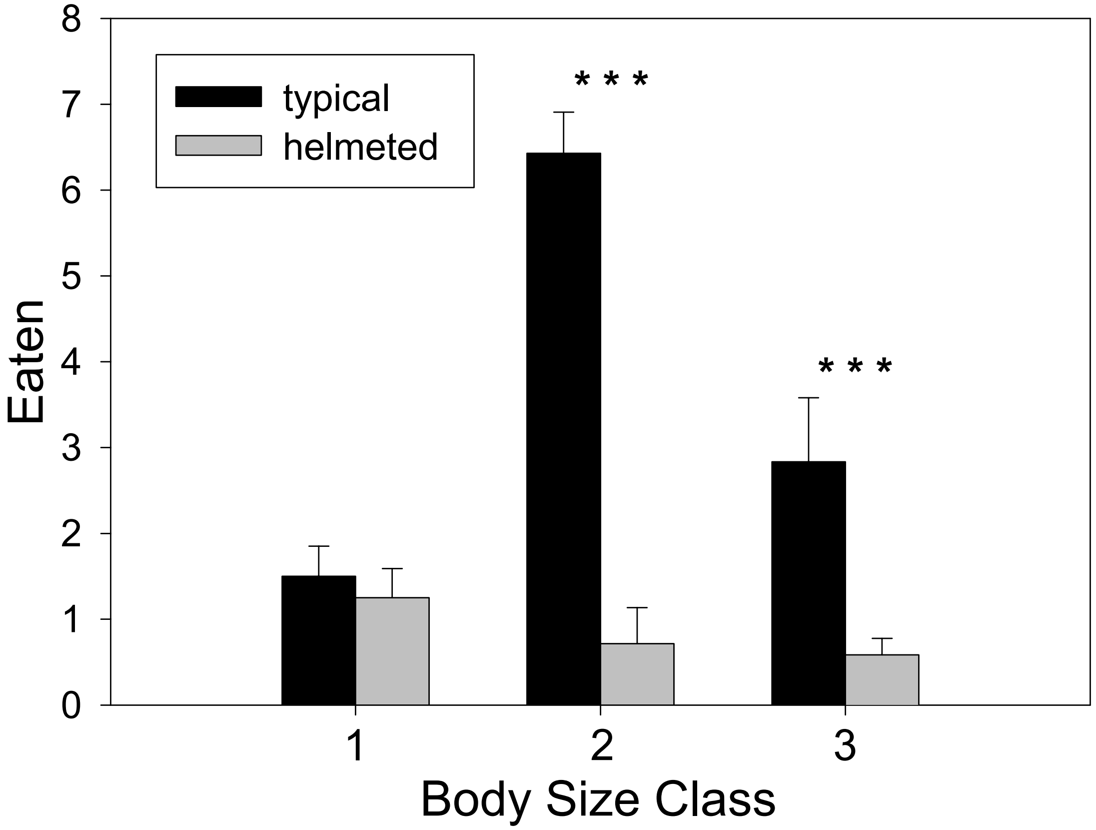

200 µm
HL
BL
SL
sc1
sc2
sc3
sc4

Fig. 2



2

INDUCIBLE DEFENSES: THE RELEVANCE OF ALARM SIGNALS IN DAPHNIDS
(Laforsch und Tollrian, *Limnology and Oceanography*, Akzeptiert in Abhängigkeit der Revision)

Inducible defenses: The relevance of alarm signals in daphnids

Christian Laforsch † and Ralph Tollrian *

†* Section of Evolutionary Ecology, Department Biologie II, Ludwig-Maximilians-Universität München, Karlstr. 23 – 25, 80333 München, Germany

† Corresponding author: Tel: +49 89 5902253, Fax: +49 89 5902461,
laforsch@zi.biologie.uni-muenchen.de
* tollrian@lmu.de

Running Head: Alarm signals in *Daphnia*

Acknowledgements

We thank W. Gabriel for encouragement and discussion; M. Kredler, Laura Beccara and Andreas Buckenmeier for help during experiments; and Scott Stevens for linguistic improvements.

Abstract

Many aquatic organisms use chemical cues to recognize predators and to activate inducible defenses. In zooplankton many of these cues are thought to be kairomones released by specific predators. However, in several other organisms, e.g., fish and amphibians, alarm signals from conspecifics also provide reliable cues. Here we tested whether alarm substances released by wounded conspecifics also act as chemical cues in *Daphnia* and assessed their relevance as compared to predator kairomones. We found that water-born cues from macerated daphnids alter plastic morphology in three *Daphnia* species (*Daphnia cucullata, Daphnia longicephala* and *Daphnia lumholtzi*). However, kairomones released by predators had distinctly stronger effects on the plastic traits in each of the species tested. In *Daphnia cucullata* the diet of the predator is relevant. The increase in relative helmet length was most pronounced when the predator was fed with conspecifics. Cross-species experiments with alarm substances revealed that *Daphnia cucullata* is more sensitive to chemical signals from macerated conspecifics than to chemical cues from *Daphnia magna*. In *Daphnia cucullata* reactions to alarm substances are concentration-dependent and there is an accumulative effect of chemical cues released by homogenized daphnids. Our study suggests that alarm cues together with predator specific kairomones may form a blend of chemicals which activates inducible defenses in nature.

Introduction

Chemical information transfer is widespread in inter- and intra-specific communication, especially in aquatic ecosystems in which turbidity can make visual cues less detectable. Sources of biotic information include factors such as food quality, mating-partner density or predation impact (Atema et al. 1988) and chemical cues are often used to trigger inducible defenses. Anti-predator responses frequently incur costs and a driving factor behind many inducible defense systems is that these costs can be saved when a particular defense is not needed (Tollrian and Harvell 1999). Thus the specificity and reliability of the information about the predation risk is crucial to maximize the benefit of a defense (Gabriel et al. unpubl.).

Both predator-released kairomones and prey-released alarm signals can provide important information about the current predation risk for prey. Chemical substances released by predators, defined as kairomones (Brown et al. 1970), lead to phenotypic changes in their prey. These chemically-mediated anti-predator responses have been shown in a variety of aquatic organisms (for review see: Tollrian and Harvell 1999). Prey-released alarm signals, which are correspondent to distress calls of birds and mammals (e.g., Staton 1978; Klump and Shalter 1984), are not related to a specific source of predation. They are important factors for the activation of anti-predator responses in terrestrial and aquatic habitats. Although numerous examples are reported to evoke behavioral changes (e.g., Brown and Godin 1997; Brown and Smith 1997; Wisenden *et al.* 2001), sparse work on the relevance of alarm substances for the induction of morphological defenses has been done so far (reviewed in Chivers and Smith 1998).

Many cladoceran species form predator-induced defenses (reviewed in Tollrian and Dodson 1999). Since no detectable effect of chemical stimuli of crushed conspecifics could be demonstrated in *Daphnia pulex* (Walls and Ketola 1989; Parejko and Dodson 1990) and *Daphnia galeata mendotae* (Stirling 1995), most researchers working on inducible defenses in daphnids focused on predator kairomones (e.g., Grant and Bayly 1981; Krueger and Dodson 1981; Hebert and Grewe 1985; Dodson 1989, Hanazato 1991; Tollrian 1990 and 1994; Krizan-Lüning 1997). But plankton organisms are often lethally or non-lethally injured during attack because larval and juvenile fish still have to learn how to feed on their prey (Ibrahim and Huntingford 1992) and several invertebrate predators crush their prey item before it can be swallowed (Branstrator 1998). Therefore, alarm signals could also provide relevant cues in *Daphnia*. Two recent studies indicated that the behavior and life history of *Daphnia magna* was affected by cues from homogenized conspecifics (Pijanowska 1997; Pijanowska and Kowalczewski 1997).

The aim of our study was to test the importance of broadly-defined alarm signals in *Daphnia* (in daphnids, potential alarm signals are most likely not released by specific glands, but rather consists of cellular fluids). We used three *Daphnia* species for our induction experiments, all well known for inducible morphological defenses. *Daphnia cucullata* develop big helmets (Tollrian 1990, Agrawal et al. 1999), *Daphnia longicephala* produces huge crests (Grant and Bayly 1981) and *Daphnia lumholtzi* show a remarkable elongation of their head and tail spines (Tollrian 1994). Using *Daphnia cucullata* as model system, we also investigated whether the cues are accumulating and whether they are species specific. Furthermore, with all species we tested the difference between predator-released kairomones and cues from homogenized daphnids to estimate a potential ecological relevance.

Material and Methods

Laboratory-cultured clones of daphnids were used for the experiments. We collected *Daphnia cucullata* from Lake Thalersee (Germany). *Daphnia magna* was isolated from Ismaninger Speichersee (Germany). We used a clone of *Daphnia longicephala* originating from Australia and a clone of *Daphnia lumholtzi* originating from North America. Additional experimental animals, *Chaoborus flavicans* and *Bosmina longirostris*, were taken from Lake Klostersee (Germany). We collected notonectids from a pond in the garden of our university. All tests were conducted in the laboratory under constant conditions at 20 °C and fluorescent light in 1.5 L glass beakers, filled with one liter of medium. The daphnids were reared in a synthetic medium (Laforsch and Tollrian unpubl.) and were fed *ad libitum* daily with *Scenedesmus obliquus*. The medium was changed weekly, beside the accumulation experiment. About 25 randomly chosen ovigerous daphnids were introduced into each beaker at the beginning of the experiment. All beakers contained a cylindrical cage made of an acryl frame (10 cm diameter and 12 cm length) covered by nylon mesh (100 µm) in order to avoid direct contact between crushed conspecifics or predators and daphnids, but to still enable the exchange of chemical cues. To produce alarm signals the daphnids were macerated, resuspended, and diluted to the appropriate concentration. Subsequently the homogenate was poured into the net cages. Alarm signals were added daily into the beakers. All treatments were replicated three times.

Induction experiment with alarm signals released by Daphnia cucullata and concentration dependent induction

In the first experiment we tested whether *Daphnia cucullata* shows a helmet induction with chemical signals released by injured conspecifics. A suspension of 100 crushed *Daphnia cucullata* was added daily to each beaker.

The second experiment was conducted to show whether the inductive effect of alarm signals is concentration dependent. Solutions of 0, 20, 40, 60 and 80 macerated conspecifics of *Daphnia cucullata* per liter were added.

Accumulative effects of chemical alarm signals

To test for accumulative effects we used a concentration of 40 crushed *Daphnia cucullata* per liter, based on our results from the concentration-dependent induction experiment. In the first treatment the medium was changed every second day (40c), whereas in the second treatment the medium was not changed during the whole experiment (40a). Accordingly, two treatments without alarm signals served as controls, one with medium exchange every second day (cc) and the other without any medium exchange (ca). We added 100µl/L Ampicillin daily to each beaker to test if the cues would be degraded by bacteria and whether they are chemically stable enough to accumulate.

Species-specificity of the cues

To test whether *Daphnia cucullata* reacts only to alarm substances released by conspecifics, we investigated the induction effect of alarm signals released by *Daphnia magna* on *Daphnia cucullata*. In the first treatment 80 crushed *Daphnia cucullata* per liter were used. This amount was based on our results from the concentration-dependent induction experiment. In the second treatment we added the suspension of 40 macerated adult *Daphnia magna* per liter. The third treatment, without any addition, served as control. 100µl/L Ampicillin were added daily to each beaker. Compared to *Daphnia cucullata*, *Daphnia magna* is a relatively large zooplankton species (approximately 25 x the dry-weight of *Daphnia cucullata*). Based on our prior experiments we chose a higher biomass of *Daphnia magna* to ensure that a stronger response to conspecific cues could not be caused by a higher *Daphnia cucullata* biomass.

Kairomones vs. alarm signals

The last experiment was designed to show whether kairomones released by a feeding predator or alarm signals from injured conspecifics have stronger effects on the magnitude of plastic morphology of different *Daphnia* species.

Daphnia cucullata: In the first treatment a solution of 40 crushed *Daphnia cucullata* was poured daily into each beaker (40c). In the second treatment 10 *Chaoborus* larvae were fed daily with 40 individuals of *Daphnia cucullata* (ch_cuc). Ten *Chaoborus* larvae were fed every day with 300 individuals of *Bosmina longirostris* in the third treatment (ch_bos), because the dry-weight of one adult *Daphnia cucullata* is almost equivalent to the dry-weight of eight *Bosmina longirostris*. A fourth treatment without supplement served as control. 100µl/L Ampicillin were added to each beaker daily.

The predators, fourth-instar larvae of *Chaoborus flavicans,* were placed into the cages described above. Pupate *Chaoborus* larvae and dead predators were replaced daily. The cages were cleaned daily. Beakers without predators served as control. In this experiment we measured daphnids of the second and third body-length size class for analysis, because *Daphnia cucullata* shows the longest absolute helmet length in these size classes.

Daphnia longicephala: Ten daphnids (10c) were crushed and added daily to the first treatment. In the second treatment a concentration of 50 homogenized *Daphnia longicephala* per liter was used (50c). One *Notonecta* was placed into a net cage and fed daily with 10 *Daphnia magna* in the third treatment (noto). The cages were covered by a net to prevent backswimmers from escaping. The cages were treated as described in the *Daphnia cucullata* experiment. Beakers without any addition served as control. The daphnids were measured after having reached maturity.

Daphnia lumholtzi: Treatments were similar to those of the *Daphnia longicephala* experiment (10c; 50c; control). We used deep-frozen fish kairomones from *Phoxinus phoxinus* for the predator treatment in a concentration of one fish per three liters. We produced the fish kairomone by keeping 10 fish for 24 hours in 10 liters of medium. Thereafter the fish were removed and the medium was filtered (Schleicher and Schuell: Excelon PES 20/3HC; 0.2 µm) and frozen (-60°C). Prior to this procedure the fish were fed exclusively with chironomid larvae, which are known to be ineffective in inducing plastic traits in cladocerans. The daphnids were measured after having reached maturity.

All induction experiments lasted three to five weeks. Daphnids from each beaker were measured under a dissecting microscope with a digital image-analysis system (Soft Imaging System, Analysis Pro, Münster, Germany). The morphological parameters, recorded from *Daphnia cucullata*, *Daphnia lumholtzi* and *Daphnia longicephala,* were the helmet length (defined as the length between the tip of the helmet to the upper edge of the compound eye) and the body length (defined as the length between the upper edge of the compound eye to the base of the tail spine). For *Daphnia longicephala* we also recorded the crest height (defined as the distance between the outer edge of the compound eye to the most dorsal point of the crest). Prior to analysis we divided *Daphnia cucullata* into four body-length size classes (sc1: <600 µm; sc2: 600 – 799 µm; sc3: 800 – 1000 µm; sc4: >1000 µm). We calculated the relative helmet length and relative crest height (helmet length or crest height / body length *100), to compensate for size-dependent changes in helmet length and crest height within the classes. The relative values were arcsin-square-root-transformed prior to analysis (Sokal and Rohlf 1995). All treatments were replicated three times. The replicates could be pooled for analysis in all cases because they showed no significant within-treatment difference in Kruskal - Wallis tests. In the first experiment a non-parametric Mann-Whitney U - test was used to compare the two independent groups. A one-way ANOVA followed by a Tamhane Post-Hoc Test (SPSS, Version 11), which is insensitive to unequal variances, was applied to test for significant treatment effects in all other experiments.

Results

Daphnia cucullata exposed to a high concentration (100 daphnids per liter) of crushed conspecifics showed a significant helmet elongation in each body-length size class (Table 1, Mann-Whitney-U; size class 1 - 4: all $p < 0.001$).

A test of the concentration dependence revealed significant inductions already at 20 crushed *Daphnia*/L. Helmet length of *Daphnia cucullata* differed significantly between concentration treatments in each body-length size class in this experiment (one-way ANOVA: sc1: $F_{4,487} = 32.8$, $p < 0.001$; sc2: $F_{4,403} = 31.0$, $p < 0.001$, sc3 $F_{4,406} = 37.3$, $p < 0.001$, sc4: $F_{4,259} = 47.86$, $p < 0.001$). Tamhane multiple-comparison tests show that helmet length of *Daphnia cucullata* was, in each induction treatment and in each body-length size class, significantly longer than that of the control, with the only exception being the first size class in the treatment with 20 crushed daphnids (Tamhane Post-Hoc Tests: control vs. 20c; sc1: $p = 0.130$, sc2: $p = 0.005$, sc3: $p = 0.010$, sc4: $p <$

0.001; control vs. 40c, 60c, 80c; sc1-4: all $p < 0.001$). The dose-response curve revealed a maximum at approximately 60 crushed *Daphnia*/L followed by a plateau (Tamhane Post-Hoc Tests: 60c vs. 80c; sc1-4: all ns; Figure 1). The accumulation experiment revealed a cumulative effect of the chemical cue. Treatment effects differed significantly in this experiment in each body-length size class (Table 2). *Daphnia cucullata* cultured without water exchange and with an addition of 40 macerated conspecifics per day showed significantly longer helmets in each size class compared to the treatment with 40 crushed daphnids and water exchange (Tamhane Post-Hoc Tests: sc1,sc2, sc4: $p < 0.001$; sc3: $p = 0.001$). Helmet length differed significantly between treatment 40a and its corresponding control (ca) in each size class (Tamhane Post-Hoc Tests: all $p < 0.001$). The relative helmet length of the daphnids induced by homogenized conspecifics (40c) with water exchange were significantly longer in body-length size class 2 and 3 compared to the control (cc) animals (Tamhane Post-Hoc Tests: sc2, sc3: $p < 0.001$; sc1, sc4: ns).

Cross-species experiments show that *Daphnia cucullata* also respond to alarm signals from other *Daphnia* species, but the inductive effect is stronger when animals are exposed to chemical cues from macerated conspecifics. Helmet induction differed significantly between treatments in each body-length size class (one-way ANOVA: sc1: $F_{2,265} = 44.4$ $p < 0.001$; sc2: $F_{2,315} = 40.9$ $p < 0.001$, sc3: $F_{2,231} = 40.1$ $p < 0.001$, sc4: $F_{2,118} = 40.6$ $p < 0.001$). Helmet length of both induction treatments with *Daphnia cucullata* and *Daphnia magna* were in each body-length size class significantly longer than that of the control (Tamhane Post-Hoc Tests: all $p < 0.001$). *Daphnia cucullata* showed in size classes 2-4 significantly longer helmets in response to conspecific cues compared to alarm signals from *Daphnia magna* (Figure 2; Tamhane Post-Hoc Tests: sc2-4 $p < 0.001$; sc1 = ns).

Experiments with predator kairomones revealed a distinctly stronger induction of the plastic traits compared to alarm signal induction in all tested *Daphnia* species.

Helmet length of *Daphnia cucullata* differed significantly between treatments in both body-length size classes in the predator kairomone experiment (Table 3). *Daphnia cucullata* showed the strongest helmet elongation in both body-length size classes in the treatment with phantom midge larvae fed with conspecifics (Tamhane Post-Hoc Test: all $p < 0.001$). Also helmets of *Daphnia cucullata* induced with kairomones released by *Chaoborus* larvae fed with *Bosmina* were significantly longer than helmets induced with alarm substances from *Daphnia cucullata* in each size class (Tamhane Post-Hoc Test: all $p < 0.001$). Helmet length of *Daphnia cucullata* induced with alarm substances differed significantly from that of the control (Tamhane Post-Hoc Test: all $p < 0.001$).

Similarly, *D. longicephala* showed in each treatment with chemical signals significantly longer helmets and crests than the control animals (Tamhane Post-Hoc Test: all $p < 0.001$). Both the relative helmet length and the relative crest height of *Daphnia longicephala* were distinctly larger in the predator treatment as compared to the other treatments (Table 4; Tamhane Post-Hoc Test: all $p < 0.001$). We observed no difference in any of the morphological traits concerning different alarm signal concentrations (Tamhane Post-Hoc Test: ns).

Additionally, in the experiment with *Daphnia lumholtzi,* the daphnids induced by predator kairomones showed significantly longer helmets than both control animals and the animals exposed to different concentrations of alarm cues (Table 5; all $p <$ 0.001). Only the treatment with 10 macerated daphnids showed a significant difference to the control (Tamhane Post-Hoc Test: $p = 0.009$). All other treatments showed no significant difference in the relative helmet length in this experiment.

Discussion

Our experiments revealed that the morphologically plastic traits of different *Daphnia* species are indeed affected by chemical cues from macerated conspecifics. All three *Daphnia* species responded with the induction of their defenses. However, although the inductive effect of the alarm signals was significant compared to the control in each experiment, all tested daphnids showed a distinctly stronger induction effect when exposed to chemical cues released by their predators. Our accumulation experiment shows that there is a bacterial degradation of the chemical alarm signals, which is analogous to that of the kairomones (Reede and Ringelberg 1995; Tollrian 1995). Therefore, because they do not linger, alarm signals may provide timely information about the actual predation risk. This is especially important for phenotypically plastic organisms, which evolved traits to react flexibly to changing environmental conditions. A particular *Daphnia* species exposed to alarm stimuli may be able to smell that it is currently under predation but the gathered chemical information is not specific to any predator. Therefore, a predator-specific response cannot be activated. In this context alarm signals are “imperfect” cues if predators with contrasting selectivity are present, e.g., invertebrates and fish. However, even if alarm signals provide no particular information about the predator, they could be relevant in situations where the predators supply insufficient information or where many predator species co-occur in low densities. Potentially, their specific cues would not reach a sufficient concentration but the combined alarm signals could indicate a significant predation risk. Another possible adaptive value of these damage-released cues could be that alarm signals lead to a general alertness state. Induced alertness has been indicated by the results of Pijanowska (1997) who showed that *Daphnia magna* induced with crushed conspecifics avoided attacks by fish significantly more often than “naïve” daphnids. Similarly, Hews (1988) considered that prey alertness induced by alarm signals may be responsible for different capture efficiencies by a predatory dragonfly on tadpoles. In this alertness state daphnids can decide how to react when the most likely source of danger is identified.

Although all examined *Daphnia* species showed a significant response to damage-released alarm signals, this effect was definitely moderate when compared to the induction by water-born cues from predators (Tab. 3-5).

Our data revealed that *Daphnia cucullata* shows significantly longer helmets in the induction treatment with *Chaoborus* larvae fed with conspecifics as compared to the treatment of phantom midge larvae fed with *Bosmina*. This could not have been a food concentration effect because prey biomass was identical in both experiments. Furthermore, our cross-species alarm signal experiments indicate that daphnids can partly distinguish between alarm cues from conspecifics and from related species. Similar cross-species responses on injury-released alarm signals have been reported in a variety of aquatic animals (e.g., crayfish, Hazlett 1994; tadpoles, Pfeiffer 1966; fish, Schutz 1956; Smith 1982). Thus, our results suggest that daphnids can differentiate between predators foraging on any kind of prey and a predator feeding on conspecifics. Predators feeding on a relatively large zooplankton species, such as *Daphnia magna*, usually show a different size selectivity as compared to predators foraging on *Daphnia cucullata*. In this context alarm signals from *Daphnia magna* should not be as relevant as alarm cues from conspecifics for *Daphnia cucullata* in assessing the predation risk.

There is no evidence that a special substance has evolved as an alarm signal for daphnids, as reported for fish tissue (Hugie *et al.* 1991; Brown and Smith 1997; Smith 2000; Bryer *et al.* 2001). It seems more likely that daphnids can detect general components of injured conspecifics. Although some progress was made to identify the chemical characteristics of kairomones for *Chaoborus* (Tollrian and Von Elert 1994) and fish (Von Elert and Loose 1996), the molecular structure still remains unknown.

Two possible explanations for the enhanced effect of damage-released alarm signals and predator kairomones can be hypothesized. Either there is a synergistic effect when both cues are given simultaneously, or predators produce a different quality of kairomone when feeding on conspecific prey. The classical form of predator labeling, where alarm substances accumulate inside the predator and are released subsequently in high concentrations (e.g., cnidaria: Howe and Harris 1978; fish: Mathis and Smith 1993; Stabell and Lwin 1997; insects: Chivers et. al. 1996; echinoderms: Hagen et. al.

2002), seems unlikely in this case. The data of our concentration-dependent induction experiments show that all tested *Daphnia* species are inducible with alarm signals up to a certain concentration. An increase in alarm signal concentration had no increasing effect on induction (Fig. 1, Tab. 4, 5). Because the maximum response was well below the response to predator released cues, an accumulative effect can be ruled out as an explanation.

Instead, a mixture of several components may constitute the active substance, including injury-released alarm signals. Similarly, Slusarczyk (1999) concluded in his experiments that kairomones and chemical alarm signals act synergistically to induce resting-egg production in *Daphnia magna*. Likewise, Appleton and Palmer (1988) revealed for snails threatened by a predatory crab that damage-released alarm signals combined with predator-released cues are more effective in inducing morphological defenses. These combined chemical cues certainly increase the information quality and the reliability of the predation risk estimation. Therefore, when planning their future experiments, researchers focusing on induced defenses should take into account that the diet of the predator matters.

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Table 1

Mean relative helmet length (%) and standard error (SE) of *Daphnia cucullata* raised with (crushed: 100 daphnids/L) and without (control) chemical cues released from crushed conspecifics. The daphnids were divided into four body-length size classes (sc1: < 600 µm; sc2 = 600 – 800 µm; sc3 = 800 – 1000 µm; sc4 > 1000 µm) for analysis. Treatments were compared using Mann – Whitney U – tests.

	Control		Crushed		
Size class	Mean ± SE	*n*	Mean ± SE	*n*	*p*
sc1	18.32 ± 0.32	79	26.32 ± 0.52	91	< 0.001
sc2	16.51 ± 0.34	97	25.39 ± 0.41	85	< 0.001
sc3	14.91 ± 0.26	85	22.56 ± 0.31	106	< 0.001
sc4	13.19 ± 0.28	38	19.56 ± 0.69	18	< 0.001

Table 2

Mean relative helmet length (%) of *Daphnia cucullata* cultured in experimental treatments (ca: control without water exchange; cc: control with water exchange; 40a: 40 crushed *Daphnia cucullata* without water exchange; 40c: 40 crushed *Daphnia cucullata* with water exchange) to test for accumulative effects of chemical alarm cues. Daphnids were divided into four body-length size classes prior to analysis (sc1: < 600 µm; sc2 = 600 – 800 µm; sc3 = 800 – 1000 µm; sc4 > 1000 µm). Data were tested using one-way ANOVA. The respective ANOVA values and probabilities are given. Letters (H) indicate homogeneous groups based on Tamhane Post-Hoc Tests.

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Size class	Treatment	Relative helmet length (%) Mean ± SE	*n*	H	ANOVA
sc1	ca	23.19 ± 0.46	74	B	$F_{3,322} = 37.36$ $p < 0.001$
	cc	21.29 ± 0.32	114	A	
	40a	27.13 ± 0.56	74	C	
	40c	21.88 ± 0.34	64	AB	
sc2	ca	19.14 ± 0.33	82	A	$F_{3,296} = 88.29$ $p < 0.001$
	cc	19.11 ± 0.30	70	A	
	40a	27.68 ± 0.59	62	C	
	40c	23.26 ± 0.41	86	B	
sc3	ca	19.06 ± 0.32	67	B	$F_{3,284} = 50.62$ $p < 0.001$

	cc	17.48 ± 0.28	67	A	
	40a	23.08 ± 0.38	83	D	
	40c	20.33 ± 0.36	71	C	
sc4	ca	16.61 ± 0.26	92	AB	$F_{3,424} = 26.55$ $p < 0.001$
	cc	16.59 ± 0.21	99	A	
	40a	19.15 ± 0.24	113	C	
	40c	17.48 ± 0.21	124	B	

Table 3

Mean relative helmet length (%) of *Daphnia cucullata* induced with chemical cues released by predators compared to alarm cues released by crushed conspecifics (ch_cuc: *Chaoborus* larvae fed daily with 40 *Daphnia cucullata*; ch_bos: *Chaoborus* larvae fed daily with 300 *Bosmina longirostris*; 40c: 40 crushed *Daphnia cucullata* per liter; control without any addition). Two body-length size classes were used for analysis (sc2 = 600 – 800 µm; sc3 = 800 – 1000 µm). Arcsin Square Root transformed data were tested using one-way ANOVA. The respective ANOVA values and probabilities are given. Homogeneous groups (H) are based on Tamhane Post-Hoc Tests.

Size class	Treatment	Relative helmet length (%) Mean ± SE	*n*	H	ANOVA
sc2	ch_cuc	30.86 ± 0.27	206	D	$F_{3,653} = 419.77$ $p < 0.001$
	ch_bos	28.74 ± 0.25	188	C	
	40c	25.38 ± 0.41	60	B	
	control	19.82 ± 0.21	203	A	
sc3	ch_cuc	29.16 ± 0.34	103	D	$F_{3,352} = 286.26$ $p < 0.001$
	ch_bos	26.94 ± 0.28	106	C	
	40c	23.52 ± 0.48	30	B	
	control	18.51 ± 0.24	117	A	

Table 4

Mean relative helmet length (%) and relative crest height of *Daphnia longicephala* induced with chemical cues released by the invertebrate predator *Notonecta* compared to different concentrations of alarm cues released by crushed conspecifics (10c: 10 crushed *Daphnia longicephala* per liter; 50c: 50 crushed *Daphnia longicephala* per liter; noto: one *Notonecta* fed daily with 10 *Daphnia magna*; control without any addition). Morphological parameters measured were the relative helmet length (relhel) and the relative crest height (relcres). Arcsin Square Root transformed data were tested using one-way ANOVA. The respective ANOVA values and probabilities are given. Homogeneous groups (H) are based on Tamhane Post-Hoc Tests

Trait	Treatment	Trait length (%) Mean ± SE	*n*	H	ANOVA
relhel	10c	14.27 ± 0.24	44	B	$F_{3,170} = 1261.89$ $p < 0.001$
	50c	14.36 ± 0.23	45	B	
	noto	36.35 ± 0.57	44	C	
	control	10.22 ± 0.16	41	A	
relcres	10c	43.70 ± 0.39	44	B	$F_{3,170} = 1278.56$ $p < 0.001$
	50c	44.33 ± 0.23	45	B	
	noto	81.13 ± 0.74	44	C	
	control	41.02 ± 0.26	41	A	

Table 5

Mean relative helmet length (relhel %) of *Daphnia lumholtzi* induced with chemical cues released by fish compared to different concentrations of alarm cues released by crushed conspecifics (10c: 10 crushed *Daphnia lumholtzi* per liter; 50c: 50 crushed *Daphnia lumholtzi* per liter; fish: one fish per three liter; control without any addition). . Arcsin Square Root transformed data were tested using one-Way ANOVA. The respective ANOVA values and probabilities are given. Homogeneous groups (H) are based on Tamhane Post-Hoc Tests.

Treatment	Trait length (%) Mean ± SE	*n*	H	ANOVA
10c	13.20 ± 0.39	24	B	$F_{4, 89} = 83.06$ $p < 0.001$
50c	12.57 ± 0.48	18	AB	
fish	24.87 ± 1.11	16	C	
control	11.24 ± 0.38	21	A	

Figure legends

Figure 1

Concentration-dependent helmet induction in *Daphnia cucullata* with chemical cues released by crushed conspecifics (20 crushed daphnids / L; 40 crushed daphnids / L; 60 crushed daphnids / L; 80 crushed daphnids / L). Symbols with standard error bars indicate the mean relative helmet length (%). The daphnids were divided into four body-length size classes (sc1: < 600 µm; sc2 = 600 – 800 µm; sc3 = 800 – 1000 µm; sc4 > 1000 µm) for analysis.

Figure 2

Helmet induction in *Daphnia cucullata* with chemical cues released by crushed daphnids (80cuc crushed = 80 crushed *Daphnia cucullata* / L; 40 magna crushed = 40 crushed *Daphnia magna* / L) from different species. Standard error bars indicate the mean relative helmet length (%). The daphnids were divided into four body-length size classes (sc1: < 600 µm; sc2 = 600 – 800 µm; sc3 = 800 – 1000 µm; sc4 > 1000 µm) for analysis. Asterisks indicate significant differences between the two treatments: 80 crushed *Daphnia cucullata* and 40 crushed *Daphnia magna* (*** = $p < 0.001$; ** = $p < 0.01$).

Figure1

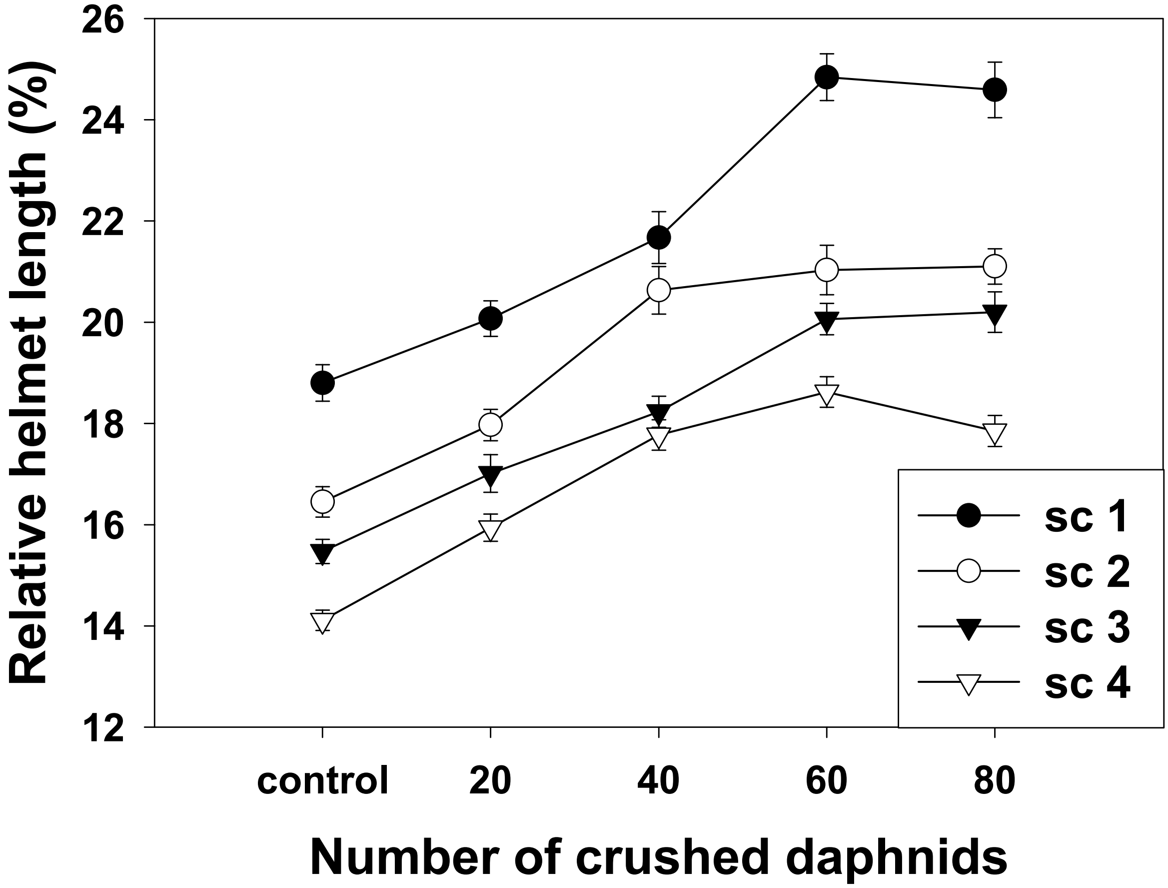
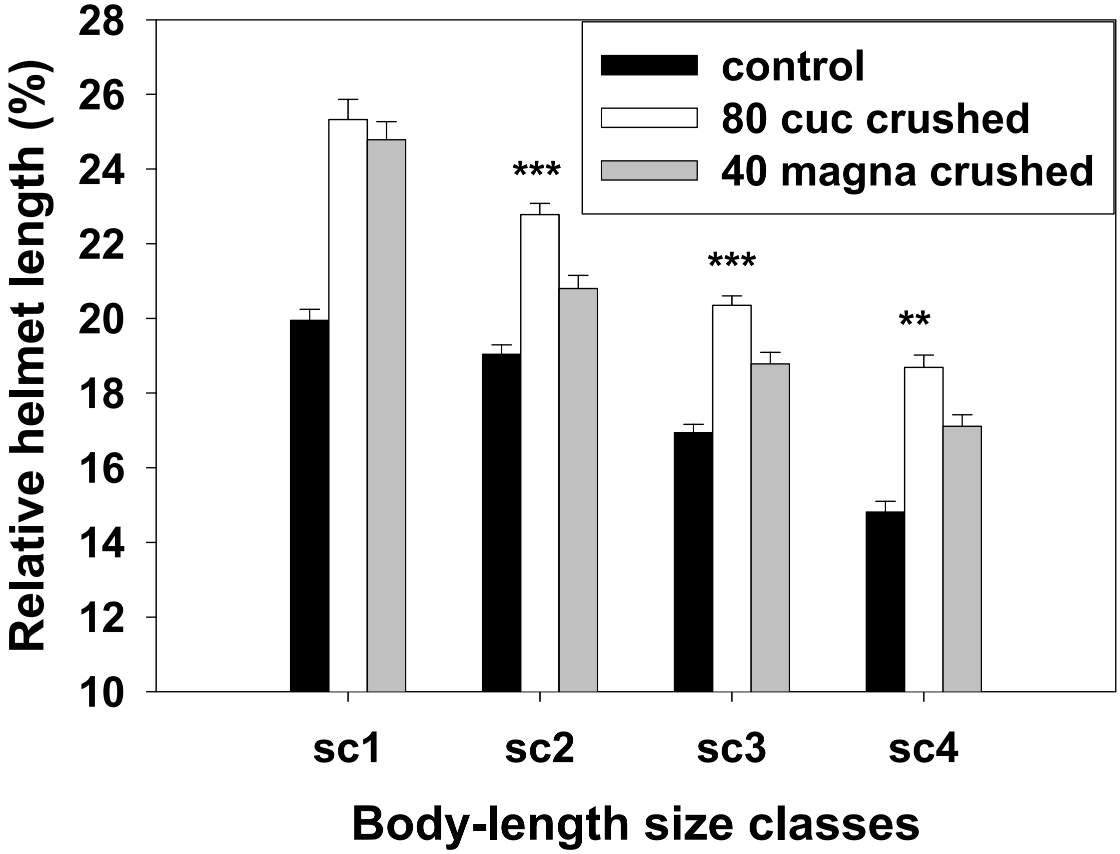

Relative helmet length (%)
26
24
22
20
18
16
14
12
control
20
40
60
80
Number of crushed daphnids
sc 1
sc 2
sc 3
sc 4

Figure 2


28
26
24
22
20
18
16
14
12
10
Relative helmet length (%)
control
80 cuc crushed
40 magna crushed
***
***
**
sc1
sc2
sc3
sc4
Body-length size classes

3

TRANSGENERATIONAL INDUCTION OF DEFENSES IN ANIMALS AND PLANTS

(Agrawal, Laforsch und Tollrian, *Nature*, 401:60-63,1999) reduces the already slim likelihood that the non-occurrence of therians before ~110 Myr is due to a preservational/sampling artefact.

Finally, in offering the first well-preserved holotherian dental remains known from the Middle to Late Jurassic of Gondwana, *A. mahabo* has important paleobiogeographic implications. Mesozoic mammal faunas of Laurasia and Gondwana were long considered to have remained isolated during much of the late Mesozoic; tribosphenidans (regarded as Laurasian in origin) were thought to have been excluded from the southern continents until latest Cretaceous or early Paleocene time, because Mesozoic tribosphenidans remained undiscovered in Gondwana[4]. To the contrary, recent finds in Morocco[25] and Australia[17] indicate the occurrence of tribosphenidans on the southern continents as early as the earliest Cretaceous (that is, as early in Gondwana as in Laurasia). The discovery of *A. mahabo* inverts this biogeographic scenario even further, showing tribosphenidans to have appeared in Gondwana by the Middle Jurassic. They may have remained isolated there until earliest Cretaceous time, when they appear in North Africa, Europe and Asia. ☐

Received 15 March; accepted 28 June 1999.

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Acknowledgements

This work was supported by the National Geographic Society, Field Museum of Natural History and the Unviersity of California. We thank the Ministre de L'Energie et des Mines of Madagascar for authorizing this work, S. Goodman and M. Ravokatra for help with our field efforts and G. Ravololonarivo Randria and A. Rasoamiaramanana. Field Museum volunteers D. Kinzig and R. Chisholm found this specimen during washing and microscope sorting of the matrix; K. Mazanek and M. Donnelly produced the illustrations; R. Andriantompohavana, L. Razafimanantso, A. Toto Volahy and A. Gandie aided us in the field; and W. Clemens and A. Smith provided advice.

Correspondence and requests for materials should be addressed to A.R.W.
(e-mail: wyss@geology.ucsb.edu).

Transgenerational induction of defences in animals and plants

Anurag A. Agrawal*, Christian Laforsch† & Ralph Tollrian†

* *Department of Entomology and Center for Population Biology,*
University of California at Davis, Davis, California 95616-8584, USA
† *Ludwig-Maximilians-University München, Zoological Institute, Karlstrasse 25,*
80333 München, Germany

Predators are potent agents of natural selection in biological communities. Experimental studies have shown that the introduction of predators can cause rapid evolution of defensive morphologies and behaviours in prey[1–5] and chemical defences in plants[6,7]. Such defences may be constitutively expressed (phenotypically fixed) or induced when predators initially attack[8–10]. Here we show that non-lethal exposure of an animal to carnivores, and a plant to a herbivore, not only induces a defence, but causes the attacked organisms to produce offspring that are better defended than offspring from unthreatened parents. This transgenerational effect, referred to as a maternally induced defence, is in contrast to the more common defences induced in single individuals within a generation. Transgenerational induction of defences is a new level of phenotypic plasticity across generations that may be an important component of predator–prey interactions.

Inducible defences may provide an adaptive defensive strategy in which non-lethal cues from predators, herbivores or parasites provide a reliable indicator about the future risk of attack. In both animal and plant systems, inducible defences allow organisms to reap the benefits of defence while saving potential costs associated with investment in the defensive strategy when it is not needed. Such phenotypic benefits and costs have been demonstrated for inducible biochemical and morphological defences in plants, and for inducible morphological, life-history and behaviour defences in animals[8–11]. If the parental environment predicts the quality of the progeny's environment, then parents may further enhance their net reproductive success by differentially endowing their offspring with phenotypes to cope with potential hazards such as predation. Such maternal effects have been reported in both animals and plants[12–14]. These effects have traditionally been ascribed to passive consequences of the resource environment and have been viewed as phenotypic noise associated with thwarting adaptive evolution. More recently, however, maternal effects have been seen as being potentially adaptive[12,15–18]. Adaptive maternal effects involve responding to information in the environment to improve the fitness of offspring by altering the offspring's phenotype. We have investigated the consequences of non-lethal exposure of wild radish plants (*Raphanus raphanistrum*) and waterfleas (*Daphnia cucullata*) to their predators, and have documented the effects on the defensive phenotypes of their offspring.

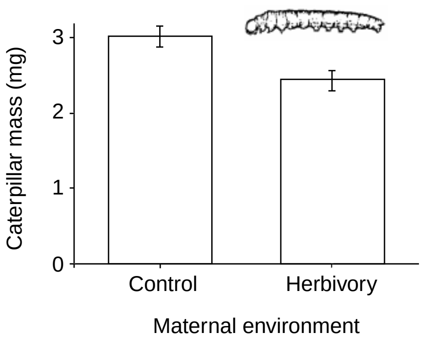

Figure 1 *Raphanus raphanistrum* resistance to herbivory as measured by growth of a specialist caterpillar, *Pieris rapae*, on the F_1 generation of seedlings from different maternal environments. Maternal plants were either subject to herbivory by *P. rapae* or left undamaged. This transgenerational induction of defences was not associated with differences in seed mass or seed concentrations of carbon or nitrogen. Error bars show s.e.

Wild radish plants damaged by a specialist caterpillar, *Pieris rapae*, induced tenfold higher concentrations of indole glucosinolates (mustard oil glycosides) and 30% higher densities of setose trichomes on newly formed leaves of damaged plants compared with undamaged control plants[19,20]. The induction of these putative defences protected them against subsequent herbivory in several field experiments, and induced plants had 60% higher lifetime seed production than uninduced controls[11,19].

To examine the consequences of herbivory for the next generation of plants, we grew unmanipulated control plants and plants with 50% of each leaf consumed by a caged *P. rapae* larva, a natural herbivore of *R. raphanistrum*. Defensive glucosinolate profiles shifted in seeds from damaged plants compared with undamaged controls (multivariate analysis of variance; Wilks' $\lambda = 0.699$, $F_{4,26} = 2.803$, $P = 0.046$). Hydroxylated glucosinolates increased in concentration, whereas other classes of glucosinolates (aliphatic and indole) decreased. Herbivory on the maternal plant also induced changes in the number of trichomes per leaf in seedlings (A.A.A., unpublished observations). Seedling progeny from damaged and undamaged plants were challenged with *P. rapae* larvae. Caterpillars gained 20% less weight on seedlings whose parents were damaged than on seedlings whose parents were not damaged (Fig. 1, Table 1). This transgenerational induced plant resistance was not explained by seed mass variation or investment in primary metabolites (Table 1). Wild radish plants with induced defences produced seeds that did not differ in percentage composition of nitrogen or carbon compared with seeds from undamaged plants (nitrogen: control, 4.37 ± 0.07 (mean ± s.e.); maternal herbivory, 4.46 ± 0.06; $t = 0.988$, d.f. $= 33$, $P = 0.330$; carbon: control, 56.93 ± 0.30; maternal herbivory, 56.77 ± 0.19; $t = 0.454$, d.f. $= 33$, $P = 0.653$). Thus, induction of plant defences persisted in the progeny of infested plants. This effect may be the result of both a maternally induced defence and a greater rapidity of induction of plant defences in the offspring of damaged mothers.

Induced chemical defences have been best studied in plants, but induced morphological defences have been best studied in animals. Helmet formation in *Daphnia cucullata* (Fig. 2) is a textbook example of cyclomorphosis[21] (seasonal variation in morphology), although the agents that maintain this polyphenism have not been adequately demonstrated[22,23]. Morphological defences in cladocerans are induced by chemicals known as kairomones that are released by predators[24]. In the presence of caged predaceous cladocerans, *Leptodora kindtii*, the relative helmet length of *D. cucullata* almost doubles (control, 15.53 ± 0.35; induced, 29.71 ± 0.49; $t = 23.72$, d.f. $= 303$, $P < 0.001$). Similar results were obtained for *D. cucullata* in the presence of caged aquatic larvae of the dipteran phantom midge, *Chaoborus flavicans* (control, 13.93 ± 0.15; induced, 27.88 ± 0.28; $t = 44.29$, d.f. $= 470$, $P < 0.001$). The induction of helmets acts as a defence by lowering the capture success by both predators: in controlled experiments, daphnids with induced helmets suffered lower mortality from both predators (mean (± s.e.) number of prey captured per feeding trial; *Leptodora*: control, 6.43 ± 0.48; induced, 0.71 ± 0.42; $n = 7$, $P = 0.018$; *Chaoborus*: control, 1.86 ± 0.33; induced, 0.71 ± 0.22; $n = 14$, $P < 0.001$). These results indicate that a proximate cue used is a chemical released by predators, and defence against invertebrate predators is a major selective force favouring cyclomorphosis in *D. cucullata*.

Table 1 Effects of maternal environment (herbivory or no herbivory) and grandmaternal and maternal family on growth of caterpillars feeding on seedling plants

Source	d.f.	MS*	*F*	*P*
Maternal environment (ME)	1	1.503	7.516	0.009
Grandmaternal family (GF)	7	0.090	0.452	0.863
Maternal family nested within ME × GF	43	0.200	1.157	0.288
Seed mass	1	0.017	0.099	0.754
Error	74	0.173		

* MS, mean square.

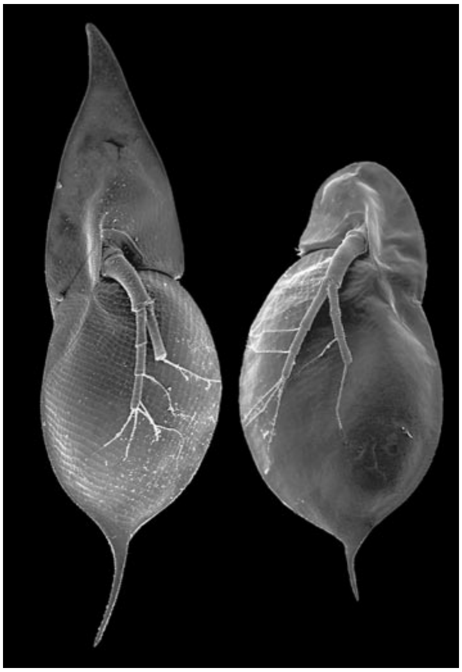

Figure 2 Scanning electron micrograph showing typical and predator-induced morphs of *Daphnia cucullata* of the same clone.

To examine transgenerational induction of defences in *D. cucullata*, we raised daphnids in either control environments or environments with the *Chaoborus* kairomone. We then imposed four treatments and compared helmet size across the treatments in the F_1 and F_2 generations: (1) control daphnids (C), which, like their mothers, had not been in contact with the predator kairomone; (2)

Table 2 Helmet lengths of *Daphnia cucullata* progeny compared with ANOVA within each brood and age class

Source	Age	d.f.	MS	*F*	*P*
F_1, first brood	Neonate	3,295	0.144, 0.004	40.51	<0.001
	Adult	3,239	0.603, 0.002	340.61	<0.001
F_1, second brood	Neonate	3,266	0.170, 0.004	47.57	<0.001
	Adult	3,208	0.414, 0.002	193.44	<0.001
F_1, third brood	Neonate	3,197	0.134, 0.002	71.65	<0.001
	Adult	3,176	0.297, 0.002	170.90	<0.001
F_2, generation	Neonate	3,241	0.334, 0.003	132.11	<0.001
	Adult	3,215	1.202, 0.002	486.14	<0.001

MS and d.f. are shown first between groups and second within groups (error).

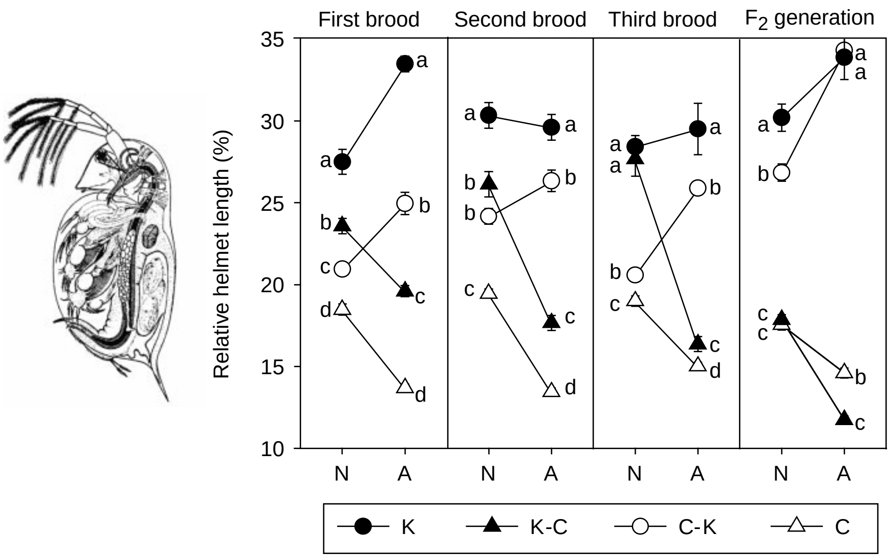

Figure 3 Relative helmet length (mean ± s.e.) of *Daphnia cucullata* (F_1 and F_2 generation) of four treatments, organized by brood number. K, kairomone treatment (*Chaoborus*); C, control; K ⟶ C, mother (F_0) had been transferred from kairomone to control treatment; C ⟶ K, mother (F_0) had been transferred from control to kairomone treatment. Mothers were transferred between environments after they had become pregnant. Daphnids are sensitive to the kairomone starting in a late embryo stage and in the juvenile stages (R.T., unpublished data). We analysed the first three broods of the F_0 mothers (for example, in the transfer treatment, three broods following the transfer). Thus, only the first brood shared the maternal environment for a very short time. Lines between neonates (N) and adults (A) show relative helmet growth within each treatment. Results of each overall ANOVA are shown in Table 2. Homogeneous groups within each brood and age class are indicated by vertical ordered letters (Bonferroni adjusted). The F_2 generation is the first brood offspring of the F_1 first brood. Where error bars are not shown, they were smaller than the symbol.

kairomone daphnids (K), which, like their mothers, had been in permanent contact with the kairomones; (3) daphnids which had been born from control mothers and were transferred to the kairomone environment (C ⟶ K); and (4) daphnids which had been born from kairomone mothers and were transferred to the control environment (K ⟶ C). The latter two treatments were used to distinguish between a maternally induced defence and environmental induction in the progeny. Pregnant mothers were transferred between environments shortly after they had deposited their first clutch into their brood pouch. At this stage, young daphnids are not yet sensitive to the kairomone (R.T., unpublished data).

F_1 neonates varied in helmet length between the four treatments (Table 2, Fig. 3), with offspring from mothers in the kairomone environment always having larger helmets than offspring from mothers in the control environment. Significant differences between daphnids with the same F_1 environment but different maternal environments (compare C with K ⟶ C, and K with C ⟶ K) demonstrate the importance of the maternal environment in helmet formation. This pattern persisted in subsequent broods that were initiated after the mothers had been transferred, which demonstrates the lasting influence of the maternal environment on the transgenerational induction of helmet defences (Fig. 3). A comparison of treatments with the same maternal environment but different offspring environments (compare K with K ⟶ C, and C with C ⟶ K) indicates that the late embryonic environment also had an influence on helmet induction, albeit a smaller effect than that of maternal environment (Fig. 3).

Postnatal helmet development in *D. cucullata* is induced by the juvenile environment, as can be seen from the interactions between helmet length and age (Fig. 3). The K ⟶ C daphnids, which were born with large helmets, reached maturity with relatively small helmets. In contrast, neonates from C ⟶ K mothers were born with small helmets but reached maturity with relatively large helmets. However, the juvenile environment could not induce phenotypes that matched those of maternally induced individuals. For example, the helmet size of adult daphnids from the kairomone treatment (K) could not be reached by C ⟶ K daphnids, which also spent their whole lives in contact with the kairomone. Similarly, K ⟶ C daphnids reached maturity with larger helmets than control (C) daphnids.

The F_2 generation from C ⟶ K daphnids had significantly smaller helmets than did K daphnids, indicating that grandmaternal environment constrained the size of helmets in the F_2 neonates (Fig. 3). However, the F_2 generation from K ⟶ C daphnids did not have a different helmet size than C daphnids. In both cases, the maternal (F_1) environment influenced helmet induction in the F_2 generation.

The greater defences of organisms whose parents were threatened by predation provides a case of adaptive phenotypic plasticity across generations. Adaptive maternal effects are distinct from other adaptations, however, in that the genes responsible are activated in the maternal generation, whereas the phenotypes on which natural selection may act are expressed in the progeny. This lagged link between parental environment and offspring phenotype may amplify or subdue responses to natural selection[18,25].

Transgenerational effects may provide tools to overcome developmental constraints acting on individuals at critical stages in the life cycle. For example, the later development of a maximally large helmet for initially short-headed daphnids might not be possible, but transgenerational effects imposed by the maternal environment may allow for such maximal defences. Furthermore, progeny born with a maternally induced defence avoid a major disadvantage of inducible defences: that the defence is not initially present when needed (there is a lag phase[26]). The immediate development of plant resistance in wild radish seedlings is important because the establishment phase has a strong influence on plant fitness[11,27]. Maternally induced progeny will be just as well protected as permanently defended individuals, and better protected than undefended individuals or individuals that respond to threats early in life. Because predation can be such a strong selective force, we posit that transgenerational induced defences are a logical extension of fitness-enhancing strategies of organisms under threat. Several other systems are strong candidates for having maternally induced defences[28–30]. That such effects are present in both animals and plants reinforces the view that the expression of adaptive phenotypes in prey may be driven by predation from higher trophic levels. ☐

Methods

Wild radish experiments.

We used *R. raphanistrum* seeds from a second generation of untreated greenhouse-grown plants. We germinated about 10 seeds from each of 13 maternal families in a greenhouse. At the four-leaf stage, each plant was randomly assigned to one of the two treatments (3–5 plants per treatment per family). The caterpillar herbivory treatment was maintained throughout the growth of the plant. Effects of herbivory on seed set are reported elsewhere[19]. Seeds from 8 of the original 13 families were chosen for the transgenerational experiment because they spanned the range of tolerance to herbivory[19], and 2–4 seeds from each of 58 maternal plants within 8 grandmaternal families (a total of 126 plants) were grown in a greenhouse and inoculated with a single newly hatched *P. rapae* larva. Caterpillars were not caged. After four days, the caterpillars were weighed.

Effects of grandmaternal family (fixed), maternal environment (control or herbivory; fixed), maternal family (nested within grandmaternal family by treatment interaction; random) and seed mass (covariate) on caterpillar growth were analysed using a mixed-model analysis of variance (ANOVA). *F*-ratios for grandmaternal family and treatment effects were calculated with maternal family nested in grandmaternal family by treatment interaction mean-square and degrees of freedom in the denominator.

For phytochemical analysis, glucosinolates were analysed using modified procedures for determination of trimethylsilyl glucosinolate derivatives with capillary gas chromatography and flame ionization detection[20]. Seed nitrogen and carbon were determined from a separate set of seeds using dynamic flash-combustion and gas-chromatographic separation and a thermal-conductivity detection system (Division of Agriculture and Nature Resources, University of California, Davis).

Daphnia experiments.

We used a *Daphnia cucullata* clone isolated from Thaler lake, Germany. The experiments were conducted in the laboratory under constant conditions at 20 °C and fluorescent light in a synthetic medium. The F_0 generation had been synchronized by always raising the third brood offspring born within 12 h, starting from a single *Daphnia*. *Daphnia* were fed *ad libitum* daily with *Scenedesumus acutus* (1.5 mg C l^{-1}).

Animals with freshly deposited eggs were placed into 0.75 l medium containing a 125-μm net cage, enclosing either 10 fourth-instar larvae of *Chaoborus flavicans* or 4 *Leptodora kindtii*, or no predators for controls. The net cages prevented direct contact between predators and prey.

Predation experiments with *Chaoborus* were conducted for 0.5 h with each prey morph separately in 100 ml medium with 10 prey organisms and a single predator. Predation trials with *Leptodora* were conducted with 10 prey organisms of each morph together in 0.5 l in 24-h experiments. In each experiment, animals of the same age and body size class (0.6–0.8 mm, from the eye to the base of the tail spine) but of different morphology were compared.

We induced helmet formation in *Daphnia* by using *Chaoborus* kairomones. We placed four *Chaoborus* larvae in net cages in 1.5-l beakers and changed the water ever day. Chaoborids were fed daily with 10–15 prey (*D. cucullata* and *Ceriodaphnia* sp.). The F_0 generation had been born and raised in these beakers in either the control or kairomone treatment. We used three beakers per treatment as replicates, which did not differ and were pooled for analysis.

Daphnids were measured with a digital image-analysis system (SIS, Münster, Germany). To compensate for small changes in body length within an age class, we calculated the relative helmet length (helmet length/body length × 100). Relative helmet length is a good predictor of the defensive effect within an age class. The relative values were arcsin-transformed for analysis. In the induction treatment, differences between control and kairomone treatment were compared using *t*-tests. Effects of induced *Daphnia* phenotypes on predation were analysed by using a Mann–Whitney-*U*-test for *Chaoborus* and with a paired Wilcoxon test for related samples for *Leptodora*. To test for transgenerational effects, we compared the treatments within each brood and age class (Table 2, Fig. 3) with ANOVA and Bonferroni adjustments.

Received 29 March; accepted 28 June 1999.

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Acknowledgements

We thank R. Karban, the plant-herbivore group at Davis and W. Gabriel for advice and encouragement; M. Morra and V. Borek for help with phytochemical analyses; R. J. Mercader, M. Kredler, S. Y. Strauss, J. Kniskern and E. Hochmuth for help with experiments; and L. S. Adler, S. Diehl, H. Dingle, R. Karban, R. E. Lenski, T. W. Schoener, S. Y. Strauss, J. S. Thaler, D. A. Thiede, and T. G. Whitham for comments on the manuscript. This work was supported by the US NSF.

Correspondence and requests for materials should be addressed to A.A.A. (e-mail: aaagrawal@ucdavis. edu) or R.T. (e-mail: Ralph.Tollrian@lrz.uni-muenchen.de).

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Genetic enhancement of learning and memory in mice

**Ya-Ping Tang*, Eiji Shimizu*, Gilles R. Dube†,
Claire Rampon*, Geoffrey A. Kerchner‡, Min Zhuo‡, Guosong Liu†
& Joe Z. Tsien***

** Department of Molecular Biology, Princeton University, Princeton,
New Jersey 08544-1014, USA*
*† Department of Brain and Cognitive Science, The Center for Learning and
Memory, MIT, Cambridge, Massachusetts 02139, USA*
*‡ Departments of Anesthesiology and Neurobiology, School of Medicine,
Washington University, St Louis, Missouri 63110, USA*

Hebb's rule (1949) states that learning and memory are based on modifications of synaptic strength among neurons that are simultaneously active. This implies that enhanced synaptic coincidence detection would lead to better learning and memory. If the NMDA (*N*-methyl-D-aspartate) receptor, a synaptic coincidence detector[1–4], acts as a graded switch for memory formation, enhanced signal detection by NMDA receptors should enhance learning and memory. Here we show that overexpression of NMDA receptor 2B (NR2B) in the forebrains of transgenic mice leads to enhanced activation of NMDA receptors, facilitating synaptic potentiation in response to stimulation at 10–100 Hz. These mice exhibit superior ability in learning and memory in various behavioural tasks, showing that NR2B is critical in gating the age-dependent threshold for plasticity and memory formation. NMDA-receptor-dependent modifications of synaptic

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EXACT PREDATION-RISK ASSESSMENT AND DEFENCE-LEVEL ADJUSTMENT TO PREY DENSITY (Tollrian, Duggen, Laforsch und Kopp, *Nature*, eingereicht)

Exact predation-risk assessment and defence-level adjustment to prey density

Ralph Tollrian, Sonja Duggen, Christian Laforsch & Michael Kopp*.

Ludwig-Maximilians-Universität München, Department Biology II, Section of Evolutionary Ecology and GeoBioCenter, Karlstraße 23-25, D-80333 München, Germany.

**Max-Planck-Institute für Limnologie, Postfach 165, 24302 Plön, Germany*

The ability to defend against attackers is a key factor for the evolutionary success of nearly all living organisms. Because most defences incur trade-offs and evolve in a cost–benefit framework [1, 2] organisms are selected to express only the level of defence that is optimal under the specific environmental conditions. Thus, the ability to accurately assess predation risk is crucial. So far, studies of predation-risk assessment have focused almost exclusively on the influence of predator density [3, 4]. However, individual predation risk is also a function of prey density, and an accurate assessment of predation risk should therefore take into account prey as well as predator density. We tested this hypothesis using six predator-prey systems with precisely measurable inducible morphological defences and covering a taxonomic range from protozoa to rotifers and crustaceans. Here we show that in all six systems the level of defence increased with predator density and decreased with prey density. This suggests that the ability to accurately assess individual predation risk and to adjust defences is a general feature of many defence systems, including responses to herbivores, to parasites, or even responses of the immune system.

Many organisms are faced with the problem of adjusting their investment in defence to the current risk of predation. In this situation, an accurate assessment of attack risk is crucial for avoiding unnecessary costs resulting from over- or under-expression of defences. In recent years, many prey organisms have been shown to estimate predation risk from the density of predators [3, 4, 2]. However, predation risk should also depend on prey density due to effects which are known from the evolution of swarming behaviour, such as the dilution effect [5, 6]. The dilution effect is present whenever the number of prey a predator consumes per unit of time is a saturating or decreasing function of prey density (e.g., the predator's functional response is of type II [7], and the number of predators is constant over the time-scale of interest (i.e., a high prey density does not attract a relatively greater number of predators). If these conditions are fulfilled, prey should reduce their investment in defences if their own density is high (see also [8]). This hypothesis has been widely ignored in the past, with the exception of one study by Wiackowski and Starońska [9], which suggests that the level of inducible defence expressed by the ciliate *Euplotes octocarinatus* is indeed negatively correlated with *Euplotes* density.

We tested the above hypothesis using six predator-prey systems with inducible defences. We chose these model systems as tools because in all six systems prey have defensive morphological traits that are induced by predator chemicals (kairomones) and can be accurately quantified: The ciliate *Colpidium kleini* changes from a cylindrical to a spherical shape in response to chemical cues from *Lembadion* [10]; the rotifer *Brachionus calyciflorus* develops posterolateral spines in the presence of kairomones from the predatory rotifer *Asplanchna* [11]; larvae of the phantom midge *Chaoborus* evoke helmets in the planktonic crustacean *Daphnia cucullata* [12] and so called 'neck teeth' in *Daphnia pulex* [13]; kairomones from the backswimmer *Notonecta* induce crests in *Daphnia longicephala* [14]; and *Daphnia lumholtzi* develop high helmets in response to fish cues [15]. In all of these systems the morphological traits act as defences. Their protective effect is correlated to the magnitude of defence formation which in turn depends on the concentration of the predator kairomones and, hence, on predator density. In all six systems the morphological dose responses follow saturation curves (e.g., [16]).

We investigated the effect of predator and prey density on the magnitude of defence formation using a two-by-two factorial design with two predator (i.e., kairomone) and two prey densities. The only exception was the rotifer system, in which we only used one predator density. We first isolated predator kairomones. Then we established the dose-response curves for the morphological reactions to the kairomones. Based on these curves we selected low and high kairomone concentrations. The low one was chosen to induce a weak but significant induction, and the high one was close to the plateau of the response curve. Thus we would be able to detect response modifications at both concentrations. We avoided potential pitfalls by using kairomones to preclude direct selection by predation, by adding antibiotics to prevent differential bacterial breakdown of the kairomones between prey density treatments, and by providing unlimited food concentrations to exclude any food effects (the only exception being the ciliate system, in which a direct contact to the predator is necessary to evoke strong responses and where we did not feed during the course of the experiment).

In all six systems the degree of defence formation increased with predator density, but decreased with prey (i.e., conspecifics) density (Fig. 1). The effect of prey density was present at both low and high predator densities. Like most predators, the predators in our study show type II (saturating) functional responses [2, 7]. Hence, a higher prey density would indeed reduce predation risk. Thus, our study suggests that organisms are able to precisely assess their individual predation risk based not only on predator density but also on prey density and to adjust their defences accordingly.

We used the *D. lumholtzi* system in order to gain further information on the cue by which the prey determine the density of conspecifics. In principle, the density of conspecifics could have been measured with a chemical or mechanical cue. To distinguish between these alternatives we again raised prey organisms at the high predator density in both conspecific densities and obtained the same results as before. Additionally, we only transferred medium (1-µm filtered) from a high prey density culture to a low prey density treatment and yet still obtained the weak morphological response. Thus, the daphnids measured conspecific density by a chemical cue (Fig. 2a).

In situations where the conspecifics density is low relative to the density of alternative prey species, it would be advantageous for prey to respond to the combined density of all prey species rather than only its own species. To test for species-specificity of the inducing cue we again raised *D. lumholtzi* in the high predator treatment at both conspecific densities as well as in a low conspecific density but together with *Daphnia magna* at a high density. The response was not significantly different from that in a high density of *D. lumholtzi*. Thus, *D. lumholtzi* reacted to chemical cues from *D. magna*, and the chemical cue is not species specific (Fig. 2b).

The chemical cue used by daphnids to measure prey density might be similar to the so called ‘crowding chemical’, which at very high concentrations leads to life-history shifts and induces resting-egg production [17, 18, 19]. In our study, however, even the high conspecific densities were relatively low (see methods) and well within the range of natural densities of the organisms.

Our findings may explain frequently observed differences between experimental results in studies on inducible defence systems [20]. Conspecific density modifies defence formation and has to be carefully controlled for in all experiments.

Our study suggests that exact risk assessment, decision-making based on multiple sources of information, and adaptive adjustment of the defence level is a common feature in inducible defence systems. Inducible defences to attackers are not limited to morphological traits but include physiological and behavioural adaptations, and can be found in nearly all groups of organisms [2]. For example, two studies suggest that woodfrog tadpoles adjust foraging activity (which increases predation risk) to conspecifics density [21, 22]. Further studies should test the validity of and possible deviations from this concept in other inducible defence systems, e.g., defences of plants against herbivores, hosts to parasites and the immune system to pathogens.

Using multiple sources of information for exact predation risk assessment may help to avoid costs resulting from investment in unnecessary defences, and will be especially relevant in situations with a high conspecific (or prey) density, where intra- and interspecific competition is intense. Performance under competition and the ability to defend against predators are key features which structure communities [23], and thus, on higher levels of biological organisation, our findings may have implications for population biology and food web structure [24]. Finally, our results are not only relevant to all inducible defence systems, but also to the evolution of all fixed defences that evolve within environments with a rather constant and predictable predation risk. The individual predation risk sets the frame for the magnitude of selected defences and for local adaptations.

Methods

Cultures of *D. cucullata* originated from Lake Thalersee (Bavaria, Germany), *D. pulex* from a pond in Canada, *D. longicephala* was from Lara pond (Australia), and *D. lumholtzi* from Fairfield reservoir (Texas, USA). Cultures of *C. kleini* and *L. bullinum* originated from Poland. *B. rubens* and *A. brightwelli* were isolated from a pond near Ismanning (Bavaria, Germany). All prey organisms coexist in their native habitat with the types of predators employed in our study.

Unless otherwise specified, all experiments were conducted in artificial medium (to exclude natural chemical signals) at 20 °C in a temperature-controlled room under fluorescent light. We added only predator kairomones, with exception of the ciliate system, to prevent any density-dependent direct selection on the induced traits. To provide a constant quality, the kairomones were prepared before the experiments (by rearing high numbers of predators for 24 h in medium), 0.45-µm filtered, pooled, frozen at -60 °C in portions for each day, and diluted for use in the experiments. As is unavoidable in most lab experiments with kairomones, the simulated predator densities were higher than under natural conditions [2]. We replaced half of the medium daily to provide constant kairomone concentrations. In all systems, the antibiotic Ampicillin was added (10 mg/L) after test experiments had proven that the organisms and the induction are not affected. We chose adequate, naturally occurring prey concentrations for each system. We had verified in control experiments that the prey densities and food effects alone did not induce defences. To avoid possible influences of food concentrations, we fed all prey organisms at non-limiting food concentrations (e.g., *D. cucullata* and *D. pulex* at 1.5, *D. lumholtzi* and *D. longicephala* at 2.5 mg C/L and rotifers at 1 mg C/L of *Scenedesmus obliquus*) or, in the ciliate system, did not provide any food at all. Unless otherwise specified, all treatments were replicated 10 times.

In all *Daphnia* systems we started with a single mother and reared age-synchronized cohorts to obtain experimental mothers. The mothers were transferred to the treatments when they carried their first brood. The third brood in the treatment was used for the experimental animals. In the *D. lumholtzi* system, we reared 1 *Daphnia* in 200 mL medium for the low and 10 daphnids for the high prey density treatment. *Daphnia* were measured when they reached maturity. The fish kairomone concentration was 1 stickleback (*Gasterosteus aculeatus*, 3.5 cm) /L x day in the high and 0.05 fish/L x day in the low predator treatment. For the experiments displayed in Figure 2, new batches of kairomone were produced. The high-density treatment in the cue experiment (Fig. 2a) had only 9 replicates.

In the *D. pulex* system, we reared 2 daphnids in 200 mL medium for the low and 20 for the high prey density. We measured and scored individual neck teeth in *D. pulex* in the second juvenile stage, when defence formation is strongest in this clone [16]. As the *Chaoborus flavicans* larvae were just in the second stage and very small compared to fourth instar larvae, we used kairomone concentrations corresponding to 20 larvae/L x day in the low and 200/L x day in the high predator treatment.

We reared 1 *D. longicephala* in 250 mL medium for the low and 10 for the high prey density treatment. Our high predator concentration was 0.75, our low concentration 0.075 *Notonecta glauca*/L x day.

For the small *D. cucullata,* we used 10 individuals as low and 25 individuals in 200 mL medium as high prey concentration. The *Chaoborus flavicans* kairomone was 10 fourth-instar larvae/L x day for the low and 25/L x day for the high concentration.

We conducted the rotifer experiments in 2 mL microtiter plates, which allowed controlling the predator and prey densities exactly, in an incubator at 15 °C in the dark. The low prey density was 2 *Brachionus* (34 replicates), the high density 20 *Brachionus* (10 replicates). We used *Asplanchna* kairomone in a concentration which had induced intermediate spines in pre-experiments. Because defences in *B. calyciflorus* are induced via the mother generation [11], we reared F_0- and F_1-generations under experimental conditions and measured the F_1-generation at the age of 5 days.

Induction of *C. kleini* by *L. bullinum* was studied with two prey densities (200 and 2000 per mL) and two predator densities (20, and 200 per mL) in 10 mL medium at 20 °C in the dark (5 replicates per treatment). Although it is possible to induce *Colpidium* with *Lembadion*-conditioned medium [10], the resulting transformation is weak. Therefore we kept both species in direct contact to each other. To minimize predation, *Lembadion* were starved for several days before the experiment. This resulted in extremely small cells which were poorly able to ingest their prey, and based on observed feeding rates it can be excluded that the observed effects have been caused by selective predation. No food was offered to *Colpidium* during the experiments (in order to avoid differential food supply per individual at the two prey densities). The experimental animals were fixed after 48 h. Lengths and widths of *Colpidium* were measured and the ratio was used as an index of the degree of defence.

All animals in all systems were measured using a digital image analysis system (AnalySIS, Soft Imaging Systems, Münster, Germany). Statistics were calculated with

SPSS (V11.5). Ratio values were arcsin square root transformed, or log transformed (ciliates) before analysis [25]. All data, beside the rotifer experiment, followed a normal distribution. We calculated two-way ANOVAs with the factors prey and predator. The interaction terms were meaningless and are not presented because they are primarily caused by the underlying dose-response curves. We analysed the rotifer experiment with a non-parametric Mann Whitney U-test.

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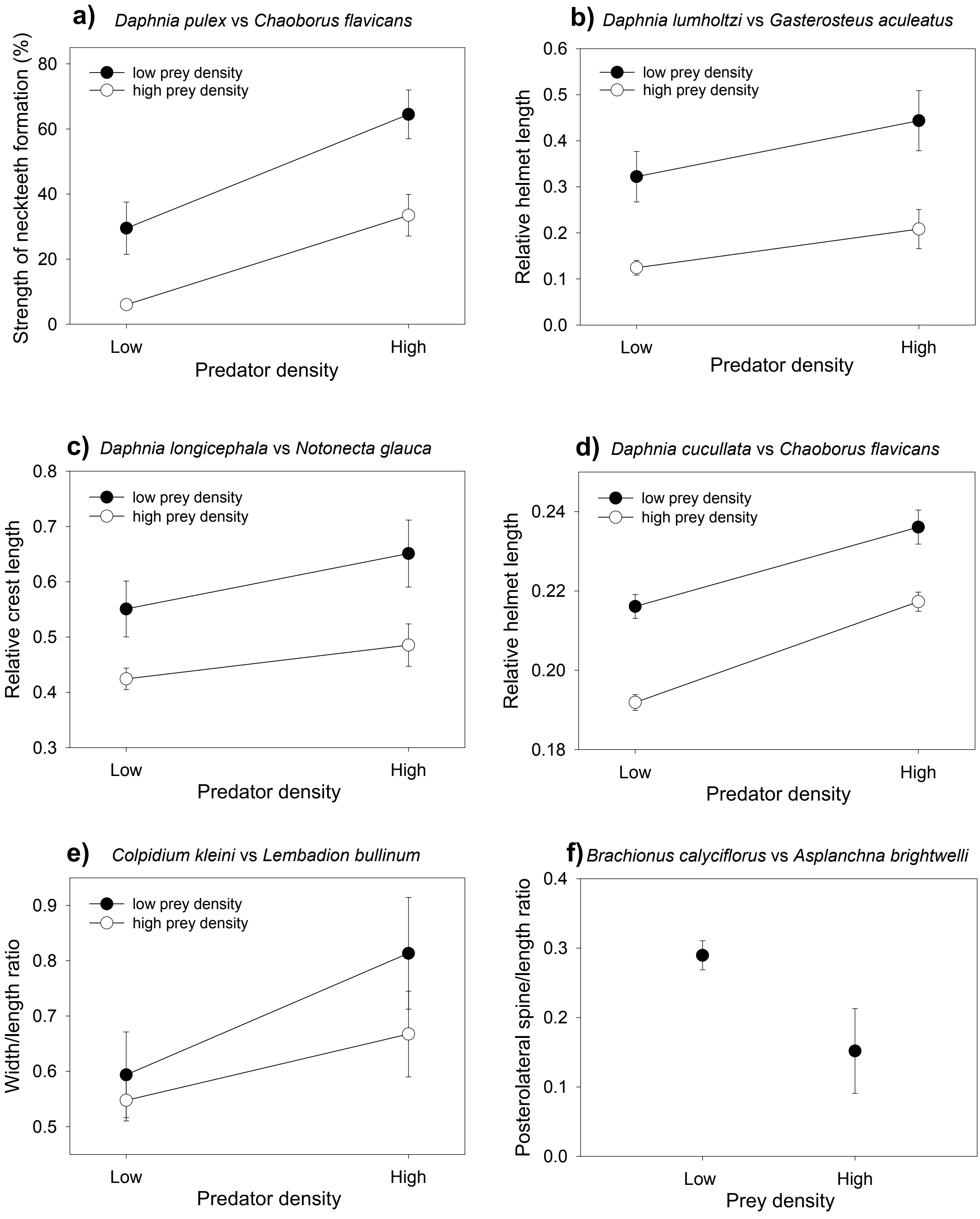
Acknowledgements

E. Gebefügi and M. Zumbusch helped with the experiments. Cultures of *C. kleini* and *L. bullinum* were kindly provided by K. Wiackowski. W. Gabriel, J. Jeschke, W. Lampert and S. Stevens gave comments to earlier versions of the manuscript and helped to improve the language. The Deutsche Forschungsgemeinschaft supported our research.

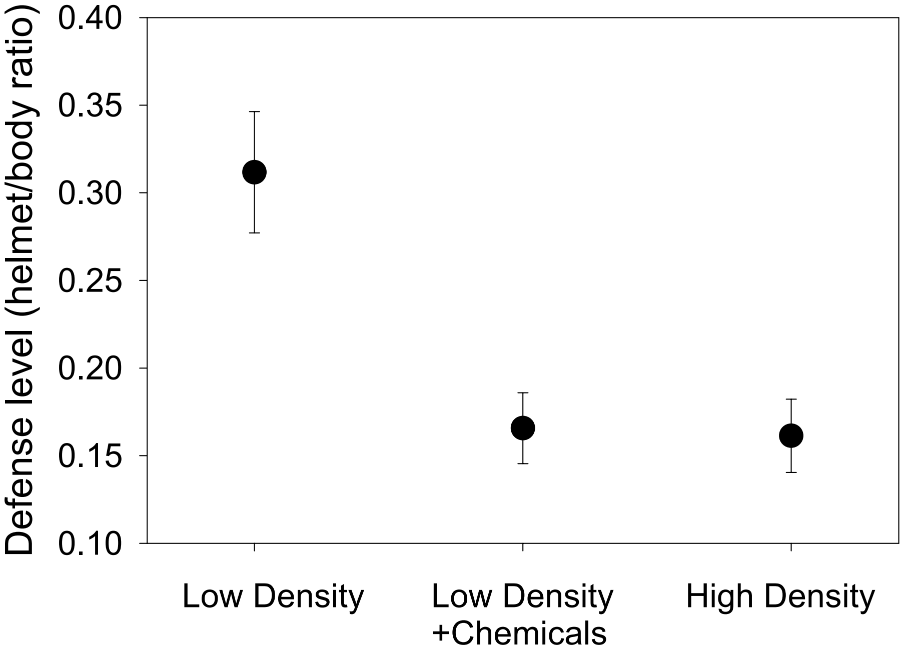
Correspondence and requests for materials should be addressed to R.T. (email: tollrian@lmu.de).

Figure 1 Formation of inducible morphological defences (shown as relative values of a trait divided by body length, or in *D. pulex* as score of neckteeth formation) increased with predator density (kairomone concentration) and at a given predator density decreased with prey density. Two-way ANOVA results are significant for predator and prey effects in all systems (all $p < 0.001$). In the *B. calyciflorus* system, the prey density effect was tested only at a single predator density and analyzed with a Mann Whitney U-test ($p = 0.023$). Shown are treatment means ± SD.

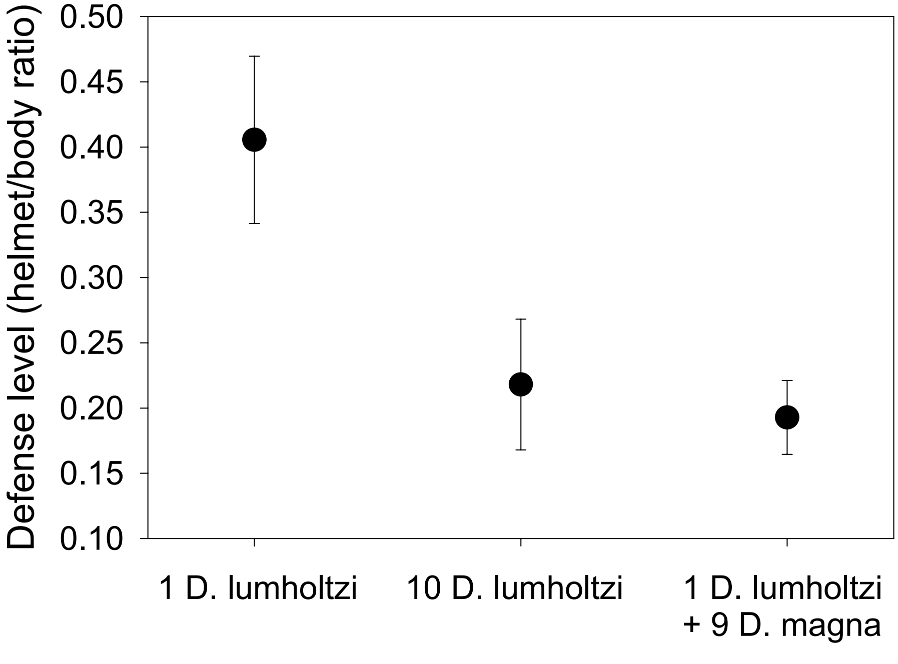
Figure 2 Nature and specificity of the cue for the prey-density effect in *D. lumholtzi*. 2a) Information about prey density is transmitted via a chemical cue. Medium from conspecifics in a high density added to a low prey density reduced the induction to a level not significantly different from that in the high-density treatment (Tamhane pairwise comparison $p = 0.921$). The low prey-density treatment differs significantly from both other treatments (Tamhane pairwise comparison, all $p < 0.001$). 2b). The chemical cue is not species specific. One *D. lumholtzi* reared together with 9 *D. magna* reduced the defence induction to a level not significantly different from the high-density treatment (10 conspecifics, Tamhane pairwise comparison $p = 0.545$). The low density treatment differed significantly from both other treatments (Tamhane pairwise comparison, all $p < 0.001$). Shown are treatment means ± SD.


a) *Daphnia pulex* vs *Chaoborus flavicans*
Strength of neckteeth formation (%)
0
20
40
60
80
low prey density
high prey density
Low
High
Predator density
b) *Daphnia lumholtzi* vs *Gasterosteus aculeatus*
Relative helmet length
0.0
0.1
0.2
0.3
0.4
0.5
0.6
low prey density
high prey density
Low
High
Predator density
c) *Daphnia longicephala* vs *Notonecta glauca*
Relative crest length
0.3
0.4
0.5
0.6
0.7
0.8
low prey density
high prey density
Low
High
Predator density
d) *Daphnia cucullata* vs *Chaoborus flavicans*
Relative helmet length
0.18
0.20
0.22
0.24
low prey density
high prey density
Low
High
Predator density
e) *Colpidium kleini* vs *Lembadion bullinum*
Width/length ratio
0.5
0.6
0.7
0.8
0.9
low prey density
high prey density
Low
High
Predator density
f) *Brachionus calyciflorus* vs *Asplanchna brightwelli*
Posterolateral spine/length ratio
0.0
0.1
0.2
0.3
0.4
Low
High
Prey density

a)


Defense level (helmet/body ratio)
0.40
0.35
0.30
0.25
0.20
0.15
0.10
Low Density
Low Density +Chemicals
High Density

b)


Defense level (helmet/body ratio)
0.50
0.45
0.40
0.35
0.30
0.25
0.20
0.15
0.10
1 D. lumholtzi
10 D. lumholtzi
1 D. lumholtzi + 9 D. magna

5

EXTREME HELMET FORMATION IN DAPHNIA CUCULLATA CAUSED BY SMALL-SCALE TURBULENCES (Laforsch und Tollrian, *Journal of Plankton Research*, Akzeptiert in Abhängigkeit der Revision)

Extreme helmet formation in *Daphnia cucullata* induced by small-scale turbulence

Christian Laforsch † and Ralph Tollrian *

†* Section of Evolutionary Ecology and GeoBioCenter, Department of Biology II, Ludwig-Maximilians-Universität München, Karlstr. 23 – 25, 80333 München, Germany

† Corresponding author: Tel: +49 89 5902253, Fax: +49 89 5902461,
laforsch@zi.biologie.uni-muenchen.de
*tollrian@lmu.de

Running title: turbulence induction (June 2003)

Keywords:
cyclomorphosis, small-scale turbulence, *Daphnia cucullata*, kairomones, inducible defenses

Abstract

Although *Daphnia cucullata* is used as a textbook example for cyclomorphosis, distinct helmet development, as shown in the field, has not been demonstrated in the laboratory until now. We show for the first time that small-scale turbulence is able to induce the maximum response of morphological plasticity in *Daphnia cucullata*. Helmet elongation reached magnitudes as extreme as observed in the field.

Different modes of generating small-scale turbulence caused different levels of helmet formation. Small-scale turbulence alone may not be responsible for cyclomorphosis in nature because field data show that two nearby lakes with similar morphometry differ in cyclomorphosis patterns, while lab experiments show that there is no difference in the ability to form helmets in the clones of each lake.

Although helmet formation in *Daphnia cucullata* is inducible with predator kairomones, helmet elongation is not as strong as that induced by turbulence. We discuss the possible role of helmets under turbulent conditions.

Introduction

Cyclomorphosis, which means seasonal variation in morphological traits, has attracted the attention of scientists since the beginning of the last century (Wesenberg-Lund 1900). A variety of *Daphnia* species is reported to show these polymorphisms. For example *Daphnia pulex* generates neck teeth, *Daphnia carinata* forms huge crests and *Daphnia galeata* and *Daphnia retrocurva* both develop helmets (for review see: Jacobs 1987). In early studies abiotic factors were predominantly discussed as proximate cues for cyclomorphosis in *Cladocera*. In this context temperature (Ostwald 1904; Wesenberg-Lund 1908; Coker & Addlestone 1938; Jacobs 1967), water turbulence (Brooks 1947; Hrbácek 1959), light (Hazelwood 1966), food conditions (Jacobs 1967) and the combined effects of these parameters were tested in field studies or laboratory experiments. However, only sparse induction effects were obtained in these studies. At that time, a parachute-effect to reduce sinking speed (Wesenberg-Lund 1908; 1939) or a balance function to facilitate horizontal swimming (Woltereck 1913; 1921) of the inducible traits was speculated. Since Gilbert (1966) showed that cyclomorphic changes in rotifers are inducible with chemical cues released by predators, research has focused on biotic interactions. In recent years many organisms have been reported to be inducible by predator kairomones and a defensive effect by the inducible traits could be demonstrated for several of these examples (for review see Tollrian & Harvell 1999). Likewise, laboratory experiments with *D. cucullata,* a very common species in eutrophic lakes across Europe (Flößner 1972), showed that elongated helmets could be induced with kairomones and act as inducible defenses against several invertebrate predators (Tollrian 1990, Agrawal et al. 1999, Laforsch & Tollrian in press). These results are in accordance with Pijanowska's (1991) suggestion that high helmets in *D. cucullata* in two

Polish lakes are linked to the abundance of invertebrate predators. However, cyclomorphic changes of the magnitude observed in the field have not previously been induced in the laboratory. Results of Hrbácek (1959) imply that helmets of *D. cucullata* are slightly inducible with water turbulence.

1. In our work we return to this old idea experimentally using two different methods for generating small-scale turbulence.
2. Additionally, we monitored cyclomorphosis in two lakes from the same area with similar morphometries and tested for clonal differences concerning turbulence induction and compared laboratory experiments with field data.
3. To test for induction strength we compared predator kairomone and turbulence induction as inducing agents for these plastic traits of *D. cucullata*.
4. Furthermore, we tested inducibility of *D. cucullata* with small-scale turbulence under cold conditions.

Methods

Clones of *D. cucullata* were isolated from Lake Thalersee and Lake Bansee, Germany. The animals were reared in artificial medium (Laforsch & Tollrian, in press) and were fed daily with *Scenedesmus obliquus* (1.5 mg C /L). The experiments were conducted in the laboratory under constant conditions in 1.5 L glass beakers at 20 °C and under fluorescent light. 20 primipar age-synchronized non-helmeted animals with freshly deposited eggs were randomly introduced into each beaker. The medium was changed weekly in each vessel.

Turbulence-1-setup:

In this setup, a PVC-disk driven by an electric motor revolves permanently. A piece of silicon tube is attached to the front of the disk. The silicon tube alternately drives three paddles made of acrylic (10 cm / 3 cm). The paddles are installed in a rack. A smooth motion is guaranteed by ball bearings. The length of the silicone tube determines the degree and the frequency of the spin of the paddles (Figure 1a). In our experiment, the paddle made a half revolution every five seconds. A strong small-scale turbulence was generated with this method, which the animals apparently survived well. The daphnids obviously had a high reproductive success and no dead animals were found during the weekly medium exchange inside the beaker. This pulse-generated turbulence was optically verified in a prior test with stained water ($KMnO_4$). Three glass beakers could be placed into this device in accordance to the paddle number. Three vessels without turbulence served as controls.

Turbulence-2-setup:

A gel-dyer (KS 501; IKA Labortechnik) was used for the second method. Three glass beakers were placed on the dyer on a 2 cm polystyrene sheet to avoid heat transfer between the dyer and the beakers. A rod made of acrylic (2 cm wide) was fixed from above over the beakers. The gel-dyer ran at 30 rpm. The rod would have generated only a laminar flow due to the permanent spin of the dyer (tested with stained water), so a plate made of acrylic (18 cm / 4 cm) was also attached at the edge of the glass beaker to break the laminar flow and to generate small-scale turbulence (Figure 1b).

Both turbulence experiments were conducted simultaneously with *D. cucullata* originating from Lake Thalersee.

Field samples

Field samples were collected from mid May to mid August of 1999 from Lake Thalersee (3.8 ha; z_{max} 7.0 m) and Lake Bansee (3.3 ha; z_{max} 4.1 m) in southern Germany. Both lakes are eutrophic and located in the same area and do not differ much in their topography. Lake Thalersee is completely surrounded by forest, but Lake Bansee is only approximately 60% surrounded, and therefore Lake Thalersee is slightly better protected from wind impact. *D. cucullata* was reported to show distinct cyclomorphosis every year in Lake Thalersee (Pawlowski, 2000), whereas no cyclomorphic change was observed in Lake Bansee (Siebeck, personal communication). Two net hauls (250 µm mesh size; 25 cm diameter; 50 cm height; Hydrobios, Germany) were drawn from the deepest point of each lake on each sampling date. The samples were conserved in sugar-formalin (4%). 50 randomly selected *D. cucullata*, with body sizes between 600 – 800 µm body length, were used for analysis at each sample date.

Clonal comparison

Because we found that daphnids from Lake Thalersee showed a distinct cyclomorphosis during the course of the year, whereas daphnids from Lake Bansee did not, we compared two clones from both lakes regarding turbulence induction.

The turbulence-1-setup was used for the clonal comparison experiment. The experiment was replicated three times.

Comparison of predator kairomone and turbulence:

Ten individuals of fourth-instar larvae of *Chaoborus flavicans* were placed in a cylindrical cage made of acrylic (10 cm diameter and 12 cm length) and 100 µm nylon mesh located inside the glass beakers. This method was used to ensure spatial separation between predators and *D. cucullata* originating from Lake Thalersee while still guaranteeing the exchange of chemical cues. The predators were fed daily with different kinds of prey. Pupate phantom midge larvae and dead predators were replaced and the cages were cleaned daily. The experiment was replicated three times. Beakers without predators served as controls.

Temperature experiment

Coker and Addlestone (1938) reported an upper threshold value of 11°C for cyclomorphosis in *Daphnia longispina.* Therefore, an additional experiment with *D. cucullata* originating from Lake Thalersee was conducted in a climate chamber at a constant level of 8°C using the turbulence-1-setup to test whether *D. cucullata* is able to form helmets in cold environments.

Data analysis

All experiments lasted three to five weeks. After that time period, approximately 100 daphnids were randomly selected from each beaker. These animals were measured under a dissecting microscope with a digital image-analysis system (Soft Imaging System, Analysis Pro, Münster, Germany). The morphological parameters recorded from *D. cucullata* were helmet length (defined as the length between the tip of the helmet to the upper edge of the compound eye) and body length (defined as the length between the upper edge of the compound eye to the base of the tail spine). For analysis, the daphnids were divided into four body-length size classes (sc1: <600 µm; sc2: 600 – 799 µm; sc3: 800 – 1000 µm; sc4: >1000 µm). In the predator kairomone induction experiment only body-length size class 2 was used for analysis, because cyclomorphic traits are most pronounced during this life stage.

To compensate for small size-dependent changes in helmet and tail-spine length within the classes, we calculated the relative helmet length (helmet length / body length *100) and the relative tail spine length (tail-spine length / body length *100) of the animals in all experiments. The three replicates per treatment could be pooled for analysis in all cases because they showed no significant within-treatment difference in Kruskal - Wallis tests. Conservative non-parametric Mann-Whitney U - tests were then used to compare the two independent groups, treatment and control. The significance level in multicomparisons was adjusted to $p \leq 0{,}017$ for three treatments according to Rice (1989). The relative values were arcsin-square-root transformed prior to analysis (Sokal & Rohlf 1995) in the predator kairomone experiment. A One-Way-ANOVA was then performed to test for treatment effects.

Results

Helmet elongation of *D. cucullata* originating from Lake Thalersee was distinctly stronger in the turbulence-1-setup than in the turbulence-2-setup (Figure 2a; Mann-Whitney-U; size class 1 - 3: all $p < 0.001$; size class 4 $p < 0.01$). Additionally, the maximum helmet length induced with the turbulence-1-setup (56.00 % relative helmet length in body-length size class: 600 – 800 µm) was longer than the maximum helmet length (44.18 % relative helmet length in body-length size class: 600 – 800 µm) observed in the field in this study. Furthermore, field data show that cyclomorphosis in *D. cucullata* differs between each lake. Daphnids from Lake Thalersee showed the typical cyclomorphic change of their plastic traits, while animals from Lake Bansee showed almost no difference in helmet formation (Table 1). Laboratory experiments with different clones of *D. cucullata* from both lakes revealed that daphnids showed significantly longer helmets in induction experiments with turbulence than did the controls (Figure 2a,b). Furthermore, daphnids from Lake Bansee show an almost identical inductive pattern as *D. cucullata* from Lake Thalersee (Figure 2b).

Daphnids induced with predator kairomones showed significantly longer helmets than control *Daphnia*. However, helmet length was significantly longer in the turbulence-1 treatment as compared to the predator-kairomone induction (Table 2). Although helmet length of *D. cucullata* differed significantly in the 8°C experiment between turbulence treatment and control (Mann-Whitney-U; size class 1 - 4: all $p < 0.05$, Figure 2c), helmet growth of the daphnids was approximately three times lower under cold turbulent conditions as compared to warm turbulent environments (Figure 2 a, b, c).

Discussion

During its cyclomorphosis, *D. cucullata* exhibits a maximum shaping of 55% relative helmet length (Hrbàcek 1959) in nature. We have shown for the first time that such extreme helmet formation can be induced by small-scale turbulence in the laboratory. Hereby, the method used to generate these turbulences seems to be crucial because different modes of small-scale turbulences induce different levels of helmet formation in *D. cucullata* (Figure 2a). Although our study confirms Hrbàceks idea that turbulence acts as an inducing agent, our results that different kinds of turbulence vary in effectiveness may also explain why he failed to induce high helmets in *D. cucullata*. He speculated that cyclomorphosis is linked to water circulation caused by wind stress on the surface. But the strongest wind effect moving the water column would be expected during lake circulation in spring and autumn, exactly at the time when *D. cucullata* usually shows its non-helmeted morphs. Even our field data indicate that water circulation caused by wind exposure seems not to be the reason for helmet formation in *D. cucullata*. The two neighbouring lakes in our study show an almost identical morphometry. Furthermore, Lake Thalersee is surrounded by forest and is therefore less exposed to wind impact. Nevertheless, animals from Lake Thalersee have higher helmets and the population in Lake Bansee showed no cyclomorphic pattern. Our laboratory induction with water turbulence revealed an almost identical helmet elongation of different clones originating from both lakes, suggesting that the patterns of cyclomorphosis in the field are not caused by clonal differences in phenotypic plasticity. Thus, large-scale turbulence caused by wind impact may not act as inductor, but rather small-scale turbulence, possibly generated by shear between stratified micro layers. Although we can induce maximum helmet size with our turbulence-1-setup, this turbulence seems to be rather strong compared to natural conditions. On the other hand, we may not conclude that we found the most effective way in generating turbulence which cause cyclomorphosis. While we identified a clear defensive effect of the induced traits in *D. cucullata* against various invertebrate predators (Laforsch & Tollrian, in press), *Chaoborus*-released chemicals failed to induce maximum head shape in *D. cucullata* in this and in previous studies, suggesting that predator cues themselves could not exclusively explain extreme cyclomorphosis in nature. Perhaps helmets alter hydrodynamics leading to a yet unknown benefit. Hrbácek (1958) revealed that *D. cucullata* only occurs in lakes and ponds containing high densities of fish. He therefore concluded (1959) that helmets provide the capability of staying in deeper layers of a circulating water column, thereby allowing daphnids to avoid fish predation. But *D. cucullata* is a very small cladoceran species which occupies the entire water column, even when fish are present (Pawlowski 1999) because *D. cucullata* does not fit into the prey-size spectrum of adult fish. Therefore, even an elongated helmet, that is completely transparent should not increase vulnerability to adult fish predation, but vice versa cyclomorphic traits could possibly be a defense against young fish. For example, the long helmets of *Daphnia lumholtzi* have been shown to act as protection against fish (Kolar & Wahl 1998). In this context, it seems possible that helmet formation in *D. cucullata* is somehow connected to the swimming activity of fish as also suggested by Lampert and Wolf (1986), because the cyclomorphosis peak of the plastic traits is concordant with the abundance and activity of young-of-the-year fish (0+). These young fish can aggregate to dense swarms of several thousands (Wanzenböck, pers. com.). Thus, it is possible that fish or invertebrate predators cause an effectively inducing small-scale turbulence pattern through their movements.

Our result that helmet elongation is just barely inducible in cold environments may suggest that in natural habitats the defense is not required, because even predators are less active in cold conditions. Relating to this, we can hypothesize that high helmets additionally restrict the maneuverability of daphnids while moving in a more viscous medium. For the evolution of phenotypic plasticity, benefits in one environment have to be offset by costs in the other environments (Tollrian & Harvell 1999); otherwise a selection on constitutive defenses should be favored. Trade-offs have not yet been identified in this system. The direct allocation of energy to the helmet development is relatively small and costs are not measurable (Tollrian 1991). Therefore, it might be speculated that other costs, e.g., an increased hydrodynamic drag when the water temperature is colder, could be relevant.

To summarize, the role of small-scale turbulence regarding cyclomorphosis in *D. cucullata* still remains unclear. It has been shown that helmets of *D. cucullata* are inducible both with biotic and with abiotic cues. Therefore it seems possible that helmets provide additional benefits beside the proven defensive effect against invertebrate predators. Hence, synergistic effects of different parameters could be responsible for extreme helmet formation in nature, indicating that inducible helmets have evolved as a multi-tool for different environmental conditions.

Acknowledgements

We thank W. Gabriel for encouragement and discussion; M. Kredler for help during experiments; and Scott Stevens for linguistic improvements and two anonymous reviewers for comments.

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Table 1

Seasonal course of mean relative helmet length (%) and standard error (SE) of *Daphnia cucullata* (body length size class: 600-800 µm) sampled in two different lakes with similar morphometry and similar wind effects.

	Bansee			Thalersee		
date	Mean	SE	N	Mean	SE	N
27.05	14.08	0.62	22	23.09	0.74	50
18.06	13.05	0.54	49	44.18	0.90	50
30.07	8.76	0.31	48	24.62	0.58	49
19.08	11.00	0.34	48	28.01	0.67	49

Table 2

Mean relative helmet length of *Daphnia cucullata* (body-length size class: 600-800 µm) originating from Lake Thalersee exposed to turbulence (setup 1), raised with (kairomone) and without (control) chemical cues released from *Chaoborus flavicans*. Data were tested using one-way ANOVA. The respective ANOVA values and probabilities are given. Letters (H) indicate homogeneous groups based on Tamhane Post-Hoc Tests. All groups differ significantly from both other treatments (all $p < 0.001$).

Treatment	Mean	SE	N	H	ANOVA
turbulence	55,94	1,10	51	A	$F_{2, 318} = 1363.3$ $p < 0.001$
chaoborus	30,61	0,29	135	B	
control	18,86	0,24	135	C	

Figure Legends

Figure 1

Schematic line drawings of both turbulence generating setups (a) Turbulence-1- setup: Abbreviations: m= electric motor; rd= permanently rotating PVC disk driven by the motor; st = piece of silicone tube attached to the frontal side of the PVC disk; bb = ball bearings; p = paddle; b = beaker; (b) Turbulence-2-setup: Abbreviations: rgd = permanently rotating gel dyer; ps = polystyrene sheet ; r= acrylic rod; pl = acrylic plate; b = beaker.

Figure 2

Relative helmet length of *Daphnia cucullata* from two lakes (Fig 2a: clone from Lake Thalersee; Fig 2b: clone from Lake Bansee) in the control and in the induction treatment with small-scale turbulence. Turbulence was generated using two different methods (turb1 and turb 2). Fig 2c: Helmet induction under cold (8°C) conditions with turbulence-1-setup and a clone from Lake Thalersee. Asterisks indicate significant differences to the control (*** = $p < 0.001$; ** = $p < 0.01$; * = $p < 0.05$).

Figure 3

Scanning Electron micrograph (for method see Laforsch & Tollrian 2000) showing frontal and dorsal view of induced individuals of *Daphnia cucullata*.

Figure 1

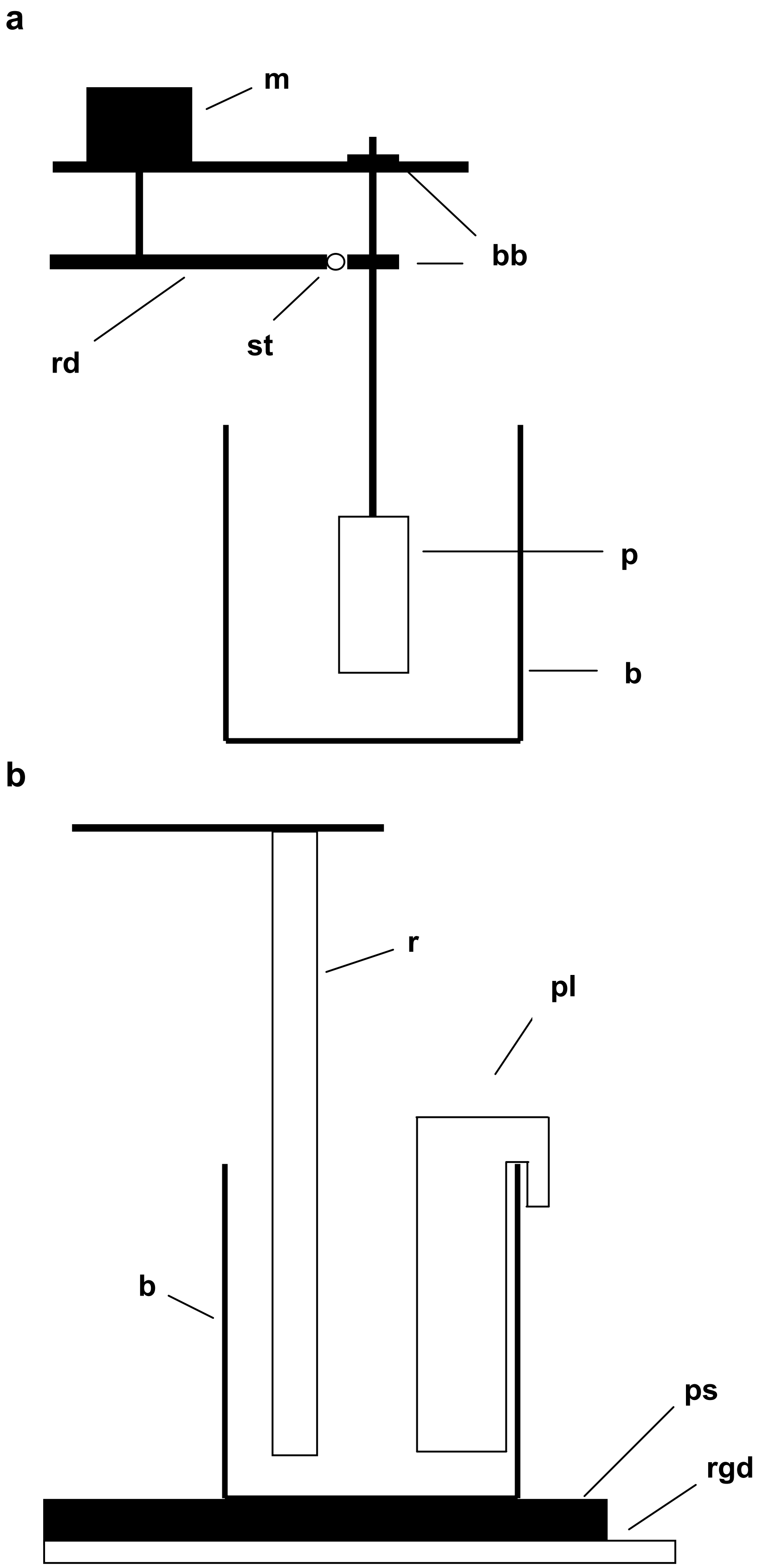
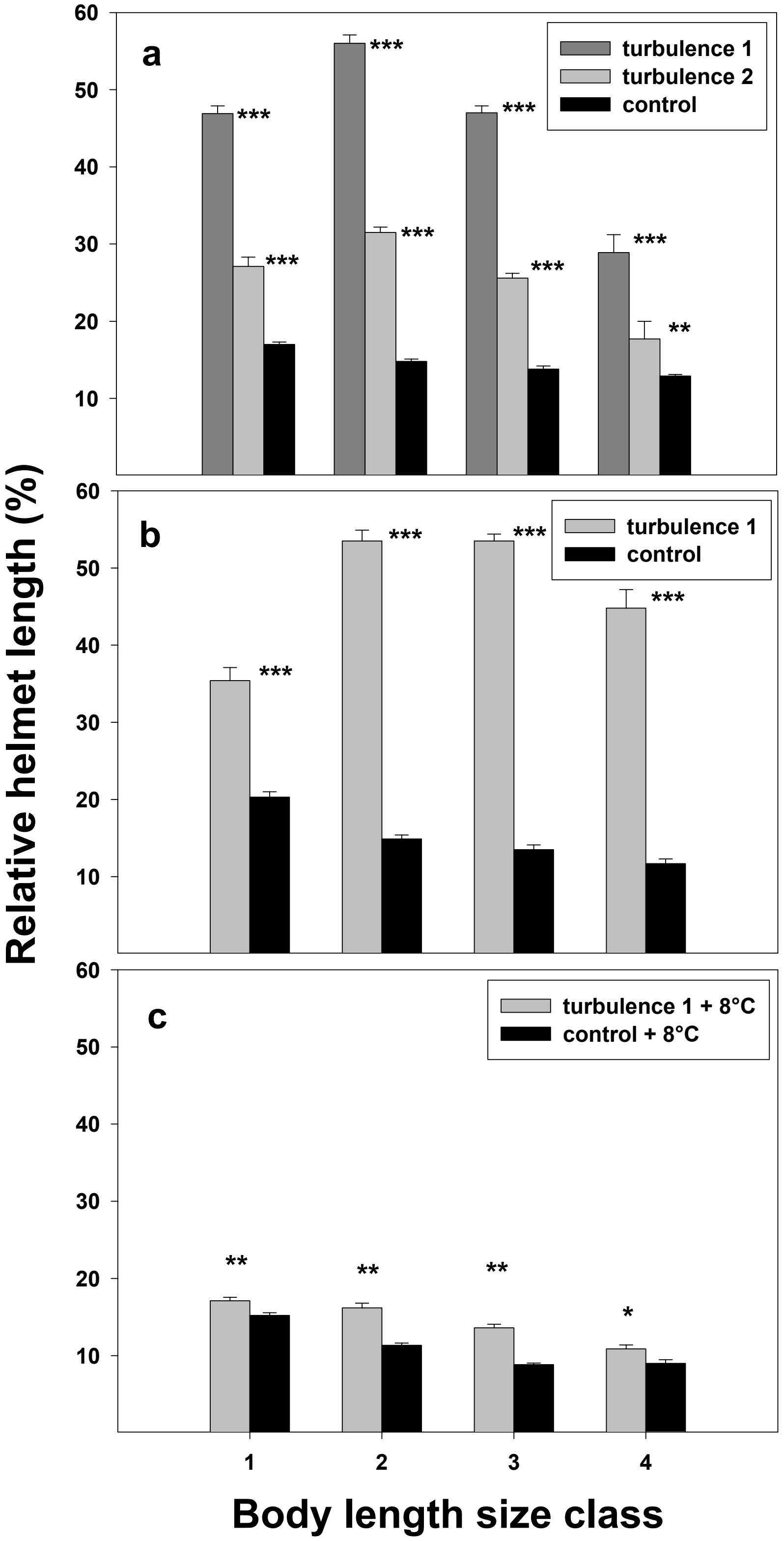


Figure 2


a
turbulence 1
turbulence 2
control
b
turbulence 1
control
c
turbulence 1 + 8°C
control + 8°C
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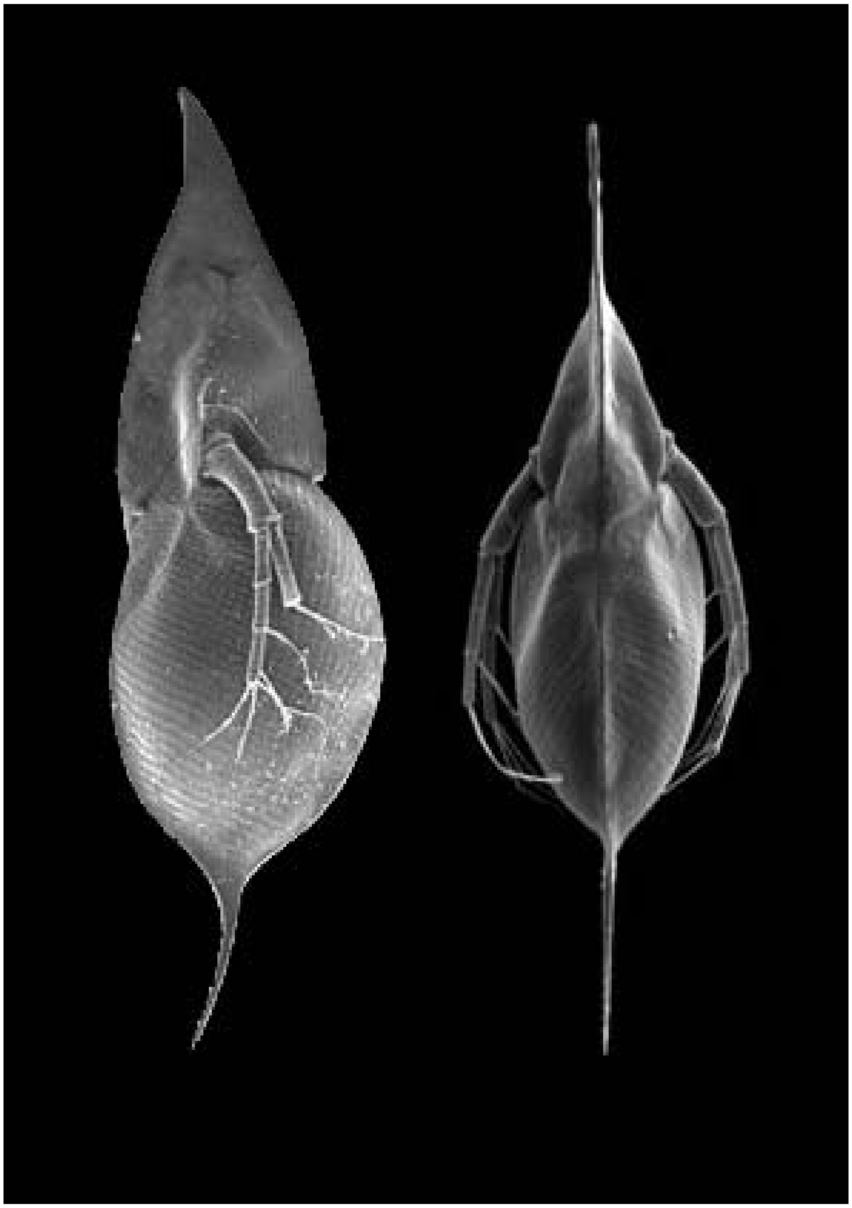
*
60
50
40
30
20
10
1
2
3
4
Relative helmet length (%)
Body length size class

Figure 3



6

A NEW PREPARATION TECHNIQUE OF DAPHNIDS FOR SCANNING ELECTRON MICROSCOPY USING HEXAMETHYLDISILAZANE (Laforsch und Tollrian, *Archiv für Hydrobiologie*, 149: 587-596, 2000)

Arch. Hydrobiol. 149 4 587–596 Stuttgart, November 2000

A new preparation technique of daphnids for Scanning Electron Microscopy using hexamethyldisilazane

Christian Laforsch* and Ralph Tollrian[1]

Ludwig-Maximilians-Universität München, Zoological Institute, Department of Ecology, München, Germany

With 5 figures

Abstract: SEM requires special preparation techniques to avoid artifacts, especially in aquatic organisms. Not all organisms can be prepared equally well for SEM. For example in *Daphnia,* preparation causes major shrinkage of the carapace in most species. We present a new preparation technique for *Daphnia* using hexamethyldisilazane (HMDS), which avoids these problems. We compared this method with the common critical point drying technique (CPD). The routinely used CPD caused shrinkage in the daphnids, predominantly in the region of the neck and the brood chamber. In contrast, all tested *Daphnia* species immersed in hexamethyldisilazane (HMDS) and dried slowly overnight in a vacuum desiccator revealed an excellent surface. The good results, low costs and easy operation without the requirement of special equipment favor HMDS as drying agent for SEM investigations in daphnids.

Key words: *Daphnia*, preparation technique, shrinkage, surface structures, critical point drying technique (CPD), HMDS.

Introduction

Scanning electron microscopy (SEM) is an important tool in many sections of biological research. SEM is the preferred technique for analyzing surface structures of many types of tissues because of its high depth of focus which leads to a three-dimensional impression of the objects. Furthermore, the high resolution of SEM reveals more details of the surface characteristics. This is especially true for transparent organisms such as cladocerans. In ultrastructural examinations of daphnids, usually only a part of the organism has been

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[1] **Authors' address:** Ludwig-Maximilians-Universität München, Zoological Institute, Department of Ecology, Karlstr. 25, D-80333 München Germany.
* Corresponding author; e-mail: laforsch@zi.biologie.uni-muenchen.de

0003-9136/00/0149-0587 $ 2.50

studied. Some authors focused on the surface structures of the ephippia (SCHULTZ 1977, GLAGOLEV 1983, DU & LI 1990). Primarily the filtering structures were investigated to answer ecologically relevant questions; for example, filter screen mesh sizes or the variability of filter structures in different *Daphnia* species (WATTS & PETRI 1981, GOPHEN & GELLER 1984, HESSEN 1985, BRENDELBERGER & GELLER 1985, BRENDELBERGER 1991, VILLALOBOS & GELLER 1997).

The drying process is the critical step in SEM preparation. Various methods have been used to prepare specimens for SEM studies. GOPHEN & GELLER (1984) dried the gut contents and the limbs of daphnids on Nuclepore filters in a heat dryer. A double fixation procedure with 4 % glutaraldehyde and 1 % osmium tetroxide was used by WATTS & PETRI (1981). Subsequently they used a graded acetone series (20–100 %) for dehydration prior to critical point drying.

Critical point drying (CPD) technique is the most common method used to dry organisms for SEM. This technique leads, in most cases, to little or no collapse of softer body parts in contrast to simple air drying which causes major shrinkage and subsequent unnatural images. BOYDE (1980) investigated a variety of vertebrate tissues. He showed that biological samples dried with CPD sometimes exhibit wrinkles due to shrinkage on their surfaces and dimensional changes during the procedure. Therefore, several alternatives have been developed. Freeze drying can result in good preservation of surface morphology of aquatic organisms, but the major disadvantage of this technique is the frequent surface damage caused by ice crystal formation (MAUGEL et al. 1980). Low vacuum SEM freeze drying (SUZUKI et al. 1995) was proposed to prepare aquatic microorganisms. Likewise some chemical agents, such as Peldri II (BROWN 1990), were successfully used for drying small flies. NATION (1983) introduced a new chemical-based drying technique: the evaporation of hexamethyldisilazane (HMDS). This method, originally exerted on soft insect tissues is not frequently used, although good results have been obtained in several taxa (*Gryllus bimaculatus,* BOCK 1987; pollen grains, CHISSOE et al. 1994; Crustacea: *Macrobrachium potiuna,* MORAES & BOUZON 1995; mammals: hepatic endothelial cells, BRAET et al. 1996; demineralized human dentine, CARVALHO et al. 1996; *Sarcocystis,* STOLTE et al. 1996).

We tested HDMS as drying agent for SEM investigations in different *Daphnia* species. We compare this method with the common CPD technique.

Materials and methods

The examined *Daphnia* species were taken from laboratory cultures. *Daphnia lumholtzi* originate from the USA, *Daphnia longicephala* from Australia, *Daphnia magna* from Germany, *Daphnia pulex* from Canada, *Daphnia ambigua* from the USA and

Daphnia cucullata was isolated from Germany. The animals were reared in an artificial medium based on ultrapure water and fed daily with *Scenedesmus obliquus*. Neck teeth, crests and helmets were induced by predator kairomones (GRANT & BAYLY 1981, KRUEGER & DODSON 1981, TOLLRIAN 1990, HANAZATO 1990, TOLLRIAN 1994). The animals were removed from their culture medium and rinsed in filtered newly prepared medium to ensure that the organisms were free of particulate matter. The daphnids were killed in a microwave (4 sec.) and immediately transferred into 70 % ethanol for fixation. Pilot experiments had shown that sugar-formalin is not a suitable agent to fix the daphnids for obtaining good SEM whole body images.

Critical Point Drying

For the CPD method, the daphnids were dehydrated in a graded acetone series (70 %, 80 %, 90 %, 10 min each; 2×10 min 98 %; 2×10 min 100 %) prior to critical point drying (Bio-Rad E4850, München). The specimens were transferred to a small CPD container (Bio-Rad, München) filled with 100 % acetone. The CPD chamber was flooded with liquid CO_2 under high pressure and low temperature (10–15 °C). After five minutes the CO_2 was slowly vented. Flooding and venting were repeated six times to substitute the acetone. Finally the temperature of the chamber was raised over 10 minutes to 42 °C and 1200 psi for critical point drying. Afterwards the chamber was slowly vented over 20 minutes.

Hexamethyldisilazane Drying

For HMDS drying, the samples were dehydrated in graded acetone solutions (70 %, 80 %, 90 %, 2×98 % and 2×100 %) for 10 min each. Subsequently the specimens were immersed in 1–1.5 ml HMDS (1,1,1,3,3,3 hexamethyldisilazane; Merck-Suchardt, Darmstadt) in 20 ml glass vials. After a soak of 30 minutes, approximately 90 % of the HMDS was removed and the vials were immediately transferred to a desiccator. The bottom of the desiccator was covered by silica gel beads (Merck-Suchardt, Darmstadt) and the desiccator itself was evacuated to avoid water contamination which would cause shrinkage in the specimens. The remaining HMDS was allowed to evaporate overnight under anhydrous conditions. All steps with HMDS were conducted under a fume hood, because HMDS is strongly irritant.

Following CPD or HMDS preparation, the samples were carefully mounted on aluminum stubs (Plano, Wetzlar) with double sticky tabs (Plano, Wetzlar), under a dissecting microscope. Afterwards, the specimens were sputter coated with gold (BIO-RAD SC510, München) for 135 sec. The samples were examined with a Philips XS – 20 scanning electron microscope at 20 kV.

Results

HDMS dried specimens showed well preserved surfaces. Almost no distortion or shrinkage was observed with this treatment. In contrast, the daphnids prepared with CPD showed partially strong shrinkages (Fig. 1). In some cases, the

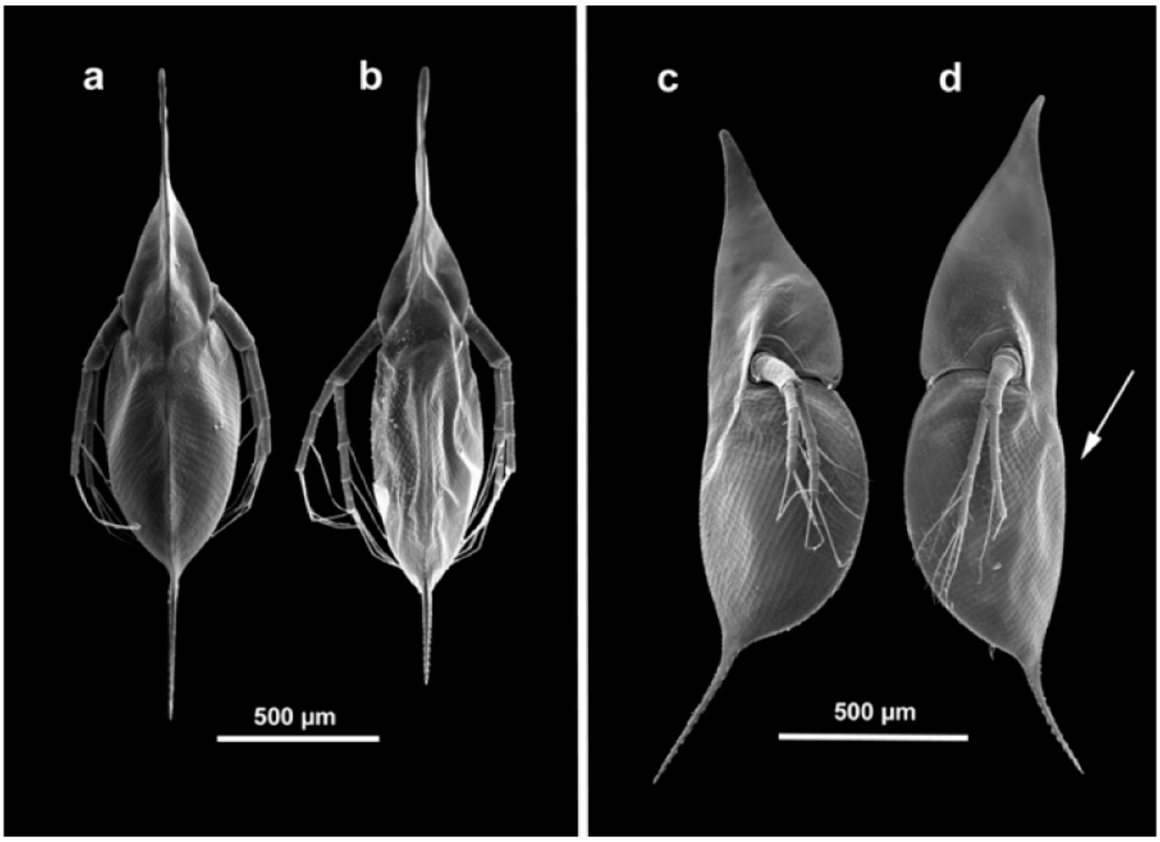

Fig. 1. Dorsal view of *Daphnia cucullata* dried with HMDS (**a**) and CPD (**b**). Lateral view of *Daphnia cucullata* dried with HMDS (**c**) and CPD (**d**). Deformations are visible after CPD, while HMDS provides a better surface structure. In particular, the carapace of the daphnids is susceptible for shrinkages in the region of the neck and the brood chamber (arrow).

difference between both treatments was marginal. However, specimens dried with CPD are more susceptible to shrinkage, predominantly in the region of the neck and the brood chamber (Fig. 1 d). The number of very good SEM whole body images was distinctly higher in the HMDS treatment. Drying with HDMS obtained good results both in fragile species such as *Daphnia cucullata* or *Daphnia ambigua* (Figs. 1 a, c, 5) and in more robust species such as *Daphnia magna, Daphnia longicephala* or *Daphnia lumholtzi* (Figs. 2, 4). Even juvenile stages of different *Daphnia* species dried with HMDS show good surface quality (Figs. 2 a, 3 d). The use of HMDS as a drying agent yields good results in the investigation of ultrastructural characteristics in daphnids. Both the neckteeth of *Daphnia pulex* (Fig. 3 c) and the thorn on the tip of the head of *Daphnia ambigua* (Fig. 5 b) showed well preserved surface structure. Likewise, the SEM recordings of the dorsal organ of the second juvenile stage of *Daphnia cucullata* (Fig. 5 a) display a presentable result when the specimens were dried with HMDS. In *Daphnia magna* good results were achieved when morphological details like the shape of the head (Fig. 2 b), the filter appendages or the chemosensillae (Fig. 3 a, b) were examined.

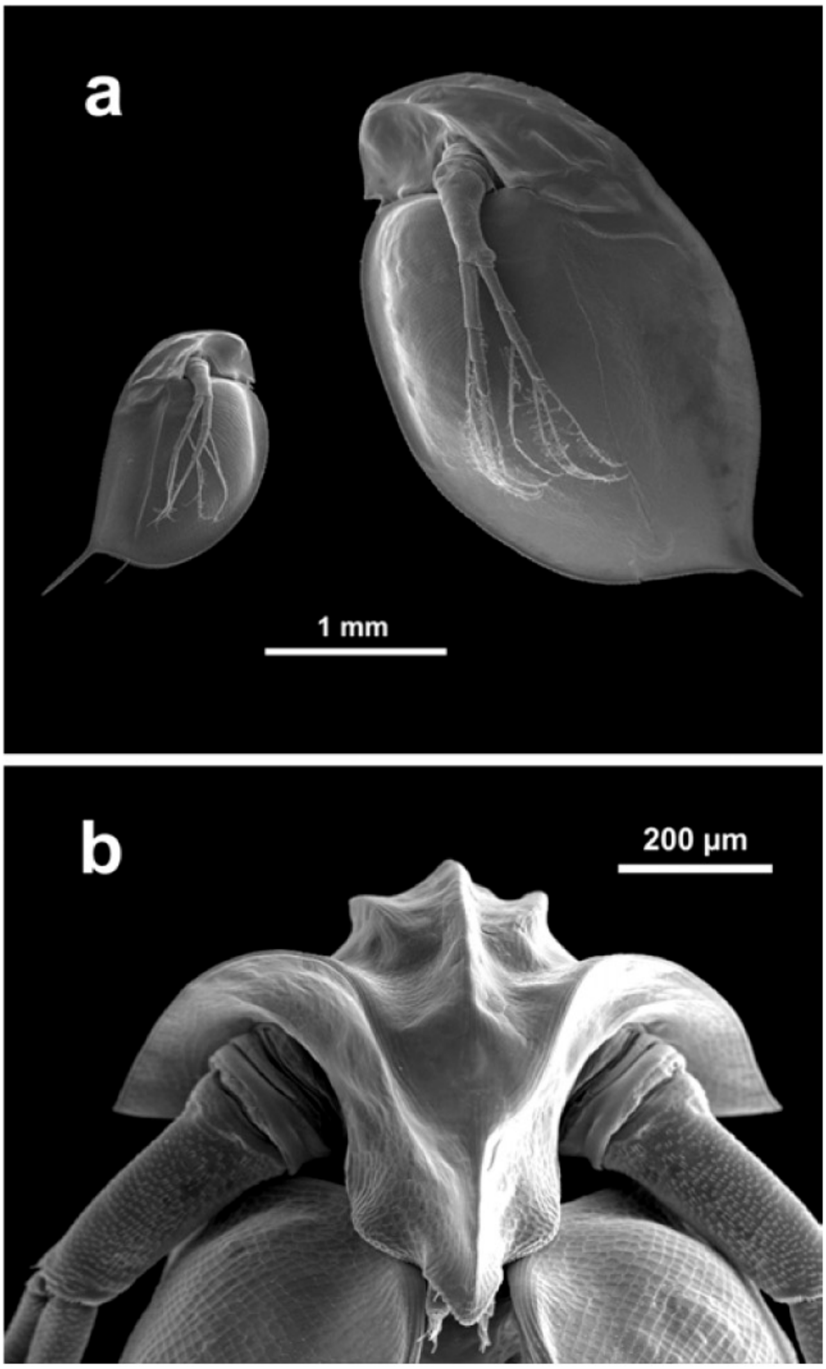

Fig. 2. *Daphnia magna,* second juvenile stage and adult female dried with HMDS **(a)**. The structures which can be seen on the head are no artifacts due to the drying procedure, as can be recognized from the frontal view of the head **(b)**.

Discussion

HMDS seems to be a convenient alternative to the established and widely used CPD method. Both in animals and plants, good results have already been achieved by using this method for SEM investigations. Chissoe et al. (1994) showed that HMDS is an excellent drying agent for pollen grains, especially

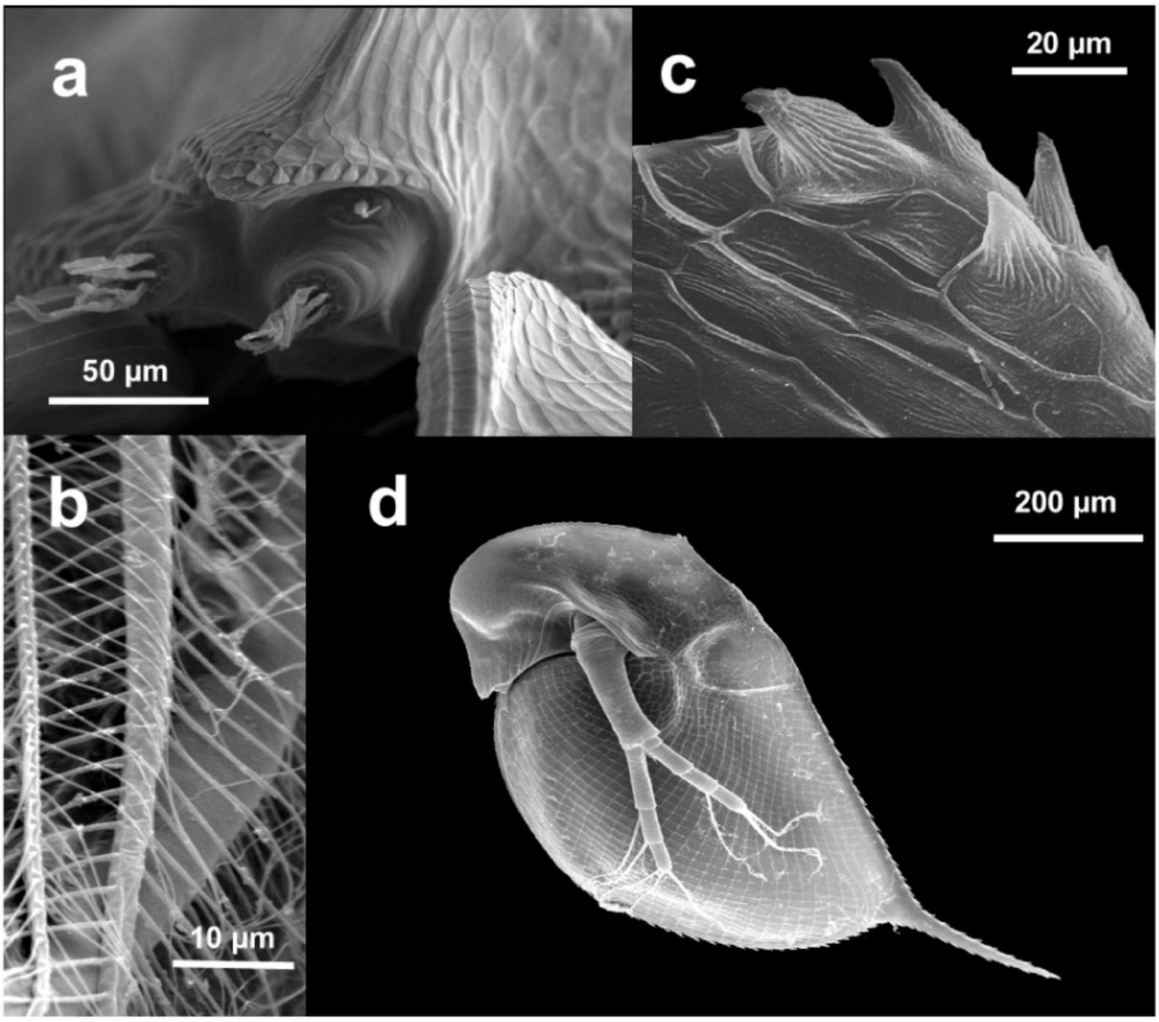

Fig. 3. Fine structure of HMDS prepared daphnids. *Daphnia magna:* **(a)** chemosensillae of the first antenna and fine structure of the carapace (HMDS); **(b)** thoracopodite appendages (HMDS); *Daphnia pulex:* **(c)** neck teeth (HMDS); **(d)** second juvenile stage with neck teeth (HMDS).

for those which are susceptible to collapse in the SEM. Furthermore sensitive internal and surface structures of insects were successfully prepared with HMDS (Nation 1983, Bock 1987, Rumph & Turner 1998). Even the dehydration of soft tissues, like hepatic cells, with HMDS was as satisfactory as the CPD method (Braet et al. 1996). Most authors working with HMDS and CPD pointed out that there is no or just a marginal (Heraty & Hawks 1998) difference between both methods. In a few taxa, such as softbodied hymenopterans, the CPD method might lead to slightly better results (Heraty & Hawks 1998).

In small and fragile species like daphnids the HMDS treatment seems to be more effective for obtaining high qualitative SEM images compared to the CPD method.

HMDS reacts with water to produce hexamethyldisiloxane and ammonia, both of which could evaporate from the object (Heraty & Hawks 1998). Na-

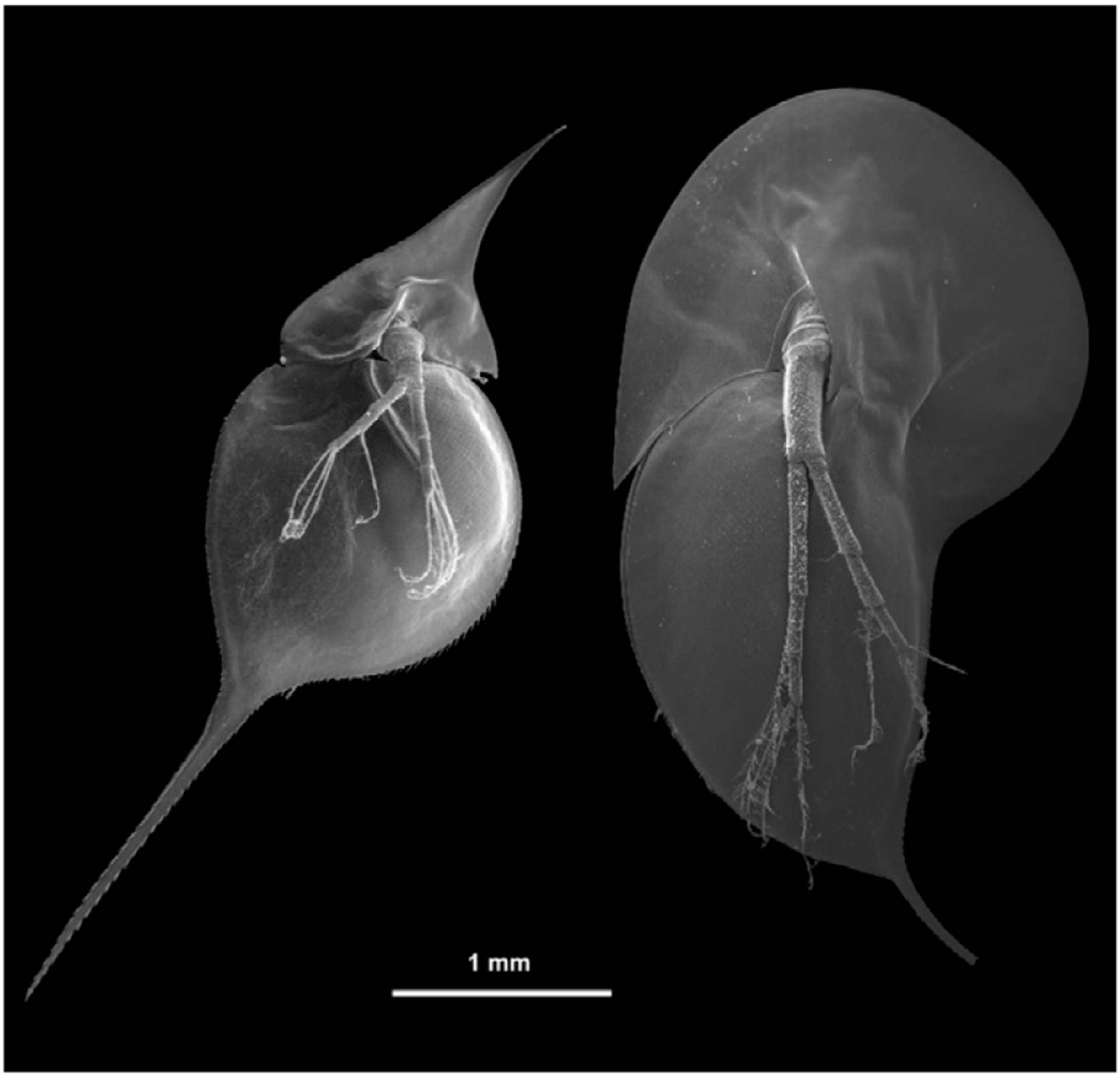

Fig. 4. *Daphnia lumholtzi* (left) and *Daphnia longicephala* (right) dried with HMDS.

TION (1983) suggested that HMDS might crosslink proteins and stiffen the tissue during the drying process. The relatively long period of time HMDS is allowed to evaporate in absolute anhydrous surroundings could be the reason for obtaining an excellent surface structure of the animals in our experiment. The extremely slow vaporizing of HMDS seems to prevent the destructive force of the surface tension during the drying procedure. Astonishingly, there is no difference between the large *Daphnia* species such as *Daphnia magna* and the small, more fragile species such as *Daphnia cucullata,* or between adult and juvenile daphnids (Fig. 2 a). Although there is sometimes a slight difference between both methods, CPD predominantly causes shrinkage in the apparently sensitive dorsal regions of the daphnids. Our results reveal that HMDS drying is successful in preserving ultrastructural features of the daphnids (Figs. 3, 5). Therefore, the HMDS method seems to be a suitable technique for a detailed investigation of the fine structure of morphological traits in daphnids.

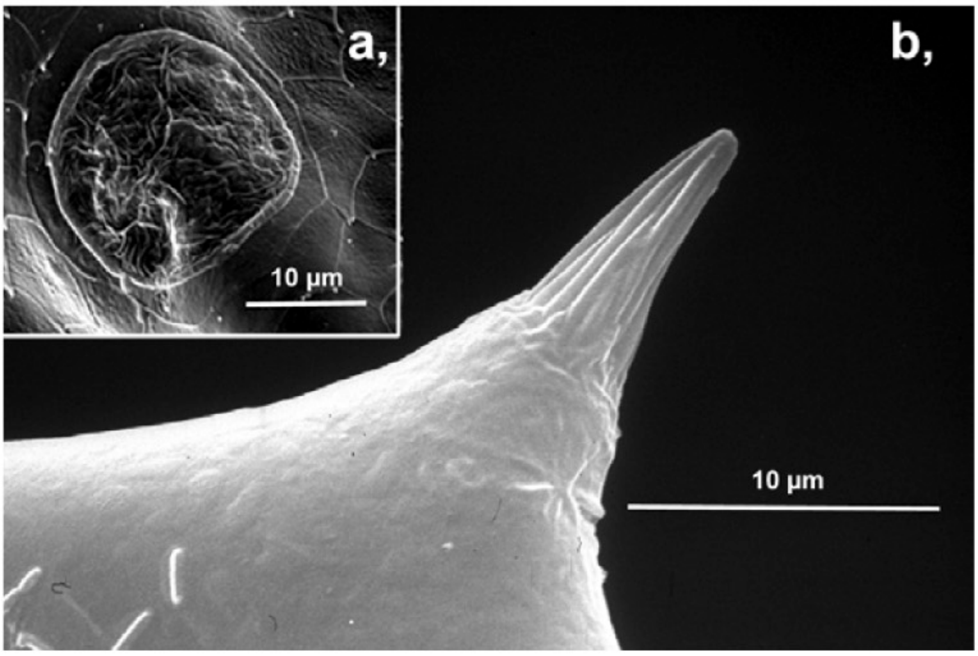

Fig. 5. Dorsal organ of the second juvenile stage of *Daphnia cucullata* (**a**) and tip of the helmet of *Daphnia ambigua* (**b**) dried with HMDS.

Another considerable advantage of HMDS is the simplicity of the method. It requires no specialized equipment, is easy to use and it is inexpensive. We are confident that the good results obtainable with this method will stimulate further investigations and lead to new insights into the link between functional morphology and ecology of cladocerans.

Acknowledgements

We thank Wilfried Gabriel for encouragement; Mechthild Kredler and Erika Hochmuth for help with experiments; Roland Melzer, Tim Vines and two anonymous reviewers for comments on the manuscript.

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Submitted: 28 February 2000; accepted: 3 May 2000.

7

EMBRYOLOGICAL ASPECTS OF INDUCIBLE MORPHOLOGICAL DEFENSES IN *DAPHNIA* (Laforsch und Tollrian, *Journal of Morphology*, eingereicht)

Embryological aspects of inducible morphological defenses in *Daphnia*

Christian Laforsch † and Ralph Tollrian *

†* Section of Evolutionary Ecology and GeoBioCenter, Department of Biology II, Ludwig-Maximilians-Universität München, Karlstr. 23 – 25, 80333 München, Germany

Number of text pages: 12

Number of figures: 2

Number of tables: 2

Running headline: embryological induction

† Corresponding author: Tel: +49 89 5902253, Fax: +49 89 5902461,
laforsch@zi.biologie.uni-muenchen.de

key words: phenotypic plasticity, embryology, inducible defenses, *Daphnia*, SEM

Abstract

Many cases of predator-induced morphological plasticity in daphnids are well studied examples of inducible defenses. However little is known about the early development of these sometimes exuberant traits. We compared for the first time in five different *Daphnia* species the embryonic development of predator-induced and non-induced animals using scanning electron microscopy (SEM). We observed significant morphological changes in the last embryonic stage in helmet formation in *Daphnia cucullata* and in neck-pedestal development in *Daphnia pulex*. In contrast, no morphological changes could be found during embryogenesis between induced and non-induced *Daphnia lumholtzi*, *Daphnia longicephala* and *Daphnia ambigua*. But the strategies to initiate the defensive traits differ between *Daphnia* species due to trade-offs between environmental requirements and developmental constraints. Furthermore, all *Daphnia* embryos have to shed at least three different membranes before leaving the brood pouch of the mother. After the embryos shed off the third membrane, chemosensillae are exposed to the olfactory environment and are likely able to detect predator-released chemicals.

Introduction

Prey organisms have evolved a variety of defensive strategies to cope with the selective force of predation pressure. An array of inducible antipredator defenses have been reported for pelagic cladocerans, including life-history shifts, behavioral adaptations and morphological changes (for review see: Tollrian and Harvell 1999). Daphnids show the most prominent examples of morphological plasticity in response to chemical cues released by predators, so-called kairomones. Helmets (e.g., *D. cucullata*, *D. lumholtzi*, *D. ambigua*), crests (e.g., *D. carinata*) and neck teeth (e.g., *D. pulex*) have been shown to be inducible with kairomones and a protective effect of these traits has been previously demonstrated (for review see: Tollrian and Dodson 1999). The inducible stages and the protective mechanisms could differ between *Daphnia* species even against the same predator. For example, *D. pulex* generates neckteeth primarily in the second instar in the presence of the predatory phantom midge larvae *Chaoborus* (Krueger and Dodson 1981), whereas *D. cucullata* can show helmet formation during all life stages (Tollrian 1990). Tests on embryonic induction in different species achieved differing ontogenetic sensitivities to the kairomones. Hanazato (1990) concluded that *D. ambigua* is only affected by water-soluble cues from *Chaoborus* in the first juvenile stages, but does not respond with helmet formation when exposed to kairomones in the embryonic stage. In contrast, several investigations in *D. pulex* showed sensitivity to *Chaoborus* kairomone during embryonic development (Krueger and Dodson 1981; Parejko 1992). Furthermore, additive effects of the chemical cues and sensitive phases of embryos and juveniles were detected in *D. pulex* (Tollrian unpublished data). In all these studies the inductive effect of the traits was exclusively scored with light microscopy techniques in juveniles which were already released from the brood pouch.

Nothing is known about trait formations during embryonic stages. Large induced traits present *Daphnia* with a logistical problem, due to the limited space in the mother's brood pouch. On the one hand extreme morphological traits should already be developed before birth, in situations in which the neonate is already threatened by a predator, to provide a protective effect from the very first moment of their neonate life. On the other hand, defensive features which often have thorn-like characteristics must somehow be defused to prevent mechanical interference with other siblings or their mother's brood pouch. In contrast, small morphological alterations which can be developed within one molt should not be observable in the last embryonic stage, especially if the defense is only needed in later life stages. Therefore, in five different *Daphnia* species, all of which show different morphological plasticity (*D. cucullata*, *D. pulex*, *D. ambigua*, *D. lumholtzi* and *D. longicephala*), we compared the embryonic development of animals reared with and without predator-conditioned medium. We used scanning electron microscopy (SEM) in order to detect small-scale differences in the morphology between both morphs.

Material and Methods:

Single clones of *D. lumholtzi*, *D. pulex*, *D. longicephala*, *D. ambigua* and *D. cucullata* were used for the experiments. Each of the laboratory-cultured clones showed distinct morphological plasticity in pilot experiments. We induced helmets (*D. lumholtzi*: Tollrian 1994, *D. ambigua*: Hanazato 1990 and *D. cucullata*: Tollrian 1990), crests (*D. longicephala*: Grant and Bayly 1981) and neck teeth (*D. pulex*: Krueger and Dodson 1981) in *Daphnia* by applying predator kairomones. The experiments were conducted in the laboratory under constant conditions at 20 °C and artificial light in 1.5 L glass beakers filled with one liter of medium at unlimited food conditions (*Scenedesmus obliquus*). The synthetic medium (1 L), based on ultra-pure water, trace-elements and phosphate buffer, was changed weekly. Twenty age-synchronized and randomly chosen daphnids with freshly deposited eggs were placed into each beaker. All beakers contained a cylindrical cage made of an acryl frame (10 cm diameter and 12 cm length) covered with nylon mesh (100 µm) in order to avoid direct contact between predators and daphnids, but to guarantee the exchange of chemical cues. The predators (1 *Notonecta*/L for *D. longicephala*; 10 *Chaoborus*/L for *D. ambigua*, *D. pulex and D. cucullata*) were placed into these cages and fed daily with different kinds of prey to ensure sufficient kairomone production. The cages were cleaned daily. We used fish kairomones from *Phoxinus phoxinus* for the predator-induction treatment with *D. lumholtzi* in a concentration of one fish per three liters. We produced the fish kairomone by keeping 10 fish for 24 hours in 10 liters of medium. Thereafter the fish were removed and the medium was filtered (Schleicher and Schuell: Excelon PES 20/3HC; 0.2 µm) and frozen (-60 °C). Beakers without predators served as control. All treatments were replicated three times. We removed randomly chosen adult daphnids from the beakers after a time period of three to five weeks and analyzed the developmental stage of their embryos under a dissecting microscope. We conducted the embryological classification according to Murugan and Sivaramakrishnan (1973), who distinguished eight different developmental stages during embryogenesis in daphnids. We combined stages 1-3 to a general egg-phase, because no developmental difference can be recognized in SEM-investigations due to the shielding egg membrane. Thus, our classification consists of five embryological stages and one egg stage. We selected 25 mothers from each

Daphnia species carrying offspring from each developmental stage. The adult daphnids containing the embryos in their brood pouch were than fixed in 70% EtOH (p.a.). We prepared the daphnids for SEM-investigation according to the methods of Laforsch and Tollrian (2000). After the chemical drying of the daphnids with HMDS, we gently opened the carapace of the mother with a dissecting needle and removed the embryos. The embryos were carefully mounted on aluminum stubs (Plano, Wetzlar) with double sticky tabs (Plano, Wetzlar), under a dissecting microscope. Afterwards, the specimens were sputter coated with gold (BIO - RAD SC 510, München) for 135 sec. The samples were examined with a LEO 1430 VP (LEO Elektronenmikroskopie GmbH, Oberkochen) scanning electron microscope at 15 kV.

To verify the activity of the kairomone, we measured the morphological parameters from approximately 50 randomly chosen adult daphnids from each treatment under a dissecting microscope with a digital image-analysis system (Soft Imaging System, Analysis Pro, Münster, Germany). The parameters recorded in all examined *Daphnia* species, except for *D. pulex*, were the helmet length (defined as the length between the tip of the helmet to the upper edge of the compound eye), the body length (defined as the length between the upper edge of the compound eye to the base of the tail spine), and the tail-spine length (defined as the length between the base of the tail spine to the tip of the tail spine). For *D. longicephala* we also recorded the crest height (defined as the distance between the dorsal edge of the compound eye to the most dorsal point of the crest). To compensate for small size-dependent changes in helmet, crest and spine length, the relative trait length (trait length / body length *100) was calculated. The relative values were arcsin-square-root transformed prior to analysis (Sokal and Rohlf 1995). Conservative non-parametric Mann-Whitney U - tests were then used to compare the two independent groups, treatment and control. In *D. pulex* we selected animals of the second instar, in which neck-teeth formation is most distinct. Neck-teeth formation was quantified according to Tollrian (1993).

Furthermore we measured morphological parameters of the fifths embryological stage of *D. cucullata* (helmet length) and *D. pulex* (neck-pedestals) and analyzed these data as described above, to test whether morphological differences existed in the embryos prior to the release out of the brood pouch. We were not able to measure the exact tail-spine length of the daphnids and helmet length of *D. lumholtzi* with our analysis system, because of the curved shape of these plastic features.

Results

We gained significant inductive effects on plastic morphological traits with all kairomones in each *Daphnia* species (Table 1), thus verifying the chemical activity of the kairomones.

Only in the last embryo stage of *D. cucullata* and *D. pulex* did our SEM-investigations on embryological development show a difference between predator-kairomone induced and non-induced animals (Fig. 1a-d). Measurements in the last embryonic stage on helmet length of *D. cucullata* and neck-pedestals in *D. pulex* confirmed the optical SEM-results. *D. cucullata* showed distinctly longer helmets and *D. pulex* exhibited higher neck-pedestals when induced with chemical cues released by predators (Table 2). In *D. longicephala, D. lumholtzi* and *D. ambigua* no difference was found on visible morphological traits between predator and non-predator exposed morphs (Fig. 1e-j).

We observed a different mode of helmet development in *D. cucullata* and *D. lumholtzi*. The helmet of *D. lumholtzi* is folded on the vertex of the head, and likewise, the fornices are also folded almost until the embryo is getting released from the brood pouch (Fig. 1i). The helmet in *D. cucullata* is already formed during embryonic development, though only to a moderate extent.

Our SEM-images showed that the basic embryonic development was almost identical in all examined *Daphnia* species. During the egg phase only the first egg membrane is visible. We could observe different structures of this membrane (Fig. 2a-c). After the hatching of the first membrane the *Daphnia* embryo has an oval shape (Fig. 2d). In the first embryonic stage the body is already subdivided into segments and the labrum, mandibles and antennae are visible under the second membrane, which covers the entire body (Fig. 2e). A dorsal organ appears in the neck of the embryo (Fig. 2f). In the second embryonic stage an advanced development of the maxillae and the postabdomen is visible. The second membrane and directly thereafter a third membrane exfoliate from the body surface (Fig. 2g, h). Both membranes burst at the ventral part of the body. After both membranes have peeled off, more detailed structures, such as rudiments of the filter appendages become visible. In the third embryonic stage remnants of the membranes are attached predominantly to the caudal part of the daphnid. The second antennae are maneuverable and a rapid development of the complete body is noticeable. Furthermore, we could observe sensillae at the tips of the first antennae (Fig. 2i).

In the fourth embryonic stage the first antennae fuse at their base. The caudal spine, which is anterior folded under the carapace, is elongated and the carapace covers almost the entire body (Fig. 2j).

In the last embryonic stage we detected a stronger differentiation of the body appendages. In this phase the daphnid is already prepared to leave the brood pouch of the mother (Fig. 2k). *D. lumholtzi* seems to be covered by a very thin fourth membrane prior to birth, visible just at the position where the folded helmet contacts the head (Fig. 1k). This membrane bursts at the moment of birth, during the enormous elongation of the helmet caused by the absorption of water (Fig. 1l). We could not detect a similar membrane in any other examined *Daphnia* species. After the release of the brood pouch a rapid morphogenesis occurs in the neonate. The shape of the daphnid changes from an embryonic appearance (Fig. 2k) to an adult daphnid-like form (Fig. 2l), in just a few seconds to minutes.

Discussion

We found effects of induction of morphological defenses to be already present in the embryonic development of two *Daphnia* species and we found differences in the embryonic development of morphological defenses between species. These differences can be regarded as adaptations to the conflicting pressures of environmental requirements and developmental constraints. Survival in predator environments calls for fully functioning defenses in certain life stages and some defenses require time to form. On the other hand, competition may require high fecundity, which could be negatively affected if large-sized defensive traits were already present in the limited space inside the brood pouch of *Daphnia*. The most obvious solution to this conflict is that all tested daphnids have “condensed” embryos which expand and harden shortly after hatching. During this post-hatching expansion, defensive morphological traits can also be expanded. For example, the tail spine is soft and folded in *Daphnia* embryos, but already shows a rigid spike-like structure in the neonate *Daphnia* (Fig. 2k-l).

D. cucullata is a very small pelagic cladoceran*,* threatened in each instar by several invertebrate predators. Helmets offer protection during all life stages against *Cyclops*, *Leptodora* and *Chaoborus* (Agrawal et al. 1999, Laforsch and Tollrian, in press). However, the full extension of helmets in *D. cucullata* cannot be developed within one molt (Fig. 1o). Hence, it is necessary for neonates of *D. cucullata* to form defenses before the moment of "birth". Our experiments show that embryos of the last developmental stage raised in predator-conditioned medium developed significantly longer helmets than did control animals (Table 2). Embryos of *D. cucullata* already show a developed helmet structure while inside the brood pouch of the mother (Fig. 1a). However, these helmets are still moderate in size.

Also in *D. pulex*, early juvenile instars have to defend against *Chaoborus* predation. Neck-teeth are formed as protective device in many clones predominantly in the second instar (e.g. Krueger and Dodson 1981, Havel 1985, Tollrian 1993). During this life stage *D. pulex* is most vulnerable to *Chaoborus* predation (Tollrian 1995) because phantom-midge larvae are size-selective predators (Pastorok 1981). Our measurements in this species revealed significantly longer traits already in the embryonic stage (Table 2). We observed "neck-pedestals", which are the base for the formation of small spikes. However, we did not detect the formation of these spikes during embryonic development. These pointed spikes could possibly interfere mechanically with safety inside the brood pouch. Tollrian (unpublished data) demonstrated a time delay in *D. pulex* of one instar between the sensitivity to the kairomone and the exhibition of the protective trait. Therefore, if an embryo is warned by the odor of a predator, it seems already to prepare itself while inside the mother's brood pouch, shortly before being released, but without developing its spike-like defensive trait already inside the brood pouch (Fig. 1n).

In *D. lumholtzi*, later instars need to defend against fish predation. They therefore require long-featured feeding deterrents as defenses. Again these long structures need several instars to grow and therefore have to be initiated in the embryonic stages. As an adaptation to the embryonic environment, helmets and fornices are completely folded and attached to the head in both predator and non-predator exposed embryos (Fig. 1i, j). Two advantages to this strategy can be hypothesized according to the limited space of the brood pouch. First, the attached thorn-like helmets and fornices save space, and second, it prevents harm to their conspecifics caused by movements within the brood pouch. In this species we would have expected much higher helmets in kairomone-induced as compared to non-kairomone-induced embryos, because predator-exposed neonates already show relatively high helmets (Fig. 1m, Tollrian 1994). It seems that the cuticle of the helmet in embryos of *D. lumholtzi* is very elastic. The final length of the helmet can only be observed after the absorption of water into the body during the rapid post-embryonic expansion. This expansion of the embryo was named “pseudomolt” in the investigation of Parjeko (1992) into the embryology of *D. pulex.* However*,* Kotov and Boikova (2001) described that *D. galeata* embryos have to peel four different membranes before being released as neonates. This is in contrast to Berill and Henderson (1971), who concluded that *D. pulex* has to shed only three membranes prior to birth. Although we failed to show a fourth membrane in all other SEM-examined *Daphnia* species, possibly due to the short time span in which this layer is detectable, it seems that the elastic helmet of *D. lumholtzi* is covered by a very thin membrane (Fig. 1k) as postulated by Kotov (1997) and Kotov and Boikova (2001). This membrane could explain the rapid alteration in the morphology of the young-borne daphnids. Before birth, the helmet of *D. lumholtzi* is still tightened by the membrane which possibly covers the entire body. During the release from the brood pouch, the thin fourth membrane seems to burst and the exfoliated helmet and tail spine can achieve their final form.

In *D. ambigua*, which usually shows its spike-like helmets in the second and third juvenile instars (Hanazato 1990), we could not detect a visible morphological difference in the last embryonic stage between daphnids exposed to predator-released chemicals and control daphnids. It seems that these helmets, as well as neck-teeth spikes in *D. pulex*, can be developed within one molt, with the exception that no morphological base of the spike could be seen in the *D. ambigua* embryos (Fig. 1e).

It is not surprising, that we could not observe any morphological difference in the fifth embryo stage in *D. longicephala* treated with and without predator cues from the predatory freshwater bug *Notonecta,* because *D. longicephala* generates its huge crests only in late juvenile and adult instars (Fig. 1p; Grant and Bayly 1981). The notonectid predators have a size preference for larger prey and thus small juvenile daphnids are relatively safe. This allows this species to form “undefended” embryos and to postpone the formation of the huge crests to later juvenile stages.

The initiation of defensive traits needs a functioning receptor system whose development is ontogenetically constrained to later embryonic stages unless there is already a transgenerational induction via the mother (Agrawal et al 1999). We suppose, considering our SEM-investigations, that the strong egg membrane is not permeable to kairomones and consequently in the early developmental stages it is not likely that the cells are able to detect the odor of predators. The different appearance of the egg surface in our SEM-investigations is likely due to drying artifacts, when we compare the results with light microscopy techniques. After hatching from the egg, the embryo is still covered by two membranes (Fig. 2e-h). Both membranes are perhaps still barriers to the predator-released chemicals. But we cannot exclude that environmental chemicals can be received through the dorsal organ of the daphnids, which is already established at the first embryonic stage (Fig. 2f, Kotov and Baikova 2001). Rudiments of the first antennae, which contain chemosensillae in adult daphnids, are already visible under both membranes. These membranes burst at the ventral part of the body due to movement of the second antennae. After shedding off both membranes, the sensillae are visible at the tip of the first antennae (Fig. 2i). It seems possible that the embryos are able to smell predator-released chemicals from this stage on. However, it is likely that the internal development of the neuro-secretory system is not yet completed in this stage and therefore not able to transmit and process the total information gathered. The entire development of the nervous system and glands is finished shortly before the release from the brood pouch of the mother. At this time period *Daphnia* embryos are most sensitive to predator-released cues (Hanazato 1991 in *D. galeata*, Tollrian 1993 in *D. pulex*).

The five *Daphnia* species in our study differed in the appearances of their inducible traits (e.g., helmets, neckteeth and crests) which have evolved under the different selective forces of different predators and different environments. Our study shows how *Daphnia* found solutions to master the trade-offs between environmental requirements to form defenses and developmental constraints regarding the formation of large traits.

ACKNOWLEDGEMENTS

We thank W. Gabriel for support and discussion; Andrea Ganal and M. Kredler for help during experiments; and Scott Stevens for linguistic improvements.

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Table1

Mean relative trait length (trait size divided by body size x 100) and standard error (SE) of different adult *Daphnia* species raised with (induced) and without (control) chemical cues released from predators. In *D. pulex* neck-teeth quantification on the second instar is shown (%). Treatments were compared using Mann – Whitney U – tests.

		control		induced		
Species	trait (adult)	Mean ± SE	*n*	Mean ± SE	*n*	*p*
D. lumholtzi	helmet	06.69 ± 0.20	62	43.97 ± 0.83	53	< 0.001
D. cucullata	helmet	14.14 ± 0.24	68	54.22 ± 0.79	61	< 0.001
D. ambigua	helmet	05.16 ± 0.19	50	13.36 ± 0.71	6	< 0.001
D. longicephala	crest	38.80 ± 0.42	56	71.84 ± 1.09	43	< 0.001
D. pulex	neck-teeth	0.0	30	74.00 ± 2.97	30	< 0.001

Table 2

Mean relative trait length (trait size divided by body size x 100) and standard error (SE) of the last embryonic stage of *D. cucullata* and *D. pulex* raised with (induced) and without (control) chemical cues released from predators. Treatments were compared using Mann – Whitney U – tests.

		control		induced		
species	trait (embryo)	Mean ± SE	*n*	Mean ± SE	*n*	*p*
D. cucullata	helmet	15.84 ± 0.39	41	20.41 ± 0.59	35	< 0.001
D. pulex	neck-pedestal	35.14 ± 0.55	29	37,02 ± 0.51	50	= 0.02

Figure legend

Figure 1

Scanning electron micrographs of the head shape of predator exposed (left hand) and non-predator exposed (right hand) daphnids in the last embryonic stage (scale bar). a,b) *D. cucullata,* (20 µm); c,d) *D. pulex*, (30 µm); e,f) *D. ambigua,* (20 µm); g,h) *D. longicephala,* (30 µm); i,j) *D. lumholtzi,* (20 µm); k) helmet of *D. lumholtzi* prior to birth, (20 µm); l) helmet of *D. lumholtzi* during the rapid development from embryo to neonate*;* m) helmet of adult *D. lumholtzi*; n) neck teeth of D. pulex; o) adult of *D. cucullata*; p) adult of *D. longicephala;* abbreviations: fc = fornices; hl = helmet; me IV = fourth embryonic membrane; nt = neck teeth; np = neck pedestal.

Figure 2

SEM-images of embryological development in *Daphnia* (scale bar). a-c) different egg morphology of *D. pulex* (20 µm); d) shedding of *D. cucullata* from the egg membrane (20 µm); e) first embryonic stage of *D. pulex*; the entire body is still covered with a membrane (30 µm); f) dorsal view of the first embryonic stage of *D. ambigua*; the dorsal organ is already visible (10 µm); g,h) second embryonic stage of *D. pulex*; the embryo peels the second and third membrane almost simultaneously (20 µm); I) third embryonic stage of *D. pulex*; chemo-sensillae are already observable at the tip of the first antenna and remnants of the third membrane still cover predominantly the post abdomen (30 µm); j) fourth embryonic stage of *D. pulex* (20 µm); k,l) embryo before birth (20 µm) and complete developed neonate (100 µm) of *D. pulex*; abbreviations: a I = first antenna; a II = second antenna; cs = chemo-sensillae; do = dorsal organ; lb = labrum; md =mandible;

me I – IV = embryonic membranes; mx = maxillae; pd = post abdomen; seg = segmentation of the embryo.

Figure 1

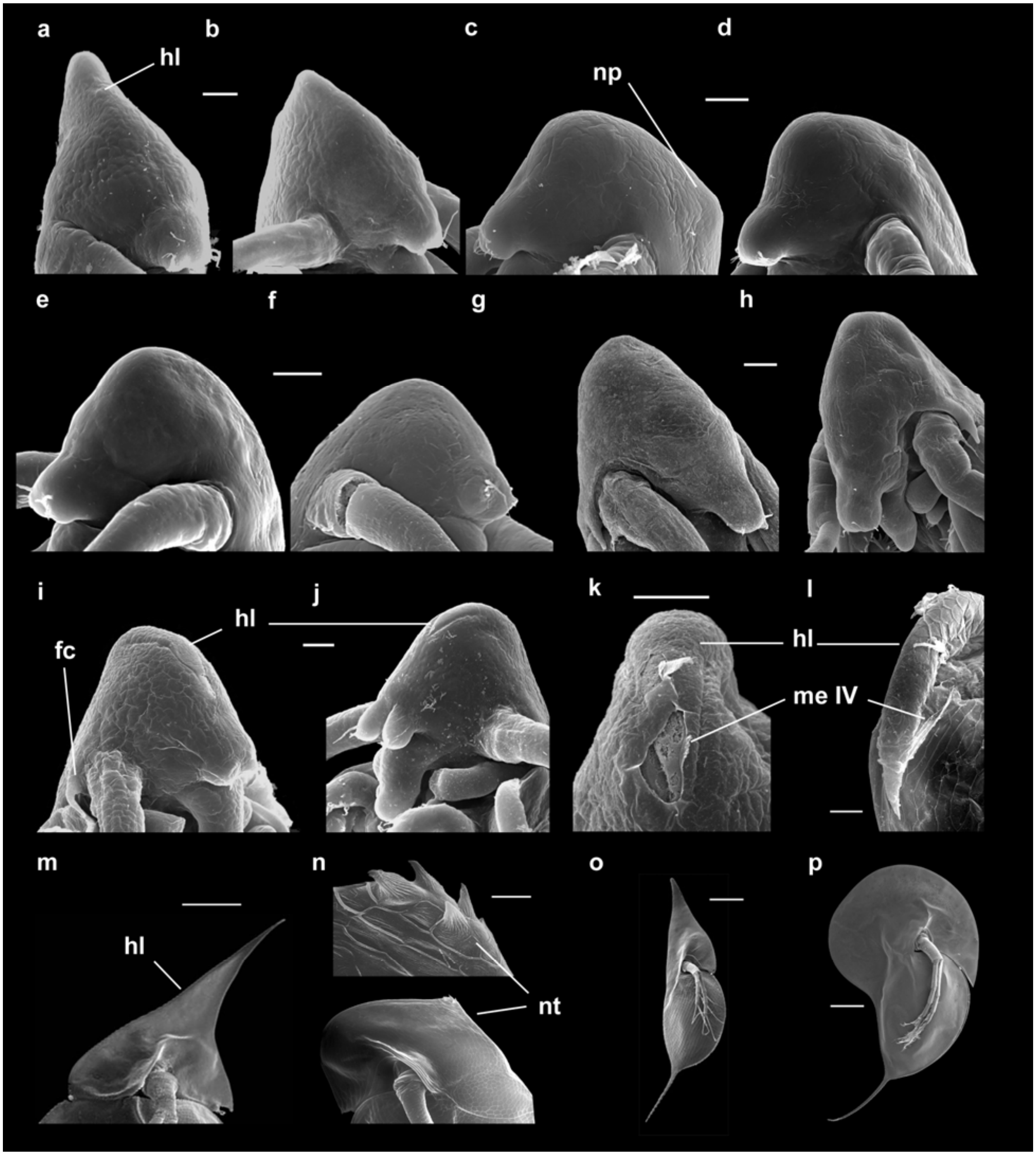
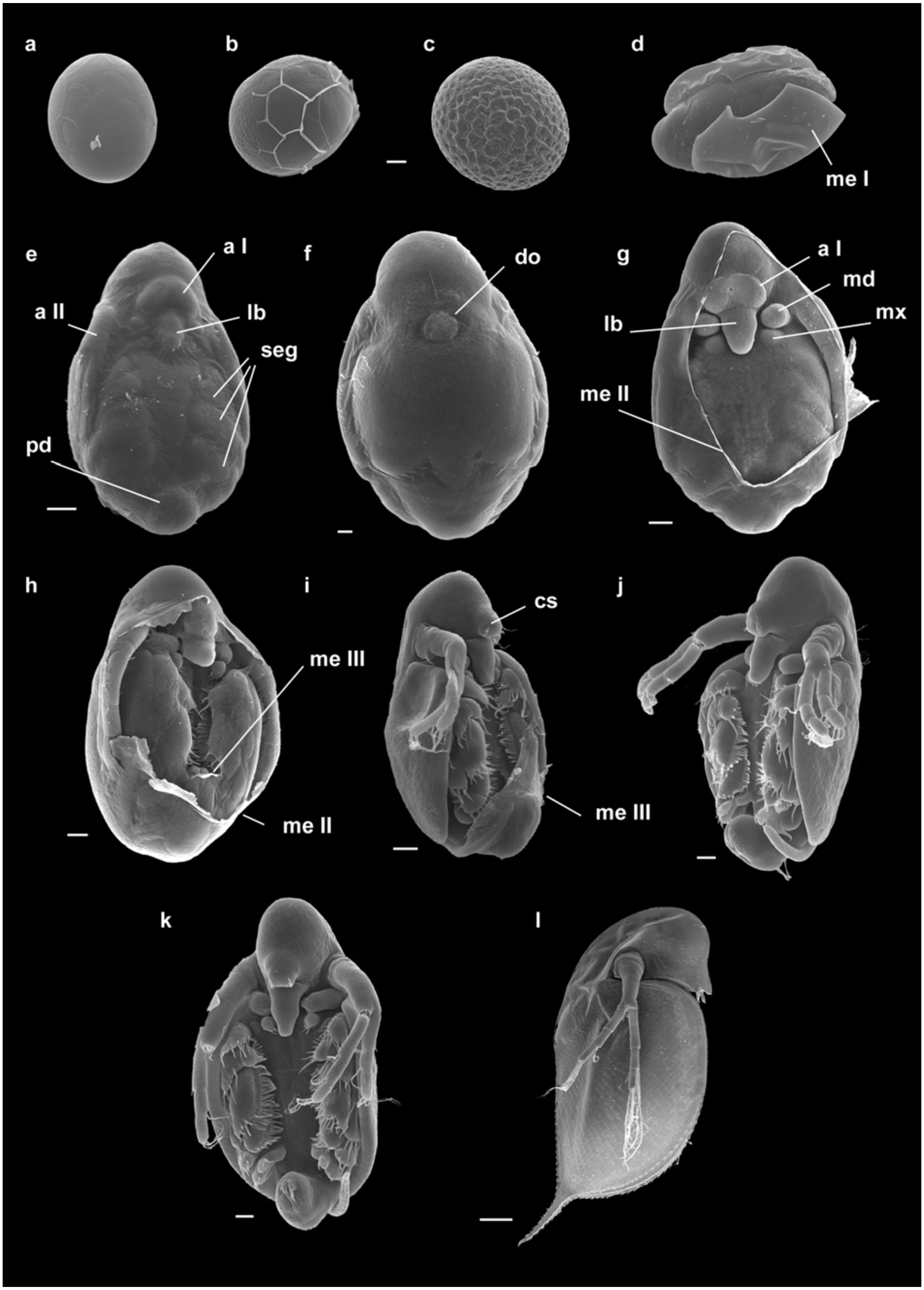


Figure 2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BEYOND THE TIP OF THE ICEBERG – A UNIQUE ACOUSTIC MICROSCOPY TECHNIQUE REVEALS HIDDEN MORPHOLOGICAL DEFENCES (Laforsch, Ngwa, Grill und Tollrian, *Nature*, eingereicht)

Beyond the ‘tip of the iceberg’ – a unique acoustic microscope reveals hidden morphological defences

Christian Laforsch *, Wilfred Ngwa #, Wolfgang Grill # & Ralph Tollrian *

** Ludwig-Maximilians-University München, Department Biology II, Section of Evolutionary Ecology, Karlstr. 25, D-80333 München (Germany)*

 # University of Leipzig, Institute for Experimental Physics II, Linnéstr. 5, D-04103 Leipzig (Germany)

Inducible defences are strategies for coping with predation in heterogeneous environments[1, 2]. In recent years the conspicuous and often dramatic morphological plasticity of several waterflea-species of the genus *Daphnia*[3-5] has been found to be activated by chemical cues released by predators. Even some minute induced traits proved to be protective against invertebrate predators, while the exact defensive mechanisms remained mysterious. Therefore, it has been suggested that the visible morphological changes are only the ‘tip of the iceberg’ of the entire protective mechanisms. In an interdisciplinary project, we applied the new method of ultrasonic microscopy with vector contrast at 1.2 GHz to probe hidden morphological defences. Here we show that induction with predator kairomones increases the stability of the carapace in two *Daphnia* species 2-3.5 –fold. This provides a major advantage for the induced morphs because invertebrate predators need to crush or puncture the carapace of their prey. Ultrasonic microscopy is a powerful tool for uncovering hidden morphological plasticity. Our result suggests that the classification in defended and undefended genotypes in *Daphnia,* and possibly in other prey organisms, should be reconsidered.

Predation is a decisive factor of natural selection [6]. In both animals and plants, different defensive mechanisms have evolved. Inducible defences have been frequently found in almost every biological community. They enable prey organisms to express a particular defence only if a reliable cue for a future attack is present[7-11]. Thereby, the organisms can minimize costs affiliated with the formation of a defence, when predation risk is low. Several *Daphnia* species form inducible defences in response to chemical cues from predators (kairomones). Although some induced morphological changes in *Daphnia* are extremely tiny they have been shown to reduce predator caused mortality [5]. Despite intense research, the exact defensive mechanisms remain unresolved. Because many invertebrate predators possess specialized mouthparts, the “anti-lock-and-key hypothesis” had been formulated [12], to state that the morphological features render the prey incompatible to the predator’s mouthparts. Other researchers suggested that the visible morphological changes are just “the tip of the iceberg” of protection and that hidden changes may be partly responsible for the defensive effects [13]. Furthermore, it has been shown that several traits may act synergistically to form a defence [4]. Similarly, changes in life history and behaviour[14, 15]can act as defences. In contrast to the more obvious traits, sparse work has been done on the armour of the animals, the carapace [16].We aimed to test for increased stability of the carapace of *Daphnia pulex* and *Daphnia cucullata* exposed to water soluble chemicals released by the predacious phantom midge larvae. We applied acoustic microscopy in this study for the first time to solve ecologically relevant questions.

Acoustic microscopy allows to image, with microscopic resolution, the interaction of acoustic waves with the mechanical properties of a sample. A diffraction-limited focus is formed on the sample by a lens, which facilitates transmission of the monochromatic acoustical signal and the receiving of the reflected signal. The final image is formed, by scanning the lens mechanically in a plane parallel to the sample surface. The acoustic wave is modulated by the surface of the sample and the reflected waves contain the specific information. Detailed theory is available to relate the elastic (mechanical) properties of the surface to the contrast, and this enables informed interpretation of the acoustic images to be made [17]. This modus operandi provides unique possibilities for probing mechanical properties of biological specimens, e.g., cells and cell compounds [18, 19]. Crucial advantages of acoustic microscopy are that it is nondestructive and the results are not altered by specific preparation methods such as embedding or sectioning. Furthermore, phase-sensitive acoustic microscopy (PSAM), which is employed in this study, presents singular advantages over conventional scanning acoustic microscopy (SAM). Three-dimensional scanning permits the acquisition of three-dimensional amplitude and phase images concomitantly [20]. The phase images contain additional sample information, and also allow for sundry image processing possibilities not possible with SAM. From the three-dimensional images, the amplitude of the signal reflected under focal conditions can be determined, and the sample's surface topography can be derived and corrected for tilt from the simultaneously acquired phase image with nanometer resolution.

For both *Daphnia pulex* and *Daphnia cucullata*, a PSAM probe of the mechanical properties (bending strength, tensile strength, hardness) of the carapace revealed that the mean reflectivity for the predator-kairomone-induced samples was significantly higher than in the non-induced samples (Fig.1a; *D. pulex*: $t = 10.77$; d.f. = 40; $p < 0.001$; *D. cucullata*: $t = 3.34$; d.f. = 27; $p = 0.002$). Higher reflectivity is tantamount to higher acoustic impedance of the carapace. The acoustic impedance of a material is defined as the product of density and acoustic velocity of that material. It is important in the determination of acoustic transmission and reflection at the boundary of two materials having different acoustic impedances (in this case *Daphnia* carapace and the coupling medium). Thus, it is a measure of material "hardness" and describes its ability to withstand penetration by a surface contact, which in our case could be the mandibles of a predator. In *Daphnia pulex* reflectivity of the induced morphs was found to be on average 3.5 fold greater than that of the non-induced samples, while in *Daphnia cucullata* it is doubled (Fig.1a). This strengthening of the carapace could explain the high escape efficiencies for induced specimens even after being caught by the phantom midge larvae [21, 22]. Although the mandibles of the predator are rigid structures, by virtue of a more than two times harder surface it requires much more strength to penetrate the armour. Moreover, the entire catching apparatus of many invertebrate predators consists of fine cuticular structures and the harder armour results in more endeavour or prevention to fold the prey's carapace. To reveal the background of the higher stability, we conducted an ultrstructural analysis. Semi-thin-sections of the carapace of *Daphnia cucullata* showed that predator-exposed animals had significantly thicker carapaces than control animals raised without chemical cues (Fig. 2a; t = -12.5, d.f.= 532, $p < 0.001$). The carapace of *Daphnia* consists of two epidermal layers connected by small pillars (Fig. 2d). The average diameter of the pillars from *Daphnia cucullata* exposed to predator kairomones was twice as large as in the control animals (Fig. 2c; nested ANOVA; d.f.= 23; F = 204.1; $p < 0.001$). This result implies that a fortification of the carapace is realized with low material expenditure. The extensions of the pillars, which are also built of small fibres (Fig. 2d), strengthen the mechanical stability of the whole carapace but yet are a "lightweight" construction. The evolutionary arms race between predator and prey drives the establishment of cost saving but effective defence mechanisms. Investments in a lightweight architecture which result in a 2 - 3.5-fold increased stability, provide an enormous advantage. They guarantee an effective defence while minimizing material investment, similar to recent results from diatoms [23].

Our finding that *Daphnia* possess "hidden" defensive traits may explain the paradox that induced but seemingly undefended daphnids still reduce their vulnerability to predators, which up to now has been attributed to the existence of yet-unidentified behavioural defences [24].

Our results indicate that induced daphnids, which have strengthened armour, are physically better protected against mechanical challenges. This likely provides the crucial advantage in struggling out of the predator's catching apparatus during an attack. While our study questions the current paradigm for the defensive mechanism, the 'anti-lock-and-key hypothesis', it does not rule out that both effects, incompatibility with the predator mouthparts and increased mechanical stability, can act synergistically. Although we have tested only the armour of two *Daphnia* species, we expect this hidden morphological plasticity to be common in *Daphnia* species threatened by small invertebrate predators and possibly in other prey organisms as well. Our study highlights the relevance of interdisciplinary projects where new physical methods lead to new insights into biological questions.

Methods

Induction treatment

Single clones of *Daphnia pulex* and *Daphnia cucullata* were used for our experiments. The laboratory-cultured animals were fed at unlimited food conditions (1.5 mg C/L) with *Scenedesmus obliquus* daily. All experiments were conducted in the laboratory under constant conditions at 20 °C and fluorescent light in a synthetic medium[22]. Daphnids were age-synchronized by collecting mothers with freshly deposited eggs. The third brood of these mothers was placed in the experimental jars.

50 *Daphnia pulex* were placed separately into each chamber of tissue plates containing 5 mL of artificial medium. Predator kairomone from *Chaoborus flavicans* (15 larvae / L) was added daily into each of the 25 chambers of the induction treatment. The rest of the chambers served as control. After the release of the first brood, the mothers were removed from the chambers. The young daphnids underwent one more molt before they were fixed in 70%-EtOH p.a. in the second juvenile stage. During this life stage *Daphnia pulex* generates most pronounced neck-teeth. Predator-kairomone-induced daphnids were checked under a microscope for neck-teeth induction. 15 *Daphnia cucullata* were introduced into each of 10 glass vessels containing 1 L of artificial medium. Helmet elongation of *Daphnia cucullata* was induced by using small-scale turbulence and predator kairomone from *Chaoborus flavicans* (15 larvae / L) simultaneously to ensure a strong induction effect. Five vessels served as the control. Adult Daphnids were removed after the birth of the first generation. Daphnids were fixed in 70%-EtOH (p.a) and stored in 50 mL glass jars after having reached maturity.

Acoustic microscopy

Prior to ultrasonic microscopy, the daphnids were also fixed with 70%-methanol for one hour on ice. Afterwards the daphnids were washed three times in artificial medium. A single randomly selected *Daphnia* was placed with a Pasteur-pipette onto a glass cover slide (ø 10 mm). A small drop of Histoacryl was applied with a dissecting needle onto the dry surface of the cover slide, because the glue polymerizes when getting in contact with water. The daphnid was attached to the glue by gently moving its body (still covered with medium) into the drop using an eyelash fixed upon a toothpick. The cover slide was mounted with double sticky tape onto the scanner.

The transducer of the phase-sensitive acoustic microscope (PSAM) [25], acting in this case like a light-sensitive receptor and coherent detector, converts the signal reflected from the sample to an electromagnetic signal. The signal is processed in a quadrature-detection scheme [25] resulting in two low-frequency signals, which are both digitized and stored (with 12-bit conversion) for each pixel of the image by a 2-channel image-processing unit. The amplitude and phases are calculated point-by-point from these data, using Pythagorean theorem and the inverse tangent. The amplitude of the reflected pulse is proportional to the acoustic reflectivity of the object at the point being investigated. The image slices obtained by three-dimensional scanning were utilized for deriving a maximum-amplitude image representing the amplitude as being at the focus. In this study, the reflectivity R_{dB} in decibels (dB) is given by: $R_{dB} = 20\log(x/k)$. Hereby, x is the average in focus amplitude of the reflected signal from a region of interest (ROI) on the carapace. The constant k = 2896.3 is the maximum amplitude obtainable from the two-frame 12-bit data acquisition. At 1.2 GHz, the resolution is about 1 micrometer. The coupling medium was composed of: 1 part aqua bidest, 2 parts tap water, SMB (salt medium for Blepharisma) 10ml/l, sea salt solution 1,5 µl/l, SeO2 100 µl/l.

Light microscopy and digital data analysis

Predator- and non-predator-exposed *Daphnia cucullata* were embedded in Durcupan and 4µm semi-thin-sections were made in horizontal alignment using a microtome. The thickness of the carapace was measured with a digital image analysis system (AnalySIS PRO; Soft Imaging System, Münster) under a light microscope at three points of the carapace in the region of the pericard (Fig. 2b). Data of control and induced animals were compared using t-tests.

In the second experiment, previously fixed animals of predator and non-predator induced *Daphnia cucullata* were stained with Hämotoxylin. A region of interest (ROI) was defined under the light microscope and the pillar diameters were analyzed in both treatments, induced and control, inside these ROI with the digital image analysis system. Each *Daphnia* was handled as replicate. Data were tested for normal distribution and homogeneity of variances. A nested ANOVA was then performed to test for treatment effects using treatment as fixed factor and replicate as random factor.

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Acknowledgements

We thank W. Gabriel for encouragement and discussion; M. Kredler for help during experiments and Scott Stevens for linguistic improvements.

Correspondence and requests for materials should be addressed to C.L. (email: laforsch@zi.biologie.uni-muenchen.de).

Figure 1 (**a**) Mean reflectivity (measurement of the strength of a material) of the carapace of *Daphnia cucullata* and *Daphnia pulex*. Plastic responses of both *Daphnia* species were induced by water-soluble chemicals released by the predacious phantom-midge larvae. Asterisks indicate significant differences to the control (*** = $p < 0.001$; ** = $p < 0.01$). (**b**) 1.2 GHz PSAM amplitude image of the carapace of *Daphnia pulex* (140 x 150 μm^2).

Figure 2 (**a**) Mean carapace thickness of *Daphnia cucullata* exposed to chemical signals released from *Chaoborus flavicans* (induced) compared to control animals. Asterisks indicate significant differences to the control (*** = $p < 0.001$). (**b**) Measurements were standardized by analyzing thickness of the armour at three defined points (indicated by arrows) in a semi-thin-section in the region of the heart (H) in each daphnid. (**c**) Mean pillar diameter of predator (induced) and non-predator exposed (control) *Daphnia cucullata*. Asterisks indicate significant differences to the control (*** = $p < 0.001$). (**d**) Scanning electron micrograph showing the lightweight construction of the armour. The pillars (arrow) connect the outer and inner carapace layer.

Figure 3 (**a**) Scanning electron micrograph (SEM) of the second juvenile stage of a predator-exposed *Daphnia pulex* (preparation [25]). The development of neck-teeth was speculated to be the “tip of an iceberg” of a defence. (**b**) SEM image of an expanded feeding basket of *Chaoborus flavicans*.

Figure 1

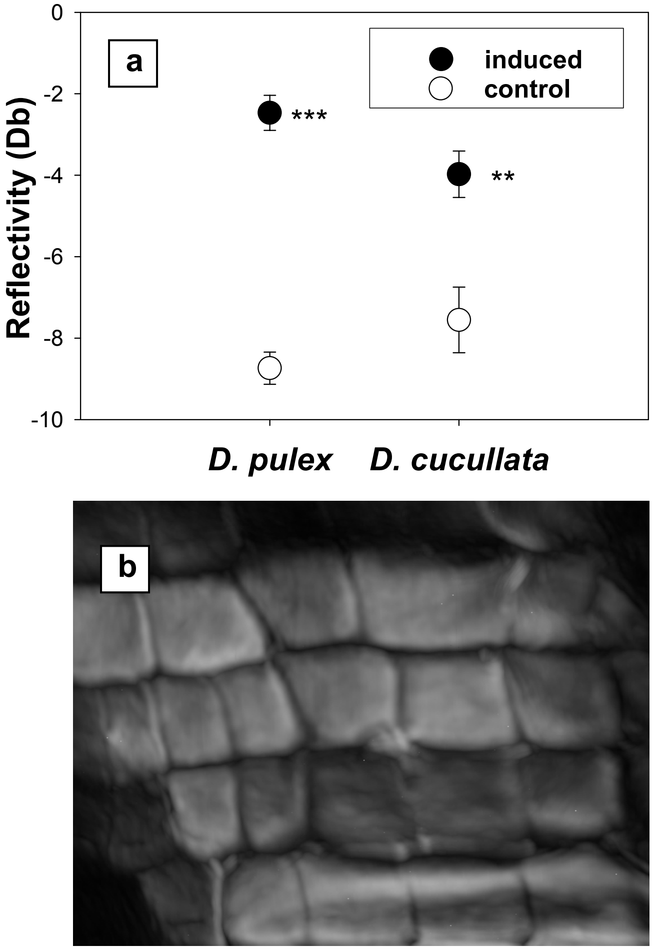

a
● induced
○ control
Reflectivity (Db)
0
-2
-4
-6
-8
-10
***
**
D. pulex
D. cucullata
b

Figure 2

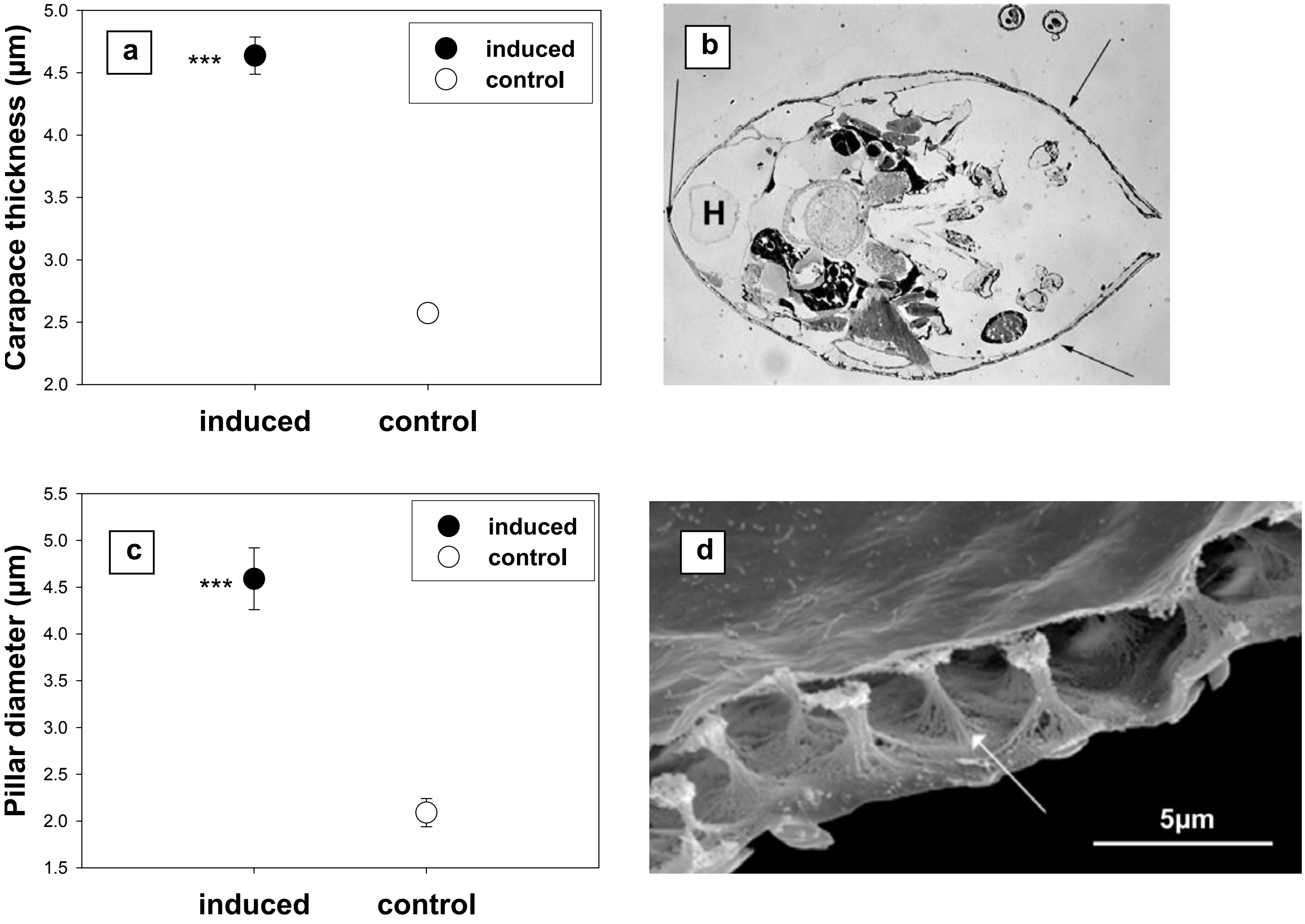
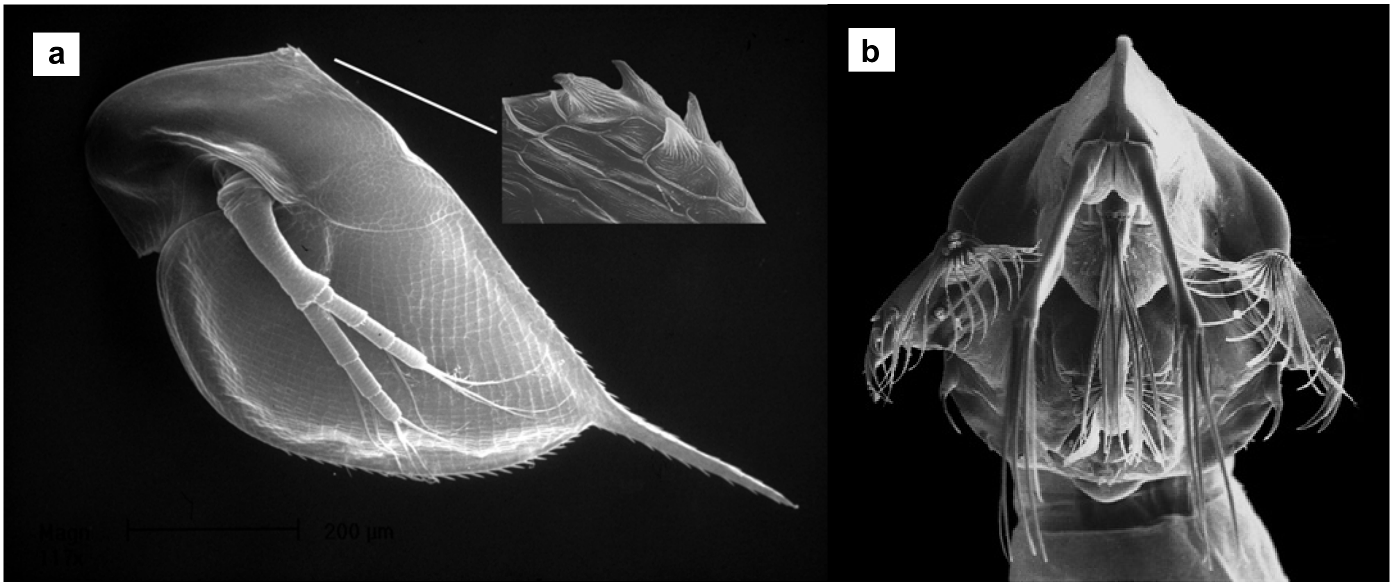

a
***
induced
control
Carapace thickness (µm)
5.0
4.5
4.0
3.5
3.0
2.5
2.0
induced
control
b
H
c
***
induced
control
Pillar diameter (µm)
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
induced
control
d
5µm

Figure 3



Danksagung

In erster Linie möchte ich meinem Betreuer und Doktorvater PD Dr. Ralph Tollrian für die äußerst kreative und immer freundschaftliche Arbeitsatmosphäre danken. Er hat mir den nötigen Freiraum gegeben meine eigenen Ideen zu verwirklichen, nahm sich dabei immer Zeit für anregende Diskussionen und hat mich jederzeit auf meinem Weg unterstützt.

Frau Mechthild Kredler hatte sehr großen Anteil daran, dass ich die Zeit meiner Promotion wahrlich genossen habe, da nicht nur die wissenschaftlichen Diskussionen und die gemeinsame Arbeit äußerst fruchtbar waren, sondern ich auch das freundschaftliche Verhältnis, gepaart mit viel Humor, angebrachter Kritik und viel Verständnis nicht missen möchte. (die Gespräche über unsere zahlreichen „Zipperlein“ werde ich wohl *nicht wirklich* vergessen)

Herr Professor Dr. Wilfried Gabriel hat mir ermöglicht meine Arbeit in seiner Arbeitsgruppe durchzuführen und hat mich dabei jederzeit unterstützt.

Weiterhin möchte ich Mitarbeitern der Ökologie und Zoologie für Ihre Hilfe bei meinen oft zahlreichen Fragen danken: Prof. Dr. Sebastian Diehl, Dr. Beate Nürnberger, Dr. Sabine Giessler; Dr. Roland Melzer; Dr. Herwig Stibor.

Der Arbeitsgruppe am Institut für experimentelle Physik II der Universität Leipzig für die Integration und „fruchtbare“ Zusammenarbeit: Prof. Dr. Wolfgang Grill, Dr. Zbigniew Kojro, Dr. Reinhold Wannemacher; Dr. Jens Jahny; Wilfred Ngwa

Jetzt wird es schwierig und ich hoffe ich habe nur wenige vergessen:
Ich möchte mich bei allen für Ihre Hilfe (füttern ect. ect.) und vor allem auch die sehr angenehme Arbeitsatmosphäre bedanken (es war eine wirklich tolle Zeit- auch wenn meine Luxuskarosse ein paar Schrammen abgekriegt hat) – ich denke der Grund meines Dankes ist euch (hoffentlich) allen geläufig:
Laura Beccara, Jens Bohn, Christian Brossmann, Peter Brunner, Andreas Buckenmeier, Ina Engel, Simone Erker, Andrea Ganal, Sybille Glöckl, Erika Hochmut, Andrea Hohner, Kathrin Huber, Stephan Hutter, Carin Jantzen, Jonathan Jeschke, Ulf Kausch, Thomas Knebelsberger, Alexander Leitner, Claudia Lemcke, Elke Meier, Michael Meisle, Elena Ossipova, Ursula Puskartz, Gertraud Schmidt, Kathrin Stein, Scott Stevens;

Meine Eltern, die mich auf meinem Weg immer liebevoll unterstützt haben.

Meiner Schwester Isabella Laforsch die jederzeit für mich da war wenn ich Ihre Hilfe benötigte und mich immer auf meinem Weg bestärkt hat.

Last but not least – meiner Lebensgefährtin Petra Kraus, die mir immer (auch im wahrsten Sinne des Wortes) den Rücken stärkte, für die nötigen Reibungspunkte zur Beflügelung meiner Kreativität sorgte und mich nicht nur wissenschaftlich inspirierte (ich hoffe Du kannst jetzt mein Grinsen sehen…)

Lebenslauf

Name:	Christian Laforsch
Geburtstag:	28.03.1968 in München
Abitur:	
1988	Dante Gymnasium München
Zivildienst:	
1989-1991	Nachbarschaft Neuhadern e. V.
Studium der Biologie:	
1991-1999	Ludwig-Maximilians-Universität München
Diplom	
1999	Fächer: Zoologie, Ökologie, Wildbiologie und Botanik; Abschlussnote von 1,0.
Promotion:	
1999 -2003	Ludwig-Maximilians-Universität München; Department Biologie II
Gastwissenschaftler:	
Seit Mai 2003	Universität Leipzig; Institut für experimentelle Physik II

Wissenschaftliche Zusatzqualifikationen:

Betreuer des meeresbiologischen Praktikums „Ökologie der Korallenriffe“ (je 2 Wo.; inklusive Tauchgruppenführung), Sinai, Ägypten; 1997-2003, mit Lehrauftrag (seit 2003)

Hilfswissenschaftliche Tätigkeit am Forschungsvorhaben 20402344 des Umweltbundesamtes: Ökologische Bewertung von UV-B Strahlung mittels Bioindikatoren; 1998

Auftragsarbeit der Wildbiologischen Gesellschaft München (WGM); 1999
Analyse des Rotwildbestandes im Odenwald

Co-Betreuung der Diplomarbeiten von Laura Beccara und Kathrin Huber; der Zulassungsarbeiten für das Staatsexamen von Andrea Ganal und Peter Brunner; 1999-2003.

Betreuung diverser Laborpraktika mit Vortragstätigkeit während des Zeitraums meiner Promotion am Department Biologie II; 1999-2003.

Organisation des „7`th Meeting of PhD Students in Evolutionary Biology“
20.3. - 25.3.2001 Bernried, Starnberg;
Das Meeting wurde von der *European Science Foundation* (ESF), dem *Bayerischen Staatsministerium für Wissenschaft, Forschung und Kunst* und namhaften Unternehmen (Smith-Kline-Beecham; BMW ect.) gefördert. Weiterhin wurden anerkannte Professoren für die einzelnen Fachgebiete als Diskussionsleiter geladen.

Betreuer der Exkursion „Hopavagen“ (2 Wo.), Norwegen; 2001

Mitglied der Deutschen Zoologischen Gesellschaft seit 2001

Dozent im ökologischen Bildungszentrum München (ÖBZ); 2001-2002

Hilfswissenschaftliche Tätigkeit (taucherische Kartierung und Probenentnahme) im DFG Projekt „Strategie von Reproduktion und Wachstum bei der Steinkoralle *Seriatopora hystrix*: Populationsgenetische Analysen einer 'unkonventionellen' life history“ auf den Forschungsstationen Heron Island und Lizard Island, Great Barrier Riff, Australien; November- Dezember 2002

Lehrauftrag für das meeresbiologische Praktikum/Exkursion Sardinien (je 2 Wo.; inklusive Tauchgruppenführung), Italien; 2002-2003

Leitung der tropischen Korallenaquariumanlage am Department Biologie II (seit 1999)

Erstellung und Betreuung der Internetseite der Abteilung Evolutionsökologie am Zoologischen Institut der LMU – München (seit 1999)

Preise und wissenschaftliche Verwendung meines Bildmaterials :

Current Biology; 1999, R3
Spektrum der Wissenschaft;11/1999
Transkript; 9/1999
Bild des Monats; Bild der Wissenschaft;8/2001
Science magazine; Vol 294 Oct. 2001
BBC wildlife; 2002,20 (9) pp7
Lehrbuch „Ökologie“; Nentwig (ed) 2003
„Bildatlas Genetik“; Harri Deutsch Verlag, voraussichtlich 2004

Kongressteilnahme:

International conference of the academy of the National Park “Hohe Tauern”: „Return of wolf, bear and lynx“. Juni 1997; Matrei i. O., Österreich.

International Lynx-Symposium of the National Park “Bayerischer Wald”. November 1997; Deggendorf, Deutschland.

International Symposium on Cladocera; 14.-18. September 1999; Plön, Deutschland.

BioLog `99 1. Münchner Studentenkongress für Biowissenschaften; 20. November 1999.

6th Meeting of PhD Students in Evolutionary Biology; 15.-18. März 2000 ;Leuven, Belgien.

The Ecological Society of America, Annual Meeting; 05. -10. August 2000; Snowbird, Utah, USA.

BioLog 2000, 2.Münchner Studentenkongress für Biowissenschaften, 18. November 2000.

7th Meeting of PhD Students in Evolutionary Biology; 21.-25. März 2001; Bernried, Deutschland.

European Society of Evolutionary Biology, VII `th Congress; 20.-25. August 2001; Aarhus, Dänemark.

Tagung der Deutschen Zoologischen Gesellschaft (dzg); 4.-8. Juni 2001; Osnabrück, Deutschland.

Tagung der Deutschen Zoologischen Gesellschaft (dzg); 20.-24. Mai 2002; Halle, Deutschland.
American Society of Limnology and Oceanography, summer meeting; 10.-14. Juni 2002; Victoria, Canada.

8th Meeting of PhD Students in Evolutionary Biology; 28. August – 1. September 2002; Lohja, Finnland.

Tagung der Deutschen Zoologischen Gesellschaft (dzg); 9.-13. Juni 2003; Berlin, Deutschland.