Proteomic analysis of stress responses in *Daphnia*

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München 2015

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Dissertation

zur Erlangung des Doktorgrades

an der Fakultät für Biologie

der Ludwig–Maximilians–Universität München

vorgelegt von

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aus Berlin

München, den 16.11.2015

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Tag der mündlichen Prüfung: 12.04.2016

Contents

Summary

English Abstract

Organisms respond to changes in their environment affecting their physiological or ecological optimum by reactions called stress responses. These stress responses may enable the organism to survive by counteracting the consequences of the environmental change, the stressor, and usually consist of plastic alterations of traits related to physiology, behaviour, or morphology. In the ecological model species *Daphnia*, the waterflea, stressors like predators or parasites are known to have an important role in adaptive evolution and have been therefore studied in great detail. However, although various aspects of stress responses in *Daphnia* have been analysed, molecular mechanisms underlying these traits are not well understood so far. For studying unknown molecular mechanisms, untargeted 'omics' approaches are especially suitable, as they may identify undescribed key players and processes.

Recently, 'omics' approaches became available for *Daphnia*. *Daphnia* is a cosmopolitan distributed fresh water crustacean and has been in research focus for a long time because of its central role in the limnic food web. Furthermore, the responses of this organism to a variety of stressors have been intensively studied e.g. to hypoxic conditions, temperature changes, ecotoxicological relevant substances, parasites or predation. Of these environmental factors, especially predation and interactions with parasites have gained much attention, as both are known to have great influence on the structure of *Daphnia* populations.

In the work presented in this thesis, I characterised the stress responses of *Daphnia* using proteomic approaches. Proteomics is particularly well suited to analyse biological systems, as proteins are the main effector of nearly all biological processes. However, performing *Daphnia* proteomics is a challenging task due to high proteo-

lytic activity in the samples, which most probably originate from proteases located in the gut of *Daphnia*, and are not inhibited by proteomics standard sample preparation protocols. Therefore, before performing successful proteomic approaches, I had to optimise the sample preparation step to inhibit proteolytic activity in *Daphnia* samples. After succeeding with this task, I was able to analyse stress responses of *Daphnia* to well-studied stressors like predation and parasites. Furthermore, I studied their response to microgravity exposure, a stressor not well analysed in *Daphnia* so far.

My work on proteins involved in predator-induced phenotypic plasticity is described in chapter 2 and 3. *Daphnia* is a textbook example for this phenomenon and is known to show a multitude of inducible defences. For my analysis, I used the system of *Daphnia magna* and its predator *Triops cancriformis*. *D. magna* is known to change its morphology and to increase the stability of its carapace when exposed to the predator, which has been shown to serve as an efficient protection against *T. cancriformis* predation. In chapter 2, I used a proteomic approach to study predator-induced traits in late-stage *D. magna* embryos. *D. magna* neonates are known to be defended against *Triops* immediately after the release from the brood pouch, if mothers were exposed to the predator. Therefore, the formation of the defensive traits most probably occurs during embryonic development. Furthermore, embryos should have reduced protease abundances, as they do not feed inside the brood pouch until release. To study proteins differing in abundance between *D. magna* exposed to the predator and a control group, I applied a proteomic 2D-DIGE approach, which is a gel based method and therefore enables visual monitoring of protein sample quality. I found differences in traits directly associated with known defences like cuticle proteins and chitin-modifying enzymes most probably involved in carapace stability. In addition, enzymes of the energy metabolism and the yolk protein vitellogenin indicated al-

terations in energy demand. In chapter 3, I present a subsequent study supporting these results. Here, I analysed responses of adult *D. magna* to *Triops* predation at the proteome level using an optimised sample preparation procedure, which was able to generate adult protein samples thereby inhibiting proteolysis. Furthermore, I established a different proteomic approach using a mass-spectrometry based label-free quantification, in which I integrated additional genotypes of *D. magna* to create a more comprehensive analysis. With this approach, I was able to confirm the results of the embryo study, as similar biological processes indicated by cuticle proteins and vitellogenins were involved. Furthermore, additional calcium-binding cuticle proteins and chitin-modifying enzymes and proteins involved in other processes, e.g. protein biosynthesis, could be assigned. Interestingly, I also found evidence for proteins involved in a general or a genotype dependent response, with one genotype, which is known to share its habitat with *Triops*, showing the most distinct responses.

Genotype dependent changes in the proteome were also detectable in the study which I present in chapter 4. Here, I analysed molecular mechanisms underlying host-parasite interactions using the well characterised system of *D. magna* and the bacterial endoparasite *Pasteuria ramosa*. *P. ramosa* is known to castrate and kill their host and the infection success is known to depend strongly on the host's and the parasite's genotype. I applied a similar proteomic approach as in chapter 3 using label-free quantification, but contrastingly, I did not use whole animal samples but only the freshly shed cuticle. It has been shown, that the genotypic specificity of *P. ramosa* infection is related to the parasite's successful attachment to the cuticle of the host and is therefore most probably caused by differences in cuticle composition. Hence, I analysed exuvia proteomes of two different genotypes known to be either susceptible to *P. ramosa* or not. Furthermore, I compared exuvia proteomes of susceptible *Daphnia* exposed to *P. ramosa* to a control group for finding proteins involved in the

infection process and in the stress response of the host. The proteomes of the different genotypes showed indeed very interesting abundance alterations, connected either to cuticle proteins or matrix metalloproteinases (MMPs). Additionally, the cuticle proteins more abundant in the susceptible genotype showed a remarkable increase in predicted glycosylation sites, supporting the hypothesis that *P. ramosa* attaches to the host's cuticle by using surface collagen-like proteins to bind to glycosylated cuticle proteins. Most interestingly, in all replicates of the susceptible genotype exposed to *P. ramosa*, such a collagen-like protein was found in high abundances. Another group of proteins found in higher abundance in the non-susceptible genotype, the MMPs, are also connected to this topic, as they may have collagenolytic characteristics and therefore could interfere with parasite infection. Furthermore, the data indicate that parasite infection may lead to retarded moulting in *Daphnia*, as moulting is known to reduce the infection success.

Contrastingly to the work presented so far, the study described in chapter 5 investigated the protein response of *Daphnia* to a stressor not well studied on other levels, namely microgravity. As gravity is the only environmental parameter which has not changed since life on earth began, organisms usually do not encounter alterations of gravity on earth and cannot adapt to this kind of change. *Daphnia* has been part of one mission to space, however, responses of the animals to microgravity are not well described so far. In addition, as *Daphnia* are an interesting candidate organisms for aquatic modules of biological life support systems (BLSS), more information on their response to microgravity is necessary. For this reason, proteomics is an interesting approach, as biological processes not detectable at the morphological or physiological level may become apparent. Therefore, a ground-based method, a 2D-clinostat, was used to simulate microgravity, as studies under real microgravity conditions in space need high technical complexity and financial investment. Subsequently, a proteomic

2D-DIGE approach was applied to compare adult *Daphnia* exposed to microgravity to a control group. *Daphnia* showed a strong response to microgravity with abundance alterations in proteins related to the cytoskeleton, protein folding and energy metabolism. Most interestingly, this response is very similar to the reactions of a broad range of other organisms to microgravity exposure, indicating that the response to altered gravity conditions in *Daphnia* follows a general concept.

Altogether, the work of my thesis showed a variety of examples of how a proteomic approach may increase the knowledge on stress responses in an organisms not well-established in proteomics. I described both, the analysis of molecular mechanisms underlying well-known traits and the detection of proteins involved in a response not well characterised. Furthermore, I gave examples for highly genotype dependent and also more general stress responses. Therefore, this thesis improves our understanding of the interactions between genotype, phenotype and environment and, moreover, offers interesting starting points for studying the molecular mechanisms underlying stress responses of *Daphnia* in more detail.

Deutsche Zusammenfassung

Organismen reagieren auf Umweltbedingungen, welche ihr physiologisches und ökologisches Optimum verändern und so Stress auslösen können, mit einer Gegenreaktion, welche als Stressantwort bezeichnet wird. Diese Stressantwort ermöglicht das Überleben des Organismus, in dem sie der veränderten Umweltbedingung, dem Stressor, entgegenwirkt und besteht normalerweise aus plastischen Veränderungen im Bereich der Physiologie, des Verhaltens oder der Morphologie. Im ökologischen Modellorganismus *Daphnia*, dem Wasserfloh, spielen Stressoren wie Räuber oder Parasiten in der adaptiven Evolution eine wichtige Rolle und wurden deswegen intensiv untersucht. Obwohl verschiedenste Aspekte der Stressantworten von *Daphnia* analysiert wurden, weiß man noch sehr wenig über involvierte molekulare Mechanismen. Um unbekannte molekulare Mechanismen zu studieren sind sogenannte ‚Omics ' Verfahren besonders gut geeignet, da sie auch die Identifikation von vorher nicht identifizierten Akteuren und Schlüsselprozessen ermöglichen.

‚Omics ' Methoden können seit kurzem auch zur Analyse von *Daphnia* benutzt werden. *Daphnia* ist ein kosmopolitisch verbreitetes Krebstier des Süßwassers, welches aufgrund seiner zentralen Rolle in limnischen Nahrungsnetzen seit langer Zeit intensiv erforscht wird. Darüber hinaus wurden auch die Reaktionen dieser Organismen auf eine Vielzahl von Stressoren wie z.B. Hypoxie, Temperaturveränderungen, ökotoxikologisch relevante Substanzen, Parasiten oder Prädation detailliert beschrieben. Von diesen untersuchten Umweltbedingungen haben Parasiten und Räuber große Aufmerksamkeit erfahren, da sie einen sehr großen Einfluss auf *Daphnia* Populationen ausüben können.

In dieser Dissertation präsentiere ich meine Arbeiten zur Charakterisierung von Stressantworten bei *Daphnia* mit Hilfe proteomischer Methoden. Die Proteomik ist besonders gut für die Charakterisierung biologischer Systeme geeignet, da Proteine

die Haupteffektoren in fast allen biologischen Prozessen darstellen. Die proteomische Analyse von *Daphnia* ist jedoch mit einigen Schwierigkeiten verbunden, da Proteinproben von *Daphnia* eine sehr hohe proteolytische Aktivität aufweisen, die höchstwahrscheinlich von Proteasen aus dem Verdauungstrakt stammt und mit einer herkömmlichen proteomischen Probenvorbereitung nicht unterbunden werden kann. Deswegen wurde in einem ersten Schritt die Probenvorbereitung für *Daphnia* dahingehen optimiert, den proteolytischen Verdau einzudämmen. Nachdem dies gelungen, war konnte ich erfolgreiche proteomische Analysen durchführen, welche einerseits anderweitig schon gut charakterisierte Stressoren wie Parasiten und Räuber einschlossen und andererseits einen noch unbekannten Stressor untersuchten, die Auswirkungen von Schwerelosigkeit.

In Kapitel 2 und 3 beschreibe ich proteomische Studien zur Räuber-induzierten phänotypischen Plastizität bei *Daphnia*. *Daphnia* ist ein etablierter Versuchsrorganismus für dieses Phänomen, da diese Tiere eine Vielzahl von induzierbaren Verteidigungen zeigen. In den hier beschriebenen Studien analysierte ich das Räuber-Beute-System von *Daphnia magna* und *Triops cancriformis*. In Anwesenheit des Räubers *T. cancriformis* verändert *D. magna* ihre Morphologie und erhöht die Stabilität der Cuticula, was als eine effiziente Verteidigung gegen den Räuber fungiert. Kapitel 2 enthält eine proteomische Studie, in der ich Räuber-induzierten Merkmalen an *D. magna* Embryonen untersucht habe. Von *D. magna* Neonaten ist bekannt, dass sie, sollte ihre Mutter bereits dem Räuber ausgesetzt gewesen sein, schon beim Schlüpfen aus der Brutkammer Verteidigungen gegen *T. cancriformis* zeigen, was für eine Entwicklung dieser Verteidigungen während der Embryonalphase spricht. Darüber hinaus sollten Embryonen einen reduzierten Proteasegehalt aufweisen, da sie noch keine Algen verdauen müssen. Um Unterschiede in den Proteomen von Tieren, die dem Räuber ausgesetzt waren und einer Kontrollgruppe aufzudecken, habe ich die 2D-

DIGE Methode angewandt. Diese Methode basiert auf 2D-Gelen und ermöglicht daher die direkte visuelle Kontrolle der Qualität der Proteinproben. Die Ergebnisse der Proteinunterschiede konnten schon bekannten Verteidigungen zugeordnet werden, wie Cuticulaproteine und Chitin-modifizierende Enzyme, die möglicherweise an Veränderungen im Carapax beteiligt sind. Außerdem wurden Enzyme des Energiestoffwechsels und das Dotterprotein Vitellogenin gefunden, welche auf Modifikationen im Energieverbrauch hinweisen. Diese Ergebnisse konnten durch eine weitere Studie bestätigt werden, die ich in Kapitel 3 vorstelle. Hier wurden durch den Räuber *T. cancriformis* ausgelöste Veränderungen im Proteom adulter *D. magna* untersucht, was durch eine Optimierung der proteomische Probenvorbereitung ermöglicht wurde. Darüber hinaus etablierte ich eine neue Methode für *Daphnia* Proteomics, eine Massenspektrometrie basierte Technik mit integrierter Label-freien Quantifizierung. Mit Hilfe dieser Technik war es mir möglich weitere Genotypen zu integrieren, um die Allgemeingültigkeit und Aussagekraft der Studie zu erhöhen. Mit dieser Methode gelang es mir, die Ergebnisse der ersten Studie an *D. magna* Embryonen zu bestätigen. Auch im Proteom von adulten *D. magna*, die dem Räuber *Triops* ausgesetzt waren, spielten Cuticulaproteine und Vitellogenine eine große Rolle. Außerdem wurden weitere mit der Cuticula assoziierte Protein wie Calcium-bindende Proteine oder Chitin-modifizierende Enzyme gefunden. Darüber hinaus konnten noch weitere involvierte Prozesse anhand der teilnehmenden Proteine identifiziert werden, z. B. die Proteinbiosynthese. Interessanterweise fand ich bei den involvierten Proteinen auch klare Hinweise auf Proteine, die eher für eine allgemeine Reaktion auf den Räuber zuständig waren, wohingegen andere Proteine an einer Genotyp spezifischen Reaktion beteiligt waren. Hierbei zeigte der Genotyp aus einem Orginalhabitat mit *Triops* Koexistenz die ausgeprägteste Reaktion auf den Räuber.

Vom Genotyp abhängige Veränderungen im Proteom fanden sich auch in der Studie,

welche in Kapitel 4 vorgestellt wird. Hier wurden molekulare Mechanismen von Wirt-Parasit Interaktionen am Beispiel des Systems von *D. magna* und ihrem bakteriellen Endoparasit *Pasteuria ramosa* untersucht. *P. ramosa* ist bekannt dafür, seinen Wirt zu kastrieren und zu töten, außerdem ist der Erfolg des Infektionsprozesses stark vom Genotyp des Parasiten und auch von dem des Wirtes abhängig. In dieser Studie verwendete ich, ähnlich wie in Kapitel 3, eine Label-freie Quantifizierungsmethode, diesmal benutzte ich jedoch keine Proben von der ganzen Daphnie, sondern nur frisch gehäutete Exuvien. Es ist bekannt, dass die genotypische Spezifität der *P. ramosa* Infektion mit der erfolgreichen Anheftung des Parasiten an die Cuticula des Wirtes zusammen hängt und deswegen vermutlich auf die Cuticulazusammensetzung zurückzuführen ist. Um diesen Zusammenhang weiter zu untersuchen, analysierte ich das Exuvienproteom von *Daphnia*, die anfällig für eine *P. ramosa* Infektion sind im Vergleich zu einem nicht anfälligen Genotyp. Außerdem analysierte ich den Einfluss der *P. ramosa* Infektion auf den anfälligen Genotyp, in dem ich die Exuvien infizierter Tiere mit denen von Kontrolltieren verglich. Tatsächlich fanden sich im Proteom der verschiedenen Genotypen interessante Veränderungen im Bereich der Cuticulaproteine und der Matrixmetalloproteinasen (MMPs). Zusätzlich wurden auch Unterschiede bei prognostizierten Glykosylierungsstellen festgestellt, hier hatten Cuticulaproteine mit höherer Abundanz im anfälligen Genotyp deutlich mehr Stellen. Dieses Ergebnis stützt die Hypothese, dass *P. ramosa* sich mit Hilfe von kollagenartigen Oberflächenproteinen an die glykosylierte Cuticulaproteine anheftet. Tatsächlich wurde auch ein solches *P. ramosa* Protein mit hoher Abundanz in allen infizierten Replikaten gefunden. Im Zusammenhang damit stehen möglicherweise auch die MMPs, welche in erhöhter Abundanz im nicht anfälligen Genotyp gefunden wurden. Da diese Proteine kollagenolytische Eigenschaften haben können, erschweren sie möglicherweise die Anheftung und damit die Parasiteninfektion. Darüber hinaus

weisen meine Daten noch darauf hin, dass die Infektion durch den Parasiten eine Verzögerung des Häutungsprozesses beim Wirt bewirkt, wahrscheinlich um den Infektionserfolg zu erhöhen.

Im Gegensatz zu den bisher vorgestellten Arbeiten beleuchtet die Studie in Kapitel 5 die Proteinantwort von *Daphnia* auf einen bisher noch nicht anderweitig gut untersuchten Stressor, das Fehlen von Schwerkraft. Da sich Schwerkraft als einzige Umweltbedingung seit dem Beginn jeden Lebens auf der Erde nicht verändert hat, erleben Organismen normalerweise keine Schwerkraftsveränderungen und können sich deswegen auch nicht daran anpassen. *Daphnia* war tatsächlich bereits Teil eines Weltraumexperiments, allerdings sind die Reaktionen von *Daphnia* auf Schwerelosigkeit bisher nicht besonders ausführlich beschrieben worden. Darüber hinaus sind Daphnien auch besonders vielversprechende Kandidatenspezies für ein *biological life support system* (BLSS), was eine Untersuchung des Einflusses von Schwerelosigkeit auf diese Tiere zusätzlich erforderlich macht. Proteomics ist eine besonders geeignete Methode für diese Fragestellung, da auch biologische Prozesse aufgedeckt werden können, die in Physiologie oder Morphologie erst einmal nicht auffindbar sind. Zur Simulation von Schwerelosigkeit wurde in dieser Studie ein 2D-Klinostat benutzt, da Experimente in echter Schwerelosigkeit hohe technische Komplexität benötigen und einen großen finanziellen Aufwand bedeuten. Zur Proteomanalyse von Tieren in simulierter Schwerelosigkeit und einer Kontrollgruppe wurde die 2D-DIGE Technik angewandt. Es zeigte sich, dass *Daphnia* nach der Schwerelosigkeitsbehandlung große Abundanzveränderungen in Proteinen verbunden mit dem Zytoskelett, Proteinfaltung und dem Energiestoffwechsel zeigte. Diese Veränderungen sind insofern besonders interessant, als dass sie mit den Reaktionen einer Vielzahl anderer Organismen gut übereinstimmen und darauf hindeuten, dass die Reaktion auf Schwerelosigkeit in *Daphnia* einem generellen Konzept folgt.

Zusammenfassend lässt sich sagen, dass die Arbeiten in meiner Dissertation eine Vielzahl von Beispielen dafür darstellen, wie Proteomanalysen das Wissen über Stressantworten vergrößern können, auch wenn es sich beim untersuchten Organismus um keinen gut etablierten proteomische Modellorganismus handelt. In meiner Arbeit untersuchte ich sowohl die Reaktion auf anderweitig gut untersuchte Stressoren als auch auf einen in *Daphnia* nicht gut charakterisierten Stressor. Darüber hinaus konnte ich Proteingruppen in stark vom Genotyp abhängigen Reaktionen sowie Proteine einer generellen Stressantwort klassifizieren. Meine Arbeit verbessert unser Verständnis von Wechselwirkungen zwischen Genotyp, Phänotyp und der Umwelt und liefert ferner interessante Ansatzpunkte für detaillierte Untersuchungen von Stressantworten und ihren molekularen Mechanismen.

Nature is not only more complex than we think.

It is more complex than we can think.

F. E. Egler

1 General Introduction

Stress refers to a state in an organism caused by changes in one or more environmental conditions in a way that they may impair its fitness in the long term (Koehn and Bayne, 1989). These changes, or stressors, can bring the organisms to or over the edge of its specific ecological niche, which describes the range of environmental conditions over which the organism can survive and reproduce (Van Straalen, 2003). Stress is usually transient and therefore organisms may survive by the induction of mechanisms which counteract the consequences of stress, the so called stress responses (see also figure 1.1). Stress responses normally consist of plasticity in traits related to physiology, biochemistry, behaviour, and sometimes, morphology (Yampolsky et al., 2014). These responses may shift the organism back into its optimum, however, a permanent shift of the ecological niche by genetic adaptation is also possible, making stress-induced variation an important factor for adaptive evolution (Badyaev, 2005). Nowadays, the expanding toolbox available to molecular ecologists holds promises for unravelling more detailed mechanisms of stress responses (Reusch and Wood, 2007). Here, especially 'omics' approaches can provide valuable insights (GarciaReyero and Perkins, 2011), as they are holistic approaches and make, in contrast to targeted approaches, no assumption about which molecules to study and therefore may discover key processes and molecular participants not already known to play a role (Feder and Walser, 2005).

To increase the knowledge on molecular mechanisms underlying stress responses,

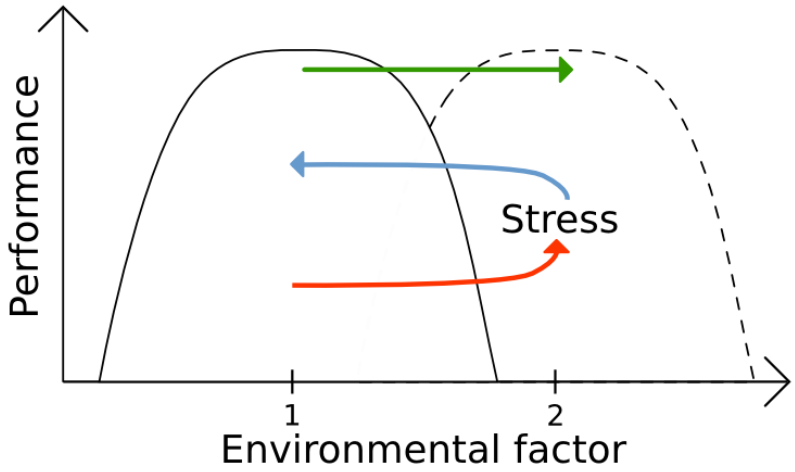

Figure 1.1: Schematic illustration showing the influence of stress on the ecological niche of an organism. When the environmental factor changes, the organism is forced out of its ecological niche (red arrow) and stress responses enable temporary survival until the factor changes again (blue arrow) or the organism adapts to the changes and therefore permanently changes its niche (green arrow). Adapted from Van Straalen (2003).

I analysed the protein responses of the ecological model organism *Daphnia magna* to different stressors. I used a proteomic approach, an approach which only recently became available for *Daphnia* and studied responses to stressors known to be important for the structuring of *Daphnia* populations (predation, chapter 2 and 3, and parasites, chapter 4). Furthermore, I analysed the effect of a stressor that organisms usually do not encounter on earth and therefore could not adapt to (chapter 5), which is microgravity.

1.1 Introducing *Daphnia*

The crustacean *Daphnia* is nowadays a genomic model species for interdisciplinary research in life science reaching from topics in ecology (Lampert, 2006) over toxicology (Denslow et al., 2007) to functional genomics (Miner et al., 2012) and was added by the American National Institute of Health (NIH) to their list of model organisms for bio-

Figure 1.2: Adult *Daphnia magna* female. Photograph by Q. Herzog.

medical research (`http://www.nih.gov/science/models/daphnia/`). These animals, also known as waterfleas, are cosmopolitan distributed species and have a key position in aquatic ecosystems due to their central role in the food web, linking autotrophic algae to higher trophic levels. Furthermore, they are versatile experimental organisms (Lampert, 2006), combining advantageous characteristics such as easy culturing in the laboratory and short generation times, therefore large populations can be produced in short periods of time and their response to environmental changes can be detected fast. *Daphnia* also has a transparent body (see figure 1.2), which enables morphological and physiological studies of the inner organs *in vivo*. In addition, they are cyclic parthenogens and the mode of reproduction is controlled by environmental conditions. Therefore, it is possible to generate and maintain clonal lines in the laboratory for extended periods, providing a defined genetic background.

Daphnia has been intensively studied over the past 250 years (Ebert, 2005) and therefore a huge amount of literature is documenting its biology, describing a wide

range of phenotypic diversity. The biggest amount of literature on environmental stressors in *Daphnia* is related to ecotoxicological studies, as *Daphnia* is a widely used organism representing around 8 % of all experimental data for aquatic animals within toxicological databases (Denslow et al., 2007). In addition, *Daphnia* is specified in the OECD Guidelines for the Testing of Chemicals as experimental animal (OECD, b,a). Furthermore, a topic that has been a subject of extensive studies over the last decades is the analysis of hypoxic stress, especially as *Daphnia* is known to express haemoglobin in response to reduced oxygen concentration, which enables the animals to cope with hypoxic conditions [e.g. Fox et al. (1951); Weider and Lampert (1985); Pirow et al. (2001); Lamkemeyer et al. (2003); Zeis et al. (2003); Gorr et al. (2004); Eads et al. (2008)]. Another well-studied subject is the influence of water temperature changes, especially on traits related to life-history and reproduction [e.g. Burns (1969); Goss and Bunting (1983); Orcutt and Porter (1984); Mckee and Ebert (1996); Mitchell and Lampert (2000); Rinke and Vijverberg (2005)]. Important stressors that are known to be important for the structuring of *Daphnia* populations and have therefore gained much attention, are predation and parasites. *Daphnia* is known to respond with a multitude of inducible defences to predation, which is among the strongest selection factors in nature [reviewed in Laforsch and Tollrian (2009) , see also section 1.2]. Furthermore, host-parasite interactions have been studied in great detail [e.g. Ebert (2005, 2008), see also section 1.2] and there is strong evidence, that parasites severely influence natural *Daphnia* populations [e.g. Ebert et al. (2000); Duncan and Little (2007)].

1.2 *Daphnia* and predator-induced phenotypic plasticity

Phenotypic plasticity, which describes the ability of one genotype to develop different phenotypes in response to changing environmental conditions (Pigliucci, 2001), is an important facet of the ecology and evolution of a broad range of organisms (Via et al., 1995). Studying phenotypic plasticity offers an exceptional opportunity to improve our understanding of the complex interplay between environment, genotype and phenotype, which are fundamental for the ecology and the evolution of species (Gilbert et al., 2010). There exist various examples for phenotypic plasticity, reaching from the response to light in flowering plants (Schmitt and Wulff, 1993) over the responses to heat-shock across kingdoms (Pigliucci, 1996) to temperature dependant sex-determination in reptiles (Janzen and Paukstis, 1991). Furthermore, phenotypic plastic responses of organisms include processes like learning, acclimation and adaptation of the immune system (Gilbert and Epel, 2009). Other prominent examples of phenotypic plasticity are inducible defences, i.e. traits showing plastic changes in response to predation. As predation is known to have a major impact on fitness and abundance of organisms (Agrawal, 2001), inducible defences are a widely distributed defensive mechanisms and occur in almost all taxa, including bacteria, protozoa, plants (here, they are usually referred to as resistance), crustaceans, insects, molluscs, amphibians and mammals [e.g. Tollrian and Harvell (1999); Kishida et al. (2010)].

The waterflea *Daphnia* shows a variety of inducible defences and therefore became a textbook example of predator-induced phenotypic plasticity (Laforsch and Tollrian, 2009). These defences include alteration of behaviour, e.g. diel vertical migration in response to fish predation (Lampert, 2007), meaning that *Daphnia* populations change their position in the water column in a day and night rhythm to avoid visually hunt-

ing fish. Furthermore, changes in life-history are a common phenomenon, consisting of changes in e.g. reproduction period, body size at maturation, clutch size and size of offspring (Riessen, 1999). Another very widespread inducible defence in *Daphnia* are alterations of the morphology, which protect the animals against the predator. The characteristic of the defensive structures depend on the predator-prey system and consist for example of spine-like structures such as neckteeth in *D. pulex* exposed to the predator *Chaoborus* (Krueger and Dodson, 1981) or the 'crown of thorns' of *D. atkinsoni* exposed to *Triops cancriformis*. *Daphnia* can also form helmets as defence against a predator, occurring e.g. in *D. cucculata* (Laforsch and Tollrian, 2004) and *D. galeata* and *D. retrocurva* (Dodson, 1988) exposed to *Chaoborus*. Recently, inducible defences were also detected in the predator-prey system of *Daphnia magna* and *Triops cancriformis*. *D. magna* responds to *Triops* predation by a distinct set of inducible morphological defences, which makes them less susceptible to *Triops* predation. These defences consist of an increase in body length, body width and tail spine length (Rabus and Laforsch, 2011) and an increase in cuticle thickness and stability (Rabus et al., 2013), altogether serving as an effective protection against *Triops* predation (Rabus and Laforsch, 2011). Furthermore, *Triops*-induced defences in *D. magna* are known to depend on the genotype (Rabus et al., 2012).

During the last years, also the analysis of molecular mechanisms underlying defensive trait formation in *Daphnia* gained more and more attention. First targeted approaches using western blot analysis detected the involvement of heat-shock proteins, actin and tubulin in inducible defences of *D. magna* exposed to fish or *Chaoborus* (Pijanowska and Kloc, 2004; Pauwels et al., 2005). Furthermore, targeted gene approaches using PCR revealed changes in expression of genes related to morphogenesis and endocrine pathways (Miyakawa et al., 2010) and protein folding (Schwarzenberger et al., 2009) in *D. pulex* exposed to fish or *Chaoborus*. More recently, also untar-

geted approaches have been applied to detect unpredicted key players in predator-induced phenotypic plasticity. The analysis of *D. magna* exposed to fish at the RNA level using microarrays revealed expression changes in genes possibly related to diel vertical migration (Jansen et al., 2013). Moreover, a transcriptomic study using RNAseq to analyse *D. pulex* exposed to *Chaoborus* was able to detect several differentially expressed genes, including cuticle genes, zinc-metalloproteinases, vitellogenin genes, genes connected to chromatin-reorganisation, cyclins, c-type lectins and several genes with unknown function (Rozenberg et al., 2015). In addition, the response of *D. magna* to fish predation was analysed at the protein level using an iTRAQ proteomic approach, finding 20 proteins altered in abundance, including proteins connected to protein synthesis, actins, globins and vitellogenins (Effertz and von Elert, 2014).

However, except from one very recent transcriptomic study (Rozenberg et al., 2015), all other studies did not use a high-throughput approach and only resulted in a limited number of detected genes or proteins. Therefore, molecular mechanisms underlying predator-induced phenotypic plasticity especially at the protein level are not well studied so far. Nowadays, progress in the availability of genomic data and *Daphnia* molecular tools enables the application of high-throughput proteomic approaches, which I applied in the work of my thesis to increase the knowledge on key proteins of inducible defences. The system of *D. magna* and *T. cancriformis* is especially suitable for this reason, as the defence is well studied, consists of distinct traits and is known to show interesting genotype dependencies.

1.3 *Daphnia* and parasites

Parasites are small organism closely associated with and harmful to a larger organism, its host (Ebert, 2005) and interactions between host and parasites are known to

be a key force driving coevolution in natural populations (Thompson and Cunningham, 2002; Harvell, 2004). Host-parasite interactions have been intensively studied in *Daphnia* [e.g. Ebert (2005, 2008)] and here, parasites are also known to strongly influence natural populations [e.g. Ebert et al. (2000); Duncan and Little (2007)].

Many invertebrates, including crustaceans, have a well-developed innate immune systems (Söderhall, 1999), which may react to parasite infection. In *Daphnia*, mechanisms related to phagocytosis (Metchnikoff, 1884) and melanisation by activation of the prophenoloxidase system (Mucklow and Ebert, 2003) are known to be part of the immune response. Furthermore, when comparing the genome sequence of *D. pulex* to genes related to the immune system in other arthropods, a variety of homologs were discovered, including genes related to the Toll pathway (McTaggart et al., 2009).

Known parasites of *Daphnia* belong to the groups of bacteria, fungi and microsporidia (Ebert, 2005). One of the best studied parasites of *Daphnia* is the Gram-positive, endospore forming bacterium *Pasteuria ramosa* (Metchnikoff), which is known to infect primarily *D. magna*, but also *D. pulex* and *D. longispina*. These bacteria infect the haemolymph and then castrate and kill their host, therefore infection occurs strictly horizontally via the release of endospores from dead conspecifics (Ebert, 2005). The susceptibility of *D. magna* to this microparasite is known to depend strongly on interactions between the genotypes of host and parasite, creating a binary infection outcome (either all animals are infected or not) for different host-parasite genotype combinations (Luijckx et al., 2011). Moreover, the attachment step of *P. ramosa* spores to the esophagus, which is part of the animal's cuticle, is thought to be responsible for this genotype specificity (Duneau et al., 2011). Studies on the molecular mechanisms involved in *Pasteuria* infection and the response of *Daphnia* to this stressor are rare so far. A candidate gene approach was not able to detect significant changes in gene expression of putative immune system related genes in *D. magna* exposed to *Pasteuria*

(Decaestecker et al., 2011) whereas a transcriptomic analysis found disturbances of ATP production after parasite infection (Jansen et al., 2013). Furthermore, a collagen-like protein of *Pasteuria* may be crucial for successful infection of the host (Mouton et al., 2009). As the infection success depends on the genotypes of host and parasites, proteomics is well suited to study if this genetic compound is also detectable at the protein level and to reveal mechanisms involved in this host-parasite interaction.

1.4 *Daphnia* and microgravity

Gravity is the only environmental parameter, which has remained constant since life on earth began and is thought to be crucial for the evolution of traits like the cytoskeleton, cell motility, gravity- and other acceleration-sensing devices, and biomineralisation (Ross, 1984). Organisms do not encounter changes in gravity on earth and therefore can not adapt to this kind of alterations, therefore the study of responses to microgravity is not only important for applied space research, but also shed light on the influence of gravity on organisms and the evolution of gravity perception. Common responses of humans to long-term spaceflight are bone demineralisation, skeletal muscle atrophy, and immune system suppression (Guéguinou et al., 2009). Furthermore, microgravity induced responses were described in a variety of biological systems reaching from alterations of cytoskeletal formation in cells (Vorselen et al., 2014) to altered plant forms in *Arabidopsis* (Link et al., 2014).

Regarding applied space research, the response of *Daphnia* to microgravity is also of interest as *Daphnia* might be a candidate organism for aquatic modules of bioregenerative life support systems (BLSS). BLSS may solve the supply problem of long duration manned space missions by providing the astronauts with essential supplies like food, water and oxygen, minimising reliability on delivered supplies and enhancing autochtonous production. Up to now, modules of such systems have been installed

on the Mir and ISS space stations (Sychev et al., 2003). Since then, additional components have been added, e.g. waste water recovery, forming a so-called ecological control and life support system (Wieland, 1998). The functions that have to be fulfilled by this system are the regeneration of atmosphere, purification of water, waste processing, food production and food processing (Schwartzkopf, 1992). Organisms integrated in these systems include bacteria and fungi for the decomposition of organic waste and excrements as well as unicellular microalgae, which produce oxygen for astronauts, but also comprise higher organisms such as vegetables or fish which may serve as food supply.

Including *Daphnia* into such a BLSS is feasible for several reasons: *Daphnia* occupies a central role in limnic food webs by being a primary consumer, hence serving as a link between oxygen producing, autotrophic producers such as algae and secondary consumers, such as planktivorous fish (Lampert, 2006). Fish, in respect, may serve as an animal protein source for the human crew. Furthermore, when integrating *Daphnia* into the system, no additional fish food has to be transported and the growth of algae populations is controlled. *Daphnia* reproduces by the mode of cyclic parthenogenesis, thus enabling *Daphnia* to reproduce asexually in favourable and sexually in unfavourable environmental conditions, which then leads to the formation of dormant eggs, the so called ephippium. Those resting eggs may serve as a backup and enable a restart of the BLSS in case of a system collapse. Furthermore, the mode of asexual reproduction is combined with short generation times and high numbers of offspring per clutch, therefore guaranteeing a high bio mass production (Sakwińska, 1998). *Daphnia* has already been part of missions to space stations and it was shown that resting eggs were viable even after exposure to outer space for more than one year (Novikova et al., 2011). Furthermore, some animals survived up to four months in space but showed changes in swimming behaviour with unusual

high looping movements (Ijiri et al., 1998).

To determine the effect of long term exposure to microgravity in space, high technical complexity and financial investment is needed. Most facilities providing free fall conditions, like parabolic flights or drop-tower experiments can only deliver short duration of weightlessness. Yet a cost-effective ground-based method is the use of a 2D-clinostat (Herranz et al., 2013), however, carefully considering the operational mode and limitation of the simulation. In the work of this thesis, such a 2D-clinostat was used to expose *Daphnia* to microgravity. Furthermore, I applied a proteomic approach to analyse the response of *Daphnia* at the protein level, enabling the detection of responses not visible in e.g. behavioural or morphological traits.

1.4.1 *Daphnia* and molecular biology

More recently, the work of the *Daphnia Genomic Consortium* (`http://wfleabase.org/`) enabled the publishing of the genome of *D. pulex* (Colbourne et al., 2011) and the pre-release of the genome of *D. magna* (`http://arthropods.eugenes.org/EvidentialGene/daphnia/daphnia_magna/`), providing vital prerequisites for high-throughput 'omics' research, namely gene and protein sequence databases for identification. However, compared to classical invertebrate model organisms like *Drosophila melanogaster* and *Ceanorhabditis elegans*, information on gene annotation is poor so far, and molecular tools that can be routinely applied to *Daphnia* are few, but growing. Studies using 'omics' techniques are now possible and have been applied to enlighten different aspects of *Daphnia* biology. There exits a variety of transcriptome studies mainly using microarray approaches analysing e.g. ecotoxicological aspects (Vandenbrouck et al., 2011; Dom et al., 2012; Asselman et al., 2012, 2015), dietary imbalance (Jeyasingh et al., 2011), thermal stress (Yampolsky et al., 2014), phosphorus supply (Roy Chowdhury et al., 2015) or the influence of multiple stressors (Jansen

et al., 2013; De Coninck et al., 2014). More recently, also RNAseq is used to perform transcriptomic analysis in *Daphnia*, studying microcystin tolerance (Schwarzenberger et al., 2014). In contrast, there are only a few proteomics studies, as proteomic analysis of *Daphnia* is a challenging task due to high proteolytic activity of whole animal lysates [see section 1.5.1]. Using different strategies to avoid proteolysis, studies on such different subjects as *Daphnia* physiology (Zeis et al., 2009; Schwerin et al., 2009; Gerke et al., 2011; Zeis et al., 2013) and ecotoxicology (Rainville et al., 2014) have been conducted. Also some metabolome studies exists, all related to environmental toxicology (Vandenbrouck et al., 2010; Poynton et al., 2011; Taylor et al., 2009, 2010; Nagato et al., 2013). The studies of Vandenbrouck et al. (2010) and Poynton et al. (2011) additionally combine metabolomic and transcriptomic approaches, enabling a more complete understanding of the analysed traits. Transcriptomics and proteomics have also been combined in the analysis of *Daphnia* haemoglobin (Zeis et al., 2013) and a combination of genomics, transcriptomics and peptidomics was conducted to analyse *Daphnia* neuropeptides and protein hormones (Dircksen et al., 2011). Predator-induced phenotypic plasticity was also studied at the level of the transcriptome using RNAseq (Rozenberg et al., 2015) and at the protein level (Effertz and von Elert, 2014) whereas host-parasite was only analysed as part of a multiple stressor approach (Jansen et al., 2013) using microarrays.

1.5 Introducing proteomics

The term proteome was introduced in 1994, describing the protein complement expressed by a certain genome in a cell, a tissue are even a whole organism (Wasinger et al., 1995; Wilkins et al., 1996). Afterwards, the process of studying this proteome became known as proteomics (Patterson and Aebersold, 2003). Proteomics is especially suitable for functional and biochemical characterisation of biological systems, as pro-

teins are key elements of almost all biological processes. Contrastingly, analysis of the transcriptome, which has of course a justification of its own, is not necessarily a good predictor of corresponding biological functions, as the abundance of individual proteins is not only influenced by their de-novo synthesis from DNA, but also by protein processing and protein degradation. Consequently, mRNA and protein abundances are not necessarily well correlated (Feder and Walser, 2005; de Sousa Abreu et al., 2009; Schwanhäusser et al., 2011; Grün et al., 2014).

Long before the analysis of global mRNA expression was possible, 2D-gel electrophoresis (2DE) was used in protein science to separate and quantify large numbers of proteins (Scheele, 1975; Klose, 1975; O'Farrell, 1975). In the first step of 2DE, proteins are separated according to their isoelectric point using pH-gradient gel strips and isoelectric focusing. This step is followed by separation according to molecular weight using polyacrylamide gel electrophoresis, resulting in protein gels which display specific spot pattern. Interestingly, common principles nowadays routinely applied in high-throughput data analysis like clustering algorithms and multivariate statistics were developed in connection with the 2DE approach (Anderson et al., 1984; Vincens et al., 1987). However, 2DE suffers from limitations in terms of reproducibility and dynamic range of detected proteins (Gygi et al., 2000). A significant improvement was the introduction of the DIGE-technique (Unlü et al., 1997), which increased reproducibility by combining two differentially labelled samples and one labelled standard on one gel.

Furthermore, other innovations were needed to enable the high-throughput identification of proteins. Progress in genomics made sequencing of whole genomes possible, with the first whole genome of yeast sequenced in 1996 (Goffeau et al., 1996), delivering complete libraries of possible protein sequences. Furthermore, advances in mass spectrometry of proteins and peptides provided an accurate method for measur-

ing molecular masses of these molecules (Aebersold and Mann, 2003), which can then be traced back to their sequence. Nowadays, it is possible to analyse the proteome of yeast with nearly complete coverage using state-of-the art mass-spectrometry based proteomics (Nagaraj et al., 2012), whereas analysis of more complex samples like human cell lines (Nagaraj et al., 2011) or mouse muscle tissue and cell lines (Deshmukh et al., 2015) revealed a deep proteome coverage with identification of around 10,000 proteins.

For protein quantification, different strategies exist. In a 2DE analysis, this step is usually separated from protein identification. Here quantification is performed on the base of the signal intensity of stained protein spots. In contrast to classical 2DE related methods, mass-spectrometry based approaches combine both, protein identification and quantification in one step. This is usually achieved by introducing MS-detectable labels at a certain point within the proteomic workflow, which typically consists of at least the following steps (Bantscheff et al., 2007): Sample preparation, protein and/or peptide separation, MS measurements and data analysis (see also figure 1.3). Quantitative approaches differ in the step of sample labelling within the proteomic workflow. An early introduction of the label is preferable, because it is afterwards possible to combine and simultaneously process different samples, therefore reducing quantification biases between samples. In metabolic labelling, stable isotopes are introduced into the organism prior to proteomic experiments, creating peptides with a mass shift which can be detected in MS-analysis. Stable isotopes are usually introduced into the organisms by enriching growth medium or food. This is done either directly by adding stable isotopes, e.g. ^{15}N labelling (Krijgsveld et al., 2003), or labelled auxotroph amino acids, e.g. stable isotopes labelling with amino acids in cell culture, SILAC, (Ong et al., 2002). Due to the early labelling event in the workflow, metabolic labelling is the most reliable quantification method. How-

ever, although this strategy was successfully applied to a variety of species (Gouw et al., 2010), labelling of more complex organisms is challenging and therefore the majority of work applying SILAC so far was conducted using unicellular organisms or cell culture. Contrastingly, labelling at the protein or peptide level is not restricted to a certain type of experimental organisms. Here, different chemical labels are available, which are detectable either in the MS or MS/MS analysis (Bantscheff et al., 2007). Current popular methods are tandem mass tags (TMTs) and isobaric tags for absolute and relative quantification (iTRAQ) (Bantscheff et al., 2012), both targeting amines and using isobaric tags (Thompson et al., 2003; Ross et al., 2004; Wiese et al., 2007), meaning that total labels have an identical mass but differences can be detected after MS/MS fragmentation. Furthermore, label-free quantification approaches become more and more successful (Bantscheff et al., 2012). In these approaches, no sample labelling is conducted and samples are not combined until the last step of the workflow, the data analysis. This strategy seems to be especially prone to quantification biases introduced during sample processing. However, experiments are also less laborious and cheaper, as no expensive chemical labels or stable isotopes are needed. Therefore it may be possible to create a higher number of replicates, increasing the power of the proteomics experiment. In addition, a recently developed software, MaxLFQ, reduces errors between different replicates by extensive sample normalisation and furthermore shows high quantification accuracy when calculating protein intensities (Cox et al., 2014).

1.5.1 Challenges of *Daphnia* proteomics

When conducting proteomics approaches with *Daphnia*, researchers realised that generating protein samples from adult *Daphnia* whole body samples is a difficult task because of the very high proteolytic activity in the samples (Fröhlich et al., 2009; Zeis

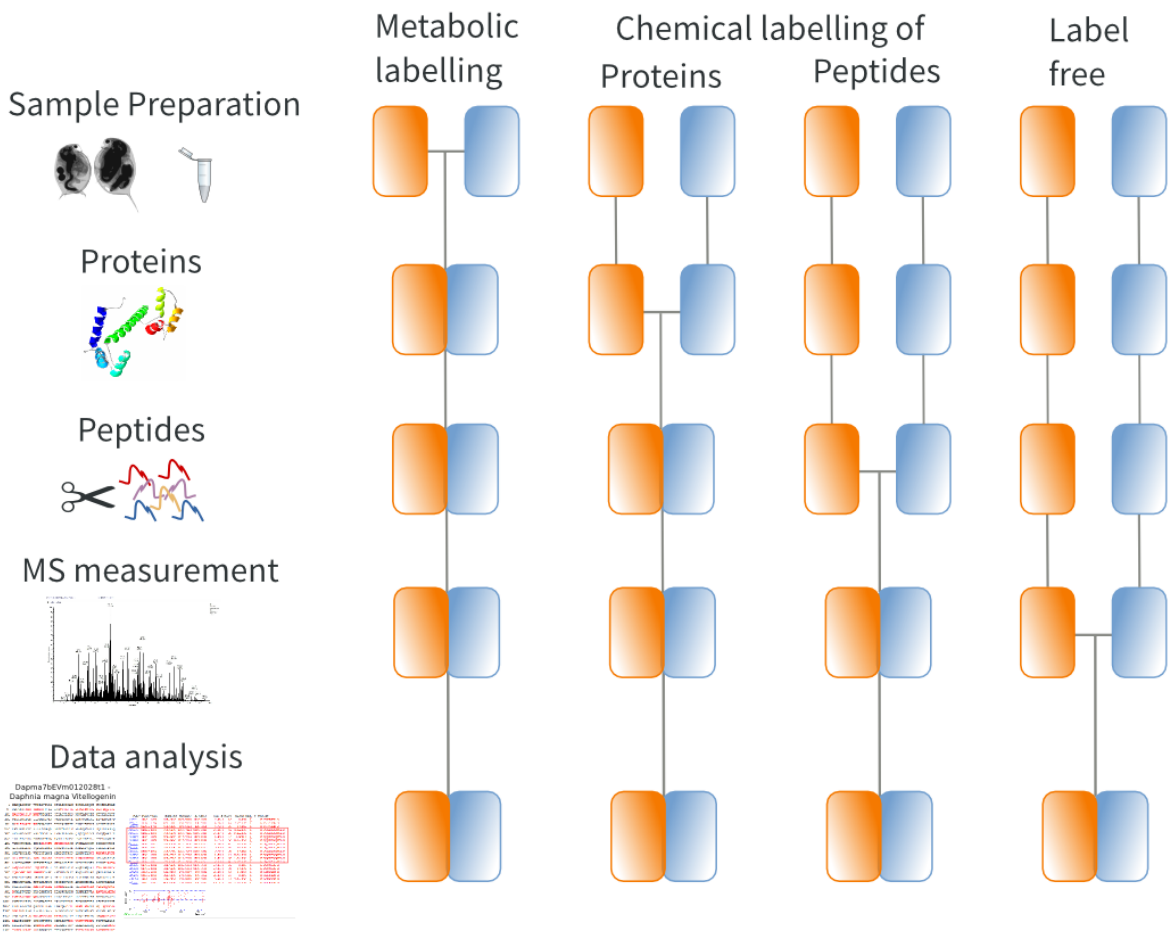

Figure 1.3: Overview on different mass spectrometry based protein quantification methods. The diagram indicates at what stage of the proteomic workflow the two experimental groups, orange and blue, are combined. Based on Bantscheff et al. (2012).

et al., 2009; Schwerin et al., 2009; Kemp and Kültz, 2012). This proteolytic activity most probably resulted from proteases originating from the gut of *Daphnia* (von Elert et al., 2004; Agrawal et al., 2005; Schwarzenberger et al., 2010) which turned out not to be blocked significantly by standard proteomic sample preparation procedures. These procedures include the usage of lysis buffer with strong chaotropic characteristics consisting of detergents, high amounts of urea and protease inhibitor cocktails (Cañas et al., 2007). 2D-Gels of adult proteins of *D. magna* showed strong characteristics of proteolysis, namely reduced protein spot number, no protein spots in the high molecular weight area and blurry appearance of protein spots (see figure 1.4). Inhibition of this proteolysis was reported after the exposure of *Daphnia* to high salinity environment prior to protein extraction (Kemp and Kültz, 2012). Furthermore, the immediate precipitation of freshly lysed *Daphnia* protein samples using trichloracetic acid (TCA) also stopped proteolytic degradation (Zeis et al., 2009; Otte, 2015) and resulted in clear protein spot pattern on the 2D-gel (see figure 1.5).

Proteomic studies on *Daphnia* tackle a variety of biological questions using different proteomic approaches. Some studies address general aspects of the *Daphnia* proteome, reaching from general proteome profiling using LC-MS/MS (Fröhlich et al., 2009) over 2DE analysis of proteome degradation (Kemp and Kültz, 2012), the analysis of neuropeptides and protein hormones using mass spectrometry (Dircksen et al., 2011) to the analysis of the *Daphnia* global phosphoproteome using phosphopeptide enrichment followed by LC-MS/MS (Kwon et al., 2014). Other proteomic studies analyse ecotoxicological questions, as *Daphnia* is a model organism in ecotoxicology. Here, changes in the proteome after exposure to potentially toxic compounds like nanoparticles or drugs were studied using 2DE or a shotgun approach with LC-MS/MS (Rainville et al., 2014, 2015; Borgatta et al., 2015). Furthermore, the protein responses of *Daphnia* to other environmental stressors like altered temper-

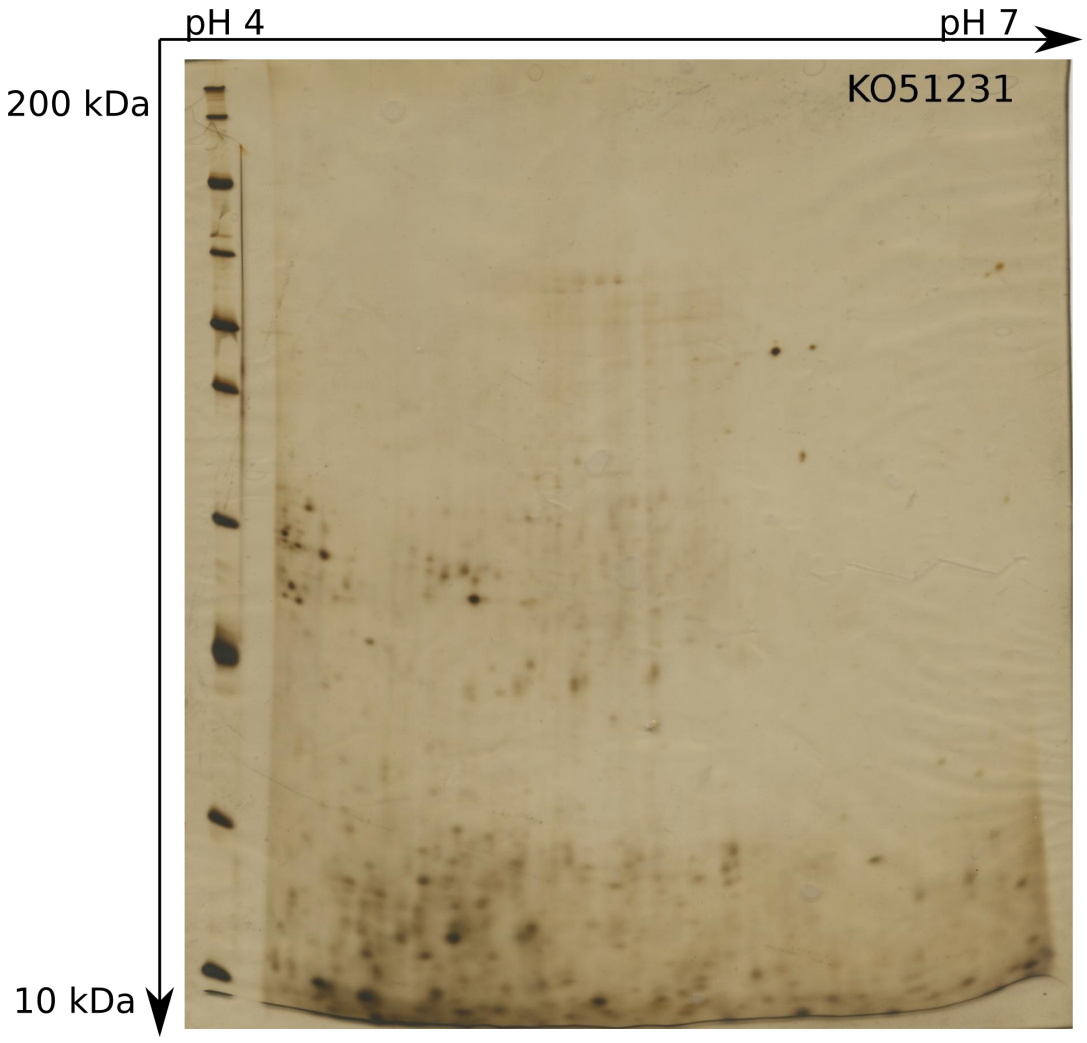

Figure 1.4: Silver stained 2D-Gel of protein lysate from whole body samples of adult *D. magna* processed with standard sample preparation protocol. Reduced spot number, no protein spots in the high molecular weight area and blurry appearance of protein spots indicate proteolysis.

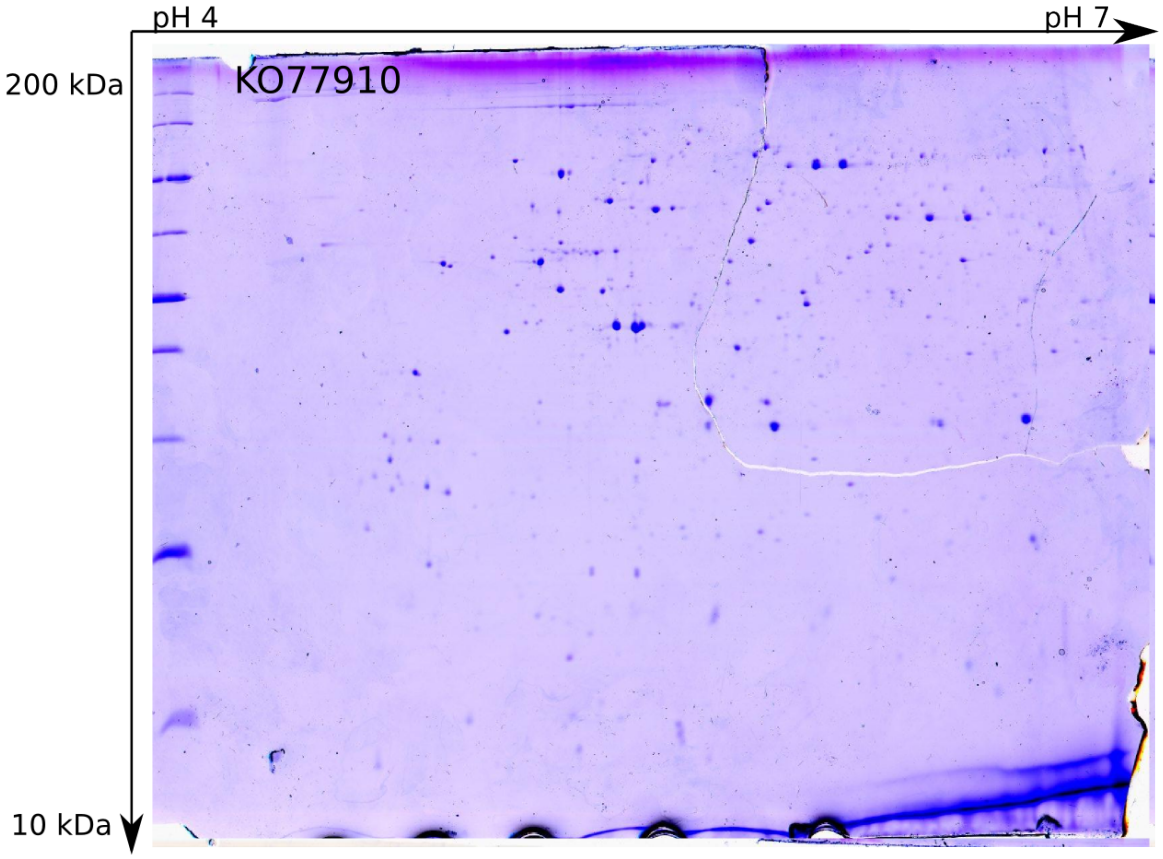

Figure 1.5: Colloidal Coomassie stained 2D-Gel of protein lysate from whole body samples of adult *D. magna* processed after TCA precipitation. Spot pattern show no indicators of proteolysis.

ature (Schwerin et al., 2009) or hypoxia (Lamkemeyer et al., 2006; Zeis et al., 2009; Gerke et al., 2011; Zeis et al., 2013), studied by 2DE, were also of interest, especially as *Daphnia* expresses haemoglobin in response to decreased oxygen availability. Haemoglobin expression is one example for the various types of phenotypic plasticity, which are common in *Daphnia*. Other phenotypic plastic traits are predator induced defences, for which *Daphnia* serve as a textbook example. Inducible defences of *D. magna* exposed to chemical cues of fish, which consist of diel vertical migration and life-history shifts, have recently been studied using a proteomic iTRAQ approach (Effertz and von Elert, 2014). In this thesis, proteins involved in the morphological defences of *Daphnia magna* exposed to the chemical cues of *Triops cancriformis* were analysed in embryos using 2D-DIGE (chapter 2) and adult females using label-free quantification (chapter 3).

1.6 Aim of the thesis

The aim of my PhD thesis was to study key proteins involved in the responses to different stressors in the ecological model species *Daphnia magna* using a proteomic approach. The detection of key proteins, processes and pathways will not only increase our knowledge on the evolution of stress responses and on the complex interplay between genotype, phenotype and environment, but is also a prerequisite for studying these traits in more detail using targeted approaches.

My very first aim was to develop proper proteomic approaches suitable for analysing the so far in proteomics not well established organism *Daphnia*. For this reason, optimisation of protein sample preparation to minimise proteolytic activity was crucial, as proteolysis strongly interferes with every kind of proteomic analysis (see also section section 1.5.1). After achieving this aim, I was able to conduct successful proteomic approaches to study stress responses in *Daphnia*. Furthermore, I wanted to

find optimal proteomic methods suitable for the different questions concerning *Daphnia* stress responses, further improving *Daphnia* proteomics.

Although predator-induced traits are well described in *Daphnia* at the behavioural, life-history or morphological level, signal pathways and molecular key players underlying these traits are not well understood so far. This knowledge is especially important, as it may elucidate possible costs and therefore can shed light on the evolution of phenotypic plasticity. I wanted to analyse the response of *D. magna* to *Triops*, which is known to consist of distinct morphological pattern and serves as an effective protection against the predator, at the protein level. As the defences against *Triops* are known to occur also in freshly born *Daphnia*, I wanted to use late-stage *Daphnia* embryos to set-up the proteomic approach. Furthermore, as proteolysis is known to be a major problem in *Daphnia* proteomics, another goal was the development of a proteomic approach inhibiting proteolysis in *Daphnia*. This work is described in **chapter 2**.

Subsequently, I aimed to create a more comprehensive study, analysing adult *D. magna* and different genotypes known to show diverse responses to the predator. Furthermore, I also wanted to develop an optimised proteomic approach, integrating high-throughput mass spectrometry based proteomics, leading to a massive increase of protein identifications. With this approach, I wanted to study differences and similarities of predator-induced phenotypic plasticity in the different developmental stages. In addition, as these defences are known to depend on the genotype, I wanted to study proteins involved in general and genotype-specific predator-induced responses. This work is described in **chapter 3**.

The aim to find genotype-specific responses also emerges in the context of host-parasite interactions in *Daphnia*. Therefore, I aimed to analyse the response to the parasite *Pasteuria ramosa*, which is known to be very important for *Daphnia* popu-

lations. As the strong genetic compound found in this system is known to depend on genotype and may be related to the cuticle composition of *Daphnia*, my goal was to analyse cuticles of two *D. magna* genotypes, either known to be susceptible for *P. ramosa* or not, to analyse if the genetic compound is visible in the different cuticle proteomes. Furthermore, I aimed to study differences in the cuticle proteome of parasite exposed and non-exposed animals of the susceptible genotype to find key players involved in the infection process and in the stress response of the host. This work is described in **chapter 4**.

Another aim of this thesis was to study also a stressor which was not well characterised in *Daphnia* so far. I wanted to analyse the response of the organisms to microgravity, which is not only interesting in terms of gravity and evolution but, in case of *Daphnia*, also because these animals are interesting candidates for biological life support systems (BLSS), enabling long-duration manned space missions. In my proteomic approach, I especially wanted to detect biological processes, which may not be visible on the physiological, behavioural or morphological level. Furthermore, I was interested in how the response of *Daphnia* may resemble the response of other organism, especially as they are not able to adapt to microgravity because this condition does not appear on earth. This work is described in **chapter 5**.

2 Proteomic analysis of *Daphnia magna* hints at molecular pathways involved in defensive plastic responses

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BMC Genomics, 15, 306, 2014.

BMC Genomics

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Proteomic analysis of *Daphnia magna* hints at molecular pathways involved in defensive plastic responses

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Abstract

Background: Phenotypic plasticity in defensive traits occurs in many species when facing heterogeneous predator regimes. The waterflea *Daphnia* is well-known for showing a variety of these so called inducible defences. However, molecular mechanisms underlying this plasticity are poorly understood so far. We performed proteomic analysis on *Daphnia magna* exposed to chemical cues of the predator *Triops cancriformis*. *D. magna* develops an array of morphological changes in the presence of *Triops* including changes of carapace morphology and cuticle hardening.

Results: Using the 2D-DIGE technique, 1500 protein spots could be matched and quantified. We discovered 179 protein spots with altered intensity when comparing *Triops* exposed animals to a control group, and 69 spots were identified using nano-LC MS/MS. Kairomone exposure increased the intensity of spots containing muscle proteins, cuticle proteins and chitin-modifying enzymes as well as enzymes of carbohydrate and energy metabolism. The yolk precursor protein vitellogenin decreased in abundance in 41 of 43 spots.

Conclusion: Identified proteins may be either directly involved in carapace stability or reflect changes in energy demand and allocation costs in animals exposed to predator kairomones. Our results present promising candidate proteins involved in the expression of inducible defences in *Daphnia* and enable further in depth analysis of this phenomenon.

Keywords: *Daphnia*, Phenotypic plasticity, Inducible defence, Predator-prey interaction, 2D-DIGE, Proteomics

Background

Phenotypic plasticity describes the ability of a genotype to express different phenotypes in response to varying environmental conditions [1,2]. Given that phenotypic plasticity is an important adaptation to face heterogeneous environments it is a fundamental aspect of the ecology and evolution of a broad range of organisms [3].

One frequently changing biotic condition, which strongly influences organisms' fitness and abundance in an ecological community context, is predation [4]. Phenotypic plasticity in defensive traits, so called inducible defences, occur in many species throughout invertebrate, vertebrate and plant taxa [5]. They are especially common in aquatic environments, where prey species can easily detect chemical cues (kairomones) released by predators [6].

Important key stone species of fresh water environments are waterfleas (*Daphnia*: Crustacea). The biology of these animals was studied over the past 250 years [7], resulting in a large amount of literature documenting their ecological diversity. With the help of the *Daphnia Genomics Consortium* (https://wiki.cgb.indiana.edu/display/DGC/Home), *Daphnia* is now one of the leading model organisms in evolutionary and ecological functional genomics. With the published genome sequence of *Daphnia pulex* [8] and the available pre-release of the *Daphnia magna* genome sequence (https://wiki.cgb.indiana.edu/display/DGC/Daphnia+magna+Genome), the American National Institutes of Health (NIH) has added *Daphnia* to their list of model organisms for biomedical research (http://www.nih.gov/science/models/daphnia/).

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Daphnia shows a multitude of inducible defences in response to changing predator regimes and hence serves as textbook example for phenotypic plasticity in defensive traits (reviewed in [9]). These defences include life history shifts like altered size or age at maturity [10-12], modifications of behaviour, e.g. diel vertical migration [13-15] and morphological changes including the formation of spine-like structures and helmets [16-18]. Also so called hidden morphological defences, which increase the stability of the carapace, were found [19-21].

The description of the *D. pulex* genome unravelled large arrays of environmental specific genes [8], which may be the key players in the formation of phenotypic plastic traits [22]. These genes often reside within the elevated number of tandem duplications, a striking feature of the *D. pulex* genome [8]. The same seems to be true for the genome of *D. magna* (Colbourne, pers. commun.). However, as molecular tools and genomic resources for *Daphnia* have only recently become available, the analysis of molecular mechanisms underlying inducible defences in *Daphnia* exposed to predator kairomones is still in its infancy (summarised in [23]). Up to date, only few studies have been conducted using either candidate gene/protein approaches [24-26] or a microarray approach based on stress and life stage specific cDNA libraries [27] in *D. magna*.

In these studies, genes involved in protein biosynthesis, protein catabolism and protein folding [26,27] showed different RNA expression patterns between *D. magna* defended against fish or *Chaoborus* and a control group. Also heat shock proteins, confirmed by western blot analysis, were found to be involved in the anti-predator defence of *D. magna*, being more abundant after short-term exposure [25] but less abundant after long-term exposure to fish kairomones [24]. Furthermore, two proteins of the cytoskeleton, actin and alpha tubulin, were affected [24].

The availability of enhanced genomic resources for *Daphnia* not only facilitates candidate gene approaches but also enables holistic approaches. In contrast to candidate approaches, holistic experiments may elucidate unpredicted key players involved in trait formation and regulation of inducible defences in *Daphnia*. Holistic proteomic analysis is especially suitable, as proteins are the typical effectors of biological functions and protein abundance is not necessarily well correlated with the corresponding mRNA level (e.g. [28,29]).

In the present study, we used the predator-prey system of *Triops cancriformis* and *Daphnia magna* for analysis of proteins involved in the formation of inducible defences. *D. magna* is a common species found in temporary and permanent ponds spreading from temperate regions to arid areas in the Holarctic and Africa [30]. This species shows inducible morphological defences in response to kairomones released by *T. cancriformis*. These morphological changes result in an increased bulkiness (increased body length, increased body width, increased tail spine length; see Figure 1) and are known to serve as an effective defence against *Triops* predation [31,32]. In addition, *D. magna* develops hidden morphological defences when exposed to *Triops* kairomones, which consist of a harder and thicker cuticle and an increased diameter of cuticle pillars, and therefore enhance carapace stability [21].

Results

We have studied differentially abundant proteins in *D. magna* exposed to kairomones of the predator *T. cancriformis*, which is known to induce phenotypic plastic defensive structures in this species [31], and a control group not exposed to predator kairomones. Performing proteomic analysis of adult *Daphnia* is a challenging task due to very strong proteolytic activity [33-36], which most likely results from proteases expressed in the digestive tract [37]. To avoid proteolytic degradation of protein lysates, we sampled late stage *D. magna* embryos featuring reduced protease activity. The sensitive period in *Daphnia* for perceiving chemical cues released by predators and for the formation of defensive traits is known to happen during embryonic development [38]. Preliminary experiments proved the same for *D. magna* exposed to *Triops* rendering late embryonic stages perfectly suitable for proteomic analysis.

Proteomic 2D-DIGE analysis and mass spectrometric analysis of abundance altered spots resulted in identification of 69 protein spots with 23 being more intense

Figure 1 Inducible defence in *D. magna*. Adult *D. magna* showing increased bulkiness after *Triops* induction (right) compared to control animal (left). (Photo: M. Rabus).

in kairomone exposed animals and 46 less intense. Mass spectrometric data, summarised spot data and further details are provided in the supplementary files (see Additional files 1, 2 and 3).

In detail, three biological replicates of *Triops* kairomone exposed animals and three biological replicates of a control group were compared using three 2D-DIGE gels. The gel images were of high-quality (see Figure 2 and also Figure 3) with all three gels showing highly reproducible spot patterns (see Additional file 4). In an unsupervised hierarchical cluster analysis, spot patterns clustered in two distinct groups, each containing solely gels from *Triops* kairomone exposed animals and controls, respectively (see Figure 4).

By software assisted image analysis of 2D-DIGE gels, 1505 spots could be matched, i.e., corresponding spots of the three replicates were assigned in a supervised manner, and the intensity of all matched spots was quantified. 179 spots were found with different intensities between *Triops* exposed and control *Daphnia* ($p \leq 0.05$, $ratio \geq |3|$). Out of these spots, 58 showed increased intensity in gels from *Triops* exposed animals whereas 121 showed decreased intensity.

87 spots were successfully identified using nano-LC MS/MS. Unambiguous identification of one single protein per spot was possible for 56 spots, while the majority of remaining spots contained contaminating fragments of the yolk protein precursor vitellogenin. The latter spots composed of peptides referring to more than one protein were only included in the bioinformatic analyses, if the total number of assigned peptides for one protein was at least three times higher than the number of all other assigned peptides. The corresponding protein was then regarded to represent the major component.

With respect to these classifications, we identified 69 protein spots in total. Out of this, 23 spots were more abundant in *Triops* exposed *D. magna* with 21 spots not containing vitellogenin (see Table 1). Of the remaining 46 spots, which were less abundant in *Triops* exposed *D. magna*, only 3 spots contained other proteins than vitellogenin (see Table 2). For vitellogenin-related spots, see the Additional file 2.

More abundant proteins of animals exposed to *Triops* kairomones (see Table 1) include proteins related to the cuticle (e.g. chitin deacetylase, different cuticle proteins), proteins involved in carbohydrate metabolism (glyceraldehyde-3-phosphate dehydrogenase, fructose-bisphosphate aldolase, ATP synthase), proteins related to the muscular system (paramyosin, troponin and actin), phosphorylation (nucleoside diphosphate kinase), glycosylation (phosphomannomutase) and a regulatory 14-3-3 ζ protein (see Table 1).

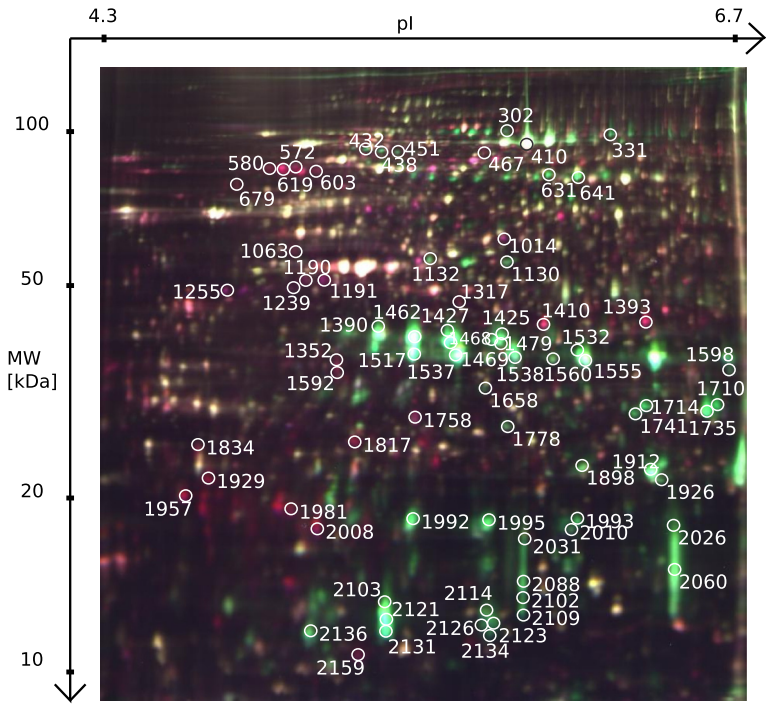

Figure 2 2D DIGE gel for comparing *Triops* exposed and control *D. magna* embryos. Spots with more abundant proteins in the kairomone exposed group are displayed in red (Cy5 labelled), spots with more abundant proteins in the control group are displayed in green (Cy3 labelled). Spots marked with Spot ID showed significantly different intensity and were successfully identified. Spot IDs not listed in Table 1 or Table 2 refer to vitellogenin-related spots.

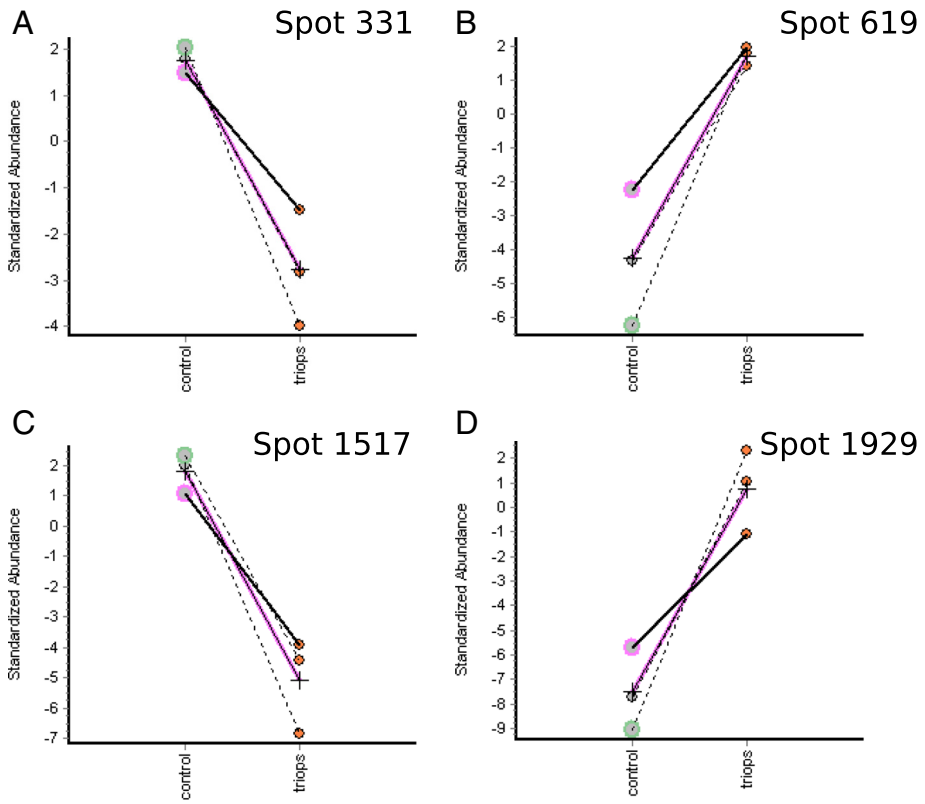

Figure 3 Examples for normalised DIGE intensity ratios. Normalisation was done according to internal pooled standard (IPS), here an abundance of e.g. 2 indicates that abundance is 2 of IPS abundance whereas -2 means 1/2 of IPS abundance. They serve as indicators for changes in protein abundance in kairomone exposed *D. magna* and in the control group for: Spot 331 – STAT Protein **(A)**; Spot 619 – Chitin deacetylase 2A **(B)**; Spot 1517 – Vitellogenin **(C)** and Spot 1929 – Cuticle Protein **(D)**.

Less abundant proteins of animals exposed to *Triops* kairomones (see Table 2) include a protein responsible for larval development called Prohibitin, a transcription activator (STAT) and a heat shock protein (HSP70).

To find grouped protein annotation terms and to visualise their relationships, ClueGO network analysis [39] was conducted using the Gene Ontology and KEGG databases of *D. melanogaster* (see Figure 5). Four functional groups could be separated, which were related to either glycolysis, actin cytoskeleton, chitin deacetylase activity or nucleoside triphosphate biosynthetic processes.

Comparison of protein data to known tandem duplicated genes in *D. pulex* with three or more duplications resulted in matching of three proteins. One cuticle protein (FBgn0033869, 33 duplications), Actin (3 duplications) and vitellogenin (4 duplications) were found to be tandem duplicated in the *D. pulex* genome.

Discussion

Predation is a key factor driving natural selection and therefore important for evolution of prey species and dynamics of prey communities [40]. As predator quantity and quality usually show heterogeneous patterns [41], prey species develop a variety of plastic defences in response to changing predator regimes [4]. Predator characteristics, e.g. prey-preference, feeding mechanism, predation strategy and habitat use, play an important role in shaping these plastic defences [42].

Particularly, *Daphnia* has to cope with a variety of size-selective predators [43]. Vertebrate predators like visually hunting fish are usually limited in the process of detecting the prey, whereas invertebrate predator like *Chaoborus* or *Triops* are often limited at the capturing, handling or ingestion step. Therefore, *Daphnia* coexisting with fish usually are smaller and more transparent [7] and show avoidance behaviour such as diel vertical migration [13-15]. In response to invertebrate predators, *Daphnia* often develops morphological defences (e.g. [16-18,31]), which impede capturing, handling or ingestion by the predator.

We studied the defensive responses of *D. magna* exposed to *T. cancriformis*, which consist of morphological changes resulting in an increased bulkiness (increased

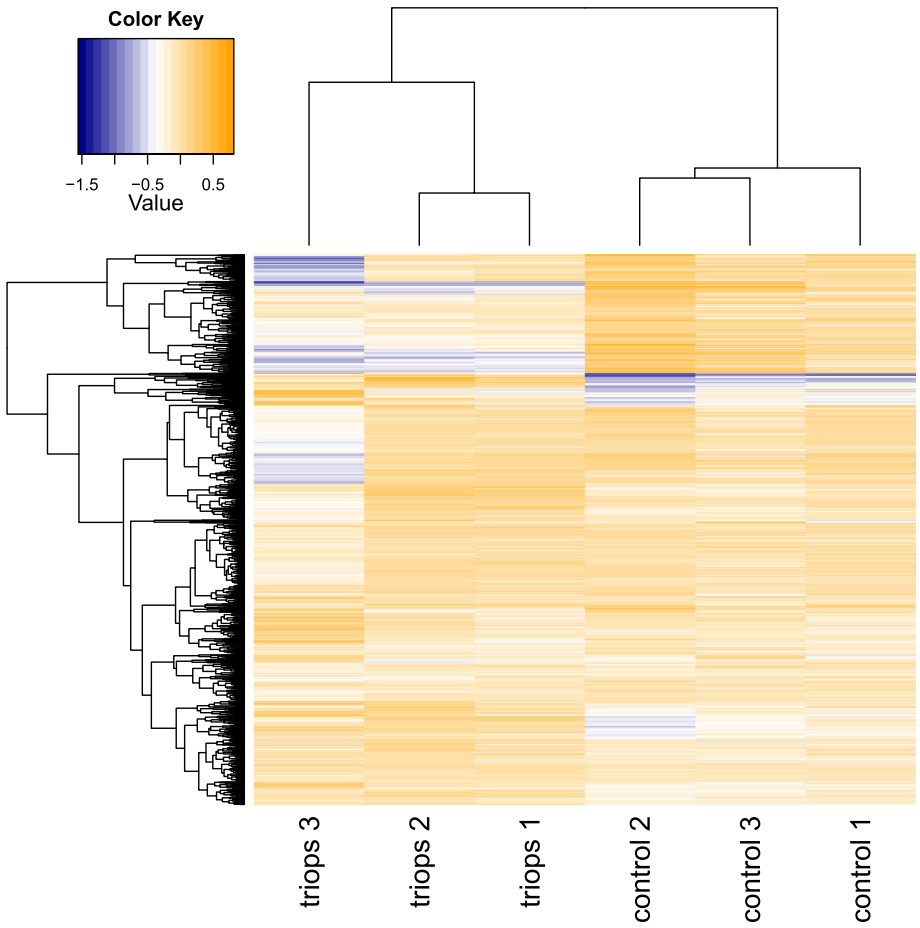

Figure 4 Hierarchical clustering heat map of all protein spots present in at least two biological replicates. Graph was created with R using the heatmap.2 function of package gplots. Rows indicate proteins whereas columns represent biological replicates of *Triops* kairomone exposed animals (*triops*) and control group (*control*).

body length, increased body width, increased tail spine length; see Figure 1).

Proteins more abundant in kairomone exposed *Daphnia* were similar to proteins connected to regulation, carbohydrate metabolism, biosynthetic processes, muscular system or the cuticle (see Table 1). The majority of less abundant proteins was identified as different isoformes of the yolk protein precursor vitellogenin. Three proteins of this data-set (cuticle protein, actin, vitellogenin) are known to be tandem-duplicated in the genome of *D. pulex*. Tandem-duplicated genes are thought to play an important role in the formation of phenotypic plastic traits [22].

Proteins involved in the formation of inducible defences regulate cell proliferation, participate in signalling pathways and facilitate protein folding

Two proteins with regulatory function, 14-3-3 ζ and phosphomannomutase, were of higher abundance in *D. magna* embryos exposed to *Triops* kairomones in our study. 14-3-3 proteins belong to a family of proteins well conserved among eukaryotes. Two of these isoformes, ϵ and ζ, have also been identified in *D. melanogaster* [44] and the silkworm *Bombyx mori* [45] and were expressed throughout a variety of life stages and in various tissues. 14-3-3 ζ binds to a large number of partners by recognition of a phosphoserine or phosphothreonine motif and is known to modulate their activity. Phosphomannomutase is an enzyme converting mannose-1-phosphate to mannose-6-phosphate and vice versa. It is therefore important for GDP-mannose synthesis, a molecule involved in glycosylation of proteins. The most similar protein in Drosophila, CG10688, is known to be involved in hypoxia-induced inhibition of protein translation [46]. In kairomone exposed *D. magna*, phosphomannomutase may therefore provide substrates important for signalling pathways involved in the formation of inducible defences.

Additionally, three proteins with regulatory characteristics, heat shock protein 78 kDa, prohibitin and a

Table 1 More intense spots for kairomone exposed *Daphnia* in 2D DIGE analysis (n=3)

Spot	GeneID	UniprotID	Protein Name (Organism)	FlybaseID	Ratio	Mw theo	Mw exp	pI theo	pI exp
1191	daphmag3mtv3l7094t1	Q9NA03	Actin (Daphnia magna)	FBgn0000046	14.3 ± 1.8	42	51	5.3	5
2008	daphmag3mtv3l18463t2	E9FZ29	Putative uncharacterized protein (Daphnia pulex) Nucleoside diphosphate kinase (Orseolia oryzae)	FBgn0000150	14 ± 0.2	17	18	6.2	5
1255	daphmag3mtv3l7094t1	Q9NA03	Actin (Daphnia magna)	FBgn0000046	13.2 ± 1.6	42	48	5.3	4.6
1929	daphmag3mtv3l7285t1	E9GDV0	Putative uncharacterized protein (Daphnia pulex) Cuticle protein (Artemia franciscana)	FBgn0033869	10.2 ± 0.7	19	22	5.7	4.5
1981	daphmag3mtv3l8582t2	E9HPK7	Putative uncharacterized protein (Daphnia pulex) Cuticle protein1c (Daphnia magna)	FBgn0086900	9.2 ± 0.6	39	19	5.1	4.9
1817	daphmag3mtv3l7094t1	Q9NA03	Actin (Daphnia magna)	FBgn0000046	8.6 ± 0.7	42	26	5.3	5.2
572	daphmag3mtv3l9358t1	E9HBN5	Putative uncharacterized protein (Daphnia pulex) Chitin deacetylase 2A (Tribolium castaneum)	FBgn0261341	7.7 ± 0.1	59	81	5.2	4.9
572	daphmag3mtv3l7734t1	E9HBN3	Putative uncharacterized protein (Daphnia pulex) Chitin deacetylase 1 (Tribolium castaneum)	FBgn0260653	7.7 ± 0.1	62	81	5	4.9
619	daphmag3mtv3l9358t1	E9HBN5	Putative uncharacterized protein (Daphnia pulex) Chitin deacetylase 2A (Tribolium castaneum)	FBgn0261341	6.2 ± 0.1	59	79	5.2	5
1957	daphmag3mtv3l20379t3	E9HPJ8	Putative uncharacterized protein (Daphnia pulex) Cuticle protein1b (Daphnia magna)	FBgn0000551	5.3 ± 0.6	22	21	5.5	4.4
2159	daphmag3mtv3l10909t1	E9FQP0	ATP synthase subunit beta (Daphnia pulex)	FBgn0010217	5.2 ± 0.2	56	11	5.4	5.2
603	daphmag3mtv3l9358t1	E9HBN5	Putative uncharacterized protein (Daphnia pulex) Chitin deacetylase 2A (Tribolium castaneum)	FBgn0261341	4.6 ± 0.2	59	80	5.2	5
603	daphmag3mtv3l7734t1	E9HBN3	Putative uncharacterized protein (Daphnia pulex) Chitin deacetylase 1 (Tribolium castaneum)	FBgn0260653	4.6 ± 0.2	62	80	5	5
1393	daphmag3mtv3l21933t1	E9GF36	Glyceraldehyde-3-phosphate dehydrogenase (Daphnia pulex)	FBgn0001092	4.2 ± 0.3	19	43	5.9	6.4
1758	daphmag3mtv3l21417t1	E9HCF1	Putative uncharacterized protein (Daphnia pulex) Probable phosphomannomutase (Drosophila melanogaster)	FBgn0036300	4.2 ± 1	16	28	7.9	5.4
1063	daphmag3mtv3l10909t1	E9FQP0	ATP synthase subunit beta (Daphnia pulex)	FBgn0010217	3.9 ± 0	56	58	5.4	4.9
1239	daphmag3mtv3l7094t1	Q9NA03	Actin (Daphnia magna)	FBgn0000046	3.8 ± 1.1	42	49	5.3	4.9
467	daphmag3mtv3l4480t1	E9HSV9	Paramyosin (Daphnia pulex)	FBgn0003149	3.5 ± 0.1	104	85	5.5	5.7
679	daphmag3mtv3l9455t1	E9HEE5	Putative uncharacterized protein (Daphnia pulex) Troponin H isoform 1 (Apis mellifera)	FBgn0004028	3.5 ± 0.3	44	74	4.8	4.7
1410	daphmag3mtv3l8855t1	E9GJ13	Fructose-bisphosphate aldolase (Daphnia pulex)	FBgn0000064	3.5 ± 0.5	40	42	6.7	6
1834	daphmag3mtv3l7635t2	E9H1W5	Putative uncharacterized protein (Daphnia pulex) 14-3-3 zeta (Artemia franciscana)	FBgn0004907	3.5 ± 0	39	25	8.5	4.5
1190	daphmag3mtv3l7094t1	Q9NA03	Actin (Daphnia magna)	FBgn0000046	3.3 ± 0.7	42	51	5.3	5
1352	daphmag3mtv3l16198t1	E9GE24	Putative uncharacterized protein (Daphnia pulex) Retinol dehydratase (Danaus plexippus)	FBgn0033887	3 ± 0.1	39	45	6.3	6.5

Spots were identified with LC-MS/MS and annotated using blastp algorithm against NCBI nr database. Spots related to vitellogenin were not shown.

Table 2 Less intense spots for kairomone exposed *Daphnia* in 2D DIGE analysis (n=3)

Spot	GeneID	UniprotID	Protein Name (Organism)	FlybaseID	Ratio	Mw theo	Mw exp	pI theo	pI exp
1658	daphmag3mtv3l7424t1	E9GTZ4	Putative uncharacterized protein (Daphnia pulex) Prohibitin protein WPH (Danaus plexippus)	FBgn0002031	-3.5 ± 0.2	30	32	5.8	5.7
331	daphmag3mtv3l10027t1	E9G1W0	Putative uncharacterized protein (Daphnia pulex) Signal transducer and activator of transcription (Artemia franciscana)	FBgn0016917	-4.1 ± 0.1	63	92	7.3	6.5
631	daphmag3mtv3l2732t1	E9GIU3	Putative uncharacterized protein (Daphnia pulex) Heat shock protein (Culex quinquefasciatus)	FBgn0026761	-10.1 ± 1.2	78	78	6.5	6

Spots were identified with LC-MS/MS and annotated using blastp algorithm against NCBI nr database. Spots related to vitellogenin were not shown.

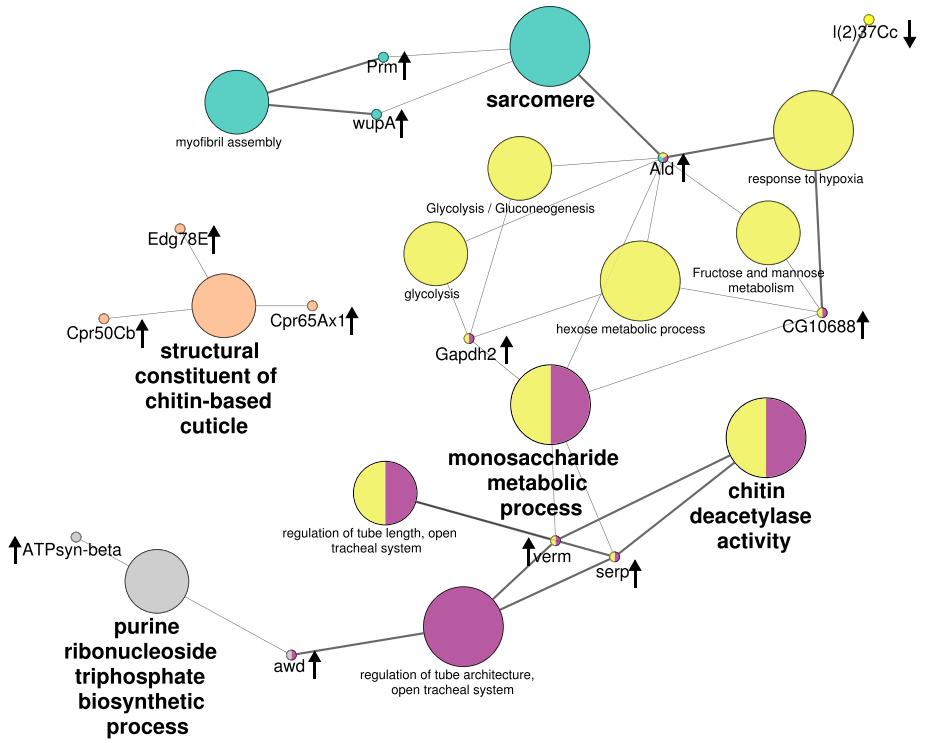

Figure 5 Annotation term network created with ClueGo using functional annotation analysis (two-sided hypergeometric test, Benjamini-Hochberg-correction, kappa-score ≥ 0.3). FlybaseIDs of proteins with increased and decreased abundance were searched against GO and KEGG databases. Small circles show involved genes and large circles refer to GO terms. Arrows next to gene names indicate decreased or increased abundance. Colours represent grouping of GO terms whereas size of circle and circle label illustrate the corrected p-value. Abbreviated Drosophila gene names correspond to the following protein names (compare also Tables 1 and 2): Ald – Fructose-Bisphosphate aldolase, ATPsyn-beta – ATP synthase beta, awd – Nucleoside diphosphate kinase, CG10688 – Phosphomannomutase, Cpr50Cb – Cuticle protein, Cpr65Ax1 – Cuticle protein 1c, Edg78E – Cuticle protein 1b, Gapdh2 – GAPDH, l(2)37Cc – Prohibitin, Prm – Paramyosin, serp – Chitin deacetylase 1, verm – Chitin deacetylase 2A, wupA – Troponin.

transcription activator (STAT), were less abundant in *D. magna* embryos exposed to *Triops* kairomones.

Heat shock proteins (HSP) act as chaperones facilitating protein folding and unfolding and play an important role in both, normal cellular homoeostasis and stress response [47]. Pijanowska and Kloc [24] found a decrease in the levels of HSP40, HSP60 and HSP70 when exposing *D. magna* from birth until first reproduction to either fish or *Chaoborus* kairomones. These findings corresponds to our findings, we also found a strong decrease in a 78 kDa HSP in *Daphnia* long-term exposed to *Triops* kairomones. Reducing HSP expression may save resources under long-term stressful conditions [48]. In addition, another study using *D. magna* shows that animals with a high tolerance against cadmium exposure display lower levels of HSP70 than animals having a lower tolerance [49]. The same may hold true for *D. magna* experiencing constant predation stress exerted by *Triops*.

Prohibitin is a ubiquitously expressed and well conserved protein, which is thought to be a negative regulator of cell proliferation in mammalian cells [50]. The similar protein in Drosophila, lethal (2) 37Cc, is most strongly expressed during late embryogenesis and may play a role in cuticle synthesis because of its presence during molts [51]. Therefore it seems possible, that the lower abundance of this protein may reflect changes of cuticle synthesis during the formation of morphological defences in *D. magna*.

The sequence of signal transducer and activator of transcription (STAT) protein is most similar to Stat92E in Drosophila. Stat92E is a signal protein and transcription factor in the well characterised JAK/STAT signalling pathway important for processes such as cellular proliferation, especially during embryonic development, immune response and stem cell maintenance [52]. Interestingly, Stat92E shows opposing influence on cell proliferation depending on developmental stage. During early development, Stat92E promotes cell proliferation whereas in later larval stages it reduces proliferation [53]. The under representation of this protein in late-stage *D. magna* embryos exposed to *Triops* kairomones may reflect a changed cell

proliferation pattern during the formation of inducible defences.

Proteomic evidence for enhanced energy demand and biosynthetic activity as a consequence of kairomone exposure

The more abundant proteins glyceraldehyde-3-phosphate dehydrogenase (GAPDH), fructose-bisphosphate aldolase (Ald), ATP synthase subunit beta (ATPsyn-beta) and nucleoside diphosphate kinase (NDK) are related to energy metabolism and biosynthetic processes (see also Figure 5). Both, GAPDH and Ald are key enzymes of the glycolytic degradation of glucose. In addition, GAPDH provides NADPH for biosynthesis of fatty acids, amino acids and nucleic acids. ATP-Syn-beta is a subunit of ATP-Synthase, which catalyses ATP synthesis within the respiratory chain. NDK provides nucleoside triphosphates for a variety of biosynthetic pathways.

Enhanced biosynthesis has already been reported in *D. magna* exposed to *Chaoborus* or fish kairomones [26]. *D. magna* showed a decreased body length when exposed to the vertebrate predator and an increase in body length when exposed to the invertebrate predator. RNA levels of protein biosynthesis related genes were increased for both treatments with higher levels in the fish kairomone treatment indicating a higher energy demand in predator exposed animals.

Another protein related to energy metabolism is vitellogenin, the precursor of the major yolk protein vitellin. Yolk proteins serve as an energy supply as well as organic building blocks throughout embryonic development of oviparous animals [54]. They are usually synthesised in extra ovarian tissues like the insect fat body [55] or non-mammalian vertebrate liver [56] and are taken up by the developing oocyte. During this process, usually referred to as vitellogenesis, vitellogenin is modified through cleavage, phosphorylation, glycosylation and lipidation [57]. At the time of embryogenesis, yolk proteins are further processed and degraded for embryo nutrition [58].

Due to the various processing steps during vitellogenesis and embryogenesis, the frequent occurrence of different vitellogenin related protein spots in 2D-gels of *D. magna* embryos found in our study is not surprising. Most of the spots were protein fragments with strong isoelectric point (pI) shifts and much smaller molecular weight (MW) compared to theoretical MW (see Additional file 2). Of the 43 vitellogenin-related protein spots found in our proteomic analysis, only 2 proteins were more abundant in *Triops* exposed *D. magna* whereas 41 were less abundant. Therefore, predator exposure seems to influence either the total amount of vitellogenin per egg provided by the mother or the yolk utilisation through the embryo because of higher energy demands.

Other studies also found yolk protein dynamics influenced by predator-released kairomones *D. magna* exposed to fish or *Chaoborus*. The proportion of total yolk used for egg production remained constant [59]. In presence of fish kairomones, *D. magna* reproduced not only earlier and at a smaller body size, but also had a higher number of offspring and this offspring had a smaller body size when compared to a control group [60,61]. In the presence of *Chaoborus*, *D. magna* reached maturity later at an increased body size and had a smaller number of offspring with larger body size [62]. *Triops* kairomones seems to increase both, the number and the size of offspring in *D. magna* [31,63]. Therefore, less yolk may be distributed to a single egg. However, the under representation of vitellogenin spots in kairomone exposed *D. magna* embryos found in this study may also indicate a higher energy demand. In addition, the higher abundance of other proteins related to energy metabolism and biosynthetic processes mentioned previously supports an increased energy demand of the embryo while building up *Triops*-induced defensive structures.

Kairomone exposure of Daphnia increases levels of proteins necessary for reinforcement of the muscular system

The muscle related proteins actin, troponin and paramyosin were all more abundant in *Triops* exposed *D. magna* embryos (see also Figure 5). Actin was found in four different protein spots with molecular weight (MW) higher than the theoretical value and acidic pI shifts, indicating posttranslational modifications. Additionally, one protein spot had a considerably smaller MW indicating a cleaved fragment (see Table 1). Actin is a major component of the cytoskeleton as well as of muscle fibres and is now one of the most abundant and highly conserved proteins in eukaryotes usually encoded in multiple genes [64].

Comparing the actin sequences using blastp algorithm, the most similar sequence in *D. melanogaster* for daphmag3mtv7094t1 is Act87E (FBgn0000046), whereas daphmag3mtv3l15317t1 was most similar to Act5C (FBgn0000042). Act87E is known to be expressed in the body wall muscles during embryonic, pupal and adult stages while Act5c is a ubiquitous cytoplasmic actin, being expressed throughout all life stages [65]. However, Röper et al. [66] showed that muscle-specific actin is incorporated into cytoplasmic structures, and cytoskeletal actin is incorporated into muscles for all actin paralogues of *D. melanogaster*. Therefore, it is not possible to deduce the function of actin only from its protein sequence.

Actin was connected to the formation of inducible defence in *D. magna* with contradictory results so far. Pijanowska and Kloc [24] reported a strong decrease of actin protein level in *D. magna* exposed to either

Chaoborus or fish predation using western blot analysis. On the contrary, Schwarzenberger et al. [26] found a moderate increase of actin mRNA expression in *D. magna* exposed to fish and only a slight decrease in *D. magna* exposed to *Chaoborus* using real-time qPCR. These inconsistent results may be a consequence of the different classes of molecules addressed in these studies, since RNA expression is not a reliable surrogate marker for protein expression.

In our proteomic analysis, strong evidence for a higher abundance of one muscle-specific actin and one cytoplasmic actin was found. In addition, two other muscle-specific proteins, troponin and paramyosin were more abundant in *D. magna* exposed to *Triops* kairomones. Troponin is an actin-binding protein found in thin filament of vertebrate and invertebrate muscle where it regulates actomyosin activity in a Ca^{2+} dependant manner [67]. Paramyosin is part of the thick filament of invertebrate muscle and a central player in regulating its diameter, with filaments of increased diameter showing an increased paramyosin:myosin ratio [68]. Predator-induced increase of muscle size has been found in other organisms, e.g. in the blue mussel *Mytilus edulis* [69] and in tadpoles of *Rana lessonae*, in the latter case it improved swimming performance. This may also be the case for defended *Daphnia*, as *D. magna* exposed to *Chaoborus* or fish kairomones show increased escape response time and higher behavioural alertness [24]. In addition, increasing muscular mass may also compensate for the consequences of carapace fortification or altered hydrodynamics resulting from a changed carapace morphology.

Cuticle proteins and chitin-modifying enzymes may cause carapace fortification in kairomone exposed *Daphnia*

In *T. cancriformis* exposed *D. magna* embryos, five proteins related to exoskeleton show a higher abundance. Out of this, three proteins were similar to cuticle proteins and two proteins were similar to chitin-modifying enzymes (see also Figure 5).

The carapace of *D. magna* consists of a chitinous integument folded back on itself with a small haemocoelic space in between. Inner and outer integument are connected by pillars as supporting structures [70]. This integument can be separated in the extracellular cuticle and the cellular epidermis. The cuticle consists of the two layers, epi- and procuticle [71]. In arthropods, epicuticle is mainly built out of proteins and lipids and procuticle is made of chitin filaments embedded in a proteinaceous matrix [72]. The properties of cuticle depend highly on the amount and combination of included proteins [73] and also on the degree of acetylation, which may influence cross-linking between protein matrix and chitin filaments [74].

Searching the sequences of the three cuticle proteins more abundant in kairomone exposed *D. magna* embryos against the prosite database for protein domains ([75], [http://prosite.expasy.org/prosite.html]) revealed chitin-binding domains in all three sequences. Consensus sequences were of the so called R&R type [76], with all proteins containing one or two RR-2 subgroups, usually associated with hard cuticles [77]. In addition, daphmag3mtv3l7285t1 also has a short consensus sequence of the RR-1 type, usually found in soft cuticles.

As further chitin modifying enzymes, we found chitin deacetylase type 1 and 2A in three different spots at around 80 kDa. These two proteins have a very similar molecular weight and pI and were therefore not well discriminated on the 2D-Gel. Molecular weight of these two proteins was 20 kDa higher than expected and pI was slightly smaller than computed pI (see Table 1), which indicates different states of post-translational modifications within the three different spots. Chitin deacetylase is a chitin modifying enzyme, which catalyses N-deacetylation of chitin and therefore changes protein binding affinity of chitin filaments. In *Tribolium castaneum*, several types of chitin deacetylase have been identified, with type 1 and 2 mainly expressed in the exoskeletal epidermis [78]. RNAi experiments revealed lethal phenotypes when using dsRNA corresponding to this chitin deacetylases. Here, animals failed to shed their old cuticles because the new synthesised cuticle lacked mechanical strength [78]. These findings support that these chitin modifying enzymes are involved in forming a harder cuticle in predator exposed *D. magna*.

Fortification of the exoskeleton in response to predator kairomones is known to play a role in inducible defences of some *Daphnia* species. *D. middendorffiana* exposed to the predatory copepod *Heterocope septentrionalis* shows increased cuticle thickness and cuticle strength [19]. Furthermore, *D. pulex* and *D. cucullata* exposed to *Chaoborus* larvae increase cuticle hardness and *D. cucullata* shows increased cuticle thickness and increased diameter of the cuticular pillars [20]. Recently, similar hidden defences were also found in *D. magna* exposed to *Triops* kairomones, revealing increased cuticle hardness, thickness and pillar diameter [21]. Carapace fortification is thought to act as protection against invertebrate predation, e.g. by increasing the escape efficiency of prey when being caught by the predator [20]. Cuticle related proteins with a higher abundance in *D. magna* exposed to *Triops*, i.e. R & R cuticle proteins as well as chitin deacetylases, may be involved in the necessary changes of chitin cross-linking with matrix proteins already in late stage *D. magna* embryos, causing increased carapace stability.

Conclusion

In our proteomic analysis, we found evidence that proteins related to cuticle, muscular system, energy metabolism and regulatory proteins are involved in the phenotypic plastic changes induced by *Triops* kairomones in *D. magna*. Cuticle proteins and the cuticle modifying enzymes chitin deacetylases 1 and 2A seem to be directly involved in the formation of morphological changes of the carapace, possibly altering chitin cross-linking with matrix proteins and therefore strengthen carapace stability. The same holds true for changes in abundance of muscle proteins (actin, paramyosin and troponin), which may adjust the muscular system to altered carapace morphology and enabling behavioural changes. Furthermore, proteins not directly involved in building up morphological traits were either involved in energy metabolism and biosynthetic processes or had regulatory functions. These proteins may reflect necessary changes in metabolism needed for the formation of inducible defences. The altered levels of regulatory proteins provide first evidence on signalling pathways possibly involved in the formation of inducible defences i.e. the Ras-mediated signalling pathways (14-3-3 ζ), glycosylation (Phosphomannomutase), protein folding (Heat shock protein), regulation of cuticle synthesis (Prohibitin) and translation regulation (STAT).

Our holistic proteomic analysis revealed promising candidate proteins involved in phenotypic plastic response of *Daphnia magna* exposed to kairomones of the predator *Triops cancriformis*. Proteins altered in abundance were either directly involved in the formation of defensive traits or reflect involved regulatory or metabolic pathways. Most interestingly, three proteins connected to this inducible defence (cuticle protein, vitellogenin, actin) belong to known tandem duplicated genes in *D. pulex*, a genetical design occurring in elevated numbers in the *D. pulex* and possibly also in the *D. magna* genome [8] which is predicted to play an important role in phenotypic plasticity [22].

Hence, our study fosters the knowledge on the molecular mechanisms of defensive trait formation, i.e. carapace fortification and – even more important – on the costs affiliated with the formation of the defence, since costs are thought to be a crucial premise for the plastic expression of a trait, and therefore a prerequisite for the evolution of phenotypic plasticity.

Methods

Induction experiment

All experiments reported in this study were conducted in agreement to the animal protection act of Germany. The induction experiment was carried out using a laboratory cultured clone of *D. magna* ($K_3 4J$) originating from a former fish pond near Munich, Germany. This clone shows strong morphological plasticity, i.e. increased body length, increased body width, increased tail spine length and increased carapace strength, in response to *Triops* predation [21,31,32]. A laboratory cultured clonal line of *T. cancriformis* provided by Dr. E. Eder, Zoological Institute, University of Vienna served as the predator. The experimental setup was installed in a climate chamber at a constant temperature of 20°C ± 1°C combined with fluorescent lighting at a constant photoperiod (15 h day : 9 h night).

The induction experiment included three biological replicates per group. For each replicate, 20 daphnids were raised in 2 L beakers containing 1.5 L semi-artificial medium [31] and a net cage (mesh width 400 μm; see Figure 6). The net cage contained one *Triops* for the kairomone exposed group allowing chemical cues to pass but preventing the daphnids from getting eaten (one Triops/1.5 L). Dead predators were replaced and feces of the predator were removed on a daily basis. For the control group, a net cage without a predator was placed into the beaker. Every second day, half of the artificial medium was exchanged. Daphnids were fed daily with *Scenedesmus obliquus* at a carbon concentration of 1 mg L^{-1}. Triops

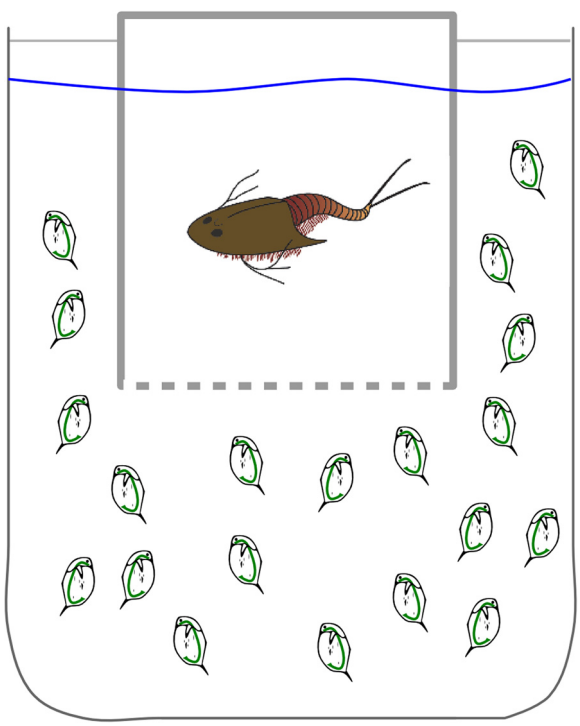

Figure 6 Setup of induction experiment. One replicate consists of one beaker with daphnids and a net cage containing the predator, so that daphnids perceive chemical cues of *Triops* but were prevented from being eaten. For the control group, the net cage was empty.

were also fed every day with living chironomids larvae, and 10 adult dead *D. magna* to take prey-specific alarm cues into account. These cues are released when prey animals are crushed by the predator and are also known to induce defensive structures in *Daphnia* [79]. *Daphnia* were killed using carbon-dioxide saturated water shortly before feeding. Preliminary experiments have shown that chironomids larvae do not induce defences in *Daphnia*.

The timetable of the induction experiment followed previous studies of inducible defences in the *D. magna* - *Triops* system [21,31,32]. The experiment was started by placing 4 age-synchronised randomly chosen primiparous daphnids and one adult *Triops* with a body length between 30 mm and 40 mm into the system. After releasing their first clutch, adult daphnids were removed and neonates were randomly reduced to 20 individuals (F_0 generation) per beaker. F_0 mothers were also removed after releasing their first clutch and F_1 neonates were again reduced to 20 individuals. The same was done after the birth of the next generation (F_2). The experiment was stopped after three generation cycles (approximately four weeks). After this duration morphological changes are known to be established in all animals of the kairomone exposed group [21,31,32]. In the end, F_2 generation animals bear their first clutch with embryos of a late developmental stage (black-eye embryos). Hence, age-synchronisation of embryos was in a time range of 12 hours. These embryos were used for proteomic analysis and therefore rinsed out of the mothers' brood pouch and washed twice using autoclaved and filtered semi-artificial medium [31] (filter pore size 0.2 μm). Subsequently, embryos were placed into one tube per biological replicate and snap-frozen using liquid nitrogen. Each replicate consisted of 300 – 400 embryos.

2D-DIGE

To prepare *Daphnia* embryos for 2D fluorescence difference gel electrophoresis (2D-DIGE) analysis, the frozen samples were homogenised in a mortar under liquid nitrogen thus preventing thawing. The resulting powder was solubilised in lysis buffer (2 mol/L Thiourea, 6 mol/L Urea, 4% CHAPS, 1 cOmplete ULTRA Tablets Mini (Roche) per 5 ml buffer) at a concentration of 1 embryo μL^{-1} buffer. Afterwards, each sample was centrifuged using a QIA Shredder Mini Spin Column (Qiagen) for 2 min at 14,000 g. Sample pH was adjusted to 8.5 using 50 mmol/L NaOH. Protein concentration was analysed by performing a Bradford Protein Assay (Coomassie Plus (Bradford) Assay Reagent, Thermo Scientific) according to the manufacturer's instructions.

50 μg protein per biological replicate were labelled with 2D-DIGE Cy3 Dye for control or Cy5 Dye for kairomone exposed group (GE Healthcare Life Sciences) following the protocol of the manufacturer. In addition, an internal standard (IPS) was prepared by pooling all biological replicates and labelling 200 μg of this IPS with 2D-DIGE Cy2 Dye.

24 cm gel strips for first dimension isoelectric focusing (IEF) were rehydrated for at least 10 h before starting of IEF with 450 μL rehydration buffer (2 mol/L Thiourea, 6 mol/L Urea, 4% CHAPS, 13 mmol/L DTT, 2% pharmalyte pH 3-10, bromphenol blue).

Prior to IEF, 50 μg of one Cy3-labelled control replicate, 50 μg of one Cy5-labelled kairomone exposed replicate and 50 μg of Cy2-labelled IPS were merged and 65 mmol/L DTT and 2% pharmalyte pH 3-10 were added. This mixed sample was applied via anodic cup loading on one gel strip. IEF was performed using an IPGPhore (Pharmacia Biotech) with a total of 60 kV h per strip.

Before second dimension gel electrophoresis, gel strips were equilibrated for 15 min in 15 mL equilibration buffer (50 mmol/L Tris-HCl pH 6.8, 6 mol/L urea, 30% glycerin, 2% SDS) containing 1% DTT on a shaker (40 min^{-1}, Certomat U, Sartorius). Afterwards, a second 15 min equilibration step in 15 mL equilibration buffer with 2.5% iodoacetamide and 200 μL saturated bromphenol blue solution was performed. For second dimension electrophoresis, lab-cast 210 × 260 × 1 mm polyacrylamide gels (1.5 mol/L Tris-HCl pH 8.8, 12.5% acrylamide/bisacrylamide (37.5:1), 0.1% SDS, 0.05% APS, 0.05% TEMED) and an ETTANDaltsix electrophoresis unit (GE Healthcare Life Sciences) were used. Equilibrated gel strips were fixed on top of the gels with the help of 0.5% agarose solved in SDS running buffer (25 mmol/L Tris, 192 mmol/L glycine, 0.2% SDS). Electrophoresis was conducted at 10°C for one hour at 5 W per gel and afterwards at 17 W per gel until the dye front reached the end of the gel.

Imaging and quantitative analysis

Gels were scanned immediately after electrophoresis using a Typhoon 9400 fluorescence scanner (GE Healthcare Life Sciences) with parameters recommended for 2D-DIGE experiments by the manufacturer. Image analysis and relative quantification were performed with DeCyder™ 2D Software version v7.0 (GE Healthcare Life Sciences). Coordinates of significantly differing protein spots ($p \leq 0.05$ with FDR correction, $ratio \geq |3|$ when comparing both treatments) were transferred to a pick list for further processing.

Excision of spots and tryptic hydrolysis

Gels were stained overnight with Coomassie Brilliant Blue (50% Methanol, 0.5% CBB R-250, 10% acetic acid) and then destained for at least 8 h. Spots of interest were cut

out automatically with a PROTEINEER spII robot (Bruker Daltonics) using the created pick list. Next, spots were digested using a DigestPro MS robot (Intavis) with the following protocol: (i) wash step with 60 µL 50 mmol/L NH_4HCO_3, (ii) wash step with 90 µL 50% acetonitrile, 25 mmol/L NH_4HCO_3, (iii) 20 min wash in 60 µL acetonitrile, (iv) 20 min wash in 60 µL 50 mmol/L NH_4HCO_3, (v) 20 min wash in 60 µL acetonitrile, (vi) 15 min wash in 60 µL acetonitrile, (vii) addition of 90 ng porcine trypsin (Promega) in 15 µL 50 mmol/L NH_4HCO_3 and incubation at 37°C for 6 h, (viii) addition of 15 µL 2.5% formic acid. Samples were than dried in a vacuum centrifuge (Vacuum Concentrator, Bachofer) and stored at -20°C until mass spectrometric analysis.

LC-MS/MS analysis

Nano-flow liquid chromatography tandem mass-spectrometry (nano-LC MS/MS) was performed with a nano LC ultra chromatographic device (Eksigent) coupled to a LTQ mass spectrometer (Thermo Scientific). Samples were resolved in 0.1% formic acid under 10 min sonication (Sonorex RK100, Bandelin). Subsequently, 10 µL of each sample were injected and loaded on a C18 trap column (C18 PepMap100, particle size: 5µm, 100 Å, column size: 300 µm × 50 mm, Dionex) for 10 min at a flow rate of 5µmin^{-1} using mobile phase A (0.1% formic acid). RP chromatography was done at a flow-rate of 280nLmin^{-1} using a Reprosil-Pur C18 separation column (Reprosil-Pur C18 AQ, 3 µm, 150 mm × 75 µm (ID), Dr. Maisch) with a 30 min linear gradient from 0% to 60% mobile phase B (A: 0.1% formic acid, B: 84% acetonitrile and 0.1% formic acid). For electrospray ionisation a distal coated Silica Tip (FS-360-50-15-D-20, New Objective) with a needle voltage of 1.4 kV was used. The MS method consisted of a cycle combining one full MS scan (Mass range: 300 – 2000 m/z) with three data dependant MS/MS events (35% collision energy). The dynamic exclusion was set to 30 s.

Bioinformatic processing

The MS/MS data were searched with Mascot Version: 2.3.00 (Matrix Science) using the following parameters: i) Enzyme: Trypsin; ii) Fixed Modification: Carbamidomethyl (C); iii) Variable modifications: Oxidation (M); iv) Peptide tol. 2 Da; v) MS/MS tol. 0.8 Da; vi) Peptide charge 1+, 2+ and 3+; vii) Instrument ESI-TRAP and viii) Allow up to 1 missed cleavages. As database, pre-released gene-predictions of *D. magna* (V2.4 effective 05/2012) were used. These sequence data were produced by The Center for Genomics and Bioinformatics at Indiana University and distributed via wFleaBase in collaboration with the Daphnia Genomics Consortium (http://daphnia.cgb.indiana.edu). Here, redundant entries of 90% similarity or more were detected through the software cd-hit [80] and removed. In addition, a common contaminants database (Max Planck Institute of Biochemistry, Martinsried, Germany: http://maxquant.org/contaminants.zip) was added. Mascot data were further processed with Scaffold 3 (Proteome Software), here "Protein Probability" and "Peptide Probability" were set to 99% and at least 2 unique peptides were used for protein identification.

Data were further processed with customised R scripts [81] (see also Additional file 5). Protein sequences were compared to data of NCBI nr [82] database using the NCBI Basic Local Alignment Search Tool (BLAST, $e - value < 0.001$) algorithm with R Package Bio3d [83].

GI numbers resulting from NCBI nr search were converted to UniProt accession numbers and further processed using the R biomaRt package [84] to gain further information on protein names and annotations, which are not yet available for preliminary *D. magna* sequence data. If no meaningful protein name was available for the first blast hit, which means that the protein name was either "uncharacterised" or a alphanumeric combination, further results were searched and added to the protein result. In addition, FlyBase Gene ID was looked up for the first blast hit related to *Drosophila melanogaster*.

Hierarchical clustering and heatmap were generated using the R package gplots. Cluster analysis of protein annotation (two-sided hypergeometric with Benjamini-Hochberg correction) and network visualisation (kappa-score ≥ 0.3) were performed using the software Cytoscape 2.8.3 [85] with the ClueGO plug-in v1.7 [39] using the Gene Ontology and KEGG databases for *D. melanogaster* and CluePedia plug-in v1.0.8 [86].

Protein data were compared to known tandem duplicated genes in *D. pulex* [8], summarised in http://wfleabase.org/genome-summaries/gene-duplicates/daphnia_tandemgene_table.html.

Additional files

Additional file 1: Spectral counting data. Spectral counting data, resulting from analysis of mass-spectrometric raw files with Scaffold Software, for all analysed spots as compressed zip file, for more details see Additional file 3.

Additional file 2: Spot data. Data of all identified spots, for more details see Additional file 3.

Additional file 3: Readme. Readme explaining contents of supporting files in more detail.

Additional file 4: Overlay images of 2D-DIGE-Gels.

Additional file 5: R-scripts.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions
CL, TF and GJA designed the study. CL conducted the induction experiment and provided samples for proteomic analysis. KAO conducted proteomic experiments, performed mass spectrometry analysis and conducted bioinformatic analysis of the data. TF supervised mass spectrometry analysis. KAO wrote the first draft of the manuscript and CL, TF and GJA contributed substantially to revisions. All authors read and approved the final manuscript.

Authors' information
Georg J Arnold and Christian Laforsch share senior authorship.

Acknowledgements
We thank M. Kredler and E. Ossipova for excellent help with the rearing of *Daphnia* and *Triops*. Members of Laforsch and Arnold group helped during induction and proteomics experiments and gave comments on the manuscript. We thank two anonymous reviewers for valuable and helpful comments on the manuscript. This work is part of the EUROCORE STRESSFLEA project funded by European Science Foundation.

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Received: 30 December 2013 Accepted: 7 April 2014
Published: 24 April 2014

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doi:10.1186/1471-2164-15-306
Cite this article as: Otte *et al.*: Proteomic analysis of *Daphnia magna* hints at molecular pathways involved in defensive plastic responses. *BMC Genomics* 2014 **15**:306.

3 Interclonal proteomic responses to predator exposure in *Daphnia magna* may depend on predator composition of habitats

Otte, K. A., Schrank, I., Fröhlich, T., Arnold, G. J., & Laforsch, C.

Molecular Ecology, 24, 15, 3901–3917, 2015.

MOLECULAR ECOLOGY

Molecular Ecology (2015) **24**, 3901–3917 doi: 10.1111/mec.13287

Interclonal proteomic responses to predator exposure in *Daphnia magna* may depend on predator composition of habitats

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Abstract

Phenotypic plasticity, the ability of one genotype to express different phenotypes in response to changing environmental conditions, is one of the most common phenomena characterizing the living world and is not only relevant for the ecology but also for the evolution of species. *Daphnia*, the water flea, is a textbook example for predator-induced phenotypic plastic defences; however, the analysis of molecular mechanisms underlying these inducible defences is still in its early stages. We exposed *Daphnia magna* to chemical cues of the predator *Triops cancriformis* to identify key processes underlying plastic defensive trait formation. To get a more comprehensive idea of this phenomenon, we studied four genotypes with five biological replicates each, originating from habitats characterized by different predator composition, ranging from predator-free habitats to habitats containing *T. cancriformis*. We analysed the morphologies as well as proteomes of predator-exposed and control animals. Three genotypes showed morphological changes when the predator was present. Using a high-throughput proteomics approach, we found 294 proteins which were significantly altered in their abundance after predator exposure in a general or genotype-dependent manner. Proteins connected to genotype-dependent responses were related to the cuticle, protein synthesis and calcium binding, whereas the yolk protein vitellogenin increased in abundance in all genotypes, indicating their involvement in a more general response. Furthermore, genotype-dependent responses at the proteome level were most distinct for the only genotype that shares its habitat with *Triops*. Altogether, our study provides new insights concerning genotype-dependent and general molecular processes involved in predator-induced phenotypic plasticity in *D. magna*.

Keywords: *Daphnia*, inducible defence, interclonal differences, label-free quantification, phenotypic plasticity, proteomics

Received 1 April 2015; revision received 9 June 2015; accepted 17 June 2015

Introduction

Phenotypic plasticity, that is the ability of one genotype to express different phenotypes in response to changing environmental conditions (Pigliucci 2001), is a fundamental aspect of the ecology and evolution of a broad range of organisms (Via *et al.* 1995). Examples for phenotypic plasticity are numerous and manifold, reaching from the response to light in flowering plants (Schmitt & Wulff 1993) to temperature-dependent sex determination in reptiles (Janzen & Paukstis 1991). In addition, processes like learning, adaptation of the immune system and acclimation belong to the repertoire of phenotypic plastic responses of organisms (Gilbert & Epel 2009). Studying phenotypic plasticity offers an

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extraordinary opportunity to gain a better understanding of the complex interplay between environment, genotype and phenotype, which are relevant for both the ecology and the evolution of species (Gilbert *et al.* 2010). This renders phenotypic plasticity, as one of the most common phenomena characterizing the living world, important for modern evolutionary thinking (Pigliucci 2005).

Prominent examples of phenotypic plasticity are inducible defences, plastic traits changing in response to predation. They are very important, as predation is known to strongly affect the fitness and abundance of organisms (Tollrian & Harvell 1999). *Daphnia*, the water flea, is known to develop a multitude of inducible defences in response to predation and serves therefore as a textbook example for predator-induced phenotypic plasticity [reviewed in Laforsch & Tollrian (2009)]. In the presence of predators, these animals can change their life history [e.g. altered size or age at maturity, Weider & Pijanowska (1993); Riessen (1999); De Meester & Weider (1999)], modify their behaviour [e.g. perform diel vertical migration Dodson & Havel (1988); Lampert (1989); De Meester (1993)] and alter their morphology [e.g. form elongated spines or helmets (Krueger & Dodson 1981; Tollrian & Laforsch 2006; Petrusek *et al.* 2009)]. In addition, some species are known to enhance carapace stability in response to predatory invertebrates (Dodson 1984; Laforsch & Tollrian 2004; Riessen *et al.* 2012).

Given their enormous plasticity, *Daphnia* have fascinated scientists over the past 250 years (Ebert 2005) and are now a model organism for interdisciplinary research reaching from ecology (Lampert 2006) over toxicology (Denslow *et al.* 2007) to functional genomics (Miner *et al.* 2012), as they combine advantageous characteristics like easy culturing in the laboratory, short generation times, a transparent body and parthenogenetic reproduction providing a defined genetic background. Because these animals have been in research focus for so long, a large amount of literature is documenting its ecological diversity. In addition, due to the work of the *Daphnia Genomics Consortium* (https://wiki.cgb.indiana.edu/display/DGC/Home), maturing genomic tools for research are available and the first *Daphnia* species genome sequence has been published (Colbourne *et al.* 2011).

As genomic data and molecular tools for *Daphnia* became more and more available during the last years, some studies on the molecular mechanisms underlying inducible defences were conducted, mainly using targeted approaches at the RNA level (Schwarzenberger *et al.* 2009; Miyakawa *et al.* 2010) or protein level (Pijanowska & Kloc 2004; Pauwels *et al.* 2005). These studies indicate that heat-shock proteins, cytoskeletal proteins, morphogenetic factors and juvenile hormone pathway genes are involved in inducible defence formation in *Daphnia*. The increasing availability of genomic resources for *Daphnia* also facilitates holistic approaches, which in contrast to targeted techniques are able to detect unpredicted key players. Proteomics is especially suitable, as proteins are the main effectors of biological functions (Fröhlich *et al.* 2009; Schwerin *et al.* 2009; Zeis *et al.* 2009).

In recent times, morphological inducible defences have also been reported in *Daphnia magna*, which is next to *Daphnia pulex* the most important *Daphnia* model species. In response to the predator *Triops cancriformis*, juvenile and adult *D. magna* show morphological changes, such as an increased body length, body width and tail spine length (Rabus & Laforsch 2011), which provide an efficient protection against the predator (Rabus *et al.* 2012). In addition, the ultrastructure of the carapace is altered, resulting in a thicker and harder cuticle (Rabus *et al.* 2013). Furthermore, the morphological defence of *D. magna* changes in a genotype-dependent manner (Rabus *et al.* 2012). In a previous proteomic analysis of *D. magna* embryos exposed to the predator *T. cancriformis*, we were able to detect changes in protein abundance directly connected to defensive structures, for example cuticle proteins, or indicating changes in energy demand and allocation costs, for example vitellogenin (Otte *et al.* 2014).

With the actual study, we want to get a more comprehensive idea of molecular mechanisms underlying inducible defences in *Daphnia* with a closer look on general and genotype-dependent responses of *D. magna* to *Triops* exposure. Hence, we analysed the response of adult females of four different *D. magna* genotypes using a high-throughput proteomic approach. These genotypes originated from European habitats with different predator compositions. We analysed both, the morphology and proteome of predator-exposed and control animals.

Material and methods

Induction experiment

We used four clonal lines of *D. magna* (Bl22, K34J, Max4, FT44-2) originating from habitats across Europe with differing predation history. Clone Bl22 originates from a permanent pond near Leuven, Belgium, with fish and notonectid but no *Triops* predation; clone K34J originates from a permanent pond in Ismaning, Germany, coexisting with various invertebrate predators not including *T. cancriformis*; clone Max4 originates from a temporary pond situated in the Camargue, France, coexisting with *T. cancriformis*, and clone FT44-2 originates from a permanent pond in Tvärminne,

Table 1 Overview on origin and habitat of genotypes. Location coordinates reflect the approximate habitat area and not necessarily the exact pond

Genotype	Origin	Location	Coexisting predators	References
K34J	Germany	48°12′ 22.4" N 11°43′03.1"E	various invertebrates (no *Triops*)	Rabus & Laforsch (2011)
Bl22	Belgium	50°51′ 53.0" N 4°41′58.5"E	fish and notonects (no *Triops*)	Rabus *et al*. (2012)
Max4	France	43°30′ N 4°40′ E	*Triops*	Rabus *et al*. (2012)
FT44-2	Finland	59°50′ 24.5" N 23°12′ 46.4"E	—	—

Finland, where no predators at all are reported (see also Table 1). All genotypes were kept in the laboratory for several years. Bl22, K34J and Max4 are known to develop an array of morphological changes when exposed to chemical cues of the predator *Triops* (Rabus & Laforsch 2011; Rabus *et al*. 2012). On the contrary, FT44-2 shows no morphological changes in the presence of *Triops* (M. Rabus, pers. commun.). *T. cancriformis* predators were taken from a laboratory cultured clonal line which originates from Dr. E. Eder, Zoological Institute, University of Vienna. The experimental set-up was installed in a climate chamber at a constant temperature of 20°C ± 1°C combined with fluorescent lighting at a constant photoperiod (15 h day : 9 h night).

We studied five biological replicates per treatment per genotype. For one biological replicate, two aquaria (30 × 20 × 20 cm) were arranged one below the other in a flow-through system (Figs S1–S2 in Supporting information). The aquaria contained 15 L of semi-artificial SSS-medium consisting of well water, ultrapure water, phosphate buffer and trace elements (Rabus & Laforsch 2011). In addition, the ground of the bottom aquarium was covered with sterilized sand (grain size approximately 1 mm, colourstone). The aquaria were connected via a silicone tube (bottom to top tube, diameter 6 mm, Roth) and a PVC tube (top to bottom tube, diameter 4 cm, Aqua Medic). Inlets of tubes were covered with gauze to keep *Daphnia* in the aquarium (mesh width 300 μm). Unidirectional flow was created by a pump (u500 proflow, JBL), which was placed in the bottom aquarium. Flow was manually adjusted to 3 L/h.

During the experiment, the top aquarium contained 60 *Daphnia*, whereas the bottom aquarium contained 2 *Triops* of 20–30 mm body size or was empty for the control group. *Daphnia* were fed daily with *Scenedesmus obliquus* at a carbon concentration of 1 mg/L. *Triops* were also fed every day with five dead chironomids larvae and five freshly killed adult *D. magna* per animal. The same number of dead chironomids and *Daphnia* were added to the control aquarium, and here they were replaced regularly. Once per week, half of the medium in the system was changed and the PVC tube and gauze were cleaned. Silicone tubes were replaced every second week.

The experiment was started with cohorts of 50 age-synchronized neonate females (F_0-Generation) per aquarium. The first clutch of these animals was removed. The F_0-females were also removed after releasing their second clutch. This second clutch was used to raise the next generation (F_1), and animals were randomly reduced to 60 individuals per aquarium. The F_1-Generation was treated like the F_0. Finally, 60 F_2 adult females were used for further analysis with their second clutch reaching the so-called black-eye stage, meaning that the offspring in the brood pouch has developed one black compound eye and will leave the pouch within the next 12 h. The whole experiment took approximately 1 month. Morphological changes are known to be established in all animals of the *Triops*-exposed group after this duration (Rabus & Laforsch 2011; Rabus *et al*. 2012, 2013).

Ten animals per biological replicate were conserved in 70% Ethanol for subsequent morphological analysis. For the other 50 animals per biological replicate, embryos were rinsed out of the brood pouch and adults were then washed twice using autoclaved and filtered (filter pore size 0.2 μm) semi-artificial medium (Rabus & Laforsch 2011) and snap-frozen using liquid nitrogen.

Morphological analysis

For morphological analysis of alcohol-preserved animals, 10 individuals per biological replicate were photographed under a stereo-microscope and images were analysed using the software cell^p (Olympus). Body length, body width and tail spine length (Figs S1–S2 in Supporting information) were determined as indicators of defensive trait formation. For each genotype, morphological parameters of predator-exposed and control animals per genotype were compared using ANOVA in R (R Development Core Team 2011) with biological replicates as blocking factor and 'Tukey Honest Significant Differences' method was applied on ANOVA results to look for differences within the genotypes.

Sample preparation

To generate samples for proteomic analysis, 50 frozen animals per biological replicates were pooled and

homogenized in a mortar under liquid nitrogen thus preventing thawing. The resulting powder was solubilized in lysis buffer (2 mol/L Thiourea, 6 mol/L Urea, 4% CHAPS, 1 complete ULTRA Tablets Mini (Roche) per 5 mL buffer) using 10 μL buffer per animal. Afterwards, each sample was centrifuged using a QIA Shredder Mini Spin Column (Qiagen) for 3 min at 16,100 *g* to get rid of debris. Then, samples were precipitated using 30% trichloroacetic acid for 20 min on ice to inhibit proteolytic activity (Zeis *et al.* 2009). Subsequently, samples were centrifuged for 10 min at 16 100 *g*, the supernatant was discarded, and the resulting protein pellet was washed three times with cold acetone (approximately 0°C, LC-MS/MS grade). The pellet was dried and then resolved in the same amount of lysis buffer as before (see above). Protein concentration was analysed by Bradford Protein Assay (Coomassie Plus (Bradford) Assay Reagent, Thermo Scientific) according to the manufacturer's instructions. Resulting protein concentrations were between 5 and 7 mg/mL.

SDS–PAGE prefractionation and tryptic digestion

Samples were prefractionated on a small SDS–PAGE gel (gel size 8 × 7 × 0.75 cm) using a 1 cm stacking gel (4% acrylamide, 0.125 mol/L 6.8 pH Tris-HCl, 0.1% SDS, 0.05% APS, 0.1% TEMED) and a separation gel (12% acrylamide, 0.375 mol/L 1.5 pH 8.8 Tris-HCl, 0.1% SDS, 0.1% APS, 0.5% TEMED). From each replicate, 50 μg protein was used. Prior to gel-electrophoresis, 2% SDS was added and then each sample was treated with 4.5 mmol/L DTT at 65°C for 30 min and subsequently with 10 mmol/L for 15 min at room temperature. Glycerol was added to give a concentration of 10% (v/v). Gels were run on a Mini-PROTEAN II device (Bio-Rad) at 80 V for 15 min and afterwards at 150 V (Running Buffer: 25 mmol/L Tris, 0.2% SDS, 192 mmol/L glycine). Gels were then stained by colloidal coomassie (Roti-Blue, Roth) according to the manufacturer's protocol.

For in-gel tryptic digestion, we first washed the gels two times with water and then cut each gel lane in 10 pieces (see Fig. 1), transferred each piece to a tube and minced it with a pipette tip. Gel pieces were washed for 30 min per step using the following solutions: 1 × 50 mmol/L ammoniumhydrogencarbonate (ABC), 2 × 25% acetonitrile (ACN)/37.5 mmol/L ABC, 1 × 50% ACN/25 mmol/L ABC, 1 × 100% ACN. Gel pieces were dried and resolved in 200 μL 50 mmol/L ABC, and 280 ng trypsin (Sequencing Grade Modified Trypsin, Promega) was added. Digestion was performed over night at 37°C. After digestion, the supernatant was taken and peptides were further eluted stepwise using 0.1% formic acid, 5% formic acid 50% acetonitrile and 100% acetonitrile, respectively. For each step, 200 μL eluent was added to the gel pieces followed by 30 min on a shaker. All corresponding supernatants were collected, pooled, dried in a vacuum centrifuge (Vacuum Concentrator, Bachofer) and stored at −20°C.

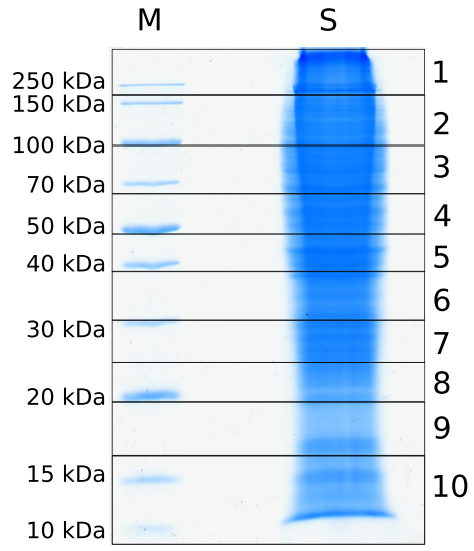

Fig. 1 Example SDS–PAGE gel for prefractionation of *Daphnia* proteins (Example). M: Marker (PageRuler Broad Range, Thermo), S: Sample, one biological replicate of FT44-2 exposed to predator. Left side: Molecular mass of marker proteins. Right side: Fractions.

LC-MS/MS

LC-MS/MS was performed with a nano-LC ultra-chromatographic device (Eksigent) coupled to a LTQ Orbitrap XL mass spectrometer (Thermo Scientific). Samples were resolved in 30 μL 0.1% formic acid. 10 μL was then injected and loaded on a C18 trap column (C18 PepMap100, particle size: 5 μm, 100 Å, column size: 300 μm x 50 mm, Dionex) for 10 min at a flow rate of 5 μL/min using mobile phase A (0.1% formic acid). RP chromatography was performed at a flow rate of 280 nL/min using a Reprosil-Pur C18 separation column (Reprosil-Pur C18 AQ, 3 μm, 150 mm × 75 μm (ID), Dr. Maisch) with a linear gradient from 6% to 30% mobile phase B (A: 0.1% formic acid, B: 84% acetonitrile and 0.1% formic acid) in 105 min, a further gradient to 45% in 25 min, followed by a 10 min step at 100% B and a 10 min step at 10% B. Overall gradient length was 150 min. For electrospray ionization, a distal coated Silica Tip (FS-360-50-15-D-20, New Objective) with a needle voltage of 1.7 kV was used. The MS method consisted of a cycle combining one full MS scan (Mass range: 300–2000 m/z) with five data-dependent MS/MS

events (35% collision energy). The dynamic exclusion was set to 30 sec.

Bioinformatic processing

Spectral data (Thermo raw files) were further processed using the software MAXQUANT (Cox & Mann 2008) version 1.4.1.2 and the implemented label-free quantification (LFQ) option (Smaczniak *et al.* 2012). Beyond that option, the 'match between runs option' was enabled (match time window 1 min, alignment time window 20 min). For protein identification, unique and razor peptides and a protein FDR of 1% were used. As database, prereleased gene-predictions of *D. magna* available at http://server7.wfleabase.org/genome/Daphnia_magna/ (V2.4 effective 05/2012) was used. Built-in contaminants database was included into the search. Carbamidomethyl was set as a fixed modification, whereas acetyl (protein N-term) and oxidation (M) were set as variable modifications. All other parameters were set according to MaxQuant default.

Similar proteins that could not be discriminated on the base of different peptides were included into one *protein group* by the software, sharing quantitative information. Subsequent protein sequence analysis was performed on sequences assigned as *majority proteins* by the software, meaning that these protein sequences had the best peptide evidence within the group. To simplify matters, we will not distinguish between proteins and protein groups when discussing quantitative data during the results and discussion part.

Further data analysis was conducted using R (R Development Core Team 2011). Label-free intensity data of precursor ions were first log2-transformed and then LOESS-normalized using the median values across biological replicates as a reference set (Papakostas *et al.* 2012). To find proteins differing significantly in abundance, a two-way ANOVA with treatment and genotype as explanatory variables was used. Furthermore, to get an FDR-based estimate for the set of significant proteins, the q-value was calculated (Storey & Tibshirani 2003) using the R package *qvalue* and 'Tukey Honest Significant Differences' method was applied on ANOVA results to look for detailed differences between all possible combinations.

To get further information on similar proteins, all significant protein sequences were blastp-searched against NCBI *nr* and *swissprot* databases using local standalone blast (Geer *et al.* 2010).

In addition to the protein database, associated *D. magna* protein annotation data were received from http://server7.wfleabase.org/genome/Daphnia_magna/ (V2.4 effective 05/2012). The GO annotation data of all proteins present in this protein database were used as background when testing for enrichment of GO terms within the significantly altered proteins using customized standalone EASE (Hosack *et al.* 2003) (Benjamini corrected *P*-value <0.05). Enriched terms were tested for redundant terms and semantic similarities using the online-tool REViGO (Supek *et al.* 2011) and visualized as treemap graphs using R.

In addition, protein sequences were also processed using the software BLAST2GO (Conesa *et al.* 2005), which uses results of NCBI blast search to map sequences directly to GO terms. These GO terms were used as additional information.

Cluster analysis and heatmap were generated using annHeatmap2 function of R HEATPLUS package.

Results

In this study, we analysed the response of different genotypes of *D. magna* to chemical cues released by the predator *T. cancriformis* at the morphological and the protein level.

We found both, significant morphological changes and also significant changes in protein abundance in response to predator exposure, the latter revealing either general or genotype-dependent alterations.

Morphological analysis

Significant differences between predator-exposed animals and the control group in three of the four genotypes were detected (see Fig. 2). For the genotypes Bl22, all three measured parameters, that is body length, tail spine length and body width, were significantly increased for the predator-exposed group. Animals of the genotype Max4 showed no significant alteration of body length in response to *T. cancriformis*; however, tail spine length and body width were significantly increased. Of the morphological parameters measured in K34J, only tail spine length increased significantly. Contrastingly, no parameters at all changed significantly in the genotype FT44-2.

Proteomic analysis

Using a label-free quantification approach, we were able to identify 1640 proteins or protein groups, which were detected in at least 3 of 5 analysed biological replicates per genotype per treatment. Out of this data set, 294 proteins show significant differences for predator exposure or predator–genotype interaction in two-way ANOVA (qvalue exposure or exposure x genotype <0.05, ratio predator-exposed/control protein intensity >1.5 or <0.8). Here, 166 proteins were significantly different between predator exposure treatment and control

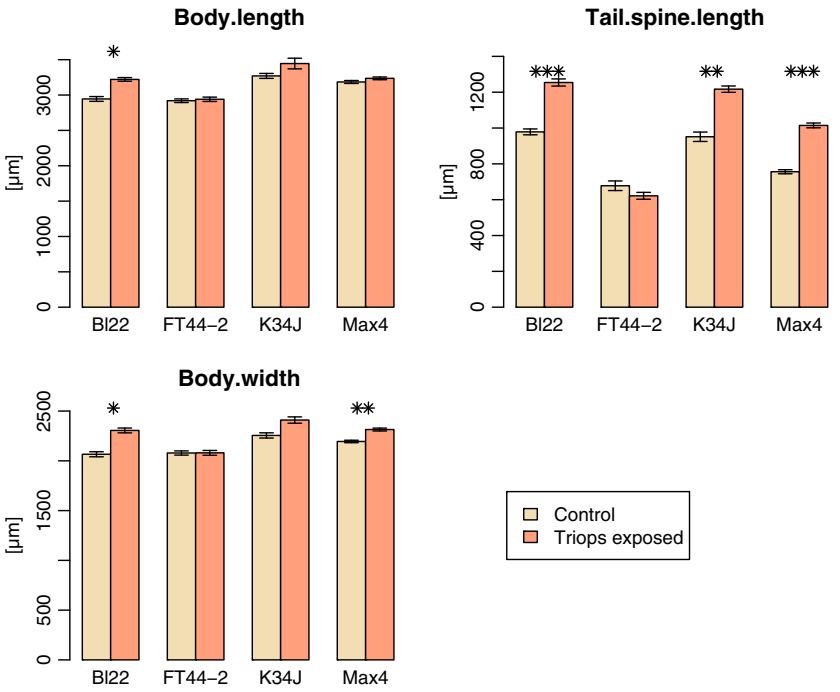

Fig. 2 Morphological analysis of three parameters in *T. cancriformis* exposed and control animals (bars: arithmetic mean, error bars: standard deviation). Ten animals were analysed per biological replicate and compared with ANOVA and Tukey *post hoc* test. Stars indicate significant difference (*P*.adjust ***<0.001, **<0.01,*<0.05) between *Triops cancriformis*-exposed and control group in one genotype.

and showed no exposure x genotype interaction, indicating genotype overlapping abundance alterations. Seventy-one proteins had a significant exposure x genotype interaction but no general exposure difference, suggesting a very genotype-dependent response to the predator. Fifty-seven proteins showed significant changes in both terms. Out of the proteins with a significant difference between predator exposure and control, 69 proteins were more abundant in predator-exposed animals, whereas 154 proteins were more abundant in the control group (Fig. 3).

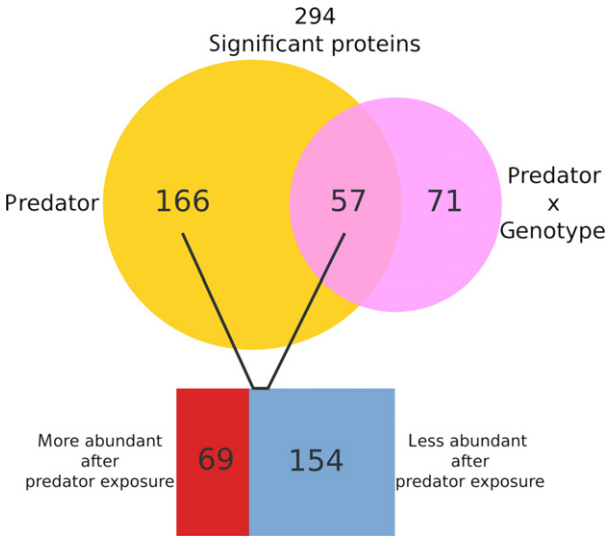

Fig. 3 Schematic overview on the distribution of significantly different proteins.

EASE analysis

To get further information on the proteins involved in the response to predator kairomones, we performed an enrichment analysis of gene ontology terms (GO). We used annotation information, which was derived from comparison of *D. magna* protein sequence data to known sequence motifs and afterwards assigning GO terms for biological processes, molecular function or cellular component.

To analyse the GO distribution within the complete data set, we computed the GO enrichment of all identified proteins. Furthermore, we tested all significant proteins for enrichment of GO terms. In addition, proteins with higher or lower abundance in the predator-exposed group were tested separately.

When analysing the whole data set (Table S3, Supporting information), we found 129 enriched GO terms. Most of these terms, for example *cytoplasm*, *translation* and *actin binding*, were related to proteins usually high abundant in the cell (Zhong *et al.* 2012). As the proteome is very dynamic and diverse in composition and therefore may span 6 to 12 orders of magnitude among abundance distribution, it is usually easier to detect high abundant proteins within a proteomic analysis (Eriksson & Fenyö 2007).

In a GO enrichment analysis of the proteins differing significantly in abundance, we found enrichment of 26 GO terms (Table 2). Most of these terms were also

Table 2 Significantly enriched GO terms (EASE analysis, *P*.adjust <0.05) with number of hits for all significant proteins (Total), more abundant proteins in the predator exposure treatment (Pred.+), less abundant proteins in the predator exposure treatment (Pred.−) and proteins having a significant predator exposure × genotype interaction (Geno.)

Gene.Category	Total	Pred.+	Pred.−	Geno.
F:structural constituent of cuticle	34	19	—	15
C:nucleosome	20	—	11	9
P:nucleosome assembly	20	—	11	9
F:structural constituent of ribosome	17	—	—	13
P:translation	17	—	—	13
C:ribosome	13	—	—	9
F:carbohydrate binding	11	—	—	6
F:pyridoxal phosphate binding	11	—	7	6
F:hydrolase activity	8	—	7	—
F:oxygen binding	6	4	—	—
P:one-carbon metabolic process	6	—	—	—
F:oxygen transporter activity	6	4	—	—
F:alpha-mannosidase activity	5	—	—	5
P:mannose metabolic process	5	—	—	5
F:acyl-CoA dehydrogenase activity	5	—	—	—
C:small ribosomal subunit	4	—	—	4
P:protein catabolic process	4	—	—	—
F:leukotriene-A4 hydrolase activity	3	—	3	—
P:leukotriene biosynthetic process	3	—	3	—
P:transport	—	9	—	—
C:lysosome	—	3	—	—
F:lipid transporter activity	—	5	—	—
F:haeme binding	—	8	—	—
P:carbohydrate metabolic process	—	—	11	—
F:metallocarboxypeptidase activity	—	—	7	—
C:prefoldin complex	—	—	—	3

present when analysing all identified proteins; however, order and significance levels of terms were different (e.g. *structural constituent of cuticle*) and highly significant terms from the whole data set (e.g. *actin binding*) disappeared completely. Therefore, correlation between the GO terms of the whole data set and the significant proteins is present, but seems not to be very strong.

Of these 26 terms, 19 terms were found when searching the whole data set. Beyond that, four terms were solely found enriched within the more abundant proteins, whereas two terms were solely found within the less abundant proteins and another one protein within the proteins showing a significant predator exposure x genotype interaction.

Here, more abundant proteins were enriched in terms related to the cuticle, oxygen and lipid transport, whereas less abundant proteins were related to the nucleosome, carbohydrate metabolic process and other enzymatic functions. Interaction proteins were also enriched in nucleosome assembly-, the ribosome- and carbohydrate-connected terms. Terms related to translation were enriched in the whole data set but not in the subgroups, meaning that the proteins related to the terms are divided between the subgroups.

As a detailed discussion of all significant proteins is beyond the scope of this research article, we focused the discussion on the following groups of proteins related to enriched GO terms: Cuticle-related proteins, calcium-binding proteins and vitellogenins- and translation-related proteins. Altogether, this set included 67 proteins (Table 4) covering 23% of all proteins significantly altered in abundance.

Genotypic differences

To visualize the different protein abundances between genotypes, we generated a heatmap by plotting significant protein abundances. We separated the row dendrogram at a height of 11 and therefore generated 4 different clusters (Fig. 4).

These four cluster were analysed according to enrichment of GO terms within the clusters using the EASE software. Semantic analysis and visualization were performed using ReviGO (Fig. 5).

Here, all control and predator exposure groups of the different genotypes clustered together with the exception of K34J. Two main clusters were formed, one containing the genotypes FT44-2 and Bl22 and the other the genotype Max4. Most interestingly, the control group of K34J seems to be more similar to Max4, whereas the *Triops*-exposed group responded similar to the other genotypes. In addition, the morphologically not responding genotype FT44-2 was missing a higher number of protein signals, which could be detected in the other groups, when compared to the morphologically responding genotypes (see white lines in Fig. 4 and Table S2, Supporting information).

GO enrichment analysis of protein clusters revealed terms also enriched in the global search (Fig. 5), indicating groups of proteins with similar abundances. Terms related to *lipid transporter activity* were enriched in cluster 1, which combined proteins with high abundances, especially in the morphologically responding genotypes. Cluster 1 also contained ribosome-related proteins, which were also found to be over-represented in cluster 3. Here, protein abundances were lower in general and especially in the nonresponding genotype FT44-2. In cluster 2, which contained lower abundant proteins and

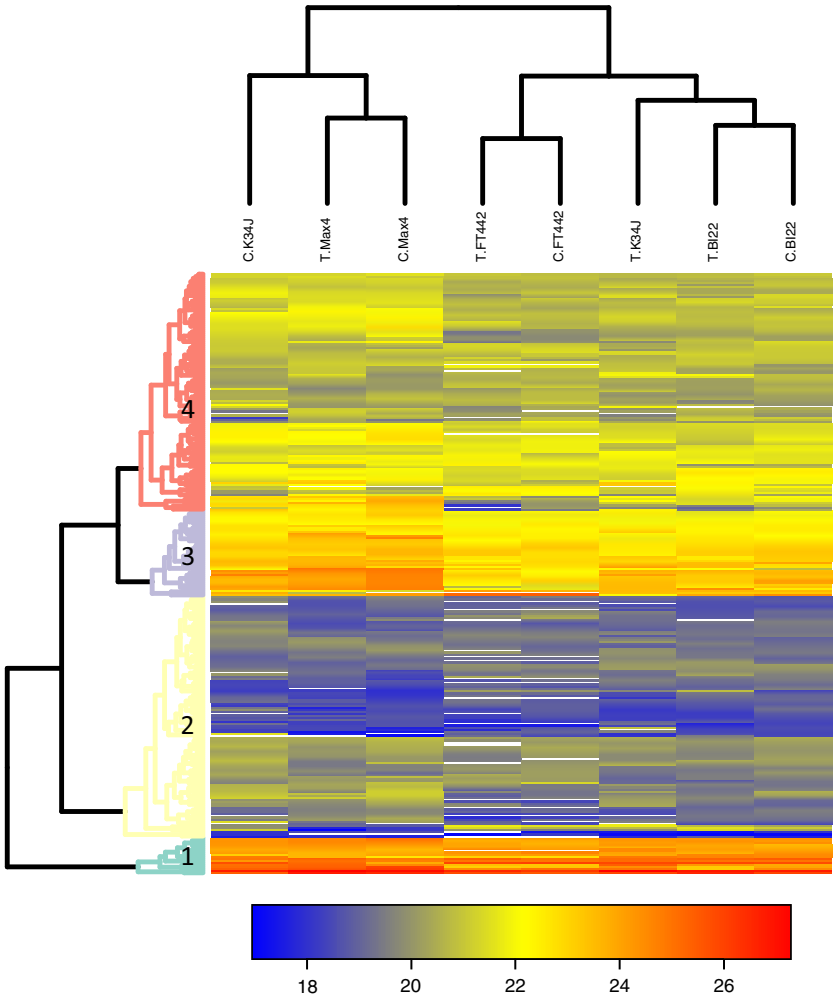

Fig. 4 Heatmap generated from all significant protein abundances (log2 transformed). Control (C) and predator-exposed (T) replicates of all genotypes were displayed. White fields indicate missing protein abundance values. The row dendrogram was cut at a height of 11 to gain 4 subclusters.

a lot of missing values for FT44-2, the term *Calcium ion binding* was enriched among others. In cluster 4, proteins of the cuticle were especially prominent.

For each genotype and each abundance altered protein, we determined the relative protein abundance level as the average value between all five biological replicates of control and predator exposure. To determine protein signatures characteristic for each of the morphologically responding genotypes, we looked for significant abundance differences when comparing morphologically responding genotypes to the nonresponding genotype FT44-2 on the basis of the *post hoc* test (Fig. 6). Here, in total, 149 proteins had a significant difference to FT44-2. Out of this, only 84 proteins were significantly different between FT44-2 and Max4, the genotype coexisting with *T. cancriformis* in its habitat. Only three proteins were significantly altered in all genotypes, whereas 11 significant protein differences were shared between Max4 and Bl22 and 19 between Max4 and K34J. Most interestingly, there was no overlap in the two central European genotypes Bl22 and K34J, but both genotypes had their own set of significantly altered proteins with 15 proteins only significant in Bl22 and 17 proteins in K34J.

Discussion

We analysed the response of four adult *Daphnia magna* genotypes with five biological replicates each, originating from habitats characterized by differences in predator composition to exposure of chemical cues released by the predator *Triops cancriformis*. We analysed both the morphological and proteomic level of the responses and found interesting differences throughout the genotypes.

The Finnish genotype FT44-2, which is not known to encounter predation in its habitat, did not show any morphological changes at all. The other three genotypes, which coexist with different predator species in their original habitat, increased at least one of the parameters with the greatest changes seen in the length of the tail spine. Furthermore, the genotype Max4,

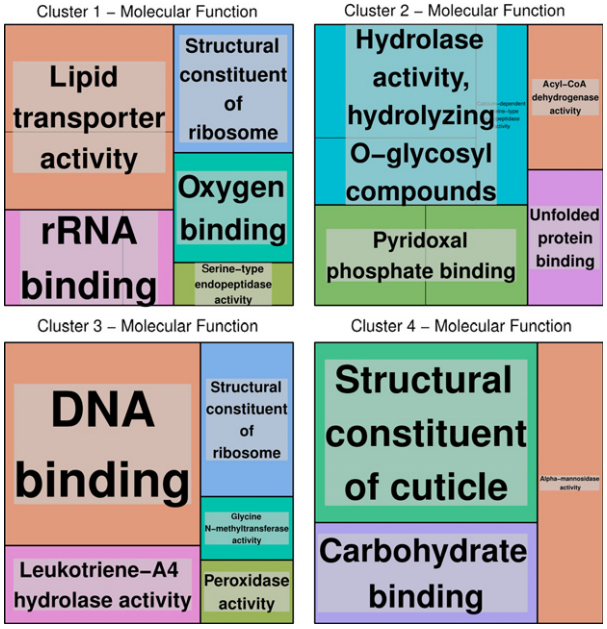

Fig. 5 Results of REViGO semantic analysis. Enrichment of GO molecular function terms is displayed. Proteins were separated according to the 4 groups found in the hierarchical clustering according to Fig. 4. Colours mark semantic similarity, whereas the size of the corresponding area reflects the *P*-value of the enrichment.

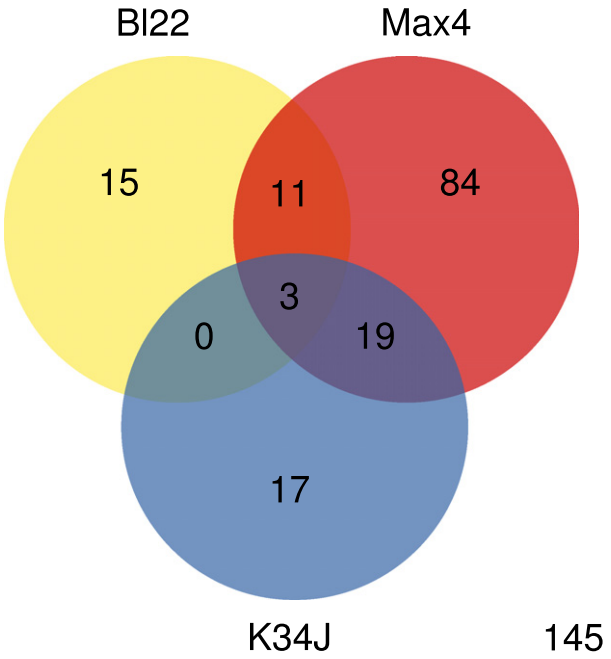

Fig. 6 Venn diagram of significantly altered proteins between genotypes. Relative average abundance level per genotype was tested using ANOVA followed by Tukey *post hoc* test and significant differences between the morphological not responding genotype FT44-2 and the other morphologically responding genotypes were noted.

which is the only genotype coexisting with *T. cancriformis* in its natural habitat, showed the most significant increases in body width and tail spine length when exposed to the predator.

At the protein level, we found both significant protein abundance alterations which were not influenced by the genotype and abundance changes which strongly depend on the genotype. While the first group consisted of proteins which could be interpreted as taking part in a general response to predator exposure, the latter group could be seen as mediators of genotype-dependent responses. Proteins of the general response were found in the groups of vitellogenin-related proteins, whereas cuticle-related, chitin-modifying, calcium-binding and translation-related proteins show genotype-dependent changes (Table 3).

Interestingly, at least half of the significantly altered proteins found in our study in the groups of cuticle-, vitellogenin-, calcium- and translation-related proteins have no shared sequence similarities with any protein sequence present in the NCBI nr database outside the *Daphnia* lineage (Fig. 7 and Table S2, Supporting information). Colbourne *et al.* (2011) found that around one third of the genes in the *D. pulex* genome have no detectable homologies, a large fraction when compared to other species. Furthermore, these lineage specific genes were significantly over-represented within genes that were affected by exposure to biotic and abiotic stressors (Colbourne *et al.* 2011), indicating that these genes were the most responsive genes to ecological challenges. This assumption is also supported by our study, as we found a high number of *Daphnia* proteins involved in the response to predator exposure.

Genotypic specificity of predator-induced phenotypic plasticity

Genotypes of *Daphnia* are known to show huge differences at the transcriptional level when exposed to environmental stressors, for example single and mixed stresses of cadmium and a toxic cyanobacterium (De Coninck *et al.* 2014), changes in temperature (Yampolsky *et al.* 2014) and changes in phosphorus supply (Roy Chowdhury *et al.* 2015). In these studies, changes in gene expression were not always well correlated with tolerant or sensitive phenotypes, indicating that different gene expression pattern may lead to a similar phenotype.

In our study, the analysed genotypes showed differences at the morphological and protein level. Max4, which is the only genotype that shares its original habitat with the predator *T. cancriformis*, showed a strong response at the morphological level and had the highest number of differences compared with the nonresponding genotype FT44-2 (Fig. 6).

Table 3 Summary of significantly altered proteins between genotypes. Relative average abundance level per genotype was tested using ANOVA followed by Tukey *post hoc* test and significant differences between the morphological not responding genotype FT44-2 and the other morphologically responding genotypes were noted. Values are numbers of proteins with ratio of protein abundance per genotype divided by protein abundance of FT44-2 displayed in parentheses

	Protein names	Bl22	K34J	Max4
Cuticle	No name available	1 (0.99)	1 (3.63)	5 (0.41–2.57)
Chitin modification	No name available	—	—	3 (2.04–9.78)
	Chitinase (Type 4, 7, 10)	—	1 (0.19)	2 (1.23–3.41)
Calcium-binding	No name available	—	1 (0.41)	1 (2.1)
Vitellogenin	No name available	—	1 (3.68)	2 (0.39–1.97)
Translation	No name available	—	1 (1.54)	6 (1.92–5.82)
	40S ribosomal protein (S16, S23)	—	—	1 (2.55)
	60S ribosomal protein (L4, L12, L15, L23)	—	1 (0.32)	3 (0.3–1.99)
	Clustered mitochondria protein	—	—	1 (0.48)
	Elongation factor 1-beta	—	1 (0.72)	—

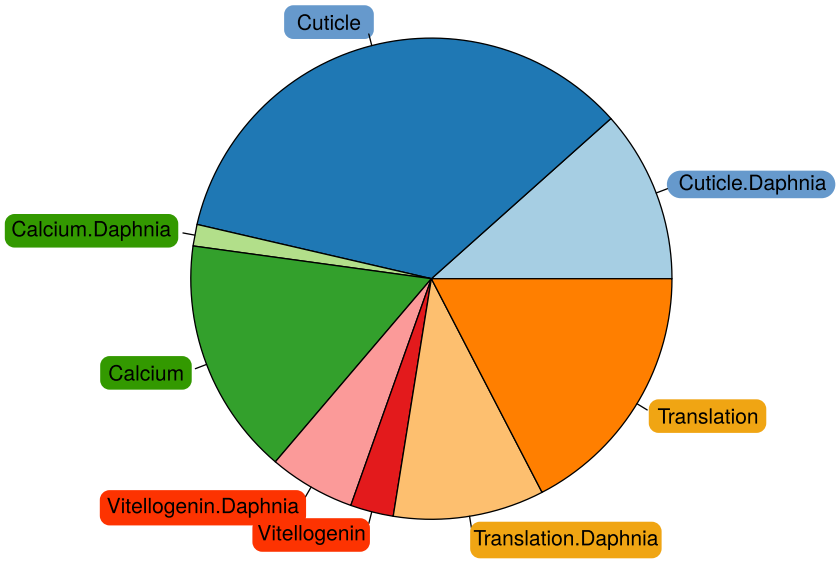

Fig. 7 Pie chart displaying number of significantly altered proteins among the enriched groups. Here, proteins only detected in the *Daphnia* group were marked separately (.Daphnia).

Furthermore, the two other morphologically responding genotypes Bl22 and K34J are known to coexist not with *Triops* but with other predators in their original habitats. Compared with Max4, they showed a reduced number of significantly altered protein abundances when tested against FT44-2. In addition, although sharing a similar number of proteins with the genotype Max4, no overlapping proteins were found between Bl22 and K34J, indicating differences in the response to *Triops* predation at the protein level. This assumption was also supported by the genotypic differences in the cluster analysis (Fig. 4). However, as there exists no information on the predation history of the habitats, it is not clear whether the responses of Bl22 and K34J are a result of former *Triops* predation [both genotypes have an overlapping distribution with *T. cancriformis* (Korn *et al.* 2006)] or of the coexistence with other invertebrate predators that might have selected for similar responses. A phenomenon like this was already described in another *Daphnia* species, *D. barbarta*; here, different invertebrate predators induced different shapes of the same morphological defensive trait (Herzog & Laforsch 2013).

Contrastingly, the Finnish genotype FT44-2, which did not coexist with any predator in its habitat, showed responses most different at both morphological and protein level. The morphology of these animals did not change in any of the measured parameters, and protein abundances were altered differently compared with the other three genotypes.

Altogether, morphological and proteome responses seems to be related to the predator regime of the original habitats. However, further analysis of more genotypes from different *Daphnia* populations and increased information on associated habitats are needed to make

Table 4 Summary of proteins significantly involved in the general and in the genotype dependent response. Total: total number of proteins. Pred.+: number of more abundant proteins with significant influence of predator exposure, pred.−: number of less abundant proteins with significant influence of predator exposure, geno: number of proteins with a significant predator exposure x genotype interaction. Values per genotype are minimal/maximal ratios within the genotype for the predator-exposed/non-exposed signal

	Protein names	Total	Pred.+	Pred.−	Geno.	FT44-2	BL22	K34J	Max4
Cuticle	No name available	19	12	6	6	0.54–1.32	0.46–2.69	0.43–4.01	0.59–2.97
Chitin modification	No name available	4	—	4	2	0.85–1.49	0.67–1	0.47–0.62	0.42–0.66
	Chitin deacetylase 9	1	—	1	—	0.68	0.68	0.79	1.12
	Chitinase (Type 4,7,10)	4	—	3	3	0.85–1.52	0.58–1.15	0.4–1.26	0.49–0.79
Calcium-binding	Juvenile hormone-binding protein	1	1	—	—	0.89	1.02	1.33	1.58
	No name available	4	2	2	4	0.78–1.24	0.92–1.25	0.52–4.5	0.93–1.33
	Calmodulin	1	1	—	—	—	2.13	2.15	1.06
	Calpain-B	1	—	1	—	0.95	0.67	0.93	0.76
	DE-cadherin	1	—	1	—	0.71	0.63	1	0.95
	Epidermal growth factor receptor substrate	1	1	—	—	—	2.27	1.17	1.16
	Fibrillin-2	1	—	1	1	—	0.64	0.93	0.56
	Myosin	2	1	1	1	0.85	1.17–1.96	0.35–1.33	0.8–0.94
	Troponin C	1	—	1	—	0.73	0.61	0.78	0.78
Vitellogenin	Vitellogenin receptor	1	1	—	—	1.25	1.53	1.21	1.35
	No name available	5	4	—	1	0.92–1.34	1.11–1.63	1.1–1.56	1.05–2.17
Translation	No name available	10	1	4	9	0.88–1.58	0.77–1.69	0.45–0.91	0.6–2.18
	40S ribosomal protein (S16, S23)	2	1	—	1	1.08–1.36	0.85–0.91	0.62–1.16	0.75–1.54
	60S ribosomal protein (L4, L12, L15, L23)	4	1	2	1	0.86–1.23	0.67–0.97	0.92–1.17	0.65–2.21
	ATP-binding cassette	1	—	1	—	1.01	0.88	0.65	0.64
	Clustered mitochondria protein	1	—	—	1	0.86	1.18	1.02	1.54
	Elongation factor 1-beta	1	—	—	1	0.92	0.95	1.6	1.19

more general statements on local adaptation of *D. magna* to *Triops* predation.

Cuticle structural component proteins and chitin-modifying enzymes may be directly involved in predator-induced morphological defences

As also shown in this study, *D. magna* changes its carapace morphology in the presence of the predator *T. cancriformis* and increases its body length, body width and tail spine length, which serve as an effective defence against *Triops* predation (Rabus & Laforsch 2011; Rabus *et al.* 2012). In addition, these large-scale morphological defences are accompanied by ultrastructural defences, which result in increased cuticle hardness (Rabus *et al.* 2013). The cuticle of the *D. magna* carapace consists, as in all arthropods, of an epicuticle which is mainly built out of proteins and lipids and a procuticle which is made of chitin filaments embedded in a proteinaceous matrix (Andersen 1995). The mechanical properties of the cuticle are determined by its components including chitin nanofibres, proteins and the degree of cross-linking of these components (Vincent & Wegst 2004).

The 19 cuticle proteins found in this study were annotated with the GO term *structural constituent of cuticle*, which was found enriched (Table 2 and Fig. 5). When comparing predator exposure and control group protein abundances per genotype, cuticle proteins minimal fold changes were similar around 0.5 and similar in all genotypes. In contrast, the three morphologically responding genotypes have much higher maximal fold changes compared to the nonresponding genotype (4 vs. 1.32, Table 4). Furthermore, ratios in the nonresponding genotype were often low when high in the morphologically responding genotypes and vice versa (Supporing information Table S2), indicating differences in cuticle composition between morphologically responding and nonresponding genotypes. In addition to the cuticle proteins, other proteins were annotated with the GO term *chitin catabolic process*. Four of them were similar to chitinases found in *Tribolium castaneum*. Chitinases are chitin-degrading enzymes which are important for the remodelling of chitinous structures (Merzendorfer 2003). Within the chitin-modifying enzymes, we additionally found one chitin deacetylase, which is known to influence chitin–protein interactions

(Vincent & Wegst 2004). Protein abundances of chitin-modifying enzymes were in most cases lower after predator exposure; however, the decrease was usually stronger within morphologically responding genotypes (Table 4). Furthermore, when looking at relative abundance levels of cuticle proteins and chitin-modifying enzymes in each genotype, all responding genotypes have significant differences to the nonresponding FT44-2 (Table 3), indicating genotype-dependent responses. The highest number of altered cuticle-associated proteins was found in the genotype Max4, which shares its habitat with *T. cancriformis*.

Chitinases and chitin deacetylases may influence alterations in the ultrastructure of the cuticle and could therefore, together with the altered cuticle protein composition, be responsible for predator-induced changes of cuticle mechanical properties, leading to increased cuticle hardness (Rabus *et al.* 2013). Predator-induced increase in the exoskeleton is known to be an effective protection in different systems, for example crab-induced increase in shell thickness in mussels (Leonard *et al.* 1999) or fish-induced increase in cuticle thickness in dragonfly larvae (Flenner *et al.* 2009). In *D. magna*, the enhanced cuticle stability is thought to increase the handling time of the predator and therefore decrease the animal's susceptibility to predation (Rabus *et al.* 2012).

Predator exposure affects calcium-binding protein abundance

An important component of the cuticle of arthropods is calcium, which also contributes to the mechanical properties (Vincent & Wegst 2004). In contrast to other freshwater zooplankton, *Daphnia* has relatively high calcium demands because of their heavily calcified exoskeleton and large loss of calcium during moulting (Hessen & Rukke 2000; Waervagen *et al.* 2002; Jeziorski & Yan 2006). Low-calcium environment is known to inhibit predator-induced morphological changes in *D. pulex* (Riessen *et al.* 2012), indicating the importance of calcium for the formation of cuticle-related defensive traits in *Daphnia*.

In our study, we found proteins associated with the GO term *calcium ion binding*, which showed significant differences in the predator exposure treatment (Table 4). Genotype-dependent abundance differences were detected in calcium-binding proteins and in some cytoskeletal components. All other proteins are not influenced by the genotype, indicating a more general response. In addition, only two proteins showed significantly different abundances between the morphologically responding genotypes and the nonresponding FT44-2 (Table 4); however, four proteins of this group were not detected in FT44-2 at all and were therefore not included in the analysis.

Less abundant calcium-binding proteins significantly altered by predator exposure are related to a variety of functions like extracellular matrix, proteolysis, cytoskeleton and muscle. In different genotypes, the abundance of calcium-binding proteins was decreased in response to the predator, perhaps to save more calcium for the formation of defensive structures. On the other hand, they increase the abundance of other calcium-binding proteins related to important processes for defensive trait formation. For example, one calcium-binding protein is also a vitellogenin receptor with higher abundance in all predator-exposed genotypes, even in the one that is not responding at the morphological level. This receptor is known to be involved in the uptake of vitellogenin by endocytosis and is regulated by juvenile hormone (Chen & Lewis 2004), which is another indicator of the involvement of the JH pathway in inducible defences of *D. magna*.

We also found one protein having a juvenile hormone-binding sequence motif (PF06585/JHBP) with increased abundance in the morphologically responding genotypes after predator exposure. Juvenile hormones (JHs) are known to regulate many aspects of insect physiology, such as development, reproduction, diapause and polyphenisms (Nijhout 1994; Riddiford 1996; Wyatt & Davey 1996). In *Daphnia*, they regulate male reproduction and are possibly involved in haemoglobin synthesis (Eads *et al.* 2008), and also regulate vitellogenin synthesis (Tokishita *et al.* 2006). In addition, JHs are able to enhance the formation of morphological defensive structures (Oda *et al.* 2011; Miyakawa *et al.* 2013; Dennis *et al.* 2014) and may therefore be involved in the regulation of predator-induced phenotypic plasticity. The JH-binding protein found in our study may bind JH during the transport in the haemolymph and protects the hormone from degradation (Kolodziejczyk *et al.* 2003) and therefore could be an indicator of higher JH titre in morphological defended animals.

Furthermore, the protein calmodulin was found to increase two-fold during predator exposure in two of the responding genotypes (Table 4). Most interestingly, calmodulin was not detected in the nonresponding genotype, indicating that this protein is connected to morphological changes of the cuticle. Calmodulin serves as intermediate messenger and transduces calcium signals to other targets. Indeed, it was shown that pharmaceutical inhibition of calmodulin in *D. magna* led to a decrease in gene expression of a cuticle protein and vitellogenin (Furuhagen *et al.* 2014). Therefore, calmodulin may also be involved in signal pathways leading to altered abundances of cuticle proteins and vitellogenins found during predator exposure in our study.

Predator exposure caused increased yolk protein abundance in all genotypes

Our data set of abundance altered proteins contained members of the yolk protein group (vitellogenins, vtgs) as well as one vitellogenin receptor. These proteins were more abundant in animals exposed to the predator with slightly higher fold changes in the morphologically responding genotypes (Table 4). Regarding global protein abundances (Table 3), three vtg proteins were significantly different between the nonresponding and responding genotypes. Here, fold changes were both higher and lower in the responding genotypes, indicating that every genotype, including the nonresponding genotype, had their own set of high abundant vtg proteins. All vtg proteins found in our study were annotated with the term *lipid transporter activity*, a term enriched in the GO analysis (Table 2).

Yolk proteins serve as substrate and energy supply for the developing embryo in most oviparous animals (Subramoniam 2010). They are synthesized in extraovarian tissue like the insect fat body (Sappington & Raikhel 1998) or non-mammalian vertebrate liver (Romano *et al.* 2004) and then transported to the developing oocyte. In *Daphnia*, fat cells which form the fat body, are the most probable place of vtg synthesis (Zaffagnini & Zeni 1986). During the formation of inducible defences in adult *D. magna*, changes in vtg abundance may result from an increasing number of eggs produced or an elevated vtg concentration per egg. Therefore, they most likely reflect life history shifts associated with predator exposure. Indeed, *D. magna* are known to have more offspring with an increased body size in the presence of *T. cancriformis* (Hesse *et al.* 2012). In our study, vtg and vtg receptor abundances increased in all genotypes, also in the genotype that did not respond to predator exposure at the morphological level. Therefore, vitellogenin content seems to be part of a general response to predator exposure.

In other studies, the exposure of *D. magna* to chemical cues of predatory fish or *Chaoborus* larvae is known to alter yolk dynamics (Stibor 2002; Effertz *et al.* 2014). In addition, our previous proteomic study on *D. magna* embryos exposed to *Triops* revealed that vtg is affected by predator exposure (Otte *et al.* 2014). Predator-exposed embryos showed a striking decrease in vtg abundance, which indicated an increased metabolism resulting in higher vtg turn-over, and may indicate the costs associated with the building of *Triops*-induced defensive structures.

In *D. magna*, one vtg gene is known to have a juvenile hormone (JH)-responsive element and is negatively controlled by juvenile hormone agonists in neonate *Daphnia* (Tokishita *et al.* 2006). In contrast, JH or JH agonists are able to positively control vtg synthesis in adult female insects (Tufail *et al.* 2014) and crustaceans (Subramoniam 2010). In our study on adult female *D. magna*, vtg as well as a JH regulated vtg receptor (Chen & Lewis 2004) and a JH-binding hormone are more abundant in animals exposed to the predator; therefore, a positive control of vtg by JH seems also likely. Therefore, JH may be involved in key processes of defensive trait formation in *D. magna*, namely changes of morphology and vitellogenin synthesis.

Ribosomal proteins and an elongation factor are involved in plastic responses

Within the protein annotations of our data set, we also found enriched GO terms connected to the ribosome and translation (Table 2 and Fig. 5). Thirteen of these 19 proteins were not only influenced by predator exposure but also change their abundance in a genotype-dependent manner. Furthermore, 14 proteins have significantly altered global protein abundances when comparing the morphologically responding genotypes to the nonresponding genotype FT44-2 (Table 4). Again, the highest number of differences, 11, were found in the genotype Max4.

Some proteins were identified as ribosomal proteins well conserved throughout species (Table 4), most of them also present in prokaryotic ribosomes (Ban *et al.* 2014). Recent structural analysis of human and fly ribosomes revealed that ribosomes of higher eukaryotes consists of a core most similar to the bacterial ribosome surrounded by extra layers of proteins specific for eukaryotes (Anger *et al.* 2013).

Among the ribosomal proteins involved in plastic defensive trait formation of *D. magna*, protein S23, which is in concordance with the bacterial protein S12, may affect accuracy of protein synthesis (Wilson & Nierhaus 2005). A decreased abundance of this protein may lead to faster translation rate, probably matching increased protein needs. Another interesting protein is ribosomal protein L12, which is more abundant in animals exposed to the predator. The corresponding protein in bacteria, L11, is involved in stress response, inducing the downregulation of components of the translational apparatus and upregulation of metabolic enzymes (Wendrich *et al.* 2002). Furthermore, ribosomal proteins contained in our data set are known to have functions beyond the ribosome (Aseev & Boni 2011) including regulation of mRNA stability and DNA repair (prokaryotic L4/eukaryotic L4, Trubetskoy *et al.* (2009)) and replication (prokaryotic L14/eukaryotic L23, Wool (1996)). In addition to the ribosomal proteins mentioned above, a translation elongation factor was also found involved in inducible defences of *D. magna*, increasing

in abundance in the morphologically responding genotypes K34J and Max4 during predator exposure.

The group of translation-related proteins resembles the largest fraction of genotype-dependent protein responses in our study. Furthermore, alterations of ribosomal protein abundances were also observed when exposing *D. magna* to chemical cues of a fish predator (Effertz *et al.* 2014). In that study, these proteins were the largest group of proteins which responded to predator exposure, indicating the importance of translation-associated proteins in predator-induced responses.

Conclusion

Analysing the morphology and the proteome of different *D. magna* genotypes exposed to chemical cues released by the predator *T. cancriformis*, we found general and genotype-dependent responses to predator exposure. Vitellogenin proteins and a vitellogenin receptor are part of a general response to predation found in all genotypes, whereas cuticle-associated, calcium-binding and translation-related proteins show genotype-dependent abundance alterations. Furthermore, a large part of these proteins was so far detected only in *Daphnia*, which supports the hypothesis that the corresponding genes are the most responsive genes to ecological challenges. Most interestingly, genotype-dependent changes at the morphological and protein level correspond to differences in predator composition of the habitats.

In addition, the finding of a juvenile hormone-binding protein and the calcium-binding protein calmodulin offers interesting candidates possibly participating in signal pathways that regulate cuticle protein expression and vitellogenin synthesis.

The results of our work offer most interesting starting points to study the molecular mechanisms underlying inducible defences in *D. magna* in more detail. Our study increases the knowledge on molecular mechanisms underlying defensive trait formation in *Daphnia*, highlighting key players and important processes.

Acknowledgements

We thank M. Kredler and E. Ossipova for excellent help with the rearing of *Daphnia* and *Triops*. Members of Laforsch and Arnold group helped during induction and proteomics experiments. This work is part of the EUROCORE STRESSFLEA project funded by European Science Foundation. Our work benefits from and contributes to the Daphnia Genomics Consortium. The authors declare no conflict of interest.

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C.L. designed and supervised research. K.A.O. and I.S. conducted predator exposure experiments. K.A.O. conducted proteomic experiments, performed mass spectrometry analysis and conducted bioinformatical analysis of the data. T.F. supervised mass spectrometry analysis. K.A.O. wrote the first draft of the manuscript, and C.L., T.F., I.S. and G.J.A. contributed substantially to revisions. All authors read and approved the final manuscript.

Data accessibility

The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium (Vizcaíno *et al*. 2014) via the PRIDE partner repository with the data set identifier PXD001787. Results of morphological analysis and protein results list (combining quantitative data and annotation information) are available in the supporting information.

Supporting information

Additional supporting information may be found in the online version of this article.

Table S1 Results of morphological analysis.

Table S2 Combined table of protein LFQ data and protein annotation data.

Table S3 Results of GO enrichment analysis computed for all identified proteins.

Fig. S1 Photograph (A) of induction experiment setup and schematic side view of one biological replicate (B).

Fig. S2 Analysis of morphological parameters. Body length, body with and tail spine length of ethanol-preserved animals were determined as indicators of defensive trait formation.

4 Analysis of genotype-genotype interactions of the parasite *Pasteuria ramosa* and its host *Daphnia magna* at the protein level

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unpublished manuscript.

Analysis of genotype-genotype interactions of the parasite *Pasteuria ramosa* and its host *Daphnia magna* at the protein level

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Keywords: *Daphnia*, *Pasteuria*, proteomics, label-free quantification, host-parasite interaction

Abstract

Parasites are known to have great influence on the structuring of natural populations. The fresh water crustacean *Daphnia magna* and its endoparasite *Pasteuria ramosa* are a well-studied system for host parasite-interactions, which is known for its strong genotype-genotype interactions. Infections can only result when the parasite is able to attach to the hosts cuticle. The molecular mechanisms underlying this mechanism are so far unknown.

We used a proteomic analysis including a label-free quantification approach to study differences in the cuticle proteome of *D. magna* genotypes known either to be susceptible or non-susceptible to *P. ramosa*. Furthermore, we compared the cuticle proteomes of infected animals to a control group. We detected genotype-specific abundance alterations within cuticle proteins and matrix-metalloproteinases, which may be responsible for differences in susceptibility to the parasite. In addition, a collagen-like protein of *P. ramosa*, which was already proposed to be important for the attachment process, was found to be highly abundant in susceptible animals exposed to the parasite. Beyond that, we found changes in chitin-modifying enzymes in this group, which may be related to retarded moulting induced by the parasite to increase its infection success.

Our proteomic approach reveals proteins involved in molecular mechanisms of host-parasite interactions, consistent with existing hypotheses and providing new insights into this topic. Furthermore, we present the first proteome of a *Daphnia* compartment.

Introduction

Interactions between host and parasites are an important factor for shaping ecology (Elton, 1927) and evolution (Haldane, 1949) as the antagonistic nature of these interactions is a key force driving coevolution in natural populations (Thompson and Cunningham, 2002; Harvell, 2004). A popular model host-parasite system for studying natural diseases is the fresh water crustacean *Daphnia magna* and its parasites (Green, 1974). Here, host-parasite interactions have been studied in great detail [e.g. Ebert (2005, 2008)] and there is strong evidence, that parasites severely influence natural *Daphnia* populations [e.g. Ebert et al. (2000); Duncan and Little (2007)].

One well-studied example parasite of *Daphnia* is the Gram-positive, endospore forming bacterium *Pasteuria ramosa*. This parasite castrates and eventually kills its host *D. magna* and infection occurs strictly horizontally via the release of endospores from dead conspecifics (Ebert, 2005). The susceptibility of *D. magna* to this microparasite is known to depend strongly on interactions between the genotypes of host and parasite, creating a binary infection outcome (animals are either susceptible or resistant) for different host-parasite genotype combinations (Luijckx et al., 2011). This high genotypic specificity is almost entirely dependent on one step of the infection process when *P. ramosa* spores attach (or not) to the esophageal cuticle of the host (Duneau et al., 2011).

Daphnia, the water flea, is a classical model organism in ecology (Lampert, 2006) and ecotoxicology (Denslow et al., 2007) because of its central position in limnic food webs, linking primary production to higher trophic levels. More recently, *Daphnia* has gained popularity as a model organism in other fields, e.g. functional genomics (Miner et al., 2012) due to a number of advantageous characteristics like easy culturing in the laboratory, short generation times and a transparent body. Furthermore, parthenogenetic reproduction enables the generation of a defined genetic background

and also the availability of molecular tools for *Daphnia* increases (Colbourne et al., 2011; Kato et al., 2011). Proteomics can now also be used to study mechanisms at the protein level in *Daphnia*, as recently emerged genomic data form the basis for these approaches (Colbourne et al., 2011). Proteomics is especially appropriate for biochemical and molecular characterisation of organisms, as proteins are involved in almost all biological processes. Proteomic methods were already applied to analyse aspects of *Daphnia* physiology (Zeis et al., 2009; Schwerin et al., 2009; Gerke et al., 2011; Zeis et al., 2013), ecotoxicology (Rainville et al., 2014) and predator-induced phenotypic plasticity (Otte et al., 2014; Effertz and von Elert, 2014; Otte, 2015).

Studies on the molecular mechanisms involved in *Pasteuria* infection and the response of *Daphnia* to this stressor are rare so far. On the host's side, a candidate gene approach was not able to detect significant changes in gene expression of putative immune system related genes in *D. magna* exposed to *Pasteuria* (Decaestecker et al., 2011) whereas a transcriptomic analysis found disturbances of ATP production after parasite infection (Jansen et al., 2013). On the parasite's side, a gene family has been described which may linked to the attachment step. These collagen-like proteins of *Pasteuria* may be crucial for successful infection of the host (Mouton et al., 2009).

In this study, we used a proteomic approach to explore molecular mechanisms underlying susceptibility to the parasite. As the genotypic specificity of parasite infection seems to be indicated by the composition of the host's cuticle, we analysed cuticles of two *D. magna* genotypes, known to be either susceptible to *P. ramosa* or not, to proof if the genetic determinate is visible in the different cuticle proteomes. Furthermore, we examined alterations in the cuticle proteome of parasite exposed and non-exposed *Daphnia* of the susceptible genotype.

Methods

Host and parasite

For exuvia sampling, we chose two genotypes of *D. magna*, FI-SK58 and FI-FA46, both originating from rockpool habitats in Tvärminne, Finland. The resistance phenotype of these clones was determined relative to clone C1 of *P. ramosa* by performing an attachment test using fluorescently labelled spores as described in (Duneau et al., 2011). The *Daphnia* genotype FI-SK58 is resistant to *P. ramosa* genotype C1, whereas genotype FI-FA46 is susceptible to *P. ramosa* genotype C1.

For each *D. magna* genotype, we raised 120 mothers with 10 individuals per 400 ml jar under standard conditions using ADaM medium (Klüttgen et al., 1994), temperature of 20 °C, light: dark cycle of 16 h:8 h and animals were fed 10^5 cells of the algae *Scenedesmus obliquus* per ml of medium per day (Ebert et al., 1998). When the mothers were 30 days old, we collected 1120 offspring born on the same day $\pm$ 24 h and distributed these offspring among 100 ml jars, 20 individuals per jar. These offspring were raised under standard conditions until they were two weeks old and were then used for parasite exposure and exuvia sampling.

Parasite exposure and exuvia sampling

Individuals were transferred to clean jars containing fresh medium and were checked every 30 min for newly shed exuvia. Exuvia were collected for 3 h and kept on ice the entire time. Only animals of the genotype FI-FA46 were exposed to spores of the parasite. Cleaned spores of *P. ramosa* (spores washed three times using 1 ml sterile ADaM, followed by centrifugation for 3 min at 16,000 RCF to pellet the spores, followed by filtration to 20 µm) were applied every 6 h (50,000 spores per individual per dose) until a sufficient number of exuvia had been collected.

We collected 100 exuvia per biological replicate, washed them twice using 1 ml ice-cold sterile PBS and snap-froze them using liquid nitrogen. In total, three biological replicates were generated for each of the three experimental groups, i.e. non exposed FI-SK58, non-exposed FI-FA46 and FI-FA46 exposed to *P. ramosa* C1 (FI-FA46-C1).

Proteomic sample preparation

To generate samples for proteomic analysis, 100 frozen exuvia per biological replicate were homogenised in a mortar under liquid nitrogen thus preventing thawing. The resulting powder was solubilised in 20 µl lysis buffer (2 mol L^{-1} Thiourea, 6 mol L^{-1} Urea, 4 % CHAPS, 1 cOmplete ULTRA Tablets Mini (Roche) per 5 mL buffer). Afterwards, each sample was sonicicated on ice with 10 s pulse followed by 20 s break for 5 min in total (Sonoplus, Bandelin). Samples were then centrifuged using a QIA Shredder Mini Spin Column (Qiagen) for 3 min at 13,200 rpm to eliminate debris. Then, samples were concentrated using Amicon Ultra 0.5 ml filter devices 3 kDa cut-off (Merck Millipore) by centrifugation for 30 min at 13,200 rpm.

The successful preparation of protein lysates from *Daphnia* samples is a challenging task due to the high degree of proteolytic activity (Fröhlich et al., 2009; Zeis et al., 2009; Schwerin et al., 2009; Kemp and Kültz, 2012). However, as the proteases originate from the gut of *Daphnia* (von Elert et al., 2004; Agrawal et al., 2005; Schwarzenberger et al., 2010), they should not be present in *Daphnia* cuticle samples. Therefore, our sample preparation protocol should be appropriate, an assumption which is also supported by gel images of our protein samples [see image 1].

SDS-PAGE pre-fractionation and tryptic digestion

Concentrated samples were fractionated on a small SDS-PAGE gel (gel size 8 x 7 x 0.75 cm) using a 1 cm stacking gel (4 % acrylamide, 0.125 mol L^{-1} 6.8 pH Tris-HCl, 0.1 %

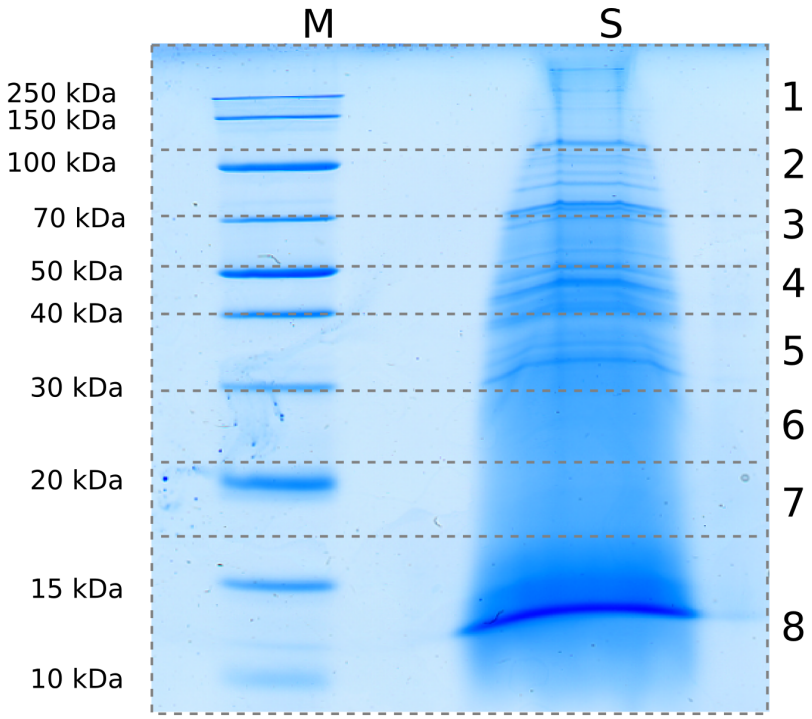

Fig. 1: Example SDS-PAGE gel for pre-fractionation of *Daphnia* Proteins (Example). M: Marker (PageRuler Broad Range, Thermo), S: Sample, one biological replicate of FI-FA46. Left side: Molecular mass of marker proteins. Right side: Fractions.

SDS, 0.05 % APS, 0.1 % TEMED) and a separation gel (12 % acrylamide, 0.375 mol L^{-1} 1.5 pH 8.8 Tris-HCl, 0.1 % SDS,0.1 % APS, 0.5 % TEMED). Prior to gel-electrophoresis, 2 % SDS was added, and each sample was treated with 4.5 mmol L^{-1} DTT at 65 °C for 30 min and afterwards with 10 mmol L^{-1} for 15 min at room temperature. Glycerol was added to a concentration of 10 % (v/v). Electrophoresis was performed on a Mini-PROTEAN II device (Biorad) at 80 V for 15 min and afterwards at 150 V (Running Buffer: 25 mmol L^{-1} Tris, 0.2 % SDS, 192 mmol L^{-1} glycine). Gels were then stained by colloidal coomassie (Roti-Blue, Roth) according to the manufacturer's protocol.

For in-gel tryptic digestion we first washed the gels two times with water and then cut each gel lane in 8 pieces (see figure 1), transferred each piece to a tube and minced

it with a pipette tip. Gel pieces were washed for 30 min per step using the following solutions: 1 x 50 mmol L^{-1} ammoniumhydrogencarbonate (ABC), 2x 25 % acetonitrile (ACN) / 37.5 mmol L^{-1} ABC, 1 x 50 % acetonitrile (ACN) / 25 mmol L^{-1} ABC, 1 x 100 % acetonitrile (ACN). Gel pieces were dried, resolved in 200 µL 50 mmol L^{-1} ABC, and 80 ng trypsin (Sequencing Grade Modified Trypsin, Promega) was added. Digestion was performed over night at 37 °C. After digestion, the supernatant was taken and peptides were further eluted stepwise using 0.1 % formic acid, 5 % formic acid 50 % acetonitrile and 100 % acetonitrile respectively. For each step, 200 µL eluent was added to the gel pieces followed by 30 min on a shaker. All corresponding supernatants were collected, merged, dried in a vacuum centrifuge (Vacuum Concentrator, Bachofer) and stored at −20 °C.

LC-MS/MS

LC-MS/MS was performed with a EASY-nLC 1000 ultra chromatographic device (Thermo scientific) coupled to a LTQ Orbitrap XL mass spectrometer (Thermo Scientific). Samples were resolved in 13 µL 0.1 % formic acid. 10 µL were then injected and loaded on a C18 trap column (Acclaim PepMap 100, particle size: 3 µm, 100 Å, column size: 75 µm x 20 mm, Thermo scientific) for 5 min at a flow rate of 5 µL min^{-1} using mobile phase A (0.1 % formic acid). RP chromatography was performed at a flow-rate of 200 nL min^{-1} using a EASY-Spray Column PepMap RSLC separation column (C18, particle size: 2 µm, 100 Å, 500 mm x 75 µm ID, Thermo scientific) with a linear gradient from 6 % to 30 % mobile phase B (A: 0.1 % formic acid, B: acetonitrile and 0.1 % formic acid) in 120 min, a further gradient to 50 % in 10 min, followed by a 10 min step at 84 % B and a 5 min step at 6 % B. Overall gradient length was 145 min. For electrospray ionisation a needle voltage of 1.9 kV was used and a column temperature of 40 °C. The MS method consisted of a cycle combining one full MS scan

(Mass range: 300 – 2000 m/z) with five data dependant MS/MS events (35 % collision energy). The dynamic exclusion was set to 30 s.

Bioinformatic Processing

Spectral data (Thermo raw files) were further processed using the software MaxQuant (Cox and Mann, 2008) version 1.5.1.2 and the implemented label-free quantification (LFQ) option (Smaczniak et al., 2012). Beyond that option, the 'match between runs option' was enabled (match time window 1 min, alignment time window 20 min). For protein identification, unique and razor peptides and a protein FDR of 1 % were used. As database, the pre-released *D. magna* genome available at `http://arthropods.eugenes.org/EvidentialGene/daphnia/daphnia_magna/` (effective 03/2015) was used. Built-in contaminants database was included into the search. Carbamidomethyl was set as a fixed modification, whereas acetyl (protein N-term) and oxidation (M) were set as variable modifications. All other parameters were set according to MaxQuant default.

Data were subsequently processed using Perseus version 1.5.1.6. Here, missing data were imputed by normal distribution as implemented in Perseus. Further data analysis was conducted using R (R Development Core Team, 2011). To find proteins differing significantly in abundance, two sample t-test was applied to compare the susceptible and the resistant genotype (FI-FA46 vs. FI-SK58) and the non-exposed to the genotype exposed to the parasite (FI-FA46 vs. FI-FA46-C1). Only proteins that had three valid quantitative values in at least one of the experimental groups were used for the statistical analysis. In addition, average protein intensity values were only computed if three valid values were available per experimental group and were labelled *NA* otherwise.

To get further information on similar proteins, all significant protein sequences

were blastp searched against NCBI *nr* and *swissprot* databases using local standalone blast (Geer et al., 2010) (e-value $< 10^{-4}$). Preliminary annotation data were received from `http://arthropods.eugenes.org/EvidentialGene/daphnia/daphnia_magna/` (effective 03/2015) and protein associated gene ontology (GO) terms were tested for enrichment using customised standalone EASE (Hosack et al., 2003) (Benjamini corrected p-value < 0.05).

Cluster analysis and heatmap were generated using annHeatmap2 function of R Heatplus package. Protein sequences were tested for predicted GalNac glycosylation sites using NetOGlyc4.0 (Steentoft et al., 2013).

Results

Using a label-free quantification proteomics approach, we were able to identify and quantify 95 proteins from *Daphnia* exuvia, which were detected in at least all three biological replicates of one of the experimental groups. The relative small number of proteins identified in this study compared to other proteomic approaches resulted from the difficult nature of exuvia because of both, low total amount of protein and challenges in protein extraction. However, a comparable study on insect exuvia identified a similar number of proteins (He et al., 2007).

The hierarchical cluster analysis of all proteins yielded good separation between the two different genotypes with FI-SK58 forming a group away from FI-FA46 (see figure 2). When testing all proteins for enrichment of gene ontology (GO) terms, we found significant terms related to chitin binding and chitin metabolism, as would be expected for a arthropod cuticle sample. In addition, the GO term *extracellular region* was found to be enriched.

Thirty-one of the 95 proteins are either cuticle proteins or probable chitin modifying enzymes. Other substantial groups of the exuvia proteome are connected to calcium

Table 1: Results of GO enrichment analysis using EASE (p-value < 0.05, benjamini corrected).

GO Term	GO Name	p-value
GO:0008061	chitin binding	3.04E-010
GO:0006030	chitin metabolic process	3.04E-010
GO:0005576	extracellular region	2.36E-009
GO:0005975	carbohydrate metabolic process	0.0307
GO:0004553	hydrolase activity, hydrolyzing O-glycosyl compounds	0.0452
GO:0004568	chitinase activity	0.0494

Table 2: **Cuticle associated proteins** showing abundance differences between the susceptible (FI-FA46) and non-susceptible (FI-SK58) genotype. First ID of *D. magna* protein groups (DmagID), log2 transformed average protein intensity per genotype (FI-FA46/FI-SK58) and results of t-test (p.value) are displayed. The number of putative glycosylation sites (glyco.no) were computed.

DmagID	FI-FA46	FI-SK58	p.value	glyco.no
Dapma7bEVm001040t1	22.64	21.64	0.039	238
Dapma7bEVm028419t1	22.47	21.1	0.042	429
Dapma7bEVm028420t1	21.45	NA	0	468
Dapma7bEVm001606t1	21.21	22.08	0.033	33
Dapma7bEVm023885t1	19.55	19	0.048	3
Dapma7bEVm028301t1	20.68	21.99	0.049	8
Dapma7bEVm029332t1	21.22	NA	0.007	NA

binding. Another substantial fraction (11 proteins) is related to proteolysis. For 13 proteins, no annotation information was available.

Exuvia of susceptible and non-susceptible genotypes

When testing for differences between the genotypes FI-FA46 and FI-SK58 ($p < 0.1$) we found 18 proteins with altered abundances. Furthermore, differences were found in cuticle associated proteins with 2 proteins more abundant in FI-SK58 and 5 proteins more abundant in FI-FA46, respectively (see table 2). Moreover, 5 proteins were char-

Table 3: **Endopeptidases** showing abundance differences between the susceptible (FI-FA46) and non-susceptible (FI-SK58) genotype. First ID of *D. magna* protein groups (DmagID), log2 transformed average protein intensity per genotype (FI-FA46/FI-SK58), results of t-test (p.value) and BLAST results are displayed.

DmagID	FI-FA46	FI-SK58	p.value	GO
Dapma7bEVm001430t1	23.42	21.92	0.098	metallopeptidase activity
Dapma7bEVm002505t1	23.64	25.45	0.009	metallopeptidase activity
Dapma7bEVm002628t1	NA	21.89	0.059	metallopeptidase activity
Dapma7bEVm010831t1	NA	18.95	0.063	metallopeptidase activity
Dapma7bEVm001206t1	23.41	24.23	0.083	serine-type endopeptidase activity

acterised as peptidases with 1 protein being a serine-type endopeptidase and 4 proteins having a metallopeptidase activity similar to matrix metalloproteinases found in *Daphnia pulex* (see table 3). Four of these peptidases were exclusively detected or more abundant in FI-SK58, the genotype not susceptible to *P. ramosa*.

Exuvia of parasite exposed and non-exposed animals

Testing FI-FA46 animals exposed to *P. ramosa* (FI-FA46-C1) against a non-exposed group of the same genotype (FI-FA46), we detected 10 different proteins. Within these proteins, we found one collagen-like protein originating from the parasite *P. ramosa*, which had high quantitative values in the parasite exposed exuvia and was also found in exuvia exposed to a another *P. ramosa* clone (C19, data not shown). However, this protein was not detected in any of the non-exposed experimental groups.

A large fraction of proteins were related to the cuticle with 3 proteins characterised as putative chitinases, 2 chitin deacetylases and 1 cuticle protein. The chitinases and one of the deacetylases were more abundant in the FI-FA46 control group whereas

Table 4: Proteins showing abundance differences between the non-exposed (FI-FA46) and *P. ramosa* exposed (FI-FA46-C1) susceptible genotype FI-FA46. First ID of *D. magna* protein groups (DmagID), log2 transformed average protein intensity per genotype (FI-FA46/FI-FA46-C1), results of t-test (p.value) and protein name from the *D. magna* database are displayed.

DmagID	FI-FA46-C1	FI-FA46	p.value	Dmag Name
Dapma7bEVm003311t1	17.52	NA	0.076	Annexin-B10
Dapma7bEVm010422t1	20.39	NA	0.087	c-type lectin
Dapma7bEVm000704t1	18.58	NA	0.031	Chitin deacetylase 1
Dapma7bEVm015014t1	19.39	20.23	0.015	Chitin deacetylase 1
P1_h138_ORF02034_1	22.8	NA	0.003	Collagen-like protein
Dapma7bEVm000987t1	21.68	23.02	0.089	Cuticle protein
Dapma7bEVm001605t1	21.88	20.24	0.071	Cuticle protein
Dapma7bEVm006394t1	20.46	NA	0.043	JH-binding protein
Dapma7bEVm001289t1	NA	21.07	0.022	Putative chitinase
Dapma7bEVm003677t1	NA	21.14	0.052	Putative chitinase

one deacetylase and the cuticle protein were more abundant in the parasite exposed FI-FA46-C1 group. Moreover, one juvenile hormone binding protein, one annexin and one c-type lectin were more abundant in the FI-FA46-C1 group.

Discussion

In this study, we compared cuticle proteomes of *D. magna* genotypes known to be either susceptible (FI-FA46) or resistant (FI-SK58) to a certain genotype of *P. ramosa* to find cuticle components possibly contributing to the specificity of the infection process. Furthermore, we analysed cuticle samples of the susceptible host genotype exposed to the parasite (clone C1) and compared them to non-exposed cuticles of the same host genotype (FI-FA46).

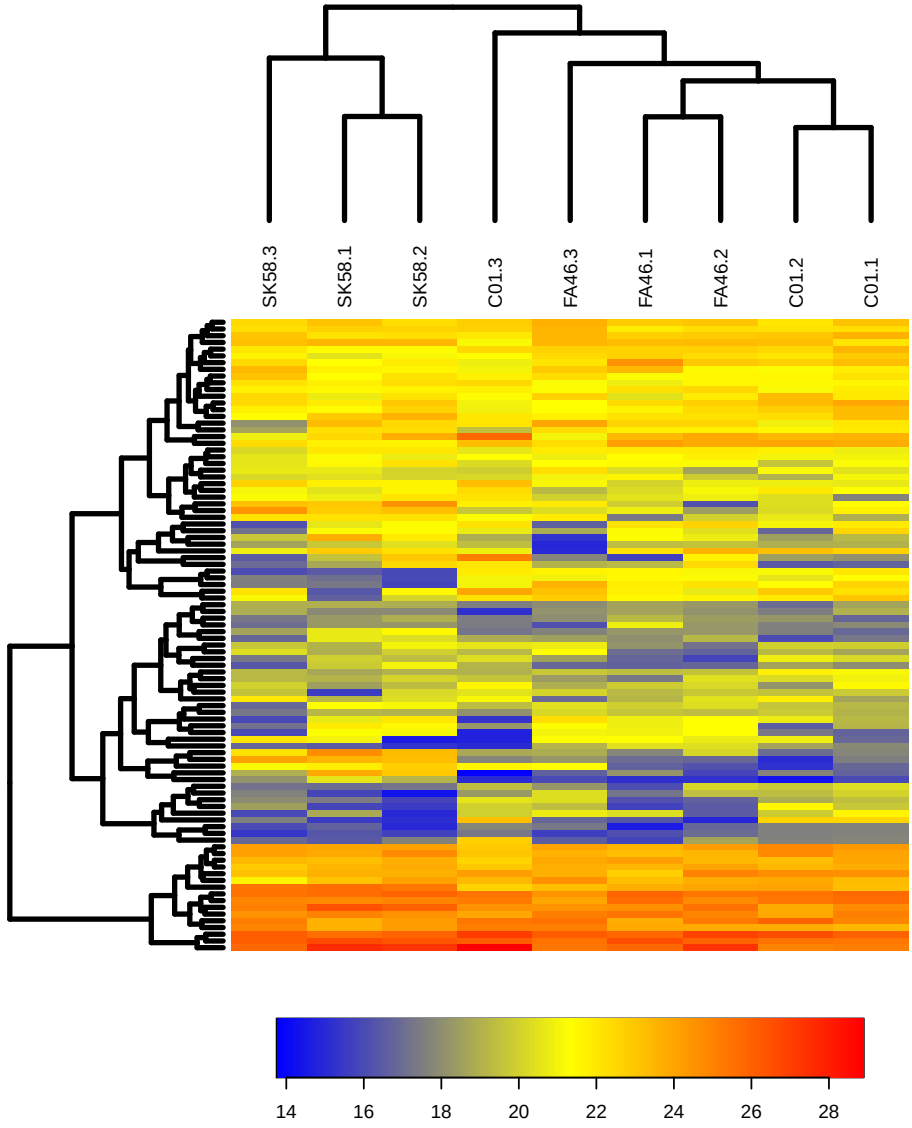

Fig. 2: Heatmap generated from all protein abundances (log2 transformed) which occur in all three replicates of at least one experimental group. Missing protein abundance values were imputed.

The exuvia proteome of *Daphnia*

To the best of our knowledge, we present here the first data-set of a crustacean exuvia proteome. Although genomic information is available for two species of *Daphnia*, *D. pulex* (Colbourne et al., 2011) and *D. magna* [`http://arthropods.eugenes.org/EvidentialGene/daphnia/daphnia_magna/`], usually not much is known about the proteins except the sequence of its amino acids. Homologs in other, better characterised species can be used to determine putative functions of a protein. However, this is difficult for a specific trait like the exoskeleton of arthropods (Giribet and Edgecombe, 2012), as only few of the classical, well-annotated model species in molecular biology belong to this group. Therefore, even though arthropods make up most of the described diversity of life on Earth (Edgecombe, 2009), molecular mechanisms underlying arthropod specific traits are not necessarily well-studied at the molecular level. Although not much information was available and for more than 10 % of our protein data-set no annotations were found, we were able to characterise the cuticle proteome of *Daphnia* in more detail. As expected, a considerable fraction of the proteins found were identified as cuticle proteins, which which usually represent, in addition to chitin fibres, a substantial part of the arthropod exoskeleton (Andersen, 1995).

Another large fraction of the cuticle proteome consisted of chitin modifying enzymes, annotated with the GO term *chitin metabolic process*. Most probably, these proteins are chitinases and chitin deacetylases. Chitinases are chitinolytic enzymes important for the remodelling of chitinous structures (Merzendorfer, 2003), whereas chitin deacetylases modify chitin-protein interactions (Vincent and Wegst, 2004). Chitinases and hexosaminidases, which we also found, have been detected in the moulting fluid of insects (Qu et al., 2014). Furthermore, various proteins of our data-set were annotated with GO terms related to peptidases, being either metallopeptidases

or serine-type endopeptidases. Most interestingly, these two types of peptidases have also been detected in moulting fluid of *Manduca sexta* (Samuels and Reynolds, 1993), as proteolytic activity is thought to be a prerequisite for the action of chitinases during moulting. As the cuticles analysed in our study were freshly shed, it can be assumed that these proteins were also involved in the moulting process of *Daphnia*.

A bacterial collagen-like protein is prominent in *Daphnia* exposed to the parasite

When comparing the moulted exuvia of animals exposed to the parasite to control samples, we found one *P. ramosa* protein (P1_h138_ORF02034_1) with strikingly high abundances in all parasite exposed replicates that was not detected in the control replicates (see table 4 and supplementary data S1). This protein was also showing high abundances in samples of the same *Daphnia* genotype that were exposed to a different genotype of *P. ramosa* (data not shown).

The discovered protein is similar to a collagen-like protein (GenBank: ADU04115.1) previously identified in *P. ramosa* (McElroy et al., 2011). Collagen-like proteins were found in a variety of bacteria, sharing the same characteristic Gly-Xaa-Yaa repeating amino acid sequence, a sequence also present in animal collagens and responsible for its unique triple-helical structure (Yu et al., 2014). These proteins were only characterised in a few pathogenic bacteria in more detail. They are believed to facilitate parasite invasion by acting as adhesins. For example, one collagen-like protein from *Streptococcus pyogenes* is able to interact with mammalian collagen receptors (Caswell et al., 2008) which facilitates adhesion to host cells and activates intracellular signalling (Humtsoe et al., 2005). Furthermore, bacterial collagen-like proteins are structural components of bacterial exosporium surface filaments of *Bacillus anthracis* (Boydston et al., 2005) and spore appendages of *Clostridium taeniosporum* (Walker

et al., 2007).

In *P. ramosa*, collagen-like proteins are part of the spore's surface coat and may be responsible for variation in the ability to attach to the host's foregut (Mouton et al., 2009; Ebert et al., 2016). Further screening for putative collagen-like proteins in the *P. ramosa* draft genome led to the discovery of 37 candidate genes, which were highly polymorphic and these polymorphisms matched patterns of infection specificity (McElroy et al., 2011). For the closely related species *Pasteuria penetrans*, which is a parasite of nematodes and also shows high host attachment specificity, it was proposed that collagen-like protein fibres on the surface of the endospore may bind to glycosylated proteins in the cuticle of the host in a Velcro-like manner (Davies, 2009). This mechanism is also supported by the high abundance values of a *P. ramosa* collagen-like protein, which were detected in our study in the exuvia of *Daphnia* exposed to the parasite.

However, we used only two host clones, one susceptible, the other resistant to the parasite P. ramosa. In our study design, arbitrary genetic differences among these clones cannot be separated from differences due to the contrasting resistance profile. To do so, it would be necessary to test multiple clones of each resistance profile and analyse for common patterns, while at the same time the genetic background is randomised. Thus, our design may overestimates the number of proteins that differ due to differences in the resistant phenotype profiles of the hosts. Follow-up studies need to take this factor into consideration.

Susceptible and tolerant genotypes differ in glycosylation state of cuticle proteins and matrix metalloproteinase abundance

A common form of glycosylation found in proteoglycans and mucins is O-N-acetylgalactosamine (GalNac). GalNac glycosylation is also present in arthropod cuticle

proteins e.g. locusts (Andersen, 1998) and crabs (Compère et al., 2002). Most interestingly, when testing for predicted GalNac glycosylation sites (Steentoft et al., 2013) within our protein data-set, we found that 3 cuticle proteins that were significantly different between the non-susceptible and the susceptible genotypes had very high numbers, over 200, of predicted glycosylation sites (see table 2). Furthermore, these proteins were more abundant or only detected in the susceptible genotype FI-FA46, indicating their possible involvement in the attachment of the parasite *P. ramosa*. In contrast, 2 cuticle proteins more abundant in the non-susceptible genotype FI-SK58 showed a distinct lower number of predicted glycosylation sites. Therefore, differences in the cuticle proteome, especially related to the protein glycosylation state, may account for genotypic differences of *D. magna* susceptibility to *P. ramosa* infection.

In addition, 4 proteins significantly altered between the non-susceptible and the susceptible genotype were annotated with the GO term *metallopeptidase activity*, with 3 proteins being more abundant or exclusively detected in the non-susceptible genotype. All of these protein sequences are similar to matrix metalloproteinase 1 (MMP-1, GenBank: EFX73051.1) found in *D. pulex*. Matrix metalloproteinases (MMPs) are evolutionarily conserved proteins found in many animal species and are known to cleave a variety of extracellular matrix proteins including collagen (Sternlicht and Werb, 2001). Within the groups of arthropods, it was shown that in *Drosophila melanogaster*, MMPs regulate larval tracheal growth and events of pupal morphogenesis (Page-McCaw et al., 2003) and are important for reepithelialisation during wound healing (Stevens and Page-McCaw, 2012). In *Tribolium castaneum*, these proteins regulate tracheal and gut development during beetle embryogenesis and pupal morphogenesis, but are also involved in innate immune defence reactions, as animals with a systemic MMP-1 knockdown were more susceptible when exposed to the entomo-

pathogenic fungus *Beauveria bassiana* (Knorr et al., 2009). Similar to *P. ramosa* (Duneau et al., 2011), entomopathogenic fungi are known to invade their host by penetration of the cuticle (Clarkson and Charnley, 1996). In *T. castaneum*, MMP-1 was also found to have collagenolytic activity (Knorr et al., 2009). As collagen-like proteins seem to be crucial for host attachment specificity (see above) in the *Daphnia- Pasteuria* systems and the non-susceptible *D. magna* genotype FI-SK58 has its own set of high abundant MMPs, these proteins may be involved in the failure of the *P. ramosa* infection process possibly by interfering with collagen-mediated parasite attachment.

Therefore, genotype-specific high abundance of glycosylated cuticle proteins in the susceptible genotype and of MMPs in the non-susceptible genotype may be crucial for genotype specificity of parasite attachment and therefore for infection process.

Indicators of *P. ramosa* infection include lectin and moulting related proteins

When comparing cuticles of parasite-exposed animals to non-exposed animals of the same genotype (see table 4) we found interesting indicators of parasite infection. One protein, which was detected in all *P. ramosa* exposed replicates is a galactose binding c-type lectin. C-type lectins are able to recognise pathogens and take part in the innate immune response of vertebrates and invertebrates (Robinson et al., 2006). They have been found to be involved in immune response of a lepidopteran (Yu et al., 2002) and in the response of *Caenorhabditis elegans* to a bacterial pathogen (O'Rourke et al., 2006). Furthermore, in *Drosophila melanogaster*, this type of lectin was shown to bind to bacteria and to take part in the immune response of the infected animals (Tanji et al., 2006). Therefore, the high abundance of a c-type lectin in *Daphnia* exposed to the parasite *P. ramosa* is an indicator for an immune response to the bacterial infection.

Most of the other proteins showing abundance differences were directly related to

the cuticle and cuticle rearrangement. Animals exposed to the parasite not only show differences in cuticle protein composition but also have decreased levels of chitinases. Chitinases are chitinolytic enzymes and when appearing in the cuticle are most probably connected to moulting events (Merzendorfer, 2003). This is especially interesting, as moulting is an important step interfering with successful parasite infection. In *D. magna* exposed to *P. ramosa*, the success of parasite infection was greatly reduced if the animals moulted within 12 h after parasite exposure (Duneau and Ebert, 2012). Therefore, it may be possible that the parasite is slowing down the moulting process to increase the infection success, which results in the decreased abundance of chitin-modifying proteins found in this experiment and in a delayed moulting in *Daphnia* exposed to the parasite (G. Bento, pers. comm.). In addition, these findings are supported by a protein having a juvenile-hormone binding domain, which is more abundant in the cuticle of parasite-exposed animals, probably indicating increased juvenile hormone concentration in the cuticle. This is especially interesting as in adult *Daphnia*, juvenile hormones may be involved in the regulation of moulting by modulating ecdysteroid activity (Mu and LeBlanc, 2004). Moulting in *Daphnia* is most probably induced by an increased level of 20-hydroxyecdysone (Martin-Creuzburg et al., 2007). Therefore, together with abundance alteration in chitinases and cuticle proteins, these data suggest that *P. ramosa* may induce a retarded moulting in its host *D. magna*.

Conclusion

In this study we analysed the cuticle proteomes of two *D. magna* genotypes known to be either susceptible or non-susceptible to the parasite *P. ramosa* to study if the genetic determinate is visible in the different cuticle proteomes. Furthermore, differences in the cuticle proteome of parasite exposed and non-exposed animals of the

susceptible genotype were analysed to find proteins related to the infection process. To the best of our knowledge, these data were the first to describe a crustacean exuvia proteome, detecting a substantial fraction of cuticle proteins and enzymes related to the moulting process. Altogether, our proteomic analysis of genotype-genotype interactions in the parasite *Pasteuria ramosa* and its host *Daphnia magna* revealed details of molecular mechanisms involved in the infection process, supporting existing hypotheses and providing new insights into this topic. However, our study suffered from limitations in both, the number of biological replicates and host genotypes. This part of the design should be improved in follow-up studies to eliminate false-positive and false-negative results. Nevertheless, our study identifies a number of promising starting-points for more detailed analysis of the molecular components involved in the response of *Daphnia* to the parasite *P. ramosa* and therefore on the evolution of host-parasite interactions.

Author contributions

KAO designed research, conducted proteomic experiments, performed mass spectrometry analysis and conducted bioinformatical analysis of the data. JA and GB performed *P. ramosa* infection experiments and exuvia sampling. CL and DE designed and supervised research. KAO wrote the first draft of the manuscript, CL, JA and DE contributed substantially to revisions.

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5 The influence of simulated microgravity on the proteome of *Daphnia magna*

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npj microgravity, 1, 15016, 2015

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The influence of simulated microgravity on the proteome of *Daphnia magna*

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BACKGROUND: The waterflea *Daphnia* is an interesting candidate for bioregenerative life support systems (BLSS). These animals are particularly promising because of their central role in the limnic food web and its mode of reproduction. However, the response of *Daphnia* to altered gravity conditions has to be investigated, especially on the molecular level, to evaluate the suitability of *Daphnia* for BLSS in space.

METHODS: In this study, we applied a proteomic approach to identify key proteins and pathways involved in the response of *Daphnia* to simulated microgravity generated by a two-dimensional (2D) clinostat. We analyzed five biological replicates using 2D-difference gel electrophoresis proteomic analysis.

RESULTS: We identified 109 protein spots differing in intensity ($P < 0.05$). Substantial fractions of these proteins are involved in actin microfilament organization, indicating the disruption of cytoskeletal structures during clinorotation. Furthermore, proteins involved in protein folding were identified, suggesting altered gravity induced breakdown of protein structures in general. In addition, simulated microgravity increased the abundance of energy metabolism-related proteins, indicating an enhanced energy demand of *Daphnia*.

CONCLUSIONS: The affected biological processes were also described in other studies using different organisms and systems either aiming to simulate microgravity conditions or providing real microgravity conditions. Moreover, most of the *Daphnia* protein sequences are well-conserved throughout taxa, indicating that the response to altered gravity conditions in *Daphnia* follows a general concept. Data are available via ProteomeXchange with identifier PXD002096.

npj Microgravity (2015) **1,** 15016; doi:10.1038/npjmgrav.2015.16; published online 24 September 2015

INTRODUCTION

Since the first moon landing in 1969, technologies have advanced and the urge to further explore space has not diminished. At the moment, the ultimate goal of manned space missions is the exploration of Mars. Yet manned missions face several key issues that have to be solved, not only concerning human health,[1] but also the provision with essential supplies, e.g., food, water, oxygen.

A solution for this supply problem, especially for long duration missions, is the development of a bioregenerative life support system (BLSS), which minimizes reliability on delivered supplies and enhances autochtonous production. Up to now, such systems have only been installed as modules on the Mir and ISS space stations.[2] Since then, new components were added to the life support system, e.g., waste water recovery, forming a so-called ecological control and life support system.[3] The functions that have to be fulfilled by this system are the regeneration of atmosphere, purification of water, waste processing, food production, and food processing. The organisms involved in these systems include bacteria and fungi for the decomposition of organic waste and excrements, as well as unicellular microalgae, which produce the oxygen for astronauts, but also comprise higher organisms such as vegetables or fish to provide the astronauts with food.[4]

The waterflea *Daphnia* (Crustacea) might be a candidate in aquatic modules of such a BLSS for several reasons: (i) *Daphnia* occupies a central role in limnic food webs by being a primary consumer, hence serving as a link between oxygen producing, autotrophic producers such as algae and secondary consumers, such as planktivorous fish.[5] Fish, in respect, serve as an animal protein source for the human crew. Positive side effects of this constellation are that no additional fish food has to be transported, as well as that the growth of algae populations is controlled. (ii) *Daphnia* reproduces by the mode of cyclic parthenogenesis, thus enabling *Daphnia* to reproduce asexually in favorable environmental conditions and sexually in unfavorable ones, which leads to the formation of dormant eggs that are encased in a protective structure, the so called ephippium. Those resting eggs could be used to restart the BLSS in case of a system collapse and it was already shown that dormant eggs of *Daphnia* are able to hatch living neonates after long-term exposure to the space environment on the ISS.[6] Combining these modes of reproduction with the short generation time and the high number of offspring per clutch, a high bio mass production can be guaranteed.[7]

Daphnia does not only serve as a model organism in the fields of ecology, evolution, and ecotoxicology,[8] but also in ecological genomics.[9] Here, especially the improved availability of genomic resources[10] facilitates untargeted holistic approaches, such as transcriptomics[11] or proteomics,[12,13] which may reveal unpredicted key players underlying biological traits.

Long-term spaceflight is known to affect human physiology leading to bone demineralization, skeletal muscle atrophy, and

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Received 21 April 2015; revised 2 August 2015; accepted 11 August 2015

npj nature partner journals

Figure 1. Cuvette clinostat provided by the German Aerospace Center (DLR).

immune system suppression.[1] Furthermore, microgravity-induced responses were described in a variety of biological systems reaching from alterations of cytoskeletal formation in cells[14] to altered plant forms in *Arabidopsis*.[15] *Daphnia* has already been part of missions to space stations, demonstrating that resting eggs were viable even after exposure to outer space for >1 year.[6] Some animals survived up to 4 months in space but showed alterations in swimming behavior with an unusual high proportion of looping movements.[16] However, more information on the influence of microgravity on *Daphnia* is needed to decide on the suitability of these animals for BLSS in long-duration missions. Studies at the molecular level are especially interesting, as they may elucidate additional biological processes not detectable at the morphological or physiological level.

To determine the effect of long-term exposure to weightlessness, high technical complexity and financial investment is needed. Most facilities providing free fall conditions, like parabolic flights or drop-tower experiments can only deliver short duration of weightlessness. Yet a cost-effective ground-based method is the use of a two-dimensional (2D)-clinostat,[17] however, carefully considering the operational mode and limitation of the simulation.

In this study, we investigated the effect of simulated microgravity on *Daphnia* at the protein level, as proteins are the main effectors of biological functions in an organism. We exposed *Daphnia* to simulated microgravity using a 2D-clinostat and subsequently performed a proteomic approach to study quantitative changes in the proteome of animals exposed to altered gravity conditions compared with a control group.

MATERIALS AND METHODS

Animal husbandry

To investigate the effect of simulated microgravity on the proteome of *Daphnia magna* the laboratory cultivated genotype K34J was used, which originated from a fishpond near Munich, Germany. The animals were kept in a density of 5–12 adult animals per 1.5-l jar filled with semi-artificial medium[18] in an illuminated climate chamber (Binder KBWF 240, Binder GmbH, Tuttlingen, Germany) at 21 ± 1 °C and a photoperiod of 12 h (L18W 865 Cool Daylight, Osram, Munich, Germany). Animals were fed every second day with the unicellular algae *Scenedesmus obliquus*, thereby reaching a carbon concentration of 1.5 mg/l.

Simulated microgravity—clinostat experiment

To simulate microgravity, we used a cuvette 2D-clinostat, designed and provided by the German Aerospace Center (Deutsches Zentrum für Luft- und Raumfahrt, DLR) in Cologne, Germany (Figure 1).

The clinorotation principle is based on the fast rotation around a small diameter thereby preventing physical sedimentation.[19] As a consequence of clinorotation, the composition of the *D. magna* proteome was investigated in comparison to the proteome under normal gravity conditions, which means in a static 1 g control. For the experiment, *D. magna* of 2 ± 0.2 mm size were used. Body size was measured with a Leica MS5 stereomicroscope (Leica Microsystems, Wetzlar, Germany). The clinostat was loaded with 10 1-ml serological BD-Falcon pipettes (BD Biosciences, Heidelberg, Germany). Each pipette contained 1 ml of medium enriched with algae (carbon concentration: 1.5 mg/l) and five randomly selected *D. magna*. Animals were able to move freely in the cuvette. Alternately one pipette was mounted on the clinostat and the next one was put next to the clinostat as a control, therefore the control pipettes were exposed to the same vibrations as the clinorotated pipettes. This process was repeated until 10 pipettes of each treatment were arranged within the experimental setup.

The rotation speed of the clinostat was set at 60 r.p.m., with a residual gravity of ~ 0.008*g*.[20] The duration of the experiment was set to 60 min at a room temperature of 20 °C. Longer exposure times of several days, as implemented for plants,[17] would not be possible without creating food limitations for *Daphnia*, especially as a higher starting amount of algae may have harmful effects on the animals and adding of additional algae is not feasible during clinorotation because of turbulence generation. However, as an exposure time of 60 min was sufficient to show effects in other studies, e.g., study by Eiermann *et al*.,[21] and the algae concentration was sufficient for this amount of time, we chose this duration for our clinorotation experiment. After 60 min, the pipettes were emptied during rotation by tilting the device into cryo tubes and excessive water was immediately removed and cryo tubes were snap frozen in liquid nitrogen. The process of water removal and freezing was performed in less than 5 s. A total number of 10 runs was performed, each consisting of 10 serological cuvettes and therefore 50 animals of each treatment.

Sample preparation

To generate samples for proteomic analysis, the frozen biological samples were pulverized in a mortar containing liquid nitrogen to prevent thawing. The resulting powder was solubilised in 330 µl lysis buffer (2 mol/l Thiourea, 6 mol/l Urea, 4% CHAPS, 1 cOmplete ULTRA Tablets Mini (Roche, Penzberg, Germany) per 5 ml buffer). Afterwards, each sample was centrifuged using a QIA Shredder Mini Spin Column (Qiagen, Hilden, Germany) for 3 min at 16,100 r.c.f. to get rid of debris. Proteins were precipitated using 30% trichloroacetic acid for 20 min on ice to inhibit proteolytic activity.[22] Subsequently, samples were centrifuged for 10 min at 16,100 r.c.f., the supernatant was discarded and the protein pellet was washed three times with cold acetone. The pellet was dried and resolved in lysis buffer. The pH of the solution was adjusted to 8 by adding 50 mM NaOH. Protein concentration was determined by Bradford Assay (Coomassie Plus (Bradford) Assay Reagent, Thermo Scientific, Braunschweig, Germany) according to the manufacturer's instructions. To reach sufficient protein concentrations for 2D-difference gel electrophoresis (DIGE), two clinorotation runs per group were pooled leading to five biological replicates.

2D-DIGE

2D-DIGE method was conducted following the general procedure described in the study by Otte *et al*.[13] Briefly, 50 µg protein per biological replicate was labeled with 2D-DIGE Cy3 Dye for control and Cy5 Dye for the clinorotated group following the protocol of the manufacturer (GE Healthcare Life Sciences, Munich, Germany). An internal pooled standard was prepared by pooling 25 µg protein of all biological replicates and labeling of 300 µg with 2D-DIGE Cy2 Dye. For each 2D-DIGE gel, 50 µg of one Cy3-labeled control replicate, 50 µg of one Cy5-labeled clinorotated replicate, and 50 µg of Cy2-labeled internal pooled standard were combined.

For first dimension separation, 24-cm gel strips (DryStrips pH 4–7, GE Healthcare) and an IPGPhore (Pharmacia Biotech, Uppsala, Sweden) were used. For second dimension separation, the gel strips were fixed on top of

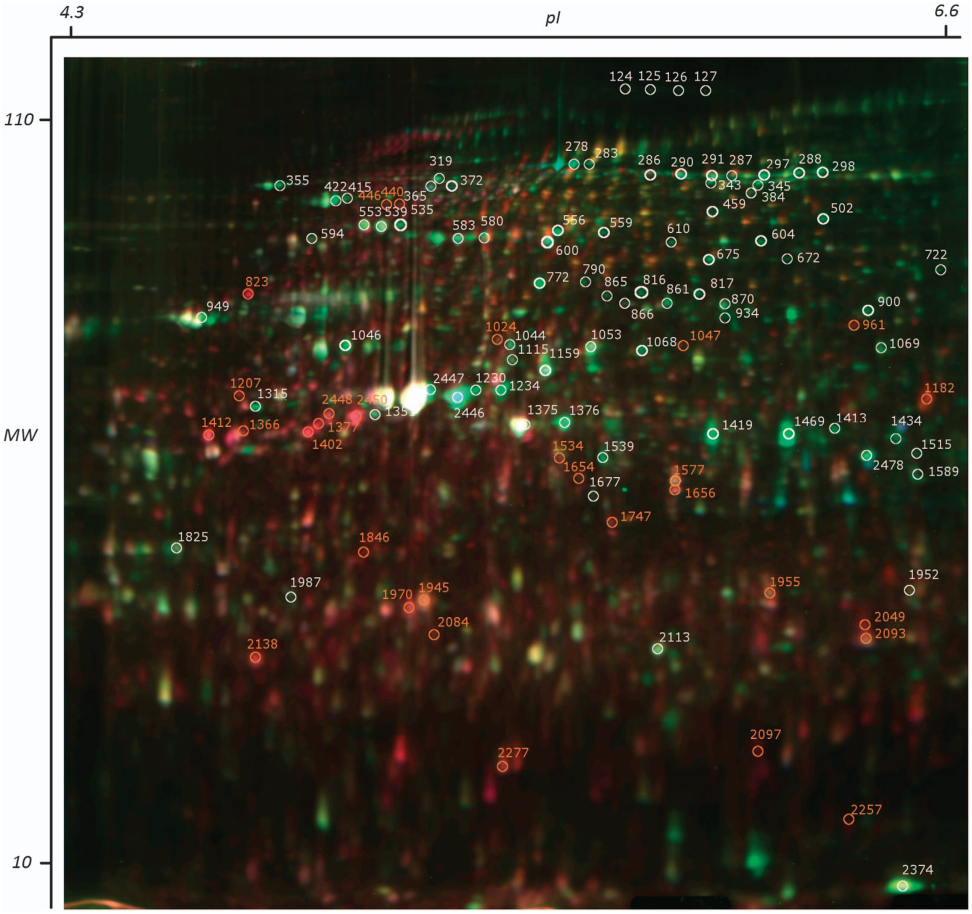

Figure 2. Example 2D-DIGE gel image. Here, protein spots which were significantly different between treatments and were identified using LC-MS/MS were marked. Red labeled spots were less abundant in the clinorotated treatment, whereas white labeled spots were more abundant in the clinorotated treatment. LC-MS/MS, liquid chromatography-tandem mass spectrometry; 2D-DIGE, two-dimensional difference gel electrophoresis.

lab cast gels and electrophoresis was performed using an ETTANDaltsix electrophoresis unit (GE Healthcare Life Sciences). During the whole 2D-DIGE procedure, all five biological replicates were processed in parallel.

Imaging and quantitative analysis

Immediately after electrophoresis, gels were scanned using a Typhoon 9400 fluorescence scanner (GE Healthcare Life Sciences) with parameter settings recommended by the manufacturers for 2D-DIGE experiments. Image analysis and relative quantification were performed with DeCyder 2D Software version v7.0 (GE Healthcare Life Sciences). Coordinates of corresponding spots differing significantly in their intensity ($P \leqslant 0.05$ after false discovery rate correction, ratio $\geqslant |2|$) were used to generate a pick list for further processing.

Excision of spots and tryptic hydrolysis

Gels were stained overnight with colloidal Coomassie staining solution (Carl Roth GmbH, Karlsruhe, Germany) and then destained using 25% methanol. Further processing of proteins spots was performed according to in the study by Otte *et al.*[13] In summary, spots of interest were cut out of the gel and digested with trypsin (Sequencing Grade–Modified Trypsin, Promega, Mannheim, Germany) to generate peptides for protein identification.

LC-MS/MS analysis

Liquid chromatography-tandem mass spectrometry (LC-MS/MS) was performed on a multi-dimensional LC system (Ettan MDLC, GE Healthcare Life Sciences) coupled to a LTQ mass spectrometer (Thermo Scientific, Braunschweig, Germany). Further settings were the same as described in the study by Otte *et al.*[13]

Bioinformatic processing

For protein identification, MS/MS data were searched with Mascot Version: 2.3.00 (Matrix Science, London, United Kingdom). As database, pre-released gene-predictions of *D. magna* (V2.4 effective May 2012) were used. These sequence data were produced by The Center for Genomics and Bioinformatics at Indiana University and distributed via wFleaBase in collaboration with the Daphnia Genomics Consortium (http://daphnia.cgb.indiana.edu). Further data processing was done as described in the study by Otte *et al.*[13] Protein spots having multiple protein identifications were not included in the final data set. To get further information on similar proteins, all significant protein sequences were blastp searched against NCBI non-redundant (nr) and Swiss-Prot databases using local standalone blast.[23] The NCBI nr database combines non-redundant protein sequences of several sources, including translations from annotated coding regions in GenBank, RefSeq, and TPA, as well as records from PIR, PRF, and PDB, whereas the Swiss-Prot database is manually annotated and therefore contains less but more reliable data. Thus, recent data of genomic studies on several organisms are found in nr database, whereas Swiss-Prot consist mainly of well-annotated protein information of a few well-studied model organisms.

Preliminary annotation data were received from http://server7.wfleabase.org/genome/Daphnia_magna/ (V2.4 effective May 2012). Protein-associated gene ontology (GO) terms were tested for enrichment using customized standalone EASE[24] (Benjamini-corrected P value < 0.05).

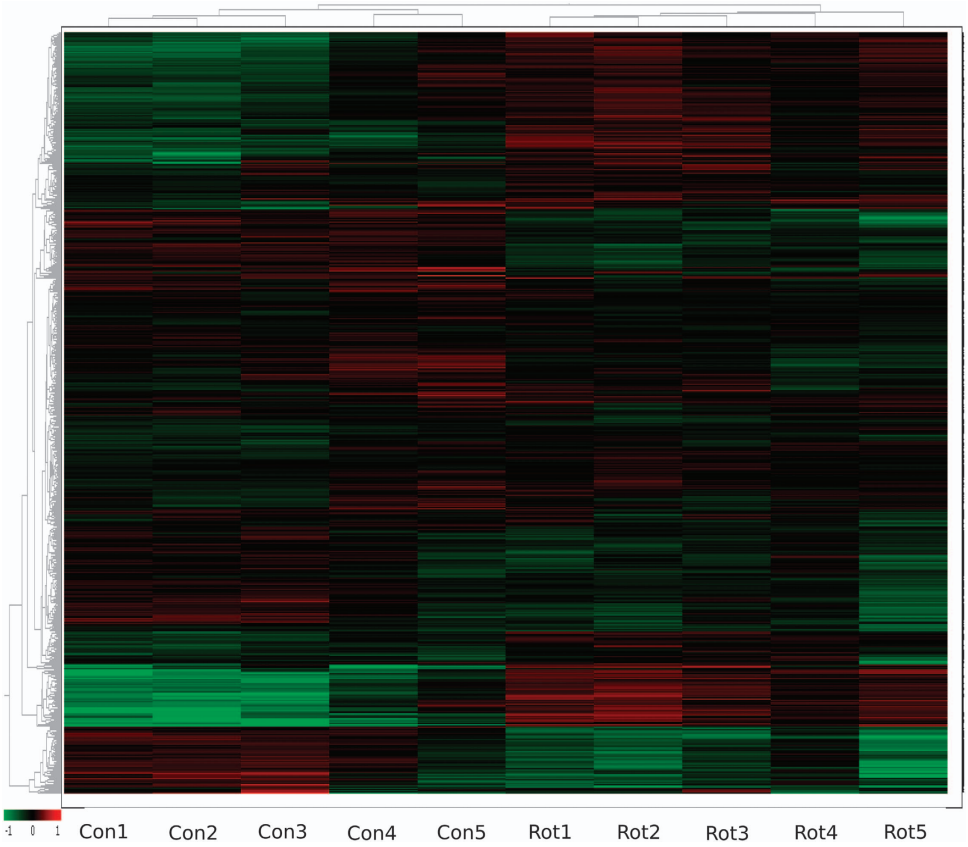

Figure 3. Heatmap and hierarchical cluster of all protein spot intensity data present in all biological replicates. Con refers to the control replicates, whereas Rot refers to the clinorotated replicates.

Enriched terms were tested for redundant terms and semantic similarities using the online-tool REViGO[25] and visualized as treemap graphs using R.[26]

In addition, protein sequences were also processed using the software Blast2go,[27] which uses results of NCBI blast search to map sequences directly to GO terms. These GO terms were used as additional information.

RESULTS

We analyzed the effects of altered gravity conditions on *D. magna* at the protein level by exposing animals for 1 h to clinorotation followed by a proteomic 2D-DIGE approach. We studied five clinorotated and five control replicates. We generated 5 2D-DIGE gels, which showed reproducible spot map patterns (Supplementary Data S2), and were able to match and quantify 1,211 protein spots in at least 4 of these 5 spot maps. Unsupervised hierarchical clustering of spot intensity data present in all spot maps showed a good clustering according to treatment (Figure 2).

About 109 of these protein spots showed significantly different intensity signals between the control and the clinorotated treatment ($P \leqslant 0.05$, ratio $\geqslant |2|$) and were identified using LC-MS/MS (Figure 3 and Table 1). Of these protein spots, 30 were less abundant, whereas 79 protein spots were more abundant in the clinorotated treatment.

As information on protein function of *D. magna* proteins is scarce so far, we performed a blastp search against NCBI nr and Swiss-Prot database to look for similar and possibly better characterized proteins in other species. Here, we used a strict BLAST bit score threshold of $\geqslant 244$, which ensures accurate prediction of protein function similarity.[28] Interestingly, nearly all hits in the nr database refer to proteins of *D. pulex*. In contrast to *D. magna*, the *D. pulex* genome is published[10] and therefore completely represented in this database. Furthermore, a majority of proteins had a similar blast hit in the Swiss-Prot database, indicating the presence of similar proteins in well-studied model organisms and therefore a good conservation of these proteins within organismal taxa (Supplementary Data S1).

Enrichment analysis of GO terms yielded five overrepresented terms in biological process and molecular function database, namely protein folding, unfolded protein binding, actin binding, ATP binding, and glycolytic process (Figure 4 and Table 2). Furthermore, nine protein spots were identified as *Daphnia* hemoglobins.

About 27 protein spots were identified as proteins connected to actin binding and they were either involved in muscular structures or the cytoskeleton. Beyond actin itself, we identified myosin, α-actinin, filamin-A, gelsolin, and advillin. Some of these proteins are present in multiple spots with isoelectric point and molecular weight shifts, and varying abundances, indicating post-translational modifications (PTMs). Most spots identified as actin, myosin, α-actinin, and filamin-a were less abundant, whereas advillin and gelsolin were identified in spots to be more abundant after clinorotation.

Furthermore, 20 protein spots were identified as proteins involved in protein folding. Here, heat shock proteins and other chaperones like endoplasmin, a protein disulfide-isomerase, and different subunits of T-complex protein 1 were found. All spots except one had a higher abundance in the animals exposed to clinorotation.

In addition, 17 protein spots were connected to different metabolic pathways involved in energy generation. Proteins were involved in glycolysis, a GO term which was also found to be

Table 1. Significantly altered proteins involved in the response of *Daphnia* to altered gravity

Spot. no.	*2D-DIGE_results*				*First_Blast_hit*		*Enriched_GO_terms*
	*ID_*D. magna*_database*	*T-test*	*Av. ratio*	*Acc*	*Name*	*Org*	
288	daphmag3mtv3l5529t1	0.001	25.87	EFX88163	Hypothetical protein DAPPUDRAFT_311761	*Daphnia pulex*	F: actin binding
556	daphmag3mtv3l7809t1	0.004	11.43	EFX86275	Hypothetical protein DAPPUDRAFT_313359	*Daphnia pulex*	F: unfolded protein binding, P: protein folding, F: ATP binding
1469	daphmag3mtv3l6920t1	0.007	9.87	BAJ72724	2-domain hemoglobin	*Daphnia magna*	—
1068	daphmag3mtv3l11111t1	0.003	9.35	EFX83276	Enolase	*Daphnia pulex*	P: glycolysis
297	daphmag3mtv3l5529t1	0.007	8.62	EFX88163	Hypothetical protein DAPPUDRAFT_311761	*Daphnia pulex*	F: actin binding
559	daphmag3mtv3l7809t1	0.006	7.67	EFX86275	Hypothetical protein DAPPUDRAFT_313359	*Daphnia pulex*	F: unfolded protein binding, P: protein folding, F: ATP binding
604	daphmag3mtv3l6730t1	0.003	7.42	EFX71530	Hypothetical protein DAPPUDRAFT_308853	*Daphnia pulex*	—
1419	daphmag3mtv3l6920t1	0.007	7.01	BAJ72724	2-domain hemoglobin	*Daphnia magna*	—
772	daphmag3mtv3l9572t1	0.008	6.94	EFX84424	Hypothetical protein DAPPUDRAFT_301074	*Daphnia pulex*	C: cytoplasm, F: ATP binding
1376	daphmag3mtv3l8231t1	0.001	6.91	EFX89163	Hypothetical protein DAPPUDRAFT_220693	*Daphnia pulex*	F: ATP binding
900	daphmag3mtv3l9835t1	0.012	6.9	EFX70620	Hypothetical protein DAPPUDRAFT_202253	*Daphnia pulex*	—
1234	daphmag3mtv3l7094t1	0.003	6.78	CAB99474	Actin	*Daphnia pulex;Daphnia magna*	C: cytoplasm, F: ATP binding
372	daphmag3mtv3l2246t2	0.013	6.13	EFX89391	Hypothetical protein DAPPUDRAFT_303199	*Daphnia pulex*	F: actin binding
817	daphmag3mtv3l10162t1	0.013	6.05	EFX77428	Hypothetical protein DAPPUDRAFT_213377	*Daphnia pulex*	F: unfolded protein binding, P: protein folding, C: cytoplasm, F: ATP binding
290	daphmag3mtv3l5529t1	0.011	5.82	EFX88163	Hypothetical protein DAPPUDRAFT_311761	*Daphnia pulex*	F: actin binding
1230	daphmag3mtv3l7094t1	0.011	5.6	CAB99474	Actin	*Daphnia pulex;Daphnia magna*	C: cytoplasm, F: ATP binding
1589	daphmag3mtv3l15212t1	0.013	5.44	EFX80600	Hypothetical protein DAPPUDRAFT_196566	*Daphnia pulex*	—
343	daphmag3mtv3l4901t1	0.009	5.31	EFX86312	Hypothetical protein DAPPUDRAFT_308519	*Daphnia pulex*	—
2446	daphmag3mtv3l7094t1	0.005	5.24	CAB99474	Actin	*Daphnia pulex;Daphnia magna*	C: cytoplasm, F: ATP binding
502	daphmag3mtv3l592t1	0.013	5.17	EFX87607	Hypothetical protein DAPPUDRAFT_192333	*Daphnia pulex*	—
2478	daphmag3mtv3l16955t1	0.013	5.1	BAA76873	Hemoglobin	*Daphnia magna*	—
298	daphmag3mtv3l11111t1	0.029	5.01	EFX83276	Enolase	*Daphnia pulex*	P: glycolysis
1046	daphmag3mtv3l10909t1	0.008	4.87	EFX90019	Hypothetical protein DAPPUDRAFT_309746	*Daphnia pulex*	F: ATP binding
675	daphmag3mtv3l10134t1	0.007	4.78	EFX75422	Hypothetical protein DAPPUDRAFT_306806	*Daphnia pulex*	F: unfolded protein binding, P: protein folding, C: cytoplasm, F: ATP binding
459	daphmag3mtv3l6920t1	0.003	4.61	BAJ72724	2-domain hemoglobin	*Daphnia magna*	—
1315	daphmag3mtv3l17606t1	0.015	4.25	EFX78249	Hypothetical protein DAPPUDRAFT_320706	*Daphnia pulex*	—
1159	daphmag3mtv3l13427t1	0.011	4.08	EFX90443	Hypothetical protein DAPPUDRAFT_299795	*Daphnia pulex*	P: glycolysis
286	daphmag3mtv3l5529t1	0.023	4.07	EFX88163	Hypothetical protein DAPPUDRAFT_311761	*Daphnia pulex*	F: actin binding
816	daphmag3mtv3l10162t1	0.013	3.82	EFX77428	Hypothetical protein DAPPUDRAFT_213377	*Daphnia pulex*	F: unfolded protein binding, P: protein folding, C: cytoplasm, F: ATP binding
1413	daphmag3mtv3l16955t1	0.026	3.82	BAA76873	Hemoglobin	*Daphnia magna*	—
535	daphmag3mtv3l7770t1	0.004	3.81	ADA79522	Heat shock protein 70	*Daphniopsis tibetana*	F:ATP binding
345	daphmag3mtv3l4901t1	0.012	3.77	EFX86312	Hypothetical protein DAPPUDRAFT_308519	*Daphnia pulex*	—
1987	daphmag3mtv3l6078t1	0.003	3.71	EFX87538	Hypothetical protein DAPPUDRAFT_192225	*Daphnia pulex*	C: cytoplasm, F: ATP binding
291	daphmag3mtv3l7067t1	0.041	3.65	EFX71215	Hypothetical protein DAPPUDRAFT_309186	*Daphnia pulex*	F: unfolded protein binding, P: protein folding, F: ATP binding
2374	daphmag3mtv3l21839t1	0.012	3.63	NA	NA	NA	—
355	daphmag3mtv3l7067t1	0.013	3.57	EFX71215	Hypothetical protein DAPPUDRAFT_309186	*Daphnia pulex*	F: unfolded protein binding, P: protein folding, F: ATP binding
287	daphmag3mtv3l5529t1	0.021	3.48	EFX88163	Hypothetical protein DAPPUDRAFT_311761	*Daphnia pulex*	F: actin binding
1515	daphmag3mtv3l15212t1	0.044	3.37	EFX80600	Hypothetical protein DAPPUDRAFT_196566	*Daphnia pulex*	—
422	daphmag3mtv3l4176t1	0.02	3.36	EFX66769	Hypothetical protein DAPPUDRAFT_302452	*Daphnia pulex*	F: unfolded protein binding, P: protein folding, F: ATP binding
861	daphmag3mtv3l11254t1	0.031	3.32	EFX74207	Cct5-prov protein	*Daphnia pulex*	F: unfolded protein binding, P: protein folding, C: cytoplasm, F: ATP binding
934	daphmag3mtv3l9835t1	0.012	3.17	EFX70620	Hypothetical protein DAPPUDRAFT_202253	*Daphnia pulex*	—
1539	daphmag3mtv3l13753t1	0.017	3.16	EFX71334	Cytosolic malate dehydrogenase	*Daphnia pulex*	—
790	daphmag3mtv3l9572t1	0.011	3.14	EFX84424	Hypothetical protein DAPPUDRAFT_301074	*Daphnia pulex*	C: cytoplasm, F: ATP binding
278	daphmag3mtv3l6051t1	0.012	3.1	EFX71787	Hypothetical protein DAPPUDRAFT_326816	*Daphnia pulex*	—
365	daphmag3mtv3l2246t2	0.027	3.09	EFX89391	Hypothetical protein DAPPUDRAFT_303199	*Daphnia pulex*	F: actin binding
865	daphmag3mtv3l11254t1	0.011	3.08	EFX74207	Cct5-prov protein	*Daphnia pulex*	F: unfolded protein binding, P: protein folding, C: cytoplasm, F: ATP binding
1069	daphmag3mtv3l5529t1	0.026	3	EFX88163	Hypothetical protein DAPPUDRAFT_311761	*Daphnia pulex*	F: actin binding
722	daphmag3mtv3l4092t1	0.013	2.97	EFX87506	Hypothetical protein DAPPUDRAFT_306375	*Daphnia pulex*	F: unfolded protein binding, P: protein folding, C: cytoplasm, F: ATP binding
415	daphmag3mtv3l4176t1	0.028	2.97	EFX66769	Hypothetical protein DAPPUDRAFT_302452	*Daphnia pulex*	F: unfolded protein binding, P: protein folding, F: ATP binding
672	daphmag3mtv3l9343t1	0.012	2.94	XP_003700942	PREDICTED: coatomer subunit delta like	*Megachile rotundata*	—
600	daphmag3mtv3l2675t1	0.023	2.84	EFX81902	Hypothetical protein DAPPUDRAFT_302856	*Daphnia pulex*	F: ATP binding
126	daphmag3mtv3l1194t1	0.013	2.81	EFX79782	Hypothetical protein DAPPUDRAFT_304363	*Daphnia pulex*	—
870	daphmag3mtv3l9792t1	0.025	2.71	EFX87987	Hypothetical protein DAPPUDRAFT_127024	*Daphnia pulex*	F: unfolded protein binding, P: protein folding, C: cytoplasm, F: ATP binding
319	daphmag3mtv3l4116t1	0.026	2.69	EFX72171	Hypothetical protein DAPPUDRAFT_308570	*Daphnia pulex*	F: ATP binding
283	daphmag3mtv3l6051t1	0.02	2.66	EFX71787	Hypothetical protein DAPPUDRAFT_326816	*Daphnia pulex*	—
580	daphmag3mtv3l5322t1	0.012	2.63	EFX90349	Hypothetical protein DAPPUDRAFT_300069	*Daphnia pulex*	F: ATP binding
1044	daphmag3mtv3l9038t1	0.023	2.62	EFX87450	Hypothetical protein DAPPUDRAFT_207615	*Daphnia pulex*	—
1115	daphmag3mtv3l12548t1	0.012	2.6	EFX81896	Hypothetical protein DAPPUDRAFT_302792	*Daphnia pulex*	—
1434	daphmag3mtv3l8815t1	0.023	2.57	EFX88463	Hypothetical protein DAPPUDRAFT_305568	*Daphnia pulex*	—
124	daphmag3mtv3l1194t1	0.019	2.56	EFX79782	Hypothetical protein DAPPUDRAFT_304363	*Daphnia pulex*	—
127	daphmag3mtv3l1194t1	0.018	2.56	EFX79782	Hypothetical protein DAPPUDRAFT_304363	*Daphnia pulex*	—
125	daphmag3mtv3l1194t1	0.016	2.54	EFX79782	Hypothetical protein DAPPUDRAFT_304363	*Daphnia pulex*	—
1053	daphmag3mtv3l11111t1	0.02	2.51	EFX83276	Enolase	*Daphnia pulex*	P: glycolysis
594	daphmag3mtv3l13753t1	0.001	2.44	EFX71334	Cytosolic malate dehydrogenase	*Daphnia pulex*	—
949	daphmag3mtv3l11112t1	0.034	2.43	EFX88851	Hypothetical protein DAPPUDRAFT_234212	*Daphnia pulex*	—
1952	daphmag3mtv3l6078t1	0.043	2.36	EFX87538	Hypothetical protein DAPPUDRAFT_192225	*Daphnia pulex*	C: cytoplasm, F: ATP binding

Table 1. (Continued)

Spot. no.	*2D-DIGE_results*				*First_Blast_hit*		*Enriched_GO_terms*
	*ID_*D. magna*_database*	*T-test*	*Av. ratio*	*Acc*	*Name*	*Org*	
539	daphmag3mtv3l7770t1	0.021	2.35	ADA79522	Heat shock protein 70	*Daphniopsis tibetana*	F: ATP binding
2113	daphmag3mtv3l14633t1	0.011	2.34	EFX88365	Hypothetical protein DAPPUDRAFT_230303	*Daphnia pulex*	—
977	daphmag3mtv3l2256t2	0.017	2.3	EFX74558	Hypothetical protein DAPPUDRAFT_307231	*Daphnia pulex*	—
583	daphmag3mtv3l5322t1	0.036	2.27	EFX90349	Hypothetical protein DAPPUDRAFT_300069	*Daphnia pulex*	F: ATP binding
866	daphmag3mtv3l4092t1	0.025	2.24	EFX87506	Hypothetical protein DAPPUDRAFT_306375	*Daphnia pulex*	F: unfolded protein binding, P: protein folding, C: cytoplasm, F: ATP binding
610	daphmag3mtv3l2732t1	0.05	2.23	EFX80327	Hypothetical protein DAPPUDRAFT_304064	*Daphnia pulex*	—
1825	daphmag3mtv3l10239t1	0.008	2.2	EFX70674	Hypothetical protein DAPPUDRAFT_256736	*Daphnia pulex*	F: ATP binding
384	daphmag3mtv3l4116t1	0.025	2.18	EFX72171	Hypothetical protein DAPPUDRAFT_308570	*Daphnia pulex*	F: ATP binding
1677	daphmag3mtv3l8231t1	0.008	2.16	EFX89163	Hypothetical protein DAPPUDRAFT_220693	*Daphnia pulex*	F: ATP binding
1375	daphmag3mtv3l8231t1	0.003	2.13	EFX89163	Hypothetical protein DAPPUDRAFT_220693	*Daphnia pulex*	F: ATP binding
553	daphmag3mtv3l7770t1	0.039	2.08	ADA79522	Heat shock protein 70	*Daphniopsis tibetana*	F: ATP binding
1351	daphmag3mtv3l12256t1	0.033	2.07	EFX68536	Hypothetical protein DAPPUDRAFT_189444	*Daphnia pulex*	—
2447	daphmag3mtv3l7094t1	0.017	2.02	CAB99474	Actin	*Daphnia pulex;Daphnia magna*	C: cytoplasm, F: ATP binding
1654	daphmag3mtv3l8855t1	0.008	−2.02	EFX80562	Hypothetical protein DAPPUDRAFT_188180	*Daphnia pulex*	P: glycolysis
1047	daphmag3mtv3l5529t1	0.026	−2.03	EFX88163	Hypothetical protein DAPPUDRAFT_311761	*Daphnia pulex*	F: actin binding
1747	daphmag3mtv3l8231t1	0.033	−2.06	EFX89163	Hypothetical protein DAPPUDRAFT_220693	*Daphnia pulex*	F: ATP binding
1534	daphmag3mtv3l11651t1	0.018	−2.09	EFX82035	Hypothetical protein DAPPUDRAFT_302845	*Daphnia pulex*	C: cytoplasm
1945	daphmag3mtv3l7094t1	0.043	−2.28	CAB99474	Actin	*Daphnia pulex;Daphnia magna*	C: cytoplasm, F: ATP binding
961	daphmag3mtv3l1503t1	0.008	−2.46	EFX86436	Hypothetical protein DAPPUDRAFT_208250	*Daphnia pulex*	F: actin binding
1412	daphmag3mtv3l7094t1	0.043	−2.46	CAB99474	Actin	*Daphnia pulex;Daphnia magna*	C: cytoplasm, F: ATP binding
1577	daphmag3mtv3l8855t1	0.017	−2.52	EFX80562	Hypothetical protein DAPPUDRAFT_188180	*Daphnia pulex*	P: glycolysis
2093	daphmag3mtv3l6920t1	0.04	−2.55	BAJ72724	2-domain hemoglobin	*Daphnia magna*	—
440	daphmag3mtv3l733t1	0.012	−2.73	EFX87106	Myosin heavy chain isoform 3	*Daphnia pulex*	F: ATP binding, F: actin binding
1024	daphmag3mtv3l5322t1	0.004	−2.87	EFX90349	Hypothetical protein DAPPUDRAFT_300069	*Daphnia pulex*	F: ATP binding
1656	daphmag3mtv3l8855t1	0.015	−2.89	EFX80562	Hypothetical protein DAPPUDRAFT_188180	*Daphnia pulex*	P: glycolysis
1955	daphmag3mtv3l6920t1	0.008	−2.89	BAJ72724	2-domain hemoglobin	*Daphnia magna*	—
1207	daphmag3mtv3l7770t1	0.012	−2.93	ADA79522	Heat shock protein 70	*Daphniopsis tibetana*	F: ATP binding
2257	daphmag3mtv3l9572t1	0.031	−2.95	EFX84424	Hypothetical protein DAPPUDRAFT_301074	*Daphnia pulex*	C: cytoplasm, F: ATP binding
2084	daphmag3mtv3l9835t1	0.012	−3.06	EFX70620	Hypothetical protein DAPPUDRAFT_202253	*Daphnia pulex*	—
1402	daphmag3mtv3l7094t1	0.027	−3.12	CAB99474	Actin	*Daphnia pulex;Daphnia magna*	C: cytoplasm, F: ATP binding
446	daphmag3mtv3l733t1	0.031	−3.13	EFX87106	Myosin heavy chain isoform 3	*Daphnia pulex*	F: ATP binding, F: actin binding
1970	daphmag3mtv3l7094t1	0.032	−3.13	CAB99474	Actin	*Daphnia pulex;Daphnia magna*	C: cytoplasm, F: ATP binding
2097	daphmag3mtv3l6920t1	0.01	−3.17	BAJ72724	2-domain hemoglobin	*Daphnia magna*	—
1366	daphmag3mtv3l7094t1	0.019	−3.2	CAB99474	Actin	*Daphnia pulex;Daphnia magna*	C: cytoplasm, F: ATP binding
2448	daphmag3mtv3l7094t1	0.012	−3.26	CAB99474	Actin	*Daphnia pulex;Daphnia magna*	C: cytoplasm, F: ATP binding
1182	daphmag3mtv3l1503t1	0.031	−3.4	EFX86436	Hypothetical protein DAPPUDRAFT_208250	*Daphnia pulex*	F: actin binding
2450	daphmag3mtv3l7094t1	0.012	−3.42	CAB99474	Actin	*Daphnia pulex;Daphnia magna*	C: cytoplasm, F: ATP binding
1846	daphmag3mtv3l8815t1	0.028	−3.49	EFX88463	Hypothetical protein DAPPUDRAFT_305568	*Daphnia pulex*	—
2049	daphmag3mtv3l6920t1	0.013	−3.69	BAJ72724	2-domain hemoglobin	*Daphnia magna*	—
1377	daphmag3mtv3l7094t1	0.019	−3.99	CAB99474	Actin	*Daphnia pulex;Daphnia magna*	C: cytoplasm, F: ATP binding
2138	daphmag3mtv3l8231t1	0.013	−4.01	EFX89163	Hypothetical protein DAPPUDRAFT_220693	*Daphnia pulex*	F: ATP binding
2277	daphmag3mtv3l733t1	0.025	−4.05	EFX87106	Myosin heavy chain isoform 3	*Daphnia pulex*	F: ATP binding, F: actin binding
823	daphmag3mtv3l2501t1	0.004	−4.6	EFX84778	Hypothetical protein DAPPUDRAFT_99081	*Daphnia pulex*	F: actin binding

Abbreviations: 2D-DIGE, two-dimensional difference gel electrophoresis; GO, gene ontology; NA, not applicable; nr, non-redundant.
2D-DIGE results, first nr Blast Hit and enriched GO terms are displayed.

overrepresented in our data set, in the tricarboxylic acid cycle, the respiratory chain, or pentose phosphate pathway. All proteins except fructose-bisphosphate-aldolase were more abundant in *Daphnia* exposed to simulated microgravity.

For a summary of identified proteins see Table 3, for details on protein spots see Supplementary Data S1.

DISCUSSION

To analyze the effects of altered gravity conditions on the waterflea *D. magna*, we exposed animals to fast clinorotation, an established method to simulate microgravity conditions on ground. Nevertheless one has to keep in mind, that this kind of simulation approach has to be verified under real microgravity conditions.[17] The clinorotated animals were compared with a control group using a proteomic 2D-DIGE approach (five biological replicates). As a general result of this study, proteins involved in actin microfilament organization were less abundant in clinorotated animals, whereas proteins connected to protein folding and energy metabolism were more abundant.

Exposure to simulated microgravity may disrupt actin microfilament organization in *Daphnia*

Around 25% of the significantly altered and identified protein spots in our data set were related to muscular structures or the cytoskeleton. All of these proteins were annotated with the GO term actin binding, which was also found to be significantly overrepresented (Figure 4).

We identified the structural protein actin in 12 spots, showing isoelectric point and molecular weight shifts on the 2D-gel. Two different kinds of spots were observed, indicating two groups of PTMs. The first group consists of four spots which were more

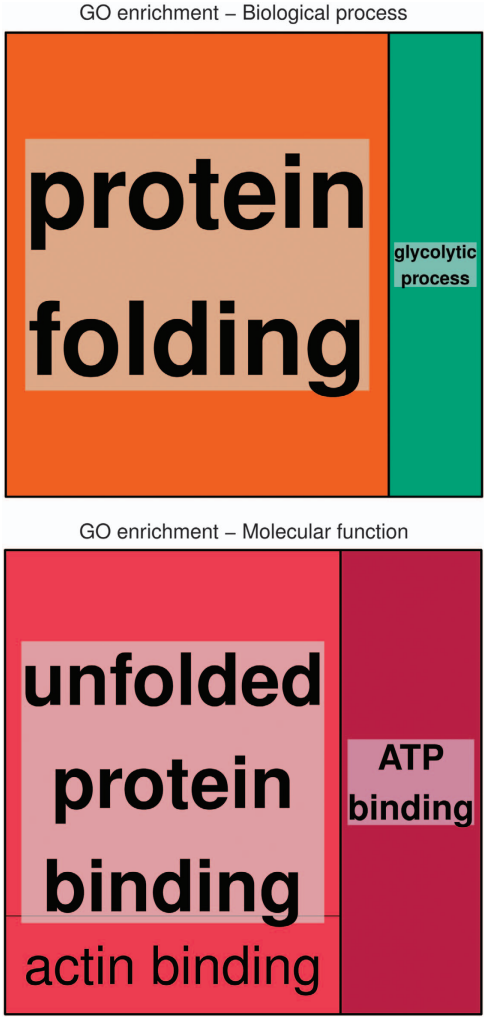

Figure 4. Results of REViGO semantic analysis of EASE results for enrichment of GO biological process terms and molecular function terms (p.adjust < 0.05). Colors mark semantic similarity, whereas the size of the corresponding area reflects the *P* value. GO, gene ontology.

abundant in the clinorotated treatment and have a pI near the theoretical value but an increased molecular mass. The second group of eight spots is less abundant in the clinorotated treatment and has a decreased pI but the molecular weight is near the theoretical value or below (Supplementary Data S1). These spots, which were located differently on the gel showing different abundance ratios, may indicate changes of actin PTMs. These changes may be related to alterations of actin microfilament organization, as PTMs are known to modulate structure and function of actin.[29]

The effect of clinorotation on actin filaments in our study is further emphasized by abundance alterations of actin-related proteins. Here, not only the actin-filament-associated motor protein myosin but also the actin-binding proteins α-actinin and filamin-A were less abundant in animals exposed to simulated microgravity. Filamin is known to be involved in the recruitment of actin filaments in *Drosophila*[30] and its decreased abundance may therefore indicate cytoskeletal disorganization. α-actinin is a cross-linker of actin filaments in muscle and non-muscle cells. *Drosophila* knock-down mutants are known to suffer from muscle weakness and atrophy,[31] which may also be the case in *D. magna* exposed to microgravity. Furthermore, α-actinin was also found to be less abundant in human neuroblastoma cells exposed to simulated microgravity.[32] In contrast to these proteins, the actin-binding proteins advillin and gelsolin were more abundant in clinorotated *Daphnia*. Both proteins are members of the gelsolin/villin family, which are involved in the regulation of actin polymer organization[33] and are also able to sever actin filaments. Therefore, it can be stated that our findings on actin-related proteins provide strong evidence for a disruption of actin microfilament organization in *D. magna* exposed to simulated microgravity, at least in the time frame of 60-min exposure.

Alterations of the cytoskeleton as a result of different gravity conditions are a phenomenon described in many studies analyzing various mammalian cell lines either exposed to real or simulated microgravity.[14] In several studies, the disorganization of the actin cytoskeleton was observed in human monocytes[34] and human neuroblastoma cells[32] using immunohistochemistry and studying cell morphology. Furthermore, changes of cytoskeletal element gene expression and morphology in human cells were detected on a short time-scale after only 2 s in real microgravity during parabolic flights.[35,36] In addition, actin protein abundance decreased after 12 days of spaceflight in *Arabidopsis*.[37]

Table 2. EASE results for enrichment of GO biological process terms and molecular function terms (p.adjust < 0.05, *P* value was Benjamini corrected)

	GO term	*p.adjust*	*Ids* D. magna *database*
GO:0051082	F: unfolded protein binding	0.000000114	DAPHMAG3MTV3L10134T1; DAPHMAG3MTV3L10162T1; DAPHMAG3MTV3L11254T1; DAPHMAG3MTV3L4092T1; DAPHMAG3MTV3L4176T1; DAPHMAG3MTV3L7067T1; DAPHMAG3MTV3L7809T1; DAPHMAG3MTV3L9792T1
GO:0006457	P: protein folding	0.00000375	DAPHMAG3MTV3L10134T1; DAPHMAG3MTV3L10162T1; DAPHMAG3MTV3L11254T1; DAPHMAG3MTV3L4092T1; DAPHMAG3MTV3L4176T1; DAPHMAG3MTV3L7067T1; DAPHMAG3MTV3L7809T1; DAPHMAG3MTV3L9792T1
GO:0005737	C: cytoplasm	0.0000684	DAPHMAG3MTV3L10134T1; DAPHMAG3MTV3L10162T1; DAPHMAG3MTV3L11254T1; DAPHMAG3MTV3L11651T1; DAPHMAG3MTV3L4092T1; DAPHMAG3MTV3L6078T1; DAPHMAG3MTV3L7094T1; DAPHMAG3MTV3L9572T1; DAPHMAG3MTV3L9792T1
GO:0005524	F: ATP binding	0.0002	DAPHMAG3MTV3L10134T1; DAPHMAG3MTV3L10162T1; DAPHMAG3MTV3L10239T1; DAPHMAG3MTV3L10909T1; DAPHMAG3MTV3L11254T1; DAPHMAG3MTV3L2675T1; DAPHMAG3MTV3L4092T1; DAPHMAG3MTV3L4116T1; DAPHMAG3MTV3L4176T1; DAPHMAG3MTV3L5322T1; DAPHMAG3MTV3L6078T1; DAPHMAG3MTV3L7067T1; DAPHMAG3MTV3L7094T1; DAPHMAG3MTV3L733T1; DAPHMAG3MTV3L7770T1; DAPHMAG3MTV3L7809T1; DAPHMAG3MTV3L8231T1; DAPHMAG3MTV3L9572T1; DAPHMAG3MTV3L9792T1
GO:0003779	F: actin binding	0.0136	DAPHMAG3MTV3L1503T1; DAPHMAG3MTV3L2246T2; DAPHMAG3MTV3L2501T1; DAPHMAG3MTV3L5529T1; DAPHMAG3MTV3L733T1
GO:0006096	P: glycolysis	0.0477	DAPHMAG3MTV3L11111T1; DAPHMAG3MTV3L13427T1; DAPHMAG3MTV3L8855T1

Abbreviation: GO, gene ontology.

Table 3. Summary of significantly altered proteins involved in the response of *Daphnia* to altered gravity

Dmag*ID*	*Swissprot accession*	*Protein name*	*No. spots*	*Average ratio*
Actin binding				
daphmag3mtv3l7094t1	P07837	Actin	12	−0.44
daphmag3mtv3l5529t1	O75366	Advillin	7	6.98
daphmag3mtv3l2501t1	P18091	α-actinin	1	−4.6
daphmag3mtv3l1503t1	Q9VEN1	Filamin-A	2	−2.93
daphmag3mtv3l2246t2	Q27319	Gelsolin	2	4.61
daphmag3mtv3l733t1	P05661	Myosin	3	−3.3
Protein binding				
daphmag3mtv3l7770t1	P29844	Heat shock protein 70	4	1.33
daphmag3mtv3l7067t1	Q66HD0	Endoplasmin	2	3.61
daphmag3mtv3l4176t1	P02828	Heat shock protein 83	2	3.17
daphmag3mtv3l7809t1	Q5R511	Stress-70 protein	2	9.55
daphmag3mtv3l2732t1	Q12931	Heat shock protein 75	1	2.23
daphmag3mtv3l10134t1	Q6P502	T-complex protein 1	8	3.62
daphmag3mtv3l9835t1	P38657	Protein disulfide-isomerase	1	6.9
Energy metabolism				
daphmag3mtv3l11111t1	P15007	Enolase	3	5.62
daphmag3mtv3l13427t1	P91427	Phosphoglycerate kinase	1	4.08
daphmag3mtv3l8855t1	P07764	Fructose-bisphosphate aldolase	3	−2.48
daphmag3mtv3l4901t1	Q9XTL9	Glycogen phosphorylase	2	4.54
daphmag3mtv3l13753t1	Q5ZME2	Malate dehydrogenase	2	2.8
daphmag3mtv3l6730t1	P50137	Transketolase	1	7.42
daphmag3mtv3l592t1	Q66HF1	NADH-ubiquinone oxidoreductase	1	5.17
daphmag3mtv3l10909t1	Q05825	ATP synthase subunit β	1	4.87
Hemoglobin				
daphmag3mtv3l6920t1	BAJ72724	2-domain hemoglobin	7	1.31
daphmag3mtv3l16955t1	BAA76873	Hemoglobin	2	4.46

Sequence ID from *D. magna* protein database and corresponding blast hit in Swiss-Prot database are displayed in addition to total number of identified protein spots and average ratio of spot intensity (clinorotated/control).

Large-scale effects of microgravity on the muscular system of humans, mice, and rats are also well-studied,[38] leading to atrophy and reduced functional capacity of the muscles. However, the source and function of actin found in our study, either cytoplasmic or muscular, is hard to predict without further experiments. Röper *et al.*[39] showed that muscle-specific actin is incorporated into cytoplasmic structures, and cytoskeletal actin is incorporated into muscles for all actin paralogues of *Drosophila Melanogaster*. Therefore, it is not possible to deduce actin function solely from its protein sequence.

Chaperones are involved in the stress response of *Daphnia* to simulated microgravity

Another substantial fraction of protein spots were associated with protein binding, a GO term found overrepresented in the enrichment analysis (Figure 4). In this group, several molecular chaperones were identified with nearly all proteins being more abundant in the clinorotated treatment.

Heat shock proteins and other chaperones facilitate protein folding, unfolding, and transportation[40] playing an important role in both normal cellular homeostasis and stress response.[41] In *Daphnia*, they were found to be involved in responses to several stressors, e.g., temperature changes,[11] presence of a predator,[13,42] exposure to the drug diclofenac,[43] and exposure to copper.[44]

Activation of the heat shock system was also observed as response to microgravity in other systems, leading to increased gene expression or higher abundance of heat shock proteins. This was reported for plant cells exposed to simulated microgravity and microgravity during spaceflight,[45] for animal cells exposed to simulated microgravity[32] and also for *Drosophila* during spaceflight.[46] Our data clearly indicate that in *Daphnia*, the heat shock system possibly reacts to stress-dependent changes in cell, tissue, or organ structures caused by altered mechanical (gravitational) forces.[47] Therefore, proteins related to protein folding seem to be involved in the response to microgravity.[46]

In addition to heat shock proteins, we identified subunits of the chaperonin containing T-complex protein, also known as CCT, in several spots, which were all more abundant in the clinorotated treatment. CCT is known to contribute to the folding of a distinct subset of cellular proteins including cytoskeletal proteins like actin and myosin.[48] Moreover, protein disulfide-isomerase was more abundant in the clinorotated animals. This protein is an essential folding catalyst and chaperone located in the endoplasmatic reticulum, which introduces disulfide bonds into proteins and catalyses the rearrangement of incorrect disulfides.[49]

Therefore, the high abundance of proteins related to protein folding in *Daphnia* exposed to clinorotation is a strong indicator for a microgravity-induced breakdown of protein structures in general.

Clinorotation leads to an increased energy demand in *Daphnia*

Further molecular consequences of simulated microgravity on *Daphnia* is seen in the field of energy related proteins. Here, proteins associated with various energy pathways were altered in their abundance, most of them more abundant in the clinorotated treatment.

We found proteins involved in glycolysis (enolase, phosphoglycerate kinase, and fructose-bisphosphate aldolase), the TCA cycle (malate dehydrogenase), and in the respiratory chain (NADH-ubiquinone oxidoreductase and ATP synthase subunit β). Most of

these proteins were more abundant in the clinorotated treatment, indicating an enhanced energy metabolism in simulated microgravity. Furthermore, the enhanced abundance of glycogen phosphorylase is also an indicator of increased energy consumption in clinorotated animals, as it catalyses the degradation of glycogen to provide an increased amount of glucose.

In contrast to the other glycolytic proteins, fructose-bisphosphate aldolase was less abundant in clinorotated animals. However, this protein is also involved in processes apart from glycolysis, e.g., pentose phosphate pathway, and fructose and mannose metabolism, which may also be affected by the stressful condition of altered gravity. Similarly, transketolase, is also involved in the pentose phosphate pathway and showed only a slightly higher abundance in clinorotated *Daphnia*.

Interestingly, proteins related to energy metabolism were also found to be affected by microgravity in other systems. In *Arabidopsis thaliana* grown for 12 days on the ISS, a lower abundance of these proteins was observed.[37] In contrast, exposure to simulated microgravity for several hours using a clinostat led to an increased abundance of carbohydrate metabolism proteins in *Arabidopsis* callus[50] and root cells.[51] The differences that were found in the abovementioned studies might be based on different exposure times (short-term versus long term) or different exposure methods (simulated versus real microgravity), which may influence the response to microgravity.[17] However, indications for an increased energy metabolism found in our study correlated well with the similar clinorotation experiments in *A. thaliana*.[50,51] Changes in protein abundance related to energy metabolism were also observed in *Pseudomonas aeruginosa*, an opportunistic pathogen, when exposed to microgravity. Here, proteins related to glycolysis were of higher abundance in ground control treatments when compared with *P. aeruginosa* exposed to microgravity.[52] Furthermore, alterations in the expression of proteins related to energy metabolism were also reported in the bacterial pathogen *Salomonella* in spaceflight environment.[53,54]

Another indicator for an increased energy demand in *Daphnia* in our study is the altered abundance of hemoglobin. *Daphnia*, is known to increase its hemoglobin concentration in response to environmental hypoxia as well as to temperature increase.[55] Here, elevated water temperature has two effects, a decrease of oxygen partial pressure due to decreased oxygen solubility and an increase of metabolic rate in the ectotherm animal.

Daphnia hemoglobin genes are located in a tandem-duplicated gene cluster, which contributes to the varying composition of the protein. Both, the abundance of different Hb subunits, as well as their post translational modifications change in an oxygen-dependant manner, most probably optimizing oxygen affinity of hemoglobin according to oxygen concentration.[56] The same may be true for our study, explaining the occurrence of both, more and less abundant hemoglobin spots in the clinorotated treatment with pI and mass shifts.

As average hemoglobin abundance is higher in clinorotated animals, oxygen consumption may be increased. Therefore, hemoglobin abundance is most probably not primarily affected by microgravity, but clinorotation may lead to a stress response in *Daphnia*, resulting in higher energy demand and therefore increased oxygen consumption. As the amount of available oxygen in the cuvette is limited due to the experimental device, this increased oxygen consumption may lead to hypoxic conditions induced by the experimental device rather than microgravity. Animals in the control cuvette did not show an increase in hemoglobin abundance although oxygen concentration in the cuvette was similar, which indicates higher oxygen consumption of clinorotated animals. However, it cannot be excluded that the increased energy demand found in our study is related to altered swimming behavior of clinorotated *Daphnia*, as it is known that *Daphnia* in space shows an increased amount of looping/summersaulting movements.[16]

CONCLUSION

Using a proteomic approach, we were able to identify molecular key processes affected by clinorotation. Assuming that this simulation approach is suitable for *Daphnia*, which has to be verified in space, our results indicate impacts of microgravity on the parameters of investigations. We found strong indicators of actin cytoskeleton disruption and breakdown of protein structures in general and an increase of energy demands. These results are in agreement with results obtained from other organisms and microgravity systems. Interestingly, most of the proteins found to be affected are well-conserved throughout taxa.

Our proteomic approach led to interesting insights into the responses to altered gravity conditions. These results not only resemble important starting points for further *Daphnia* space research concerning life support systems but also increase the knowledge on the influence of gravity on biochemical processes. In addition, our data strongly suggest that a lack of gravity affects similar molecular processes in a variety of organisms.

ACKNOWLEDGMENTS

Our work benefits from and contributes to the Daphnia Genomics Consortium. We thank Quirin Herzog for providing *D. magna* photographs. We gratefully acknowledge the financial support of BMWi and DLR (Grant No. 50WB1029), as well as the ESA-GBF-program (Contract No. 4000103571).

CONTRIBUTIONS

CL, RH, and KS designed the study. BT performed clinorotation and proteomic experiments. BT and KAO analyzed proteomic data. KAO conducted further bioinformatical analysis. TF supervised mass spectrometry analysis. KAO and BT wrote the first draft of the manuscript, and CL, TF, RH, KS, and GJA contributed substantially to revisions. All authors read and approved the final manuscript.

DATA DEPOSITION

The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium[57] via the PRIDE partner repository with the data set identifier PXD002096.

COMPETING INTERESTS

The authors declare no conflict of interest.

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Supplementary Information accompanies the paper on the *npj Microgravity* (http://www.nature.com/npmgrav)

6 General Discussion

The aim of the work presented in this thesis was to shed light on molecular mechanisms underlying stress responses in the ecological model organism *Daphnia*. Here, different stressors either known to have a big impact on *Daphnia* populations (predation and parasites) or a stressor that organisms usually do not encounter on earth and therefore could not adapt to (microgravity) were analysed using different proteomic approaches. Proteomics is particularly suited for functional characterisation of biological systems, as proteins are the main effectors of most biological processes.

6.1 *Daphnia* as challenging source for the generation of protein samples

As depicted in section 1.5.1, successful preparation of protein lysates from adult *Daphnia* whole body samples is a difficult task due to the high degree of proteolytic activity in these samples. Most probably, proteases originating from the gut of *Daphnia* (von Elert et al., 2004; Agrawal et al., 2005; Schwarzenberger et al., 2010) are the reason for this proteolytic activity and furthermore, they are not inhibited by proteomic standard protocols (Cañas et al., 2007). This problem was documented in the first studies applying proteomics to *Daphnia* (Fröhlich et al., 2009; Zeis et al., 2009; Schwerin et al., 2009; Kemp and Kültz, 2012) and most probably hindered the establishment of

Daphnia proteomics as a broadly used method, leading to a minor number of publications although protein sequence databases were already available (Lemos et al., 2010). However, although special care is needed, the successful preparation of *Daphnia* protein lysates is possible by reducing the proteolytic activity to a minimum. For the work presented here, I optimised the sample preparation protocol by the combination of freezing of the samples with liquid nitrogen, a lysis buffer with high concentration of protease inhibitor, a precipitation step and minimising of total handling time (see section 1.5.1). However, the quality of *Daphnia* protein samples should be monitored in any case, for example by generating a protein gel (e.g. SDS-PAGE) and checking the gel image for indicators of proteolysis like missing bands in the high molecular weight area like it was demonstrated in the work presented in this thesis.

Furthermore, the advance of LC-MS/MS techniques and also further optimisation of the workflow can increase the number of identified and quantified proteins in a *Daphnia* sample. This was achieved by replacing the gel fractionation step by an in solution peptide fractionation using an OFFGEL fractionator (Agilent) and analysing these peptide fractions with a new chromotographic UHPLC EASY-nLC 1000 (Thermo scientific), which became available only recently in our laboratory, coupled to an OrbitrapXL mass spectrometer (Thermo scientific). Further processing using the MaxQuant software version 1.5.2.8 with similar parameters as in chapter 3 and 4 (FDR 1 %, at least 2 unique/razor peptides per protein) and using the newly available *D. magna* protein database as in chapter 4 led to an identification of 2023 proteins in a single sample of pooled *D. magna* (data have been deposited in the PRIDE database via proteomeXchange (Vizcaíno et al., 2014), see supplementary information for details). This is around 400 proteins more than in the approach described in this thesis (see chapter 3).

6.2 Annotation information of *Daphnia* proteins

With the published genome sequence of *D. pulex* (Colbourne et al., 2011) and the preliminary *D. magna* genome (`http://arthropods.95eugenes.org/EvidentialGene/daphnia/daphnia_magna/`) protein sequence databases were available which are a necessary prerequisite for protein identification. However, although it is now possible to identify protein sequences involved in a certain trait, usually not more than the protein sequence is known about the protein. In the publicly available protein database UniProt (The UniProt Consortium, 2014), 32,401 *Daphnia* sequences were listed with 28,368 named as *uncharacterized*, which is nearly 90 % (effective 08/2015).

To get more information about a protein I used different approaches. One possibility was to look for homologous sequences in better characterised organisms using the blastp algorithm (Geer et al., 2010). Here, certain thresholds should be applied, as functional similarity can only be assumed for sequences having a certain degree of similarity (Louie et al., 2009). This approach of course only works if a homolog in a better characterised organism exists. As *Daphnia* is a crustacean, the arthropod group most closely allied with insects (Giribet and Edgecombe, 2012), a source for more information are sequence similarities in *Drosophila melanogaster*. I used *Drosophila* homologs for protein characterisation in the work presented in chapter 2. This approach also enables the usage of a lot of software tools e.g. ClueGO (Bindea et al., 2009) or DAVID (Da Wei Huang et al., 2008) for interpretation of annotation information.

However, there are biological processes in *Daphnia* that are not shared by *Drosophila*, e.g. haemoglobin synthesis, and cannot be handled by this approach. Therefore, I decided to use sequence motif information in addition to sequences similarities provided by blast in my subsequent work (chapter 3 – 5). For this reason, I used the pfam database (Finn et al., 2014) and the InterPro database (Hunter et al., 2012).

Pfam and InterPro terms can sometimes be mapped to gene ontology terms (Ashburner et al., 2000), giving more information on associated biological processes, molecular functions and cellular compartments. Part of this information is available at the *Daphnia* genome repositories. However, for gaining additional annotation information, a high degree of customisation is necessary. I faced this problem by combining an extensive BLAST search of the *D. magna* sequences using two different databases and GO enrichment analysis using the available annotation information. As additional information source, I used my BLAST results for mapping to GO terms using Blast2GO, gaining more terms probably associated with the *Daphnia* proteins. However, compared to classical molecular model organisms, not only the identification of key process members but also the detection of connections between these members is very difficult. Nevertheless, this approach is inevitable to reveal interesting biological traits, especially as in *Daphnia* lineage specific genes are thought to be the most responsive genes to ecological challenges (Colbourne et al., 2011), a hypothesis which is also supported by my own work (chapter 3). As progress in the application of molecular tools to *Daphnia* is going to stride ahead and molecular analysis of *Daphnia* will increase, more information will hopefully be available soon, which will, together with the progress in LC-MS/MS techniques (see section 6.1), facilitate the detection of molecular key processes.

6.3 Key proteins of stress responses in *Daphnia*

With the work presented here I was indeed able to reveal key proteins involved in the response of *Daphnia* to the analysed stressors. When comparing the different experiments, it is very interesting to see that the stressors which are known to be very important for *Daphnia* populations create a specific response, including proteins related to already known traits but also uncharacterised, *Daphnia* specific proteins,

which usually have only limited annotation information. The two studies of predator-induced phenotypic plasticity of late-stage *Daphnia* embryos and adults exposed to the predator *Triops* [chapter 2 and 3] revealed proteins related to similar traits, although the proteomic analysis was conducted using different techniques. In both data-sets, cuticle proteins and chitin-modifying enzymes played a substantial role which corresponds very good to the inducible defences known so far in the *D. magna*-*T. cancriformis*-system, as they are connected to the carapace and its cuticle (Rabus and Laforsch, 2011; Rabus et al., 2012, 2013) and the mechanical properties of the cuticle are determined by chitin nanofibres, proteins and the degree of cross-linking of these components (Vincent and Wegst, 2004). Furthermore, the yolk protein vitellogenin (vtg) is very clearly involved in the response to the predator. Yolk proteins serve as substrate and energy supply for the developing embryo in most oviparous animals (Subramoniam, 2010). In *Daphnia*, fat cells which form the fat body, are the most probable place of vtg synthesis (Zaffagnini and Zeni, 1986). In my experiments, *D. magna* embryos showed a reduced abundance of vitellogenin when exposed to the predator compared to the control group. This interesting finding is either related to a reduced vtg starting concentration per egg or reflect changes in energy demand and allocation costs in animals exposed to the predator, indicating an increased energy demand of the embryo while building up predator-induced defensive structures. In the proteomic analysis of adult *D. magna* exposed to *Triops*, vtg was part of the general response to the predator, meaning that it increased in abundance in all genotypes, even in the genotype that did not change its morphology when exposed to *Triops*. These changes in vtg abundance may result from an increasing number of eggs produced or an elevated vtg concentration per egg. Therefore, they most likely reflect life history shifts associated with predator exposure. Indeed, *D. magna* are known to have more offspring with an increased body size in the presence of *T. cancriformis* (Hesse

et al., 2012). In other studies, the exposure of *D. magna* to chemical cues of predatory fish or *Chaoborus* larvae is known to alter yolk dynamics (Stibor, 2002; Effertz and von Elert, 2014). As vtgs are not only a very abundant protein group in adult *Daphnia* females under non stressful environmental conditions but are also connected to *Daphnia* reproduction and life-history, also other proteomic studies in *Daphnia* found vtg abundance affected by changes in environmental conditions. Here, ecotoxicological stressors like the exposure to pharmaceuticals, heavy metals or nano-particles (Borgatta et al., 2015; Rainville et al., 2014, 2015) or a decrease in water temperature (Schwerin et al., 2009) have been shown to alter vtg abundance. Furthermore, vtgs have also been proposed as potential biomarkers for ecotoxicological approaches in invertebrates (Jubeaux et al., 2012) and, more specific, as indicator for the exposure to oestrogenic compounds in aquatic invertebrates (Matozzo et al., 2008).

The results of the second study on predator-induced phenotypic plasticity in *Daphnia* (chapter 3) confirm the results of the first study (chapter 2). Furthermore, due to the change of the proteomic approach, a lot of additional proteins involved in the response could be assigned. These additional information and also the use of different genotypes broaden the knowledge on the studied trait. Again, several proteins related to already known traits concerning the carapace were found. In addition to cuticle proteins and chitin-modifying enzymes, as have been found in the first study, also proteins related to calcium binding were found to be involved. This is also interesting, as calcium is an important component of the cuticle of arthropods (Vincent and Wegst, 2004). In addition, low-calcium environment is known to inhibit predator-induced morphological changes in *D. pulex* (Riessen, 2012), highlighting the importance of calcium for the formation of cuticle-related defensive structures in *Daphnia*. Although not much information were available on *Daphnia* proteins, some information on involved regulatory pathways could be gathered from the data. I found one

protein having a juvenile hormone-binding sequence motif with increased abundance in the morphologically responding genotypes after predator exposure. In Daphnia, juvenile hormones are known to regulate male reproduction and are possibly involved in haemoglobin synthesis (Eads et al. 2008), and also regulate vitellogenin synthesis (Tokishita et al. 2006). In addition, these hormones are able to enhance the formation of morphological defensive structures (Oda et al. 2011; Miyakawa et al. 2013; Dennis et al. 2014) and may therefore be involved in the regulation of predator-induced phenotypic plasticity. Furthermore, the protein calmodulin was detected to increase in abundance in animals exposed to the predator. Most interestingly, calmodulin was not detected in the non responding genotype, indicating that this protein is involved in morphological changes of the cuticle. Indeed, it was shown that pharmaceutical inhibition of calmodulin in *D. magna* reduced cuticle protein and vitellogenin expression (Furuhagen et al. 2014). Furthermore, a lot of the significantly altered proteins in this study have no shared sequence similarities with any protein sequence outside the *Daphnia* lineage. Colbourne et al. (2011) found that around one third of the genes in the *D. pulex* genome have no detectable homologies and were significantly over-represented within genes that were affected by exposure to biotic and abiotic stressors. This finding also supported by the data presented here. Beyond that, my study also delivers interesting insights into the influence of the genotype on predator-induced phenotypic plasticity. The analysed genotypes showed differences at the morphological and protein level which seem to be related to their habitat. Genotype-dependent protein changes were related to the cuticle, protein synthesis and calcium binding. Furthermore, genotype-dependent responses at the proteome level were most distinct for the only genotype that shares its habitat with *Triops*. This genotype had more significantly altered proteins when compared to the other genotypes, probably indicating a more specific adaptation against this pred-

ator. In general, genotypes of *Daphnia* are known to show huge differences e.g. at the transcriptional level when exposed to environmental stressors, for example single and mixed stresses of cadmium and a toxic cyanobacterium (De Coninck et al., 2014), changes in temperature (Yampolsky et al., 2014) and changes in phosphorus supply (Roy Chowdhury et al., 2015).

Genotypic differences were also analysed in the response of *D. magna* to the parasite *P. ramosa* as described in chaper 4. Genotype-genotype interactions between host and parasite are known to determine the outcome of the infection process (Luijckx et al., 2011). In contrast to the other proteomic studies presented in this thesis, I analysed here not whole body samples but only the cuticle of the animals, a challenging task because protein amount of cuticle is not very high. I only used the cuticle, as the attachment step of *P. ramosa* spores to the esophagus, which is part of the animal's cuticle, was found to be responsible for this genotype specificity (Duneau et al., 2011). Indeed, when comparing cuticles of a susceptible to a non-susceptible genotype, I found differences in the cuticle proteomes, especially within the cuticle proteins. Most interestingly, some cuticle proteins of the susceptible genotypes had a very high number of predicted glycosylation sites, whereas cuticle proteins more abundant in the non-susceptible genotype showed a distinct lower number of predicted glycosylation sites. This is especially interesting, as for the closely related species *P. penetrans*, which is a parasite of nematodes and also shows high host attachment specificity, it was proposed that collagen-like protein fibres on the surface of the endospore may bind to glycosylated proteins in the cuticle of the host in a Velcro-like manner (Davies, 2009). Furthermore, another set of proteins which is more abundant in the non-susceptible genotype is similar to matrix metalloproteinases (MMPs). MMPs are involved in innate immune defence reactions of *Tribolium castaneum*, as animals with a systemic MMP-1 knockdown were more susceptible when exposed

to the entomopathogenic fungus *Beauveria bassiana* (Knorr et al., 2009). Similar to *P. ramosa*, entomopathogenic fungi are known to invade their host by penetration of the cuticle (Clarkson and Charnley, 1996). Furthermore, MMP-1 was also found to have collagenolytic activity (Knorr et al., 2009) which is also very interesting as in *P. ramosa*, collagen-like proteins are part of the spore's surface and may be responsible for virulence alterations of different *P. ramosa* strains (Mouton et al., 2009). Altogether, genotype specific high abundance of glycosylated cuticle proteins in the susceptible genotype and of MMPs in the non-susceptible genotype may be responsible for genotype specificity of parasite attachment and therefore for infection in the *D. magna*–*P. ramosa* system. Furthermore, I also directly studied the stress response of the susceptible genotype to parasite infection at the proteome level. Within this dataset, I found very clear evidence for the involvement of one collagen-like protein in the parasite's attachment to the cuticle as this protein had a very high abundance in the cuticle replicates exposed to the parasites and was absent in the control replicates. As mentioned above, this *P. ramosa* protein is thought to be crucial for successful infection of the host. Furthermore, I found interesting indicators of parasite infection. One protein, which was present in all *P. ramosa* exposed replicates is a galactose binding c-type lectin. C-type lectins are known to recognise pathogens and participate in the innate immune response of vertebrates and invertebrates (Robinson et al., 2006). They were found in a lepidopteran (Yu et al., 2002), in the response of *Caenorhabditis elegans* to a bacterial pathogen (O'Rourke et al., 2006) and are known to bind bacteria and take part in the immune response of infected *D. melanogaster* (Tanji et al., 2006). Therefore, the high abundance of a c-type lectin in *Daphnia* exposed to the parasite *P. ramosa* is most probably an indicator for an immune response to the bacterial infection. In addition, there were differences in cuticle protein composition but also in the levels of chitinases, possibly indicating a slowing down of the moulting process by

the parasite to increase the infection success. It was shown that the success of parasite infection was greatly reduced if *Daphnia* moulted within the next 12 h after parasite exposure (Duneau and Ebert, 2012), therefore moulting is an important step interfering with successful parasite infection. A parasite-induced delayed moulting was further supported by a protein having a juvenile-hormone binding domain, which is more abundant in the cuticle of parasite exposed animals, probably indicating increased juvenile hormone concentration in the cuticle. This is especially interesting as in *Daphnia*, juvenile hormones may be involved in the regulation of moulting by modulating ecdysteroid activity (Mu and LeBlanc, 2004).

In contrast to the experiments described above, the analysis of *Daphnia* exposed to simulated microgravity unravelled a stress response which included a set of evolutionary well conserved proteins (chapter 5). Within the data-set of significantly altered proteins, a majority of proteins had a homolog with high blast identity characteristics when searched against the Swiss-Prot database. The Swiss-Prot database is manually annotated and therefore contains reliable data with most of them consisting of annotated proteins originating from a few well-studied model organisms. Evolutionary well-conserved proteins are thought to carry out essential housekeeping roles (Zhong et al., 2012), one example is the group of heat shock proteins which are molecular chaperones maintaining the correct folding of other proteins (Sörensen et al., 2003). Indeed, I found chaperones making up a substantial fraction of proteins involved in the stress response of *Daphnia* to simulated microgravity. Furthermore, my results indicate that simulated microgravity leads to the disruption of actin microfilament organisation in *Daphnia*, which is indicated by abundance alterations of actin, but also of different actin-related proteins. In addition, the exposure to simulated microgravity leads to abundance alterations in proteins associated with various energy pathways and also in haemoglobin, therefore indicating an increased energy demand

in *Daphnia*. These results are in agreement with results obtained from other organisms and microgravity systems, as there are several examples for the involvement of chaperones (Zupanska et al., 2013; Zhang et al., 2013; Taylor et al., 2014), breakdown of the cytoskeleton (Meloni et al., 2011; Vorselen et al., 2014; Zhang et al., 2013; Ulbrich et al., 2011; Grosse et al., 2012; Ferl et al., 2015) and changes in energy demands (Wang et al., 2006; Wilson et al., 2007, 2008; Tan et al., 2011; Crabbé et al., 2011; Herranz et al., 2013; Ferl et al., 2015) in microgravity. Altogether, our data strongly suggest that a lack of gravity affects similar molecular processes in a variety of organisms, indicating a generalised stress response. In *Daphnia*, this pattern is very different from the responses to predation and parasite infection described above, which include very specialised sets of proteins specific for the arthropod group or even only present in the *Daphnia* lineage. These different sets of proteins involved in the responses very good reflect the grade of adaptation *Daphnia* accomplished to the different environmental changes.

6.4 Conclusion and future directions

With the work of my PhD project, which was presented in this thesis, I was able to enhance the knowledge on the response of *Daphnia* to different stressors, highlighting key players of involved processes. My work shows suitable proteomic approaches for *Daphnia* even in a high-throughput approach and gives examples of how to perform bioinformatic analysis of data in a organism not well-established in proteomics. Furthermore, I was able to detect proteins involved in molecular mechanisms underlying well-studied traits in *Daphnia* like the response to predation or to the parasite *P. ramosa*. Here, especially traits connected to *Daphnia* carapace and cuticle were clearly reflected on the protein level. Furthermore, also elements of general stress responses similar to responses in other organisms could be found in the response to

microgravity. The results of my work offer most interesting starting points to study the molecular mechanisms underlying stress responses of *Daphnia* in more detail for example using targeted approaches and the growing *Daphnia* molecular toolbox, enabling e.g. absolute quantification of key molecules, gain- and loss-of-function approaches or immunohistochemical analysis.

Supplementary information

Mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository (Vizcaíno et al., 2014) with the dataset identifier PXD002974 and can be accessed using the following account details:

Username: reviewer96155@ebi.ac.uk Password: w3WWoxnG

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Author Contributions

Otte KA, Fröhlich T, Arnold GJ, and Laforsch C (2014). Proteomic analysis of *Daphnia magna* hints at molecular pathways involved in defensive plastic responses. *BMC genomics* 15(306).

Kathrin Otte designed research, conducted proteomic experiments, performed mass spectrometry analysis and conducted bioinformatic analysis of the data. Christian Laforsch, Thomas Fröhlich and Georg Arnold designed the study. Christian Laforsch supervised research. Christian Laforsch conducted the induction experiment and provided samples for proteomic analysis. Thomas Fröhlich supervised mass spectrometry analysis. Kathrin Otte wrote the first draft of the manuscript and Christian Laforsch, Thomas Fröhlich and Georg Arnold contributed substantially to revisions.

Otte KA, Schrank I, Fröhlich T, Arnold GJ, and Laforsch C (2015). Interclonal proteomic responses to predator exposure in *Daphnia magna* may depend on predator composition of habitats. *Molecular Ecology*, 24, 3901-3917.

Kathrin Otte designed research, conducted proteomic experiments, performed mass spectrometry analysis and conducted bioinformatical analysis of the data. Christian Laforsch designed and supervised research. Kathrin Otte and Isabella Schrank conducted predator exposure experiments. Thomas Fröhlich supervised mass spectro-

metry analysis. Kathrin Otte wrote the first draft of the manuscript, and Christian Laforsch, Thomas Fröhlich, Isabella Schrank and Georg Arnold contributed substantially to revisions.

Analysis of genotype-genotype interactions of the parasite *Pasteuria ramosa* and its host *Daphnia magna* at the protein level, Otte, K. A., Fröhlich, T., Arnold, G. J., Andras, J., Bento, G., Ebert, D., and Laforsch, C., unpublished manuscript.

Kathrin Otte designed research, conducted proteomic experiments, performed mass spectrometry analysis and conducted bioinformatical analysis of the data. Jason Andras and Gilberto Bento performed *P. ramosa* infection experiments and exuvia sampling. Christian Laforsch and Dieter Ebert designed and supervised research. Kathrin Otte wrote the first draft of the manuscript, Christian Laforsch, Jason Andras and Dieter Ebert contributed substantially to revisions.

Trotter B, Otte KA, Schoppmann K, Hemmersbach R, Fröhlich T, Arnold GJ, and Laforsch C (2015). The influence of simulated microgravity on the proteome of *Daphnia magna*. *npj Microgravity*, 1, 15016.

Christian Laforsch, Ruth Hemmersbach, and Kathrin Schoppmann designed the study. Christian Laforsch supervised research. Benjamin Trotter performed clinorotation and proteomic experiments. Benjamin Trotter and Kathrin Otte analysed proteomic data. Kathrin Otte conducted further bioinformatical analysis. Thomas Fröhlich supervised mass spectrometry analysis. Kathrin Otte and Benjamin Trotter wrote the first draft of the manuscript, and Christian Laforsch, Thomas Fröhlich, Ruth Hem-

mersbach, Kathrin Schoppmann and Georg Arnold contributed substantially to revisions.

Christian Laforsch

Kathrin Otte

Acknowledgements

I would like to thank my PhD supervisor Christian Laforsch and my proteomics supervisor Georg Arnold for giving me the opportunity to be part of this special project and opening the *Daphnia* and the proteomics world to me. Furthermore, I would like to thank them for their support and advice and for giving me a lot of freedom. I also thank EUROCORE and DFG for the funding of the project.

Another very big thank you goes to all my colleagues in the Laforsch Animal Ecology Group first in Munich and afterwards in Bayreuth and in the Arnold proteomics group in Munich: Quirin Herzog, Robert Sigl, Hannes Imhof, Wolfgang Engelbrecht, Olivia Hesse, Bernard Wolfschoon Ribeiro, Sandy Weidlich, Kathrin Schoppmann, Mechthild Kredler, Elena Ossipova, Marjiana Belic, Dorothea Wiesner, Marion Preiß, Ursula Wilczek, and Miwako Kösters.

Special thanks go to Thomas Fröhlich for bringing me close to the mass spectrometers, to Isabella Schrank for her big and profound help with *Daphnia* experiments and to Benjamin Trotter for being brave enough to do his Master thesis in *Daphnia* proteomics and to Jessica Fischer for lively scientific discussions. Furthermore, I would like to thank my awesome office mates Daniela Deustch, Katrin Meyer and my long-term ally Florian Flenkenthaler for the good time. I am also very grateful to my parents and would like to thank them for their generous support throughout my life. Last but not least, I want to thank my husband Matthias Kreuschner for his interest in my work and for his understanding and support in good times as in bad.

Eidesstattliche Erklärung

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