Molecular mechanisms of adaptive life-history changes in *Daphnia magna* induced by predator kairomones

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Für *Willi Effertz*

(04.07.1954 – 15.03.2013)

“Let's drink to the hard working people

Let's drink to the salt of the earth

Let's drink to the two thousand million

Let's think of the humble of birth”

Jagger & Richards

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General introduction and aim of the study

Planktonic crustaceans of the genus *Daphnia* (order: *Cladocera*) play an important role as keystone species in almost all standing freshwater ecosystems. As unselective filter feeders, *Daphnia* are typically the major herbivorous grazers of phytoplankton and are a major prey for predators at higher trophic levels. Due to their important position in freshwater food webs, many studies of the past decades focussed on elucidating the ecological background of *Daphnia* (Ebert 2005). Further, due to their inherent phenotypic plasticity as a response to various organisms ranging from cyanobacterial phytoplankton to fish predators, *Daphnia* have become an important model organism for the understanding of links between an organism and its environment (Lampert 2006). In a fluctuating environment, an individual´s probability of survival depends on its ability to cope with environmental changes and phenotypic plasticity describes the ability of an individual (genotype) to express different phenotypes in response to these environmental changes. If the modified phenotype exhibits a higher fitness than the unmodified one, then the phenotypic plasticity is adaptive (Stearns 1992). One potent agent of natural selection, which affects fitness und abundance of organisms in biological communities, is predation. The introduction of predators has been shown to cause rapid evolution of defensive traits in prey (Seeley 1986; Magurran et al. 1992; Gliwicz 1986; Cousyn et al. 2001). Although it was shown that predation pressure in freshwater systems is fluctuating over the season in predator type and intensity (Brooks and Dodson 1965; Ringelberg et al. 1991; Weider and Pijanowska 1993), it has led to a variety of adaptive phenotypic defences in aquatic prey organisms (Dodson 1989; Harvell 1990) such as *Daphnia*.

Daphnia are important prey organisms for both invertebrate predators and vertebrate predators like planktivorous fish, and show a wide range of defences against these predation threats. These anti-predator defences include changes in life-history (LHC), behavioural responses and changes in morphology (Lampert 1994), which were shown to be inducible by chemical cues released by predators (Larsson and Dodson 1993). In particular, these, so-called kairomones released by fish affect the resource allocation in *Daphnia* leading to LHC, e.g. reduced size at first reproduction (SFR, Weider and Pijanowska 1993). A reduced body size of *Daphnia* decreases the

predation pressure by visually hunting fish (Brooks and Dodson 1965). Another defence against fish predation in *Daphnia* is the diel vertical migration (DVM) that has been shown to be enhanced by fish kairomones (Loose 1993). DVM is a widespread adaptive migration behavior in *Daphnia* that results in a reduction of the encounter probability with visually hunting fish: daphnids select the refuge of the deep and dark hypolimnion of stratified lakes at daytime and migrate up into the epilimnion at night (Stich and Lampert 1981; Lampert 1989). The induction of this DVM behavior depends on a relative change in light intensity (Ringelberg 1991; van Gool and Ringelberg 1995).

The inducible character of anti-predator defences like LHC and DVM suggests that anti-predator traits are associated with substantial costs. The inducible character of these defences allows the daphnids to reap the benefits of defence while avoiding potential costs associated with investment in the defensive strategies when they are not needed. These phenotypic benefits and costs have been demonstrated for LHC and DVM. The adaptive reduction of size at first reproduction during LHC goes along with a lower quality of eggs (Stibor and Navarra 2000) and a smaller body size was shown to play a role in a lower competition for food in *Daphnia* (Gliwicz 1990). The daytime residence of daphnids in cold water layers during DVM results in demographic costs by decreasing growth and development (Dawidowicz and Loose 1992; Loose and Dawidowicz 1994).

A comparison of different *D. magna* clones exposed to fish kairomones revealed that all tested genotypes had the potential to respond with more than one plastic trait (Boersma *et al.* 1998), but *de Meester & Pijanowska* (De Meester and Pijanowska 1996) argued that the exhibition all available defences at the same time may not be adaptive, as selecting one or the most efficient defences against a predator. Although it might be possible that all available defences at a maximum extent could provide the highest protection for an individual, they might be too costly. Under varying predation pressure and to avoid unnecessary costs the accurate adjustment of anti-predator defences to a current predation risk (indicated and induced by the predator kairomones) seems to be crucial for prey organisms. Between *Daphnia* populations

variability was found with respect to the extent of LHC and DVM in response to fish kairomones was found (De Meester *et al.* 1995), which suggests coexistence of clones that exhibit one or the other defence. *Vos et al.* showed in a theoretical model that DVM is the preferred defence under high predation risk by fish compared to a reduction in body size (Vos *et al.* 2002), whereas a reduced body size might be more effective under low predation pressure (Fiksen 1997). However, further support for a coadaptation of both defences in *Daphnia* comes from a study in which lakes were compared. In habitats in which a *Daphnia* population is prevented from migration in deeper strata due to lake morphometry or anoxia in the hypolimnion, daphnids underwent LHC, resulting in a shift to an earlier reproduction at smaller size (Sakwinska and Dawidowicz 2005). These findings might hint to a coupling of LHC and DVM in *Daphnia*. Further evidence for a plastic coupling of LHC and DVM comes from a population of *D. catawba*, which performed DVM when exposed to fish kairomones; however, when a net barrier prevented migration into deeper strata, a decrease in SFR was observed (Slusarczyk and Pinel-Alloul 2010). Taken together, these findings suggest that DVM and LHC are not strictly coupled; *Daphnia* appear to be plastic with respect to the degree of response with either defence and it is still an open question, which additional factor besides the fish kairomones might control a possible plastic coupling of anti-predator defences (DVM and LHC) in *Daphnia*.

In **chapter 1**, I examined the role of light for the induction of life-history changes (LHC) in *Daphnia* exposed to fish kairomones. I conducted life-history experiments in the absence and presence of fish kairomones extracts exposed to different light regimes under standardized laboratory conditions. I further explored the relevance of light for kairomone-induced effects on the gene expression of known target genes for fish kairomones in *Daphnia*. Despite the well-known relevance of light for the induction of DVM, investigations of the induction of LHC have been confined to predator kairomones only (Weider and Pijanowska 1993; Stibor and Lüning 1994; Von Elert and Stibor 2006). I hypothesized that the light level a given *Daphnia* genotype is exposed to during a large part of the day, determines the magnitude of the LHC. Accordingly, low light levels should lead to no or very little LHC, and high light levels would lead to stronger LHC in response to kairomones from fish.

Chapter 1 describes for the first time how a well-known anti-fish defence in *Daphnia* is controlled by light and provides a mechanism that allows for a plastic coupling of multiple defences (i.e. LHC and DVM) in a fluctuating environment.

In **chapter 1**, the experimental animals were exposed to absence and presence of light only to observe the putatively strongest effects, whereas in the field animals are subjected to alternating periods of light and dark and different light intensities. **Chapter 2** examines the question whether LHC in *Daphnia* are controlled by different light intensities and if a light intensity threshold for the fish kairomone induced LHC exists. Therefore, I performed life-history experiments in the absence and presence of fish kairomone extracts and the experimental animals were exposed to five different light intensities, which were supposed to be comparable with light intensities in the field (Tilzer *et al.* 1995). While **chapter 1** aims to reveal the mechanism of LHC controlled by light intensity in *Daphnia*, **chapter 2** continues the research with respect to the specific role of light intensity in the coupling of anti-predator defences under more natural conditions.

Although the *Daphnia* genome and the molecular tools are available (Colbourne *et al.* 2011), there is not much known about the molecular mechanisms, that may underlie the anti-predator defences induced by fish kairomones in *Daphnia*. In **chapter 1** I conducted a candidate gene approach for the fish kairomones effects on the gene expression of *Daphnia* in the absence and presence of light, with genes (*actin*, *cyclophilin* and *HSPs*) that were recently shown to play a role in the response of *Daphnia* exposed to fish kairomones (Schwarzenberger et al. 2009; Pijanowska and Kloc 2004; Pauwels et al. 2007). I further explored the analyses on the gene level in *Daphnia* exposed to fish kairomones under five different light intensities in **chapter 2**. It is hypothesized that, compared to results of the life-history experiments, the expression level of candidate genes for fish kairomones will be affected by light intensities in the same way as the LHC.

I continued the research on the underlying molecular mechanisms of LHC in *Daphnia* via proteomics approach described in **chapter 3**. The actual phenotype of an organism is reflected in its proteome and it is known that the mRNA level does not

necessarily serve as an accurate predictor for the respective protein abundance (Anderson and Seilhamer 1997; Gygi et al. 1999; Nie et al. 2006). In contrast to the candidate gene approach conducted in **chapter 1**, a holistic proteomics approach might elucidate the role of unpredicted functional proteins that could explain phenotypic plasticity in *Daphnia*. It had previously been demonstrated that LC-MS/MS based proteome profiling in *Daphnia* using high throughput proteomics based upon the *Daphnia* genome database is feasible (Froehlich *et al.* 2009). I performed life-history experiments in the presence and absence of highly purified fish kairomone extract under different light conditions and only animals that showed the expected anti-fish response were used for the proteome analysis. **Chapter 3** describes for the first time the application of differential peptide labelling (iTRAQ) in LC-MS/MS based proteomics in *Daphnia* with the aim to identify kairomone-responsive proteins in *Daphnia*, which might explain the fish-kairomone mediated phenotypic changes.

In summary, this study presents novel and deep insights into the mechanisms underlying a well-known inducible anti-predator response in *Daphnia*. Many studies described defences like DVM or LHC separately and only a few studies were conducted that indicate a possible coupling of these defensive strategies. The present study elucidates for the first time the role of the environmental factor light as a mediator between migration behaviour and changes in life-history in *Daphnia* exposed to fish kairomones. Such a light-mediated coupling allows for a plastic adjustment to fluctuating environments and simultaneously minimizes the associated costs of multiple alternately deployable defences. Furthermore, the present study considers the effects of fish kairomones and different light intensities on the molecular level of *Daphnia* to gain knowledge of kairomones-responsive genes, functional proteins and metabolic pathways, which yield a mechanistic understanding of the observed phenotypic changes.

Chapter 1

Light intensity controls anti-predator defences in *Daphnia* - the suppression of life-history changes

Abstract

A huge variety of organisms respond to the presence of predators with inducible defences, each of which is associated with costs. Many genotypes have the potential to respond with more than one defence, and it has been argued that it would be maladaptive to exhibit all possible responses at the same time. Here we test how a well-known anti-fish defence in *Daphnia*, the changes in life-history (LHC) is controlled by light. We show that the kairomone-mediated reduction in size at first reproduction (SFR) is inversely coupled to the light intensity. A similar effect was found for the kairomone-mediated expression of candidate genes in *Daphnia*. We argue that the light intensity an individual is exposed to determines the degree of LHC, which allows for plastic adjustment to fluctuating environments and simultaneously minimizes the associated costs of multiple alternately deployable defences. It is hypothesized that this allows for a coupling of multiple defences, i.e. LHC and diel vertical migration (DVM).

Chapter 1

Introduction

In a fluctuating environment, an individual's probability of survival depends on its ability to cope with environmental changes; a high degree of phenotypic plasticity enhances the fitness of an individual in such a fluctuating environment. If the modified phenotype exhibits a higher fitness than the unmodified one, phenotypic plasticity is adaptive (Stearns 1992). Predators are potent agents of natural selection in biological communities. The introduction of predators has been shown to cause rapid evolution of defensive behaviours in prey (Seeley 1986; Magurran et al. 1992; Gliwicz 1986; Cousyn et al. 2001). Although predation in freshwater systems is fluctuating seasonally in intensity and manner (Brooks and Dodson 1965; Ringelberg et al. 1991; Weider and Pijanowska 1993), it has led to a variety of adaptive phenotypic defences in aquatic prey organisms (Harvell 1990; Dodson 1989). In particular *Daphnia* - an important prey organism for planktivorous fish in standing freshwaters - shows a wide range of antipredator defences, e.g. changes in life-history, morphology and behavior (Lampert 1994). These defences have been shown to be inducible by chemical cues released by fish, so-called kairomones (Larsson and Dodson 1993). Fish-produced kairomones (hereafter referred to as 'fish kairomones') affect the resource allocation in *Daphnia*, which leads to changes in their life-history (LHC), e.g. a reduced body size at first reproduction (SFR) (Weider and Pijanowska 1993). A smaller body size increases the chance of survival due the selection for large prey by visually hunting predators such as fish (Brooks and Dodson 1965). Furthermore, fish kairomones enhance diel vertical migration (DVM) in *Daphnia (Loose 1993a)*, a widespread adaptive migration behavior, which reduces the encounter with visually hunting fish: due to DVM, *Daphnia* reside in the deep, dark hypolimnion of stratified lakes during the day and spend the night in the epilimnion (Stich and Lampert 1981; Lampert 1989). The induction of DVM depends on a relative change in light intensity (Ringelberg 1991; van Gool and Ringelberg 1995).

Inducible defences allow prey to reap the benefits of defence while avoiding potential costs associated with investment in the defensive strategy when it is not needed. Such phenotypic benefits and costs have been demonstrated for inducible life-history

and behavioural defences in *Daphnia*: The inducible character of LHC and DVM allows *Daphnia* to adjust the defence strategy only when its required saving substantial costs. LHC lead to an adaptive reduction in SFR (Stibor and Lüning 1994), which goes along with a lower quality of the eggs (Stibor and Navarra 2000), and DVM results in demographic costs due to the daytime residence in the deep and cold water layers of stratified lakes (Loose and Dawidowicz 1994; Dawidowicz and Loose 1992). The finding that the amplitude of DVM increased by chemically mediated predator density (Loose and Dawidowicz 1994; Von Elert and Pohnert 2000) indicates that the extent of the defence is the result of integrating costs and benefits of inducible defences.

A comparison of different clones of *D. magna* revealed that all genotypes had the potential to respond with more than one plastic trait to the presence of fish kairomones (Boersma et al. 1998). However, exhibiting multiple defences at the same time may not be adaptive if a single defence is sufficient for protection against fish (De Meester and Pijanowska 1996). De Meester *et al.* (1995) found variability within populations with respect to the extent LHC and DVM by fish kairomones, which suggests the coexistence of clones that exhibit the one or the other defence.

Further support for a coadaptation between size-related life-history traits and DVM comes when different lakes are compared. Under high risk of predation by visually hunting predators, DVM seems to be preferred as a defence over the reduction of SFR (Vos et al. 2002). In habitats in which DVM was not possible due to lake morphometry or hypolimnetic anoxia which prevent migration into the deeper strata, daphnids underwent LHC resulting in an earlier reproduction at a smaller size (Sakwinska and Dawidowicz 2005). Despite this strong evidence for a coadaptation of DVM and size-related LHC, it remained to be tested whether the degree of performing DVM and LHC as defence against fish was fixed for a given genotype, or if genotypes would be plastic with respect to the coupling of these anti-predator responses.

Evidence for a plastic coupling of size-related life-history responses and DVM comes from a population of *D. catawba* which performed DVM when exposed to fish kairomones; however, when a net barrier prevented migration into deeper strata, a decrease in SFR was observed (Slusarczyk and Pinel-Alloul 2010). These findings demonstrated that DVM and LHC were not strictly coupled; instead, the animals were plastic with respect to the degree of responding with either defence.

Here we have investigated how LHC are suppressed under low light levels. We hypothesize that the light level a given *Daphnia* genotype is exposed to during a large part of the day determines the degree of LHC. Accordingly low light levels would lead to no or very low LHC, and high light levels would lead to stronger LHC in response to kairomones from fish. We therefore performed life-history experiments in the presence and absence of fish kairomone extracts under different light conditions and used the size at first reproduction as parameter for the LHC. We further explored the relevance of light for kairomone-induced changes in *Daphnia* by analyzing effects on gene expression of *actin*, *cyclophilin* and *HSPs*, which are all known to play a role in the response of *Daphnia* to fish (Pauwels et al. 2005; Pijanowska and Kloc 2004; Schwarzenberger et al. 2009).

Material & Methods

Test species and cultures

Daphnia magna clone B from Lake Binnensee, Germany (Lampert and Rothhaupt 1991) was cultured at 20 °C in aged, membrane-filtered (pore size: 0.45 µm) tap water under dim light. It has been shown earlier, that *D. magna* clone B responds to fish kairomones with LHC (Weider and Pijanowska 1993) and with DVM behaviour in indoor experiments (Bentkowski et al. 2010; Loose and Dawidowicz 1994) similar to more pelagic *Daphnia* species (Loose 1993b). Twelve animals per liter were kept under non-limiting food concentrations: 2 mg C_{part} L^{-1} of the green algae *Chlamydomonas klinobasis*, strain 56, culture collection of the Limnological Institute at the University of Konstanz. *C. klinobasis* was grown in 5 L semi-continuous batch cultures (20 °C; illumination: 120 µmol m^{-2} s^{-1}) by replacing 20 % of the culture with fresh, sterile Cyano medium (Von Elert and Jüttner 1997) every other day. The test animals originated from mothers which had been raised under control conditions (saturating concentrations of *C.* sp.) for at least five generations.

Experimental set-up

Fish kairomone extract

Three *Perca fluviatilis* (body size: 10-12 cm) were pre-conditioned for 24 h without food and then kept for 24 h in 8 L of aged tap water at 18 °C without feeding. The water containing the fish kairomones was filtered through membrane filters (pore size: 0.45 µm). For bulk enrichment of the kairomones, a C_{18} solid-phase cartridge (10 g of sorbent, volume 60 mL, endcapped, Varian Mega Bond Elut, Agilent Technologies) was preconditioned with 50 mL methanol and 50 mL ultrapure water prior to adding the sample. Methanol was added to the filtered incubation water containing the fish kairomones to obtain a 1 % concentration, and 2 L of sample were passed through the cartridge. The loaded cartridge was washed with 50 mL of ultrapure water and then eluted with 50 mL of methanol. The eluates originating from 10 L fish incubation water were pooled and evaporated to dryness using a rotary evaporator, re-dissolved in 1 mL of absolute ethanol and tested for biological activity. Water without fish was used for the production of a control extract. The same

standardized extracts of control water and fish incubation water in the concentration equivalent of three fish in 8 L of water were used for all experiments.

Life-history experiments

Test animals originated from the 3rd clutch and had been released within 12 h. The neonates were maintained at 24 ind. L^{-1} and were transferred daily to new water supplemented with algal food (2 mg C L^{-1}) and kept under permanent dim light at 20 °C until day three, at which time the cohort was divided and kept either under dim light (0.48 µmol s^{-1} m^{-2}) or dark (<0.1 µmol s^{-1} m^{-2}) conditions. On the fifth day, five animals were exposed to either control water extract or fish water extract in 250 mL in the presence (0.48 µmol s^{-1} m^{-2}) and absence (<0.1 µmol s^{-1} m^{-2}) of light without a photoperiod until the first clutch was visible. The preconditioned animals from the dim light cohort were used for the light treatment and the animals from the dark cohort were used for the dark treatment in the experiment. All treatments were run in triplicate, and *D. magna* were fed daily. The size at first reproduction (SFR) was measured for each egg-bearing individual (from the top of the eye to the base of the tailspine) with the aid of a dissecting microscope equipped with a digital camera (Imaging Source) and image analysis software. A mean SFR was calculated for each replicate; these mean values were used to calculate the respective mean value and the variance for the treatment. Somatic growth rates were calculated as according to Wacker & Von Elert (2001) (Wacker and Von Elert 2001):

$$g = [(\ln (dw_t) - \ln (dw_0)]/d,$$

in which *dw* is the body dry weight of a subsample of the animals at the beginning (dw_0) and end (dw_t) of the experiment and *d* is length of the experiment in days. Mean individual dry weights were mean values of three individuals (dw_0 is the same for all treatments, and dw_t is specific for each replicate).

RNA extraction and reverse transcription

The test animals were kept as described above. On day five, ten animals were transferred into 200 mL containing either fish water or control water extract in the presence or absence of light. After 1h, 2h and 4 h, subsamples of three animals were used for RNA extraction. The experiment was run in triplicate. RNA was extracted

and purified using the NucleoSpin® RNA II Kit (Macherey & Nagel). The integrity of each RNA sample was verified using the 2200 Tape Station (Agilent Technologies) on a High Sensitivity Screen Tape®, and the RIN values ranged from 7.5 to 8.5. The RNA concentration was determined using the Nanodrop ND-1000 v3.7 (Thermo Scientific). 1 µg of RNA was reverse-transcribed using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). The cDNA was stored at -20 °C.

Quantitative PCR (qPCR)

28S ribosomal RNA (*28S*), *alpha-tubulin* (*α-tubulin*), *glyceraldehyde-3-phosphate dehydrogenase* (*GAPDH*) and *TATA-box binding protein* (*TBP*) were used as endogenous control genes of constitutive expression in *D. magna* (Schwarzenberger et al. 2009; Heckmann et al. 2006). *Cyclophilin* was used as a candidate gene in our qPCR analysis. To discover the other candidate genes, the *D. magna* sequences for *actin* (Accession no. AJ292554) and HSPs (Accession no. EU514494, DQ845268) were used as queries for sequence similarity searches using BLASTn against the *D. magna* assembly v2.4 in wFleaBase. All gene sequences with a significant hit were aligned with Geneious® v6.0.3 (Biomatters Ltd.). Primers were designed with Primer 3 v2.3.5 and the quality checked with NetPrimer (PREMIER Biosoft) (Tab 1). Melting curve analyses confirmed specific amplification without primer dimer formation. The data acquisition for the relative expression was performed on a 7300 qPCR system (Applied Biosystems). Each reaction contained 5 ng of cDNA template, 10 µL SYBR® Green PCR master mix and 2.5 µM of each primer in a final volume of 20 µL. Cycling parameters were 95 °C for 10 min for the initial start of the DNA polymerase, followed by 40 cycles of 95 °C for 15 s, 55 °C for 30 s, 68 °C for 30 s and a final dissociation step with 95 °C for 15 s, 55 °C for 30 s, 68 °C for 30 s and 95°C for 15 s. The baseline and threshold for the Ct (Cycle threshold) was set automatically, and the each gene was tested in triplicate. Amplification efficiencies for every primer pair of each candidate gene were determined.

Table 1. Gene IDs and primers for the qPCR analysis

Gene ID	wFleaBase *D. magna* assembly v2.4	GenBank Accession no.	Forward primer (5´-3´)	Reverse primer (5´-3´)
Actin 1	WFes0011739	**AJ292554**	CCTCCTCTCCCCCTTTCATA	GATGTGGATCTCCAAACAGGA
Actin 2	WFes0005512		GAATTCATGTCACTTCCAAGTCC	TTAATTGGCCGTTCTCTTGA
Actin 3	WFes0009949		GGGAGCTTCAGTCAGGAGAA	CCCAGTCCAAACGTGGTATT
Actin 4	WFes0003283		CGATCCATACGGAGTACTTGC	AGGATCTGTACGCCAACACC
Cyclophilin	WFes0012034		GACTTTCCACCAGTGCCATT	AACTTTCCATCGCATCATCC
HSP 10	WFes0002064		CATGGTTGTTGCAGTTGGAC	AGTGCCACCATACTCAGGAA
HSP 70	WFes0008791	**EU514494**	AAGATGAAGGAGACGGCTGA	CTGCATCCTTTGTTGCTTGA
HSP 90	WFes0106938	**DQ845268**	CAAGGCTGATTTGGTCAATAAC	GCAACCAGGTAAGCCGAGTA
Alpha-tubulin	WFes0007807		TGGAGGTGGTGACGACT	CCAAGTCGACAAAGACAGCA
GADPH		**AJ292555**	GGCAAGCTAGTTGTCAATGG	TATTCAGCTCCAGCAGTTCC
TBP	WFes0002485		GCAGGGAAGTTTAGTTTCTGGA	TGGTATGCACAGGAGCAAAG
28S		**AF532883**	GAGGCGCAATGAAAGTGAAG	TGTTCGAGACGGGATCA

Data analysis and statistics

After qPCR, raw data were analyzed with qBasePLUS v2.0 (Biogazelle) based on qBase (Hellemans et al. 2007) and geNorm (Vandesompele et al. 2002). The relative gene expression of candidate genes was normalized with the generated normalization factor, and standard errors were calculated. A repeated-measurement analysis of variances (RM-ANOVA) for the qPCR data of each candidate gene generated with the qBasePLUS software was conducted to analyze the effects of the factors “treatment” and “time” on the mean relative expression (Tab. 2). The dependent variable was checked for homogeneity of variances (Levene test). A single analysis of variance (one-way ANOVA) was carried out for the qPCR data generated with the qBasePLUS software of each gene after 2 h of exposure to extract of fish incubation water in the absence and presence of light. The dependent variable was checked for homogeneity of variances (Shapiro-Wilk). A two-way analysis of variance (Two-way ANOVA) was conducted for the life-history experiments. The dependent variables were checked for homogeneity of variances (Shapiro-Wilk). The effect of single treatments was tested by post hoc tests (Tukey’s HSD multiple comparison test) at the same probability level as the respective analysis of variance. A significance level of $p = 0.05$ was applied to all statistical analyses. All statistics were performed with SigmaPlot v11.0 (Systat Software) and STATISTICA v6.0 (Starsoft Inc.).

Results

Life-history and growth experiments

In full factorial life-history experiments, *D. magna* was grown in the presence of control water extract or extract of fish incubation water (*Perca fluviatilis*) in the presence or absence of light. Size at first reproduction (SFR) decreased from 2.59 mm to 2.48 mm in the presence of fish kairomones and light (Fig. 1A), but did not in the control extract. This kairomone-mediated reduction in SFR was not observed in the absence of light (Fig. 1A). SFR was affected by the factors “light” (two-way ANOVA, factor light: $F_{1,11}$: 5.805, $p < 0.05$) and “fish water extract” (two-way ANOVA, factor extract: $F_{1,11}$: 8.883, $p < 0.05$), and both factors together (two-way ANOVA: $F_{1,11}$: 6.087, $p < 0.05$). Juvenile somatic growth rates of *D. magna* were neither affected by light (two-way ANOVA, factor light: $F_{1,11}$: 0.956, $p = 0.357$; Fig 1B) nor by fish water extract (two-way ANOVA, factor extract: $F_{1,11}$: 0.376, $p = 0.557$; Fig 1B). The daphnids were exposed either to control water extract or fish incubation water extract on day five of the experiment, and one molt per individual was observed in all treatments prior to deposition of eggs to the brood pouch.

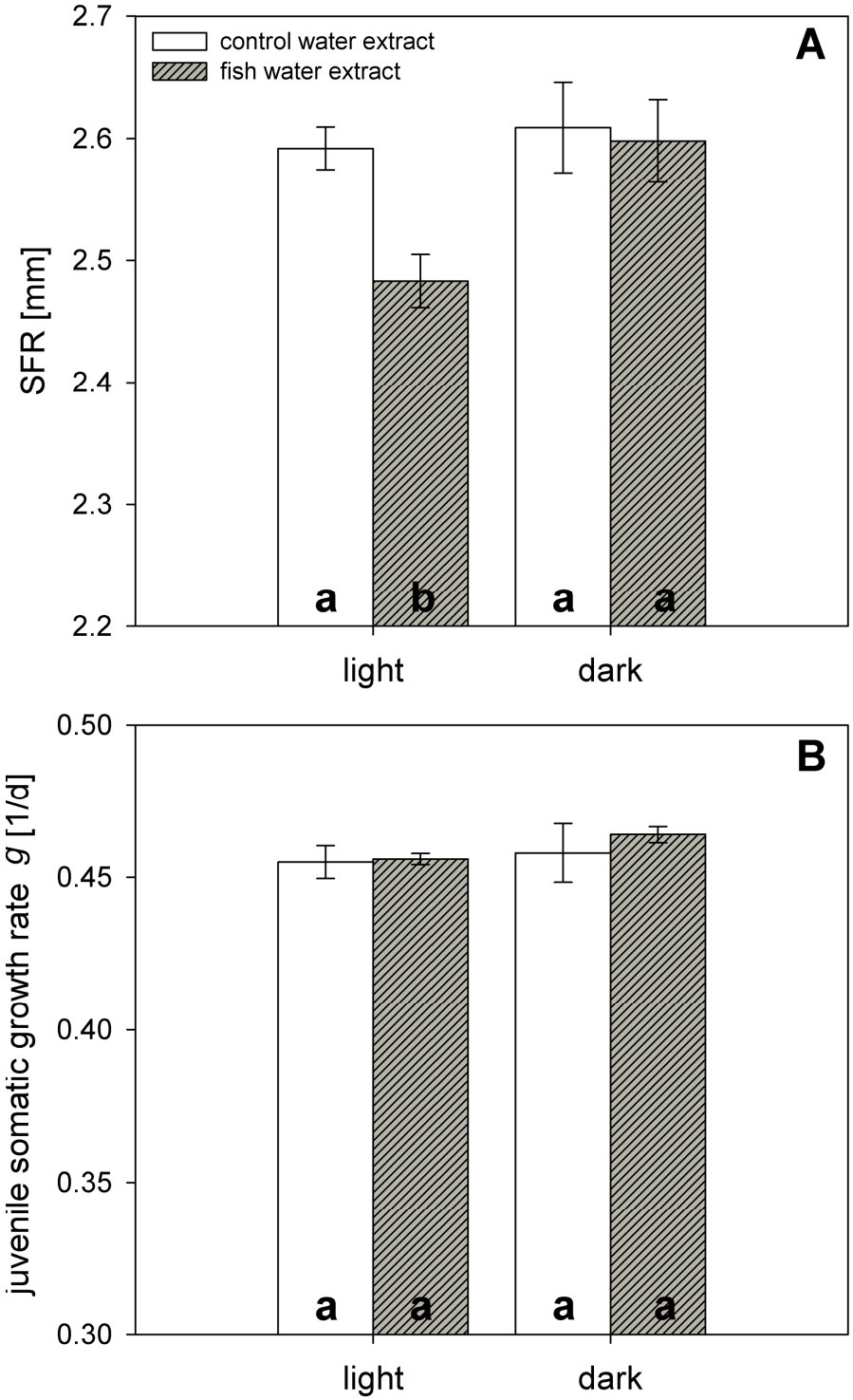

Figure 1. Size at first reproduction (SFR) and juvenile somatic growth rate *g* of *Daphnia magna* grown in the presence or absence of fish kairomones. *Daphnia* were exposed to control water extract (white bars) or to fish incubation water extract (shaded bars) in light or dark. A: size at first reproduction (SFR) (n= 3, mean ± SE), B: juvenile somatic growth rate *g* (n = 3, mean ± SE). Different letters denote a significant difference ($p < 0.05$) between the treatments.

Relative expression of candidate genes

The relative expression of the candidate genes *actin* 1-4 and *cyclophilin* in five-day-old *D. magna* was monitored after 1, 2 and 4 hours after exposure to control water extract or extract of fish incubation water. The genes *28S*, *TBP*, *α-tubulin* and *GAPDH* served as endogenous control genes in all qPCR analyses and were used for calculation of the normalization factor. The qPCR analysis revealed no significant difference in expression levels of candidate genes after one hour of exposure to fish water extract compared to control water extract. After two hours of exposure, a significant increase in expression in response to kairomones was found for the genes *actin 3* (RM ANOVA: $F_{1,6} = 25.450$, $p < 0.01$; Fig 2) and *cyclophilin* (RM ANOVA: $F_{1,6} = 19.908$, $p < 0.05$; Fig 2). The gene *actin 1* was not tested, because the assumption of variance homogeneity was not fulfilled. After four hours of exposure to fish incubation water extract, a significant decrease in expression for *cyclophilin* compared to the control water extract was observed (RM ANOVA: $F_{1,6} = 19.908$, $p < 0.05$; Fig 2). Expression of the HSPs genes (*HSP 10*, *HSP 70*, *HSP 90*) which served as proxy genes for a more general stress response (Sorensen et al. 2003) was not differently affected by exposure to extract of fish incubation water or control water after 1 h, 2 h or 4 h (data not shown). The results of this time series indicated that effects of fish kairomones on gene expression were most likely to be observed after 2 h of exposure.

Table 2. Results of repeated-measurement analyses of variances (RM-ANOVA) of the mean relative candidate gene expression in *D. magna*. All analyses compared the treatment (exposure of animals either to extract of control water or to fish incubation water) to a time series (1h, 2h and 4h). Asterisks indicate significant differences (* = $p < 0.05$; ** = $p < 0.01$).

	SS	df	F	p	
Actin 2					
Treatment	3.484	1	5.729	0.096	n.s.
Error	1.825	3			
Time	11.946	2	7.730	0.022	*
Time x treatment	2.866	2	1.854	0.236	n.s.
Error	4.636	6			
Actin 3					
Treatment	0.461	1	25.450	0.015	*
Error	0.054	3			
Time	0.999	2	15.839	0.004	**
Time x treatment	0.951	2	15.079	0.005	**
Error	0.189	6			
Actin 4					
Treatment	0.006	1	0.334	0.604	n.s.
Error	0.056	3			
Time	0.080	2	1.740	0.254	n.s.
Time x treatment	0.077	2	1.675	0.264	n.s.
Error	0.139	6			
Cyclophilin					
Treatment	0.338	1	19.908	0.021	*
Error	0.051	3			
Time	1.435	2	17.352	0.003	**
Time x treatment	1.451	2	17.544	0.003	**
Error	0.248	6			

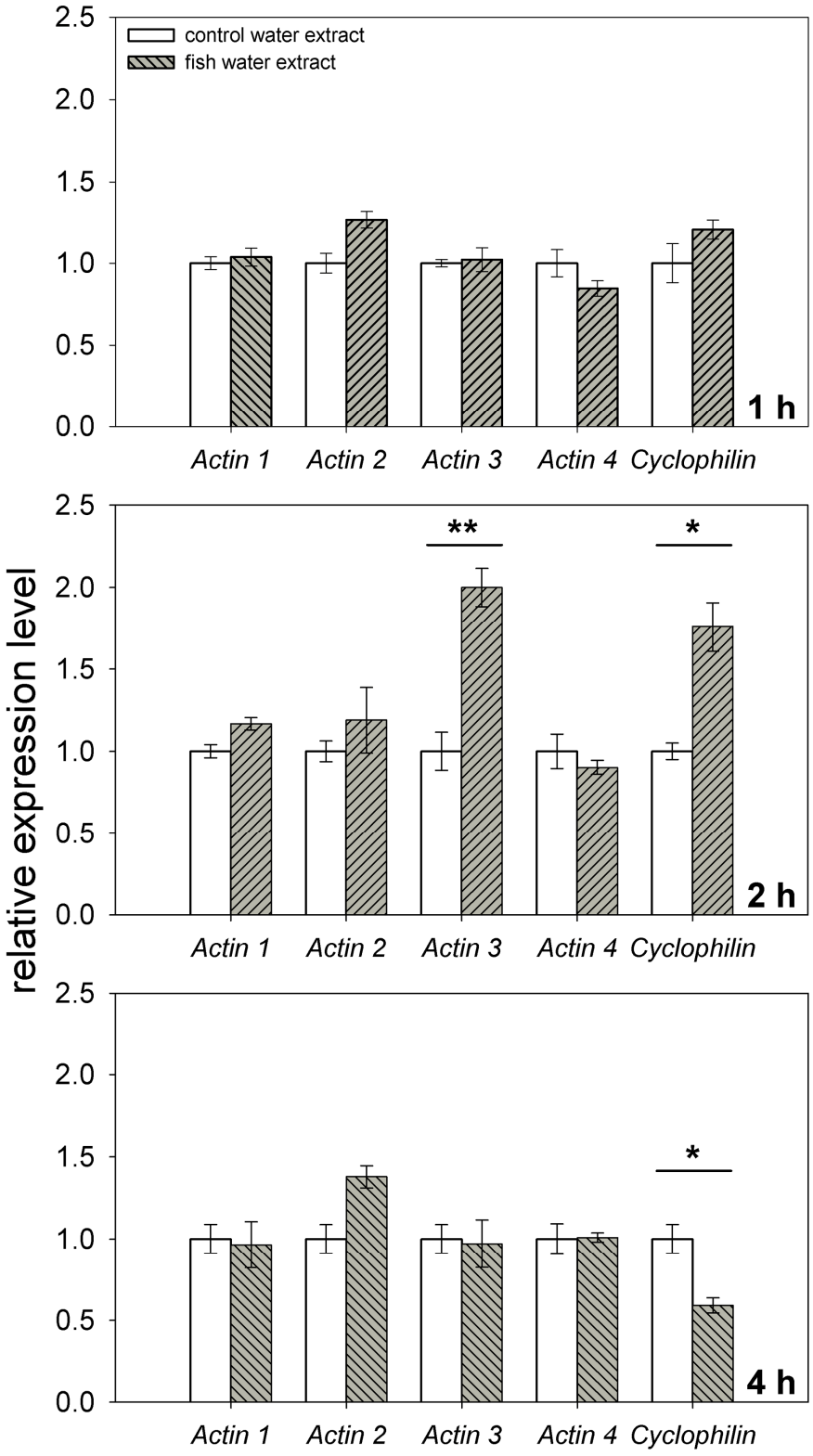

Figure 2. Mean relative expression of candidate genes in *Daphnia magna.* The expression pattern of the candidate genes *actin 1-4* and *cyclophilin* (n = 3, mean ± SE) was determined 1 h, 2 h and 4 h after exposure to extract of control water (white bars) or to fish incubation water (shaded bars). Asterisks indicate a significant difference between control and kairomones (* = $p < 0.05$; ** = $p < 0.01$).

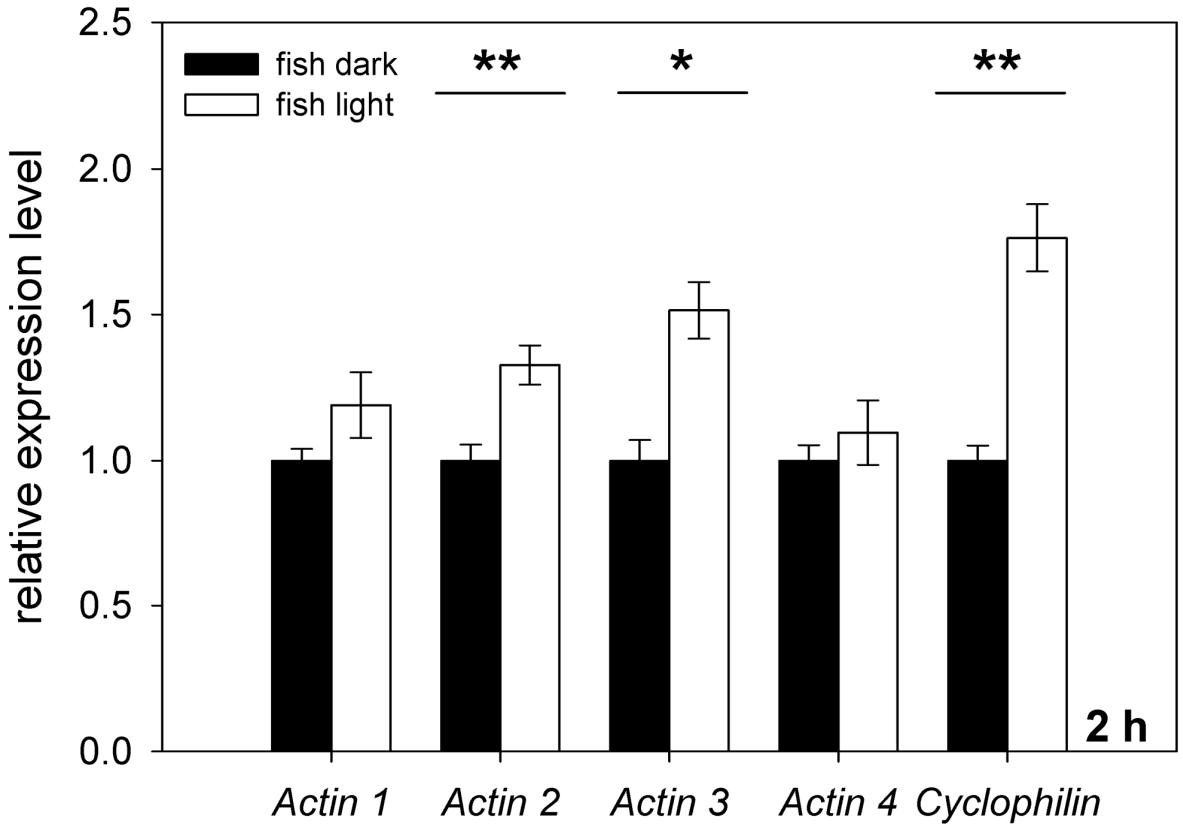

Figure 3. Mean relative expression (n = 3, ± SE) of candidate genes in *Daphnia magna* after 2 h of exposure to extract of fish incubation water in the absence (black bars) or presence (white bars) of light. Asterisks indicate a significant difference between the control and kairomone treatment (* = $p < 0.05$; ** = $p < 0.01$).

Building on the results of the time series described above, the effect of light on the kairomone-mediated increase of gene expression in *D. magna* was quantified after two hours of exposure to fish incubation water extract in the presence and absence of light. The genes *actin 2* (one-way ANOVA: $F_{1,5} = 45.230$, $p < 0.01$), *actin 3* (one-way ANOVA: $F_{1,5} = 21.305$, $p < 0.05$) and *cyclophilin* (one-way ANOVA: $F_{1,5} = 23.673$, $p < 0.01$) were significantly higher expressed in the presence of light compared to the dark treatment (Fig. 3). Similar to these significant effects, mean expression values for *actin 1* and *actin 4* were higher in the presence of light, although this difference was not significant. These results revealed that the kairomone-mediated effects on gene expression in *D. magna* were affected by light.

Discussion

Despite the well-known relevance of light for the induction of DVM, investigations of the induction of life-history changes (LHC) have been confined to predator kairomones only (Weider and Pijanowska 1993; Stibor and Lüning 1994; Von Elert and Stibor 2006). It has been demonstrated that fish kairomones do not affect carbon assimilation in *Daphnia* (Stibor and Machacek 1998) but lead to earlier allocation of assimilated resources into vitellogenin (Stibor 2002). This earlier onset of vitellogenin synthesis happens at the expense of allocation to somatic growth and thus results in smaller SFR. The role of light as an environmental factor for LHC in response to predator kairomones might easily have been overlooked in the aforementioned studies, as all these investigations were performed in the presence of light.

Here we show that the absence/presence of light has no immediate effect on somatic growth and SFR, a fact which indicates that resource allocation is not affected by light intensities *Daphnia* individuals are exposed to. Similarly, fish kairomones affected neither growth nor resource allocation in the absence of light; only in the presence of both light and kairomones was a reduction of SFR observed, demonstrating the interactive effect of the two cues. The significant reduction of SFR in the presence of light and kairomones clearly points towards changes in resource allocation as a cause for the smaller SFR observed. Despite the effect on the SFR no effect on the clutch size was found. Similar to our findings, fish kairomones did not affect clutch size but other life-history parameters (e.g. SFR, size of neonates) in several clones of *D. magna* (Boersma et al. 1998), which demonstrates that clones differ in their responsive traits to fish kairomones.

Our result that LHC in *D. magna* (Fig. 1) occurred only in the presence of light suggests that the coupling between fish kairomone and LHC is modulated by light. Plasticity with respect to DVM (Leibold 1991; Gliwicz and Pijanowska 1988) and LHC (Leibold and Tessier 1991; Gliwicz and Boavida 1996) caused by e.g. food conditions and different predation threats has been described. When *Daphnia* clones that are capable of using both defences were prevented from migration, they showed a decrease in SFR (Slusarczyk and Pinel-Alloul 2010), indicating that a higher light

intensity leads to a higher degree of life-history shifts. However, in the present study we elucidated the effect of presence and absence of light. The vertical migration in *Daphnia* is initiated by a relative rather than by an absolute change in light intensity (Ringelberg 1991), and the velocity of upward or downward migration in DVM is linearly related to a decrease or increase of this relative rate of change in light intensity (van Gool and Ringelberg 2003; van Gool and Ringelberg 1997; Ringelberg 1991). If fish are present, the kairomones are dispersed over the epilimnion all the time indicating predator presence and, thus provide neither a cue for the timing nor for the direction of migration. However, it was shown that the presence of kairomones released by fish leads to an increase in vertical displacement velocity (van Gool and Ringelberg 1998b; van Gool and Ringelberg 1998a) and thus alters the amplitude of DVM (van Gool and Ringelberg 2003; van Gool and Ringelberg 1997). In the absence of changes in light intensity (i.e. permanent darkness), the presence of kairomones did not induce DVM (Loose 1993b), indicating that the pattern of DVM is not triggered by an endogenous rhythm in *Daphnia* but is rather caused by the diel changes in ambient light. Thus the fish kairomone only does not cause DVM in *Daphnia* but it is enhancing the amplitude of migration caused by changes in the relative light intensity.

Based on our findings, we hypothesize that the light intensity an individual *Daphnia* is exposed to results from its daytime residence depth, i.e. its DVM amplitude, which thereby determines the degree of life-history changes. Taking into account earlier observations that DVM is preferred over LHC (Sakwinska and Dawidowicz 2005; Vos et al. 2002), this coupling of DVM and life-history changes nicely explains the observations that *Daphnia* clones that were collected at daytime from the epilimnion showed a smaller SFR (De Meester and Weider 1999). It remains to be seen if *Daphnia* genotypes differ with respect to light intensity thresholds for the induction of life-history. However, in this study the experimental animals were exposed to constant light conditions to observe the putatively strongest effects, whereas in the field animals are subjected to alternating periods of light and dark under non-migrating conditions.

We conducted our approach for the fish kairomone effects on the gene expression of *Daphnia* with genes that were shown to be involved in the LHC response. These genes were announced to be candidate genes and expected to show a specific response in our experiments. Pijanowska *et al.* (2004) found that *Daphnia* exposed to fish kairomones show a strong decrease in actin protein concentration. As a dynamic component of the cytoskeleton, actin could play a major role in the decrease of SFR (LHC) (Weider and Pijanowska 1993). Here we assumed that differences in actin protein level result from differences in gene expression, and we therefore investigated effects on the expression of *actin* in a time series after exposure to fish kairomones. For one *actin* gene a significant increase in expression in the presence of fish kairomones was shown (Schwarzenberger et al. 2009). Here we tested for effects of fish kairomones on expression of four *actin* paralogs in *D. magna*. The qPCR results revealed a strong response to fish kairomones and light on candidate gene expression in *Daphnia* after two hours of exposure (Fig. 2) for *actin 3* but not for the other three *actin* paralogs, indicating that the different paralogs are differently affected by kairomones from fish and for *cyclophilin*. It has been shown that the mRNA level of HSP genes in the arthropod *Tribolium* exposed to UV radiation was strongly affected after 2 h and the mRNA level decreased after 3.5 h (Sang et al. 2012) supporting our observed gene response over time in *Daphnia*. The significant increase of expression of one actin gene observed here and the decrease of overall actin protein (Pijanowska and Kloc 2004) may be due to the different methods that were used or due to the involvement of different *actin* paralogs: we were able to design specific primers for four out of the ten *actin* genes present in the *D. magna* genome, and a different *actin* paralog could be responsible for the effect observed on the protein level. Cyclophilins are highly conserved and have been described as peptidyl-prolyl *cis-trans* isomerases. Acting as chaperones, they are involved in protein folding during protein biosynthesis (Schiene-Fischer et al. 2013). Interestingly, in *Drosophila* the gene *ninaA* encodes a cyclophilin that resides in the ER and is associated with secretory vesicles, where it co-localizes stably with the photoreceptor Rh1 (Colley et al. 1991). In addition, *ninaA* belongs to a group of genes (*Nina* group) that is involved in the biogenesis of G protein-coupled receptors (GPCRs) in *Drosophila* influencing photoconversion and transduction in the optical system of flies (Ferreira and Orry 2012). These well-known functions of cyclophilin in

Drosophila, especially the role in the signal transduction of the optical system, might indicate the involvement of cyclophilin in the response of *Daphnia* to fish kairomone. We have shown that the presence of light is crucial for the induction of LHC, and therefore the response of a gene that is putatively involved in the perception of the light intensity differences in the optical system of *Daphnia* deserves further investigation.

HSPs are known to protect organisms against a wide array of environmental stressors and also to be involved in the response of *Daphnia* to fish (Pauwels et al. 2005; Pijanowska and Kloc 2004). We used three HSP genes (*HSP 10*, *HSP 70*, *HSP 90*) in our candidate gene approach to examine the effects on the expression of HSP genes. Surprisingly, none of these genes was affected by the presence of fish kairomones after 1 h, 2 h or 4 h exposure (data not shown). Reports of effects of fish kairomones on HSP levels in *Daphnia* are confined to the protein level (Pauwels et al. 2005; Pijanowska and Kloc 2004), and it remains to be tested whether or not the effects of fish kairomones on these three HSP genes investigated here occur after more than four hours of exposure.

We conducted life-history experiments with *Daphnia* using standardized extracts of either control water or fish incubation water. Effects of fish kairomones on life-history (SFR) and the expression of candidate genes, which are involved in the life-history response of *Daphnia,* were observed only in the presence of light, whereas somatic growth was not affected. Our observations point at a plastic coupling of anti-predator defences in *Daphnia* modulated by the factor “light”, as our experimental treatments ‘light’ and ‘darkness’ were meant to simulate weak migrators with a daytime residence in the epilimnion (`light´) and strong migrators with a daytime residence in the hypolimnion (`darkness´). We assume that in a scenario in which Daphnia are forced to descend deep under heavy fish pressure the amplitude of DVM alters the light level that a given genotype is exposed to. Such a proposed inverse coupling of LHC and DVM would be especially adaptive in habitats that allow for DVM only during parts of the season, i.e. spring; later in the season, when a deep-water refuge is no longer available due to hypolimnetic anoxia, alternative defences such as the decrease in the SFR (as is frequently observed in eutrophic shallow systems) may be

required. The potential to deploy more than one defence is widespread, in *Daphnia* (Slusarczyk and Pinel-Alloul 2010; Boersma et al. 1998) and the mechanistic explanation for a coupling of multiple defences suggested here describes a new mode of possible adaptation of multiple alternately deployable defences.

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Chapter 2

Coupling of anti-predator defences in *Daphnia*: the importance of light

Abstract

Here we test the coupling of two kairomone-induced, anti-fish defences in *Daphnia*, i.e. changes in life-history (LHC) and diel vertical migration (DVM) mediated by the environmental factor light. We used a gradient of five different light intensities that represents naturally occurring light intensities in the water column of a lake and we show that LHC in *Daphnia* are inversely coupled to the ambient light intensity. A similar light-dependence was found for the kairomone-mediated expression of a candidate gene in *Daphnia*. We argue that the DVM amplitude alters the ambient light intensity an individual is exposed to and thus inversely determines the degree of LHC. This coupling of these two anti-fish defences allows for plastic adjustment to fluctuating environments of standing freshwater ecosystems.

Chapter 2

Introduction

Predation is a major factor affecting natural selection in biological communities. The presence of predators has been shown to cause rapid evolution of defensive behaviours in prey (Seeley 1986; Magurran et al. 1992; Gliwicz 1986; Cousyn et al. 2001). Predation pressure in freshwater systems is fluctuating seasonally in intensity and manner (Brooks and Dodson 1965; Ringelberg et al. 1991; Weider and Pijanowska 1993) and has led to a variety of adaptive phenotypic defences in aquatic prey organisms (Dodson 1989; Harvell 1990). The keystone species *Daphnia* is an important prey organism for planktivorous fish in standing freshwaters and shows a wide range of antipredator defences, e.g. changes in life-history (LHC), morphology and behaviour (Lampert 1994), which have been shown to be inducible by chemical cues released by fish, so-called kairomones (Larsson and Dodson 1993). *Daphnia* showed a reduced body size at first reproduction (SFR) (Weider and Pijanowska 1993) due to a resource allocation that is affected by fish-produced kairomones. Only the preliminary chemical nature of the LHC inducing fish-produced kairomone has recently been characterized (Von Elert and Stibor 2006). These LHC resulting in a smaller body size of *Daphnia* increases the survival probability owing to the selection for large prey by visually hunting fish (Brooks and Dodson 1965). Furthermore, fish kairomones enhance diel vertical migration (DVM) in *Daphnia* (Loose et al. 1993), a widespread adaptive migration behaviour, which reduces the encounter probability with visually hunting fish: due to DVM, *Daphnia* reside in the deep, dark hypolimnion of stratified lakes during the day and spend the night in the epilimnion (Stich and Lampert 1981; Lampert 1989). The vertical migration in *Daphnia* is initiated by a relative rather than by an absolute change in light intensity (Ringelberg 1991), and the velocity of upward or downward migration in DVM is linearly related to a decrease or increase of this relative rate of change in light intensity (van Gool and Ringelberg 2003; van Gool and Ringelberg 1997; van Gool and Ringelberg 2003). However, it was shown that the presence of kairomones released by fish leads to an increase in vertical displacement velocity (van Gool and Ringelberg 1998a; van Gool and Ringelberg 1998b) and thus alters the amplitude of DVM (van Gool and Ringelberg 2003; van Gool and Ringelberg 1997; van Gool and Ringelberg 2003). In the absence of changes in light intensity (i.e. permanent darkness), the presence of

kairomones did not induce DVM (Loose 1993), indicating that the pattern of DVM is not triggered by an endogenous rhythm in *Daphnia* but is rather caused by the diel changes in ambient light if kairomones are present. Under high predation risk by visually hunting predators, DVM seems to be the preferred defence over LHC (e.g. decrease of SFR) (Vos *et al.* 2002). Interestingly, a comparison of *Daphnia* populations from different lakes revealed that in habitats, which do not allow for DVM due to lake morphometry or anoxia in the hypolimnion, daphnids responded with LHC (earlier reproduction at smaller body size) (Sakwinska and Dawidowicz 2005). These findings point towards a coadaptation between DVM and LHC. Slusarczyk et al. (Slusarczyk and Pinel-Alloul 2010) showed that a single population of *D. catawba* performed DVM when exposed to fish kairomones, but a decrease of SFR was observed if a net migration of the animals into deeper strata. These observations together suggested that a single *Daphnia* genotype might be plastic with respect to performing either DVM or LHC, and it remains to be investigated how these two anti-predator defences are coupled. Evidence for a coupling of these anti-predator defences comes from a study, which showed that LHC in *Daphnia* exposed to fish kairomones are suppressed in the absence of light (Effertz and Von Elert 2014). In the light of these results it was hypothesized that the DVM amplitude alters the light level that a given genotype is exposed to during a large part of the day and thereby determines the degree of LHC. The DVM amplitude of strong migrators would lead to very low ambient light levels (weak LHC), and the amplitude of weak migrators would lead to high individual light levels (strong LHC). The experimental animals of that study (Effertz and Von Elert 2014) were exposed to the presence or absence of light only, and it remained unclear whether the suppression of LHC in the presence of light is a gradual process that increases with light intensity or whether LHC are simply switched on by light intensities that exceed a certain absolute threshold intensity.

Here we have investigated how LHC in *Daphnia* are affected by different light intensities. We hypothesized that a higher light intensity in the presence of fish-kairomones would lead to stronger LHC (strong reduction in SFR) and a lower light intensity will lead to weaker LHC (low reduction in SFR). We therefore performed life-history experiments in the presence and absence of fish kairomone extracts with a gradient of five different light intensities that represented naturally occurring light

intensities in the water column of a lake. We used size at first reproduction (SFR), clutch size and juvenile somatic growth rate as parameters for the LHC. A new index for the anti-predator response in *Daphnia* was introduced to combine all relevant life-history changes in a single parameter. We further explored the relevance of different light intensities for kairomone-induced changes in *Daphnia* by analyzing effects on the expression of one candidate gene that earlier has been shown to be highly responsive to fish kairomones (Effertz and Von Elert 2014).

Material & Methods

Test species and cultures

Daphnia magna clone B from Lake Binnensee, Germany (Lampert and Rothhaupt 1991) was cultured at 20 °C in aged, membrane-filtered (pore size: 0.45 µm) tap water under dim light. Twelve animals per litre were kept under non-limiting food concentrations: 2 mg C_{part} L^{-1} of the green algae *Chlamydomonas klinobasis*, strain 56, culture collection of the Limnological Institute at the University of Konstanz. *C. klinobasis* was grown in 5 L semi-continuous batch cultures (20 °C; illumination: 120 µmol m^{-2} s^{-1}) by replacing 20 % of the culture with fresh, sterile Cyano medium [35] every other day. The test animals originated from mothers that had been raised under control conditions (saturating concentrations of *C.* sp.) for at least five generations.

Experimental set-up

Fish kairomone extract

Thirty *Perca fluviatilis* (body size: 10-12 cm) were pre-conditioned for 24 h without food and then kept for 24 h in 8 L of aged tap water at 18 °C without feeding. The water containing the fish kairomones was filtered through glass fibre filters (pore size: 0.45 µm). Bulk enrichment of the kairomones was performed according to Loose & Von Elert (1996). Briefly, a C_{18} solid-phase cartridge (10 g of sorbent, volume 60 mL, endcapped, Varian Mega Bond Elut, Agilent Technologies) was preconditioned with 50 mL methanol and 50 mL ultrapure water prior to adding the sample. Methanol was added to the filtered incubation water containing the fish kairomones to obtain a 1 % concentration, and 2 L of sample were passed through the cartridge. The loaded cartridge was washed with 50 mL of ultrapure water and then eluted with 50 mL of methanol. The eluates originating from 10 L fish incubation water were pooled and evaporated to dryness using a rotary evaporator, re-dissolved in 1 mL of absolute ethanol and tested for biological activity. Water without fish was used for the production of a control extract. The same standardized extracts of control water and fish incubation water in the concentration equivalent of three fish in 8 L of water were used for all experiments.

Life-history experiments

Experimental animals originated from the 3rd clutch and had been released within 12 h. The neonates were maintained at 24 ind. L^{-1} and were transferred daily to new water supplemented with algal food (2 mg C L^{-1}) and kept under five different light intensities (total photon flux: 5.31 x 10^{18} s^{-1} m^{-2}, 6.62 x 10^{17} s^{-1} m^{-2}, 2.29 x 10^{17} s^{-1} $m^{-2,}$ 8.43 x 10^{16} s^{-1} m^{-2} and 4.81 x 10^{16} s^{-1} m^{-2}) without a photoperiod at 20 °C until day three, when the cohort was split and subsequently subjected to five light conditions: Light intensity 1: 5.31 x 10^{18} s^{-1} m^{-2}, light intensity 2: 6.62 x 10^{17} s^{-1} m^{-2}, light intensity 3: 2.29 x 10^{17} s^{-1} m^{-2}, light intensity 4: 8.43 x 10^{16} s^{-1} m^{-2} and light intensity 5: 4.81 x 10^{16} s^{-1} m^{-2}. From day three to day five the animals were transferred daily to new water (400 ml) supplemented with algal food (2 mg C L^{-1}). The transfer of animals was done under dark conditions to prevent any disturbance by changing the light intensities. On the fifth day, five animals from each light intensity treatment were exposed to either control water extract or fish kairomone extract in 400 mL. From day five to the end of the life-history experiment, when the eggs were released into the brood pouch of the daphnids, the media containing the fish kairomone extract or the control extract was not renewed and one molt per individual was observed in all treatments prior to deposition of eggs to the brood pouch. The light intensity of a Lumilux® DeLuxe Daylight fluorescent tube (OSRAM), that was used for the life-history experiments was measured with a LI-192 Underwater Quantum Sensor (LI-COR Biosciences). All treatments were run in triplicate, and *D. magna* were fed daily. The clutch size was determined for each egg-bearing individual , and the size at first reproduction (SFR) of each individual was measured from the top of the eye to the base of the tailspine with the aid of a dissecting microscope equipped with a digital camera (Imaging Source) and an image analysis software. A mean SFR was calculated for each replicate; these mean values were used to calculate the respective mean value and the variance for the treatment. Somatic growth rates were calculated as according to Wacker & Von Elert (2001) (Wacker and Von Elert 2001):

$$g = [(\ln (dw_t) - \ln (dw_0)]/d,$$

in which *dw* is the body dry weight of a subsample of the animals at the beginning (dw_0) and end (dw_t) of the experiment and *d* is length of the experiment in days.

Mean individual dry weights were mean values of three individuals (dw_0 is the same for all treatments, and dw_t is specific for each replicate).

Calculation of the anti-fish index

This index was calculated to combine all relevant life-history changes (LHC) in a single parameter:

Anti-fish index = clutch size (egg number)/SFR (mm) x juvenile somatic growth (g)

RNA extraction and reverse transcription

The test animals were kept as described above. On day five, five animals were transferred into 250 mL containing either fish water or control water extract in the presence or absence of light. After 2 hours, subsamples of three animals were used for RNA extraction. The experiment was run in triplicate. RNA was extracted and purified using the NucleoSpin® RNA II Kit (Macherey & Nagel). The integrity of each RNA sample was verified using the 2200 Tape Station (Agilent Technologies) on a High Sensitivity Screen Tape®, and the RIN values ranged from 7.5 to 8.5. The RNA concentration was determined using the Nanodrop ND-1000 v3.7 (Thermo Scientific). 1 µg of RNA was reverse-transcribed using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). The cDNA was stored at -20 °C.

Quantitative PCR (qPCR)

28S ribosomal RNA (*28S*), *alpha-tubulin* (*α-tubulin*), *glyceraldehyde-3-phosphate dehydrogenase* (*GAPDH*) and *TATA-box binding protein* (*TBP*) were used as endogenous control genes of constitutive expression in *D. magna* (Schwarzenberger et al. 2009; Heckmann et al. 2006). To discover the other candidate genes, the *D. magna* sequences for *actin* (accession no. AJ292554) were used as queries for sequence similarity searches using BLASTn against the *D. magna* assembly v2.4 in wFleaBase. All gene sequences with a significant hit were aligned with Geneious® v6.0.3 (Biomatters Ltd.). Primers were designed with Primer 3 v2.3.5 and the quality was checked with NetPrimer (PREMIER Biosoft) (Tab 1). Melting curve analyses confirmed specific amplification without primer dimer formation. The data acquisition for the relative expression was performed on a 7300 qPCR system (Applied

Biosystems). Each reaction contained 5 ng of cDNA template, 10 µL SYBR® Green PCR master mix and 2.5 µM of each primer in a final volume of 20 µL. Cycling parameters were 95 °C for 10 min for the initial start of the DNA polymerase, followed by 40 cycles of 95 °C for 15 s, 55 °C for 30 s, 68 °C for 30 s, and a final dissociation step with 95 °C for 15 s, 55 °C for 30 s, 68 °C for 30 s and 95°C for 15 s. The baseline and threshold for the Ct (Cycle threshold) was set automatically, and the each gene was tested in triplicate. Amplification efficiencies for every primer pair of each candidate gene were determined.

Data analysis and statistics

After qPCR, raw data were analyzed with qBasePLUS v2.0 (Biogazelle) based on qBase and geNorm. The relative gene expression of candidate genes was normalized with the generated normalization factor, and standard errors were calculated. A single analysis of variance (one-way ANOVA) was carried out for the qPCR data generated with the qBasePLUS software after 2 h of exposure to extract of fish incubation water in the five light intensities. The dependent variable was checked for homogeneity of variances (Shapiro-Wilk). A one way analysis of variance (one-way ANOVA) was conducted for the life-history experiments. The dependent variables were checked for homogeneity of variances (Shapiro-Wilk). The effect of single treatments was tested by post hoc tests (Tukey's HSD multiple comparison test) at the same probability level as the respective analysis of variance. A significance level of $p = 0.05$ was applied to all statistical analyses. All statistics were performed with SigmaPlot v11.0 (Systat Software) and STATISTICA v6.0 (Starsoft Inc.).

Results

Life-history and growth experiments

In the life-history experiments, *D. magna* was grown in the presence of control water extract (hereafter referred to as `control´) or extract of fish incubation water (hereafter referred to as `fish kairomones´) in the presence of five different light intensities. Size at first reproduction (SFR) decreased in the presence of fish kairomones and In light intensity 1 (total photon flux: 5.31 x 10^{18} s^{-1} $m^{-2)}$ the presence of kairomones resulted in a decrease of SFR from 2.80 mm (control) to 2.58 mm (kairomone) (Tukey's HSD multiple comparison test, $F_{1,5}$: 16.950, $p < 0.05$) (Fig. 1). A decrease of SFR in the presence of fish kairomones compared to the control was observed for light intensity 2 (total photon flux: 6.62 x 10^{17} s^{-1} m^{-2}) led to a kairomone-mediated reduction in SFR from 2.77 mm (control) to 2.67 mm (Tukey's HSD multiple comparison test, $F_{1,5}$: 16.618, $p < 0.05$). A decrease of SFR in the presence of fish kairomones compared to the control was observed for light intensity 3 (total photon flux: 2.29 x 10^{17} s^{-1} m^{-2}) to 2.51 from 2.68 mm (Tukey's HSD multiple comparison test, $F_{1,5}$: 8.470, $p < 0.05$), but SFR was not affected in the presence of light intensity 4 (total photon flux: 8.43 x 10^{16} s^{-1} m^{-2}, Tukey's HSD multiple comparison test, $F_{1,5}$: 1.298, $p = 0.318$), and light intensity 5 (total photon flux: 4.81 x 10^{16} s^{-1} m^{-2}, Tukey's HSD multiple comparison test, $F_{1,5}$: 0.387, $p = 0.854$, Fig. 1). Juvenile somatic growth rates and clutch size (number of eggs) of *D. magna* were not affected by fish kairomones in any of the five different light intensities, although we observed a tendencies towards an increased clutch size under exposure to fish kairomones (data not shown).

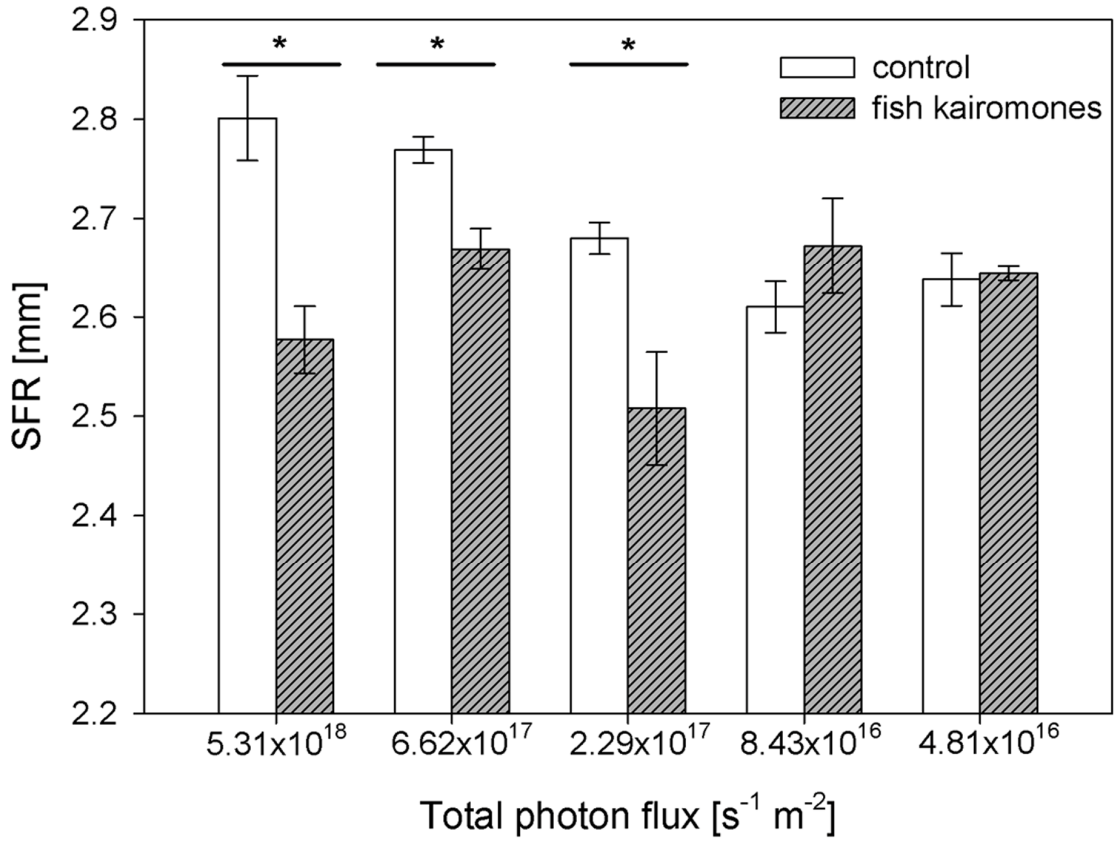

Figure 1. Size at first reproduction (SFR) of *Daphnia magna* grown in the presence or absence of fish kairomones (n = 3, mean ± SE). *Daphnia* were exposed to control water extract (open bars) or to fish incubation water extract containing fish kairomones (filled bars) in five light intensities. Asterisks indicate a significant difference between control and kairomones of a pair wise comparison (* = $p < 0.05$).

When we calculated the change of body size derived from the SFR of animals exposed to control extract or fish kairomones for every light intensity (Fig. 2), a significant difference was found between the three higher light intensities (5.31×10^{18} s^{-1} m^{-2}, 6.62×10^{17} s^{-1} m^{-2} and 2.29×10^{17} s^{-1} m^{-2}) and the group of the two lower light intensities (8.43×10^{16} s^{-1} m^{-2} and 4.81×10^{16} s^{-1} m^{-2}; one-way ANOVA, $F_{1,14}$: 12.314, $p < 0.001$).

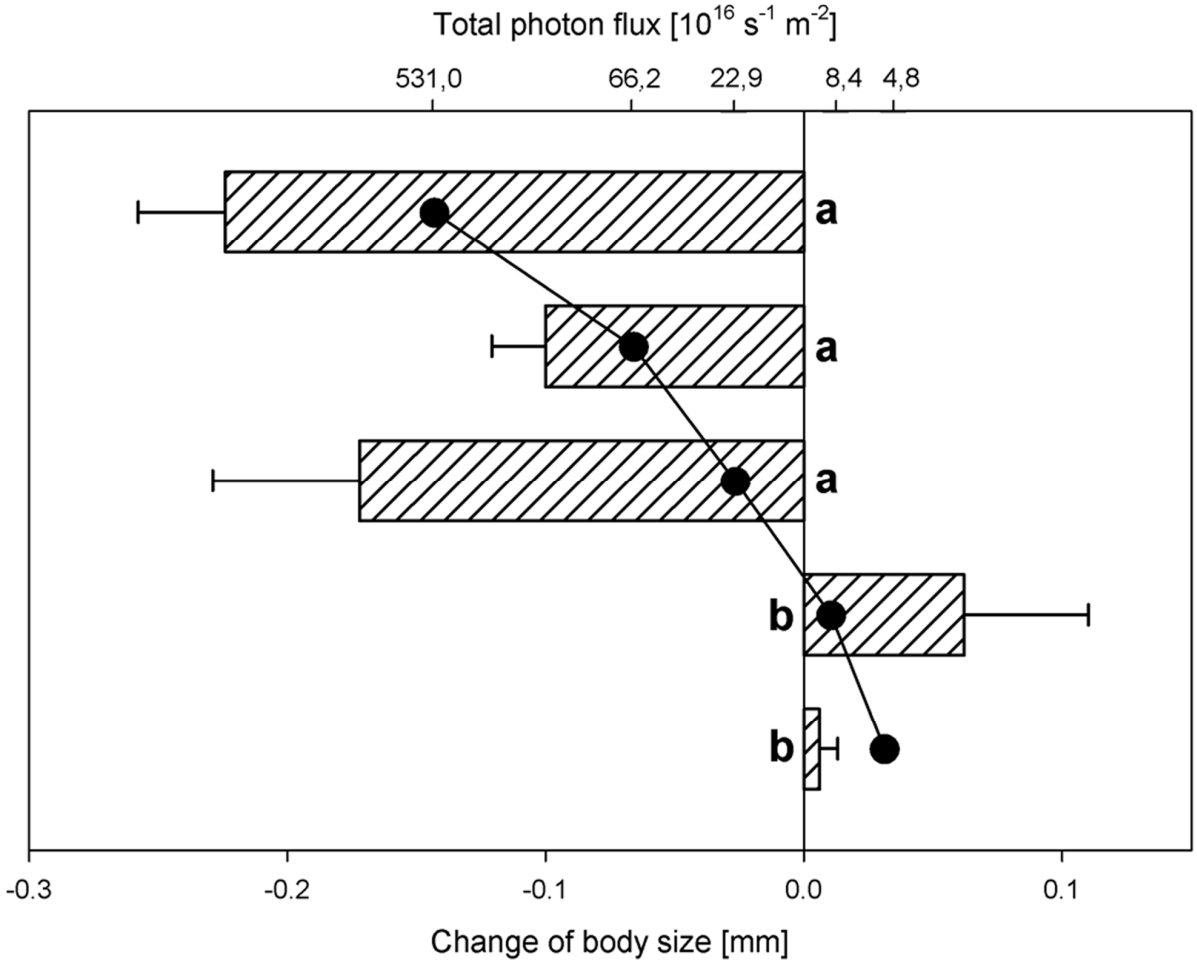

Figure 2. Change of body size derived from the difference in size at first reproduction (SFR) of *Daphnia magna* grown in the presence or absence of fish kairomones (horizontal bars, n = 3, mean ± SE) in five light intensities. Different letters denote a significant difference ($p < 0.05$) in SFR between the light intensity treatments.

A multiple linear regression (multiple linear regression, $F_{2,14}$: 2.960, $p = 0.090$) between the calculated anti-fish index (see methods) of the treatments control and fish kairomones (Fig. 3) showed that the anti-fish index significantly increased with the ambient light level in the presence of fish kairomones ($p < 0.05$), whereas the light level had no effect on the anti-fish index in the absence of kairomones ($p = 0.92$).

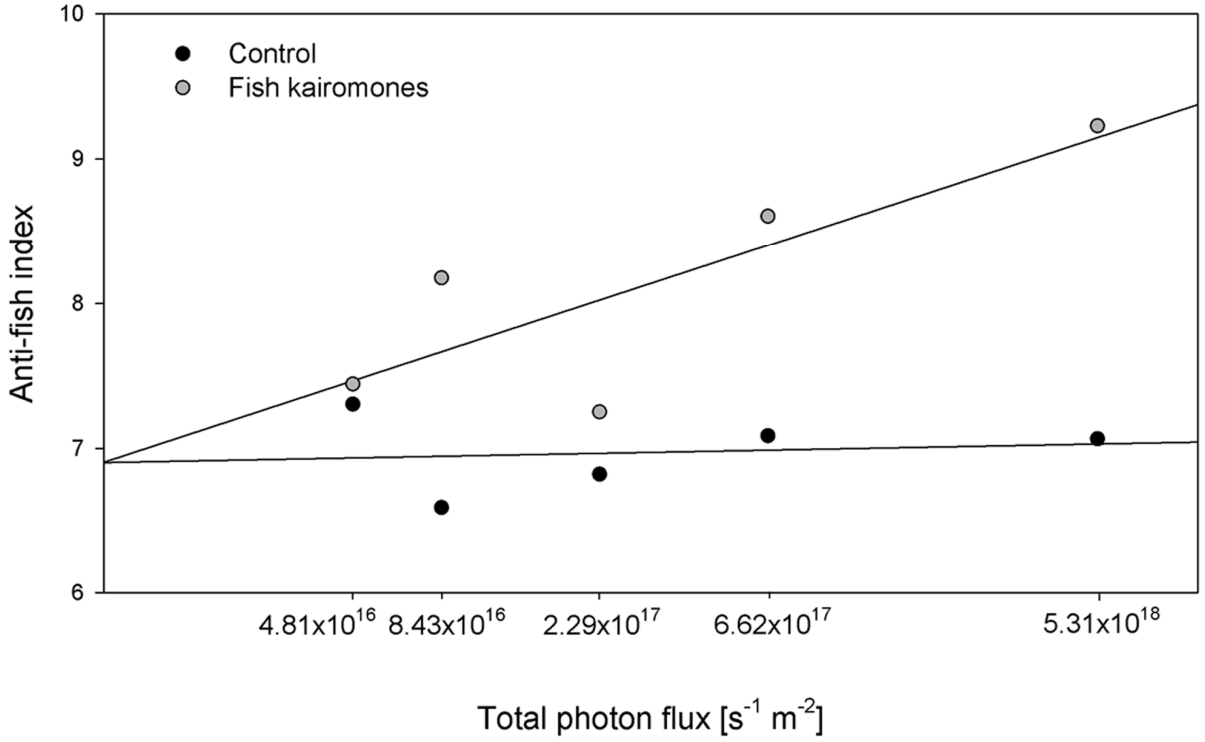

Figure 3. A multiple linear regression between the calculated anti-fish index (anti-fish index = clutch size (egg number)/SFR (mm) x juvenile somatic growth (g)) of *Daphnia magna* in the presence or absence of fish kairomones exposed to five different light intensities.

Relative expression of the candidate gene

The relative expression of the candidate gene *actin* 3 in five-day-old *D. magna* was monitored after 2 hours of exposure to control or fish kairomones under five different light intensities. The genes *28S*, *α-tubulin* and *GAPDH* served as endogenous control genes in all qPCR analyses and were used for calculation of the normalization factor. The qPCR analysis showed a significant difference in expression levels of exposure to fish kairomones after 2 h (one-way ANOVA, $F_{1,14}$: 6.148, $p < 0.05$, fig. 4) between two groups of light intensities (low light intensities: 8.43 x 10^{16} and 4.81 x 10^{16} s^{-1} m^{-2}; high light intensities: 5.31 x 10^{18}, 6.62 x 10^{17} and 2.29 x 10^{17} s^{-1} m^{-2}). These results revealed that the gene expression of *actin 3* in *D. magna* exposed to fish kairomones was affected by the same set of light intensities, which were shown to affect the parameter SFR of *D. magna* exposed to fish kairomones in the life-history experiment (Figs. 1 and 2).

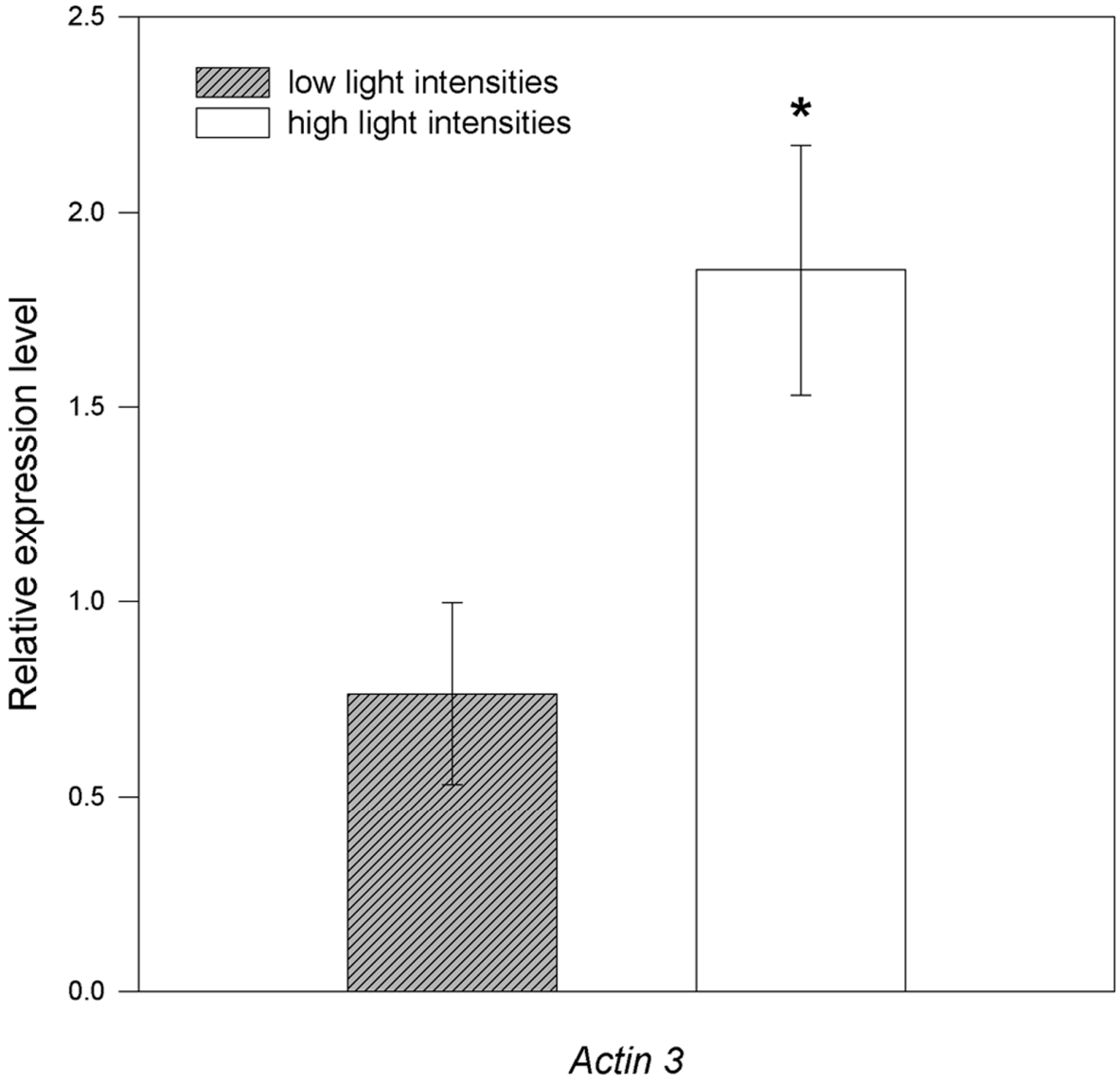

Figure 4. Mean relative expression of the candidate gene *actin 3* in *Daphnia magna.* The expression of actin 3 was determined after 2 h in the presence of fish kairomones (n = 3, mean ± SE). The expression results of *actin 3* in the presence of fish kairomones were grouped to low light intensities (light intensity 4: 8.43 x 10^{16} s^{-1} m^{-2}, light intensity 5: 4.81 x 10^{16} s^{-1} m^{-2}) and high light intensities (light intensity 1: 5.31 x 10^{18} s^{-1} m^{-2}, light intensity 2: 6.62 x 10^{17} s^{-1} m^{-2}, light intensity 3: 2.29 x 10^{17} s^{-1} m^{-2}) according to the light intensities, which were shown to affect the size at first reproduction (SFR) in the life-history experiments. Asterisks indicate a significant difference ($p < 0.05$) between low and high light intensities.

Chapter 2

Discussion

Inducible defences are widespread in plants and animals. *Daphnia magna,* a well-established model organism in ecology, ecotoxicology and evolutionary research has become a textbook example for inducible defences in response to chemical cues released from predators. Fish kairomone induced life-history changes (LHC) in *Daphnia* have only recently been shown to be suppressed in the absence of light (Effertz and Von Elert 2014). Despite the well-known relevance of light for the induction of DVM, investigations of the induction of life-history changes (LHC) have been confined to predator kairomones only (Weider and Pijanowska 1993; Stibor and Lüning 1994; van Gool and Ringelberg 1997). It has been demonstrated that fish kairomones do not affect carbon assimilation in *Daphnia* (Stibor and Machacek 1998) but lead to earlier allocation of assimilated resources into vitellogenin (Stibor 2002). This earlier onset of vitellogenin synthesis happens at the expense of allocation to somatic growth and thus results in smaller SFR. Here we show that different light intensities have no immediate effect on somatic growth, which indicates that resource allocation is not affected by the ambient light intensities that *Daphnia* individuals were exposed to. We show fish kairomones led to a reduction in SFR in *Daphnia* only in the three higher light intensities (Fig. 1). Furthermore, when the net effect of kairomone on SFR was related to the light intensity gradient (Fig. 2), it became obvious that a certain light intensity threshold seems to be crucial for the induction of LHC in *Daphnia* exposed to fish kairomones. Despite the effect on the SFR no significant effect on the clutch size was found. Similar to our findings, fish kairomones did not affect clutch size but other life-history parameters (e.g. SFR) in *D. magna* clone B (Boersma et al. 1998). Nevertheless, we could observe a tendency towards an increased clutch size of *Daphnia* exposed to fish kairomones and therefore, we calculated an anti-fish index, which combined the measured life-history parameters (SFR, clutch size and juvenile somatic growth rate) in this study. This multi linear regression revealed a linear correlation between the anti-fish index of *Daphnia* and the gradient of experimental light intensities (Fig. 3). However, this calculation included all values of measured life-history parameters in this study even without significance between the treatments and therefore, should not be contrary to the observed light intensity threshold for the induction of LHC (decrease in SFR, Fig. 2).

It was hypothesized that the light intensity an individual *Daphnia* is exposed to results from its daytime residence depth, i.e. its DVM amplitude, which thereby determines the degree of life-history changes (Effertz and Von Elert 2014) and it was shown that the DVM amplitude differed between single genotypes (De Meester *et al.* 1994) and between natural populations (Sakwinska and Dawidowicz 2005) of *Daphnia*. However, light intensities between lakes and even within a lake were shown to vary seasonally (Tilzer *et al.* 1995) and are highly affected by the water transparency (Wissel and Ramacharan 2003). The experimental animals of this study were exposed to different light intensities, which were supposed to be comparable to light intensities in the field. In our experiments we used light intensities that were measured in an oligotrophic lake in central-europe during a season (Tilzer *et al.* 1995). The five light intensities ranging from 5.31×10^{18} s^{-1} m^{-2} to 4.81×10^{16} s^{-1} m^{-2} (measured in total photon flux) that were used in this study, correspond approximately to natural light regimes in a range from the surface to a depth of 20 meters in February and to a depth of 8 m in May in Lake Constance (Tilzer *et al.* 1995). Stich and Lampert (1981) showed that in May the mean daytime depth of DVM-performing *D. hyalina* in Lake Constance is well below 8 m, which suggests that the low ambient light levels of migrating *D. hyalina* suppress LHC in these genotypes. In the same lake *D. galeata* does not perform DVM (Stich and Lampert 1981), and it remains to be tested if D. galeata undergoes LHC. Animals in the field are subjected to alternating periods of light and dark under non-migrating conditions and it deserves further investigation on which extent LHC are controlled by a certain light period.

In order to test if the observed effect of ambient light levels on LHC are as well reflected on the level of gene expression, we used *actin 3* that has earlier been shown to be highly responsive in the LHC response (Effertz and Von Elert 2014) and that therefore could serve as an indicator for the response on the gene level under exposure to five different light intensities. This gene was regarded as a candidate gene and expected to show a specific response in our experiments. Pijanowska *et al.* (2004) found that *Daphnia* exposed to fish kairomones showed a strong decrease in actin protein concentration. As a dynamic component of the cytoskeleton, actin could play a major role in the decrease of SFR (LHC, Weider and Pijanowska 1993). It

was also shown that actin is involved in the anti-predator response of *Daphnia* on the protein level against the invertebrate *Triops* (Otte *et al.* 2014) indicating a general role of actin in the anti-predator response of *Daphnia*. In line with findings of Effertz & von Elert (Effertz and Von Elert 2014), the qPCR results revealed responses to fish kairomones and light intensities on *actin 3* expression in *Daphnia* already after two hours of exposure (Fig. 4). The significant increase of *actin 3* expression between the two groups of light intensities indicated that the gene expression affected by fish kairomones showed the same pattern of dependence on light intensity as changes in SFR did (Figs. 1 and 2).

Daphnia exposed to fish kairomones showed a LHC response in the two high light intensities (5.31 x 10^{18} s^{-1} m^{-2}, 6.62 x 10^{17} s^{-1} m^{-2}) and the results of the life-history experiments under the exposure to the intermediate light intensity (2.29 x 10^{17} s^{-1} m^{-2}) observed in this study corroborate with the findings of Effertz und Von Elert (2014). In line with the finding that the LHC response is suppressed in the absence of light (Effertz and Von Elert 2014), we here find neither a LHC response nor a change in gene expression under the exposure to the low light intensities (8.43 x 10^{16} s^{-1} m^{-2} and 4.81 x 10^{16} s^{-1} m^{-2}). Almost identical results were obtained for the light-mediated expression of *actin 3*, so that for kairomone-mediated LHC and for changes in *actin 3* expression, light intensities > 8.43 x 10^{16} s^{-1} m^{-2} seem to be required. This indicates that a light intensity threshold for kairomone-mediated LHC and gene response exists, and it remains to be tested if this light intensity threshold differs among *Daphnia* genotypes with different DVM patterns in the field.

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Chapter 3

Differential peptide labeling (iTRAQ) in LC-MS/MS based proteomics in *Daphnia* reveal mechanisms of an anti-predator response

Abstract

Daphnia, an important model organism for studies on ecology and evolution, has become a textbook example for inducible defences against predators. Inducible defences are widespread in nature, and the underlying molecular mechanisms for this plasticity in general and in particular in *Daphnia* are not fully understood. Here, we provide for the first time a combination of established life-history changes (LHC), which are induced by chemical cues of a predator (fish kairomones), in *Daphnia* with differential peptide labelling (iTRAQ) in LC-MS/MS based proteomics. The aim of the present study is the elucidation of proteins involved in specific anti-predator responses in a predator-prey system of ecological relevance by high-throughput proteomics. We were able to identify functional proteins, which are likely to explain the kairomone-mediated LHC in *Daphnia*.

Introduction

Daphnia play a role as keystone species in aquatic ecosystems, and their inherent phenotypic plasticity allows to cope with a fast changing environment. This phenotypic plasticity and the well-known ecological background of daphnids led to the role as important model organism for the understanding of links between the organism and its environment (Lampert 2006). Colbourne et al. (2011) have attributed the well known ecological success of *Daphnia* to the high proportion of genes unique to the *Daphnia* linage with unknown homology, which were shown to be significantly over-represented under exposure to biotic and abiotic environmental stressors (Colbourne et al. 2011). The high proportion of genes in the *Daphnia* genome without homologues in other species (Colbourne et al. 2011) may severely constrain the assignment of functions to genes that are responsive to environmental conditions (eco-responsive). The mRNA level has been shown not to serve as an accurate predictor for the respective protein abundance (Anderson and Seilhamer 1997; Gygi et al. 1999; Nie et al. 2006). The actual phenotype of an organism is reflected in its proteome, and it is crucial for the understanding of links between organism and environment to gain information about the population of functional proteins. A LC-MS/MS based proteome profiling in *Daphnia* has demonstrated that high throughput proteomics based upon the *Daphnia* genome database are feasible (Froehlich et al. 2009), and the application of mass spectrometry based proteomics to study inducible phenotypes in the model system *Daphnia* is a powerful approach to elucidate the role of functional proteins that might explain phenotypic plasticity.

Daphnia have been shown to respond to chemical cues released by predators (kairomones) with a wide range of defences, e.g. life-history changes (LHC, Effertz and Von Elert 2014), morphology and behavior (Lampert 1994; Larsson and Dodson 1993). The inducible character of these anti-predator defences and their defined characteristics in *Daphnia* provide an insight into the expression of phenotypes and the underlying molecular mechanisms in a fast and specific manner. *Daphnia* that were exposed to fish-produced kairomones respond with a change in resource allocation leading to a reduced body size at first reproduction

(SFR, Weider and Pijanowska 1993; Von Elert and Stibor 2006). This smaller body size of *Daphnia* decreases the predation pressure by fish as visually hunting predators like fish select for larger individuals (Brooks and Dodson 1965). Furthermore, fish kairomones enhance diel vertical migration (DVM) in *Daphnia (Loose 1993)*, a widespread adaptive migration behavior, which reduces the encounter probability with visually hunting fish: due to DVM, *Daphnia* reside in the deep, dark hypolimnion of stratified lakes during the day and spend the night in the epilimnion (Stich and Lampert 1981; Lampert 1989). Plasticity with respect to DVM (Gliwicz and Pijanowska 1988; Leibold 1991) and LHC (Gliwicz and Boavida 1996; Leibold and Tessier 1991) caused by different predation threats has been described. When *Daphnia* clones that are capable of using both defences, i.e. DVM and LHC, were prevented from migration, they showed a decrease in SFR (Slusarczyk and Pinel-Alloul 2010), indicating that a higher light intensity leads to a higher degree of life-history shifts. It was shown that this reduction of SFR in *Daphnia* is not only mediated by fish kairomones but also controlled by the light intensity (Effertz and Von Elert 2014), which allows for a specific factorial experimental set-up to identify functional proteins, dependent on light intensity and fish kairomones, which might explain the phenotypic changes.

Here, we analyzed for the first time changes in the proteome of *Daphnia* in response to fish kairomones. We therefore conducted life-history experiments in the presence and absence of purified fish kairomones under different light conditions. We used SFR and clutch size as parameters for the life-history changes (LHC). The clutch size was used as additional parameter for LHC in this study as is has been shown that the number of eggs in *Daphnia* increased under exposure to fish kairomones (Reede 1995). Only animals that showed the expected anti-fish response of LHC were used for the proteome analysis. This is the first study that implements differential peptide labeling (iTRAQ) in LC-MS/MS based proteomics in *Daphnia* with the aim to identify kairomone-responsive proteins in *Daphnia*, which might explain the fish kairomone mediated LHC.

Material & Methods

The aim of this study was the differential analysis of three different proteome states in *D. magna* exposed to either fish kairomone extract or control extract under different light intensities. Based on the analysis of three biological replicates, the study was designed to identify functional proteins that may explain fish-kairomone mediated effects on LHC.

Test species and cultures

Daphnia magna clone B from Lake Binnensee, Germany (Lampert and Rothhaupt 1991) was cultured at 20 °C in aged, membrane-filtered (pore size: 0.45 µm) tap water under dim light. Ten animals per litre were kept under non-limiting food concentrations: 2 mg C_{part} L^{-1} of the green algae *Chlamydomonas klinobasis*, strain 56, culture collection of the Limnological Institute at the University of Konstanz. *C. klinobasis* was grown in 5 L semi-continuous batch cultures (20 °C; illumination: 7.23 x 10^{19} photons m^{-2} s^{-1}) by replacing 20 % of the culture with fresh, sterile Cyano medium (Von Elert and Jüttner 1997) every other day. The test animals originated from mothers that had been raised under control conditions (saturating concentrations of *C.* sp.) for at least five generations.

Experimental set-up

Fish kairomone extract

Three *Perca fluviatilis* (body size: 10-12 cm) were pre-conditioned for 24 h without food and then kept for 24 h in 8 L of tap water (aerated with ambient air for 4 days) at 18 °C without feeding. The water containing the fish kairomones was filtered through membrane filters (pore size: 0.45 µm). For bulk enrichment of the kairomones, a C_{18} solid-phase cartridge (10 g of sorbent, volume 60 mL, endcapped, Varian Mega Bond Elut, Agilent Technologies) was preconditioned with 50 mL methanol and 50 mL ultrapure water prior to adding the sample. Methanol was added to the filtered incubation water containing the fish kairomones to obtain a 1 % concentration, and 2 L of sample were passed through the cartridge. The loaded cartridge was washed with 50 mL of 1% MeOH and then eluted with 50 mL of methanol. The eluates

originating from 10 L fish incubation water were pooled and evaporated to dryness using a rotary evaporator, re-dissolved in 1 mL of absolute ethanol, tested for biological activity and used for subsequent chromatography.

Liquid chromatography (LC) of fish kairomone and control extract

All chromatography was carried out with an Accela Ultra high pressure liquid chromatography (UHPLC) system (Thermo Fisher) consisting of a 1250 psi pump, an autosampler (AS) and a photo diode array (PDA) detector. A C_{18} column (Nucleosil, 250/4, 120-5) was used as the stationary phase and a gradient of methanol (MeOH) and ultrapure water, at a temperature of 30 °C and a flow rate of 1000 µL min^{-1} as mobile phase: 0 min: 60% MeOH; 14 min: 60% MeOH; 15 min: 100% MeOH; 18 min: 100% MeOH; 19 min: 60% MeOH; 20 min: 60% MeOH. The injection volume of each sample was 100 µL. Fractions were collected from the eluate, evaporated to dryness, and were tested for biological activity. Biological activity was confined to one fraction (retention time 10 – 11 min), and this fraction was used for the further experiments. Other fractions showed no activity (data not shown). We used the Xcalibur software package (Thermo Fisher) for qualitative analysis.

Water without fish was used for the production of a control extract, which was treated in exactly the same way as the fish kairomone extract. The same standardized and fractionated extracts of control water and fish incubation water in the concentration equivalent of three fish in 8 L of water were used for all experiments.

Life-history experiments

Test animals originated from the 3rd clutch of D. magna mothers and had been released within 12 h. The neonates were maintained at 25 ind. L^{-1} and were transferred daily to new water supplemented with algal food (2 mg C L^{-1}) and kept under permanent dim light at 20 °C until day three, when the cohort was divided and kept either under permanent dim light (2.89 x 10^{17} photons s^{-1} m^{-2}) or permanent dark (< 3.01 x 10^{16} photons s^{-1} m^{-2}) conditions. The light intensity of a Lumilux® DeLuxe Daylight fluorescent tube (OSRAM, Munich, Germany) that was used for the life-history experiments was measured with an LI-192 Underwater Quantum Sensor (LI-COR Biosciences). From day three to day five the animals were transferred daily to

new water supplemented with algal food (2 mg C L^{-1}). On the fifth day, thirty preconditioned animals from the dim light cohort were exposed to either control water extract or fish kairomone extract in 1000 mL in permanent dim light. As well on the fifth day, thirty preconditioned animals from the dark cohort were exposed to fish kairomone extract in 1000 mL in the absence of light. From day five to the end of the life-history experiment, when the eggs were released into the brood pouch of the daphnids, the media containing the fish kairomone extract or the control extract was not renewed. All treatments were run in triplicate, and *D. magna* were fed daily. The size at first reproduction (SFR) and the clutch size was measured for each egg-bearing individual (from the top of the eye to the base of the tailspine) with the aid of a dissecting microscope equipped with a digital camera (Imaging Source) and image analysis software. A mean SFR was calculated for each replicate; these mean values were used to calculate the respective mean value and the variance for the treatment. Somatic growth rates were calculated as according to *Wacker & Von Elert* (2001) (Wacker and Von Elert 2001):

$$g = [(\ln (dw_t) - \ln (dw_0)]/d,$$

in which *dw* is the body dry weight of a subsample of the animals at the beginning (dw_0) and end (dw_t) of the experiment and *d* is length of the experiment in days. Mean individual dry weights were mean values of three individuals (dw_0 is the same for all treatments, and dw_t is specific for each replicate).

Sample preparation

Thirty individuals of D. *magna* per biological replicate for each treatment (exposed to control extract in the presence of dim light, exposed to fish kairomone extract in the presence of dim light or exposed to fish kairomone in the absence of light) were homogenized with a pestle in 200 µl lysis buffer containing protease inhibitors (Tab 2) for the protein extraction. The *Daphnia* homogenate was centrifuged (14000 rpm, 4 °C, 15 min), and the supernatant containing the proteins was immediately used for a chloroform-methanol precipitation (Wessel and Flugge 1984) to purify the protein samples. The obtained pellet was resuspended in 20 µl 0.1 M triethylammonium bicarbonate buffer (TEABC, with 2% SDS, pH 8.5) and quick-frozen in liquid nitrogen. All protein samples were kept on ice during the extraction process to decrease the

proteolytic activity. The protein concentration was measured with the Pierce 660 nm Protein Assay (Thermo Scientific) prior to the digestion and normalized to 70 µg in 20 µl for every sample.

Table 2. Lysis buffer

Substance	Concentration	Amount
Urea	7 M	21.0 g
Thiurea	2 M	7.6 g
TRIS	10 mM	60.5 mg
DTT	20 mM	
Protease Inhibitor Cocktail (Roche)		1 tablet
Pharmalyte (pH 3-10)	2%	1.0 ml
CHAPS	4%	2.0 g
	add 50 ml ultrapure water	
	DTT was added before use	

Western Blot for the documentation of proteolytic activity

For this documentation of the proteolytic activity the protein samples were treated like described above (sample preparation) and samples were spiked with Sulfit oxidase (SO) to a concentration of 30 ng µl $^{-1}$ prior to the protein extraction. We used the following samples: The homogenate of thirty individuals *D. magna* (D), *E. coli* lysate (E), only lysis buffer (C) (see supplementary Figure 1). For SDS-PAGE samples were denatured with 5x SDS-loading dye (50% glycerol, 3.5% SDS, 15% β-mercaptoethanol, 0.02% bromophenol blue) and heating for 5 min at 95°C prior to electrophoresis. The gel was loaded with 30 ng SO per sample and 30 ng of pure SO without any sample preparation was added as control (SO, see supplementary Figure 3). Proteins were electrophoretically transferred from the acrylamide gel to an Immobilon Polyvinylidenedifluoride (PVDF) membrane with a semi-dry blotting system (C.B.S. Scientific). Blotting was performed in transfer buffer (25 mM Tris, 192

mM glycine, 10% methanol) with three sheets of whatman paper on each side of the gel and membrane. After blocking in 5% (w/v) milk powder for 1 hour the blots were incubated with a primary antibody. SO was detected using secondary antibodies coupled to horseradish peroxidase and visualized by Super Signal West Pico Chemiluminescent Substrate (Thermo Scientific) according to the manufacturer. The signals were detected with an Enhanced Chemiluminescence Camera System (Decon) and protein bands were quantified with ImageJ software.

Digestion

Samples normalised to 70 µg protein in 20 µl were sonicated three times for 10 s. Two µl 100 mM DTT were added, and the samples were boiled to 96 °C for 10 min in TEABC buffer. After cooling down to room temperature, cysteines were alkylated with 2 µl 250 mM iodine acetamide. Samples were stored for 30 min in the dark at room temperature. Excess iodine acetamide was quenched with 0.5 µl 100 mM DTT. 200 µl 8M Urea with 0.1 M TEABC were added and the samples were transferred to Amicon Ultra™ centrifugal filter units (0.5 ml, 10K). Filter assisted sample preparation (FASP) with sequential Lys C (0.5 µg) and trypsin (1.75 µg) digestion was performed according to (3) with minor modifications: Briefly, 0.1 M TrisHCl was replaced by TEABC in all buffers used. After digestion the samples were centrifuged and filtrates were collected. 150 µl 5 % acetonitrile in water were added to the filter, mixed with the residual sample and the units were centrifuged once more. C18 SPE cartridges (Strata-C18, 1 ml, Phenomenex) were activated with Methanol, washed with 80 % acetonitrile and equilibrated to 5 % acetonitrile in 0.1 M TEABC. Combined filtrates were applied, the cartridge were washed two times with 300 µl 5 % acetonitrile in 0.1 M TEABC and the peptides were eluted with 300 µl 80 % acetonitrile in 0.1 M TEABC. One third (100 µl) of the eluates were dried in a vacuum centrifuge and redissolved in 10 µl 0.5 M TEABC for ten minutes at room temperature

iTRAQ labelling

All reagents were used at room temperature. 70 µl ethanol were added to the iTRAQ reagents (AB Sciex) and 25 µl were used for each precipitated sample containing approximately 6 µg peptides in 10 µl 0.5 M TEABC. After 2 h in the dark the reaction was quenched with 50 µl water and the labelled samples were combined. Volumes

were reduced to approximately 20 µl in a centrifugal evaporator and 200 µl ultrapure were added. STRATA C18 cartridges were used for sample clean-up as described above. However, this time, TEABC was replaced by 0.1 % formic acid in all steps. Desalted peptides were dried in a centrifugal evaporator and resuspended in 40 µl of ACN in 0.1 % formic acid.

SCX HPLC

Strong Cation Exchange (SCX) Chromatography was performed on a Biobasic SCX column (1 mm x 150 mm, Thermo) installed to an Ettan micro LC system (GE Healthcare) operated at a flow rate of 50 µl/min and 214 nm fixed wavelength detection. Buffer A was 25 % acetonitrile in 0.1 % formic acid adjusted to pH 3.5 with NaOH. Buffer B was 0.5 M NaCl in buffer A. Samples redisolved in 40 µl of 40 % acetonitrile in 0.1 % formic acid were injected onto the column equilibrated in buffer A. Peptides were separated using a gradient of 0 % B for 1.5 min, 0%- 10 % B in 4 min and 10% - 80 % B in 8 min. Fractions were collected every 60 sec. 6 fractions were dried in a vacuum centrifuge, redissolved in 50 µl 5 % acetonitrile in 0.1 % TFA and used for subsequent LC-MALDI analysis.

MALDI spotting

Reversed phase nanoHPLC of SCX fractionated peptides was carried out on an Eksigent nanoLC 1D plus system (Axel Semrau GmbH, Sprockhövel, Germany) using a vented column setup comprising a 0.1-mm-by-20-mm trapping column and a 0.075-by-200-mm analytical column, both packed with ReproSil-Pur C18-AQ, 5 µm (Dr. Maisch, Ammerbuch, Germany) and operated at 40 °C. 18 µl sample were aspirated into the sample loop and a total of 30 ul was loaded onto the trap column using a flow rate of 6 µl/min. Loading pump buffer was 0.1 % TFA. Peptides were eluted with a gradient of 5 % to 35% acetonitrile in 0.1% TFA over 70 min and a column flow rate of 300 nl/min. 0.7 mg/ml alpha-Cyano-4-hydroxycinnamic acid (HCCA) in 95 % acetonitrile in 0.1 % TFA, 1 mM ammonium phosphate were fed in using a syringe pump operated at 150 µl/h and a post column T-union. 384 fractions (10 seconds) were deposited onto a MTB AnchorChip 384-800™ MALDI target (Bruker Daltonics, Bremen, Germany) using Eksigent MALDI spotter (Axel Semrau GmbH, Sprockhövel, Germany).

LC-MALDI MS/MS analysis

MALDI MS and MS/MS analysis were carried out on an Ultraflextreme™ MALDI Tof Tof mass spectrometer (Bruker Daltonics) operated with a laser repetition rate of 1 Ghz. The process of data acquisition was controlled by Flexcontrol 3.0 and WarpLC. MALDI MS spectra were acquired over a mass range from 700 Da – 4000

Da. Spectra were calibrated externally using the Peptide Calibration Standard II (all Bruker Daltonics) on the designated target calibration spots. The laser was used with fixed energy setting and 3000 shots/spectrum were collected from random raster points. Precursor ions with signal to noise ratios equal or better than 10 were selected for MS/MS analysis. Identical peaks in adjacent spots were measured only once, preferentially from the spot with maximum peak intensity. The maximum number of MS/MS spectra per spot was limited to 20. However, this value was exceeded if no alternative positions had been available. Peaks appearing on more than 40 % of all spots were ignored. MS/MS spectra (3500 shots) were acquired with the instrument calibration and iTRAQ reporter ions as well as peptide immonium ions were used for internal recalibration.

Database searches and analysis of identified proteins

Daphnia Pulex (Tax ID 6669) entries (32413 sequences, 10481177 residues) were extracted from the NCBInr (Release of February 2. 2014). A composite database was generated with the Perl script “makeDecoyDB” (Bruker Daltoniks, Bremen, Germany), which added a shuffled sequence and a tagged accession number for each entry. The tagged decoy entries were used for the calculation of false positive rates in Proteinscape 3.0. Searches were submitted via Proteinscape (Bruker Daltoniks, Bremen, Germany) to MASCOT 2.2 (Matrix Science) and the following parameter settings were used for database searches: enzyme “trypsin” with 1 missed cleavage; fixed modifications “carbamidomethyl” ,“iTRAQ4plex (K)”, “iTRAQ4plex (N-term)”; optional modifications “Methionine oxidation”, “iTRAQ4plex (Y)”, “Gln->pyro-Glu (N-term Q)”. The mass tolerance was set to 15 ppm Da for MS and 0.8 Da for MS/MS spectra. Protein lists were compiled in Proteinscape. Peptide hits were accepted when the ion score exceeded a value of 25. Protein hits required at least

one peptide hit exceeding a peptide score of 40. Using this set of search parameters, the false positive rate was below 0.5 % on the protein level.

For the analysis of the identified hypothetical *Daphnia* proteins, all accession numbers were searched in the NCBI Conserved Domain Database (CDD) (Marchler-Bauer et al. 2011), which allows for the annotation of functional proteins.

Data analysis and statistics

A one-way analysis of variance (one-way ANOVA) was conducted for the life-history experiments. The dependent variables were checked for homogeneity of variances (Shapiro-Wilk). The effect of single treatments was tested by post hoc tests (Tukey's HSD multiple comparison test or Student-Newman-Keuls method) at the same probability level as the respective analysis of variance. A significance level of $p = 0.05$ was applied to all statistical analyses. All statistics were performed with SigmaPlot v11.0 (Systat Software) and STATISTICA v6.0 (Starsoft Inc.). All statistics for the results of LC-MS/MS analysis were performed with Excel® (Microsoft® Office 2007).

Results

Life-History and growth experiments

D. magna were grown in light in the presence of purified extract from control water or in the presence/absence of light in the presence of purified extract from fish incubation water. The experimental animals showed a reduction in the size at first reproduction (SFR) from 2.66 mm (control extract) to 2.58 mm (kairomone extract) in the presence of light. No reduction in SFR in reponse to fish kairomones was observed in the absence of light (Figure 1a). The SFR was affected by the kairomone only in the presence of light (one-way ANOVA, $F_{1,8}$: 10.533, $p < 0.05$). A kairomone-mediated increase in the clutch size from 7.80 to 9.23 eggs was observed in the presence of light (one-way ANOVA, $F_{1,8}$: 5.280, $p < 0.05$). The clutch size was not affected in the absence of light (Figure 1b). The juvenile somatic growth rates of *D. magna* were neither affected by the presence of kairomone nor by the light conditions (one-way ANOVA, $F_{1,8}$: 1.127, $p = 0.384$, data not shown).

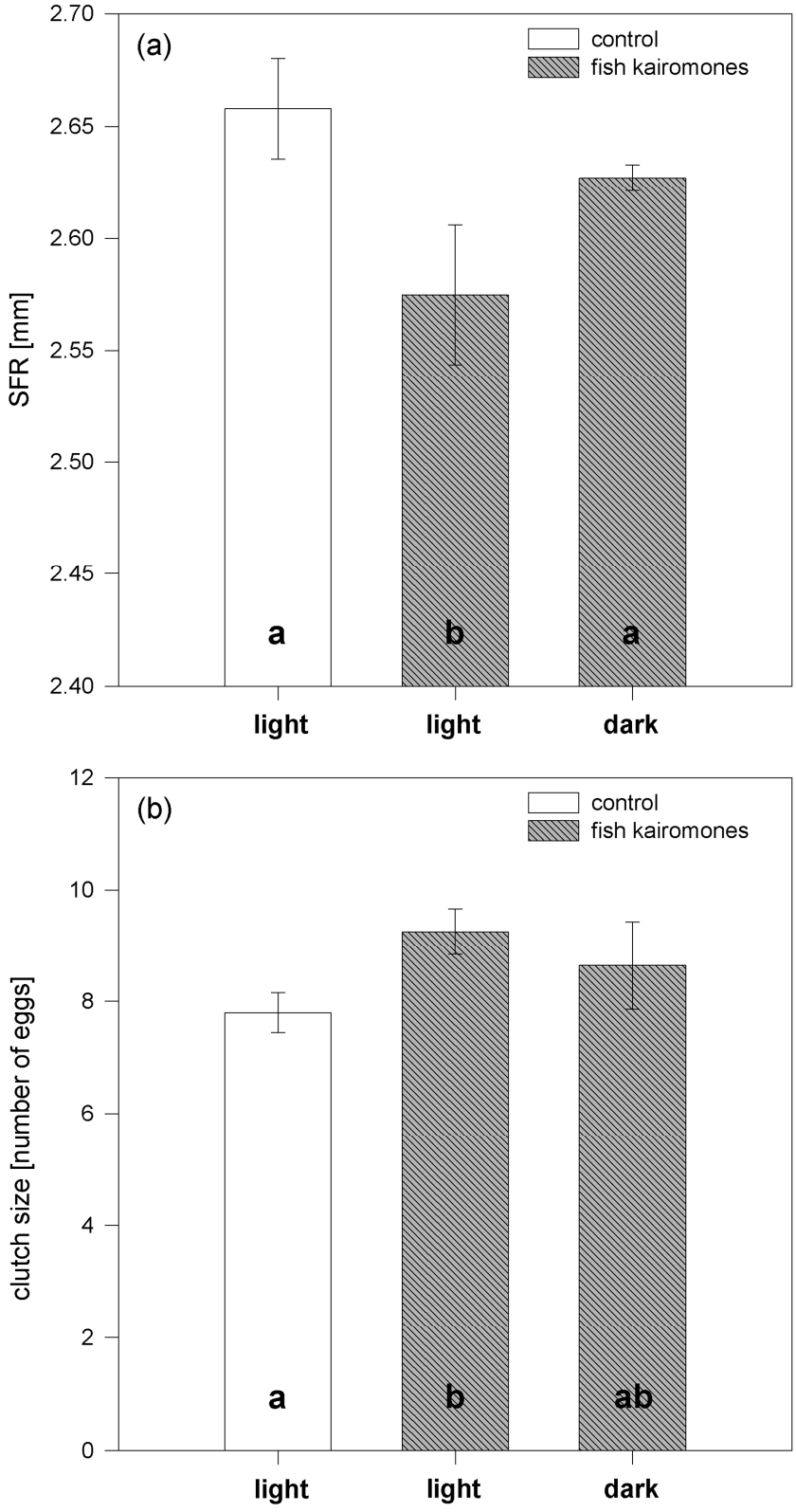

Figure 1. Size at first reproduction (SFR) and clutch size of *D. magna* grown in light without kairomones (control) or with fish kairomones in the presence/absence of light. *Daphnia* were exposed to control extract (open bars) or to fish kairomone extract (shaded bars) in light or dark. (a) SFR (n = 3, mean ± SE), (b) clutch size (n = 3, mean ±SE). Different letters indicate a significant difference ($p < 0.05$) between the treatments.

Proteomics

Qualitative analyses

The treatments were run in triplicate. The proteins of thirty *D. magna* individuals per biological replicate were extracted, and one set for the LC-MS/MS analyses consisted of protein samples of experimental animals that were exposed to three treatments: control extract and light, fish kairomone extract and light, or fish kairomone extract and no light. The iTRAQ labeling strategy was that these protein samples were differentially labeled with an iTRAQ reagent according to their treatment to observe the differences between the three proteome states. For every set of samples, the proteins were identified (set A: 601, set B: 741, set c: 593) (Figure 2), and only the 428 proteins that were present in all sets were used for the subsequent quantitative analyses (Figure 2). Quantitative analyses were based on in vitro labeling of the three proteomes of *D. magna* that showed different responses in the life-history experiments mediated by kairomones and light conditions (Figure 1) to gain information about the relative proportion of proteins that are involved in the observed anti-predator response.

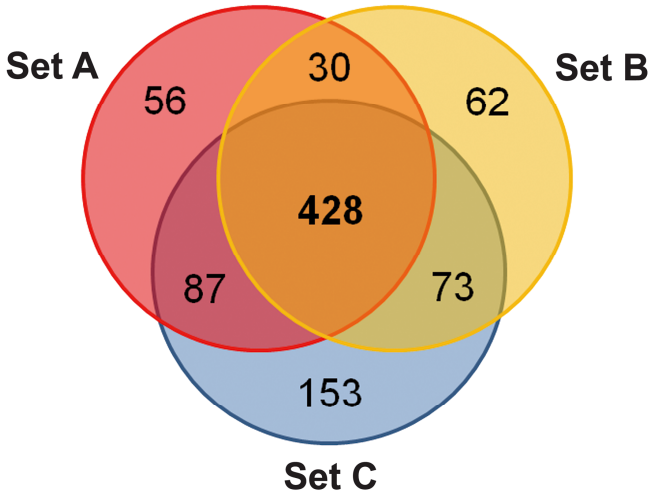

Figure 2. Venn diagram showing the overlaps of identified proteins of the qualitative comparative proteomics of three *D. magna* proteome sets. One set represents biological replicates each consisting of one proteome of *D. magna* exposed to fish kairomones and control extract in the presence of light and exposed to fish kairomones in the absence of light.

Quantitative analyses

Tryptic peptides from the samples extracted from the experimental animals of each of the three treatments were used for the iTRAQ protocol. A considerable number of proteins were identified (Fig. 2), and an average change ratio (CR) was calculated. This change ratio was derived from the $\log_2$ of all iTRAQ ratios for the identified proteins for every biological replicate of the treatments fish kairomones in the presence of light (FL) and fish kairomones in the absence of light (FD) compared to the control in the presence of light (C) (see Tab. 1). To ensure that the observed results were robust across the biological variations, we excluded all proteins that were not identified in all three sets, and only those proteins were included, which showed a CR of more than 2 SD in at least two of the three biological replicates (Fig. 3).

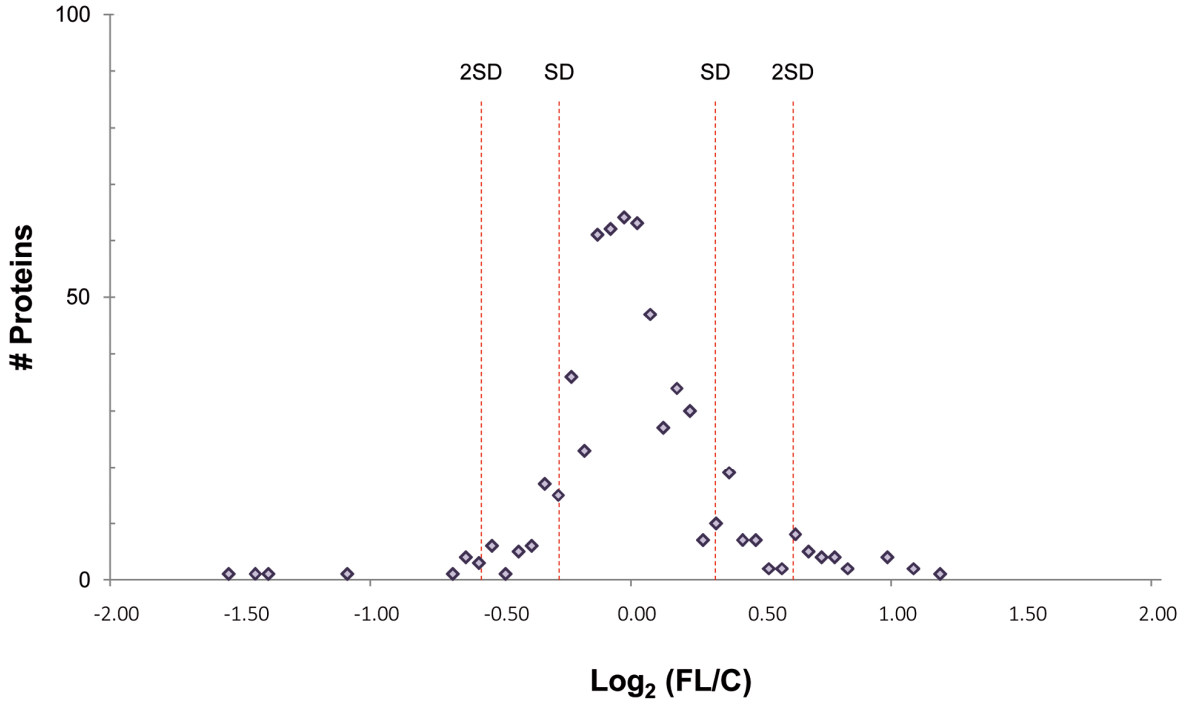

Figure 3. A diagram exemplarily showing the $\log_2$ ratio (change ratio) of proteins of one biological replicate, which were expressed between one proteome of *D. magna* exposed to fish kairomones (FL) and control extract (C) in the presence of light. The vertical lines indicate the one and twofold standard deviation. Only proteins that exceeded the ± 2SD were included in the quantitative analyses for the calculation of the average change ratio (CR) between the treatments.

In total, twenty proteins were found, which were differentially expressed in the proteome of *D. magna* upon exposure to fish kairomones in comparison to the control extract in the presence of light (Tab. 1). Among these protein sets, five proteins proved to be differentially expressed in the proteome of *D. magna* exposed to fish kairomones and no light compared to the control extract in the presence of light (Tab. 1).

The largest group of proteins that showed kairomone-mediated higher differential expression belonged to the group of ribosomal proteins and proteins that are involved in the vitellogenesis (Tab. 1).

Table 1. Quantitative proteomics of *D. magna* exposed to fish kairomones in the presence of light (FL) and exposed to fish kairomones in the absence of light (FD) normalized to control extract in the presence of light (C). The iTRAQ analyses were performed in triplicate.

Proteins		Average change ratio (CR)[c]	
Accession[a]	Conserved domains[b]	FL/C (SD)[d]	FD/C (SD)[d]
	Ribosomal proteins		
EFX77166	Ribosomal protein L15	1.00 (0.28)	0.34 (0.03)
EFX75660	60S ribosomal Protein L36	1.00 (0.23)	
EFX70638	60S ribosomal Protein L37	0.94 (0.10)	
EFX67939	Ribosomal protein L19	0.90 (0.02)	
EFX88196	60S ribosomal protein L21	0.73 (0.05)	-0.24 (0.47)
EFX89085	Ribosomal protein L32	0.71 (0.18)	
EFX86254	Ribosomal protein S6	0.71 (0.43)	
EFX70971	Ribosomal protein L13	0.69 (0.16)	
EFX73371	Ribosomal protein L7	0.67 (0.46)	0.13 (0.29)
EFX84946	60S Ribosomal protein L23	0.62 (0.17)	
EFX89778	Ribosomal protein L25	0.59 (0.46)	
	Vitellogenesis		
EFX71833	Vitellogenin	0.92 (0.57)	0.46 (0.29)
EFX71901	Lipoprotein N-Terminal	0.60 (0.37)	
	Others		
EFX88086	Globin/Myoglobin	0.74 (0.38)	0.50 (0.05)
EFX80557	Guanine nucleotide binding protein	-0.37 (0.21)	
EFX66583	Peptidase M14	-0.39 (0.18)	
EFX75804	Rag GTPase	-0.41 (0.33)	
EFX79335	Helicase	-0.50 (0.10)	
EFX82001	elF3_N	-0.62 (0.32)	
EFX73805	Actin	-0.63 (0.24)	

a) Accession number of the identified proteins in the NCBI database (released Feb 2.2014).
b) Functional proteins derived from the NCBI Conserved Domain Database (CDD).
c) Average change ratio of each protein against the control treatment, calculated by the average between three biological replicates.
d) SD for the average change ratio.

Proteolytic activity

After the Western Blot (Figure 4) the pure control protein sulfit oxidase (lane SO) and the control protein in lysis buffer (lane C) showed only one protein band. This pattern was not affected when the sulfit oxidase had been incubated in *Daphnia* homogenate (lane D), which demonstrates the absence of detectable proteolytic activity in the homogenate. For reference proteolysis a lysate of the widely used *E. coli* was included as protein sample (lane E). Here as well no evidence for degradation of the control protein was visible. Note, that the additional upper band in the lane with the *E. coli* lysate (E) at 70 kDa corresponds to another unprocessed sulfit oxidase that is present in *E. coli* (Kappler 2011).

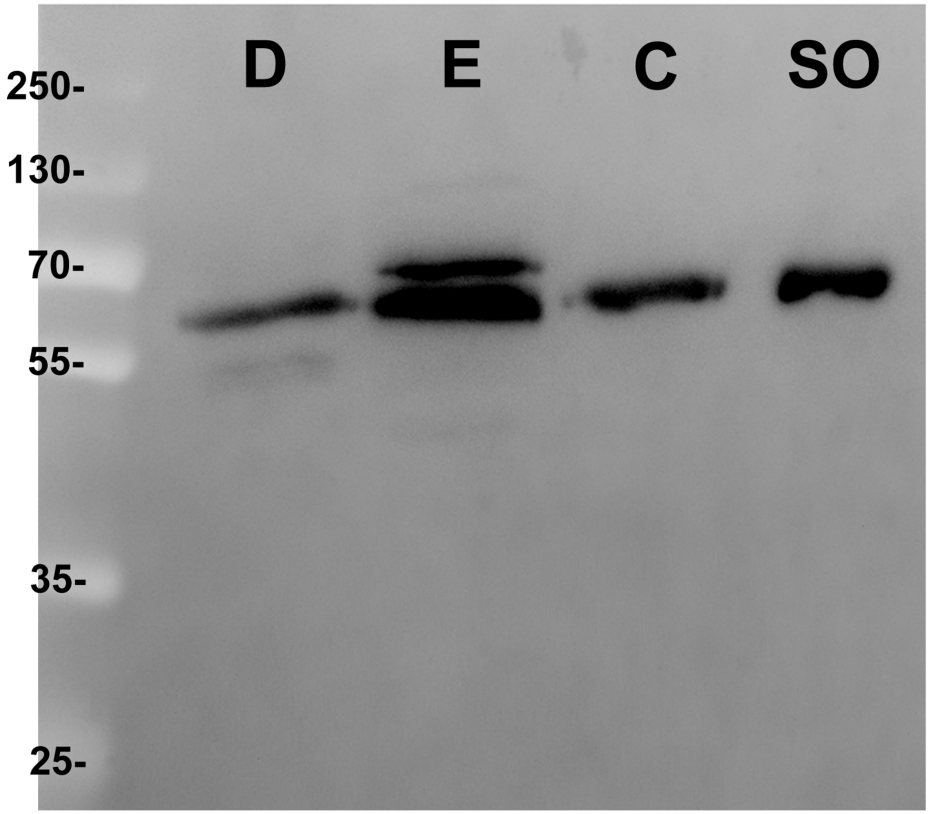

Figure 4. A Western Blot demonstrating the absence of proteolytic activity during the protein extraction protocol that was used for the proteome study presented here. Lane D corresponds to *Daphnia* homogenate with the control protein (sulfit oxidase). Lane E corresponds to E. coli lysate with control protein. Lane C corresponds to control protein with lysis buffer only. All samples were spiked with the control protein (sulfit oxidase) before and treated like it is described in the protocol. Lane SO corresponds to pure control protein (sulfit oxidase).

Chapter 3

Discussion

Inducible defences are widespread in plants and animals. *Daphnia magna*, an important component of freshwater food webs and a well-established model organism in ecology, ecotoxicology, and evolutionary research has become a textbook example for inducible defences in response to chemical signals released from predators. *Daphnia* as major prey organism for planktivorous fish show a wide range of anti-predator defences, e.g. changes in life-history (LHC), morphology and behaviour (Lampert 1994). These defences were shown to be inducible by chemical cues released by fish, so-called kairomones (Larsson and Dodson 1993). Despite these known phenotypic responses in *Daphnia*, the underlying molecular biology of inducible defences against fish has not been investigated. This study was designed to find an explanation for the phenotypic changes on the protein level of a known kairomone-induced defence strategy in the model organism *Daphnia*, that elicits the ecological role of phenotypic plasticity in this specific environmental conditions (Miner et al. 2005). Fish kairomone induced life-history changes (LHC) in *Daphnia* have only recently been shown to be suppressed in the absence of light (Effertz and Von Elert 2014). Here, in accordance with the literature, the experimental animals exposed to fish kairomones showed the expected significant decrease in SFR in the presence of light (Weider and Pijanowska 1993; Stibor and Navarra 2000), and in accordance with the recent findings by *Effertz & von Elert* (2014), this life-history change was not observed in the absence of light (Fig. 1), whereas the somatic growth was not affected (data not shown). Interestingly, we observed a significant increase in the clutch size of daphnids that were exposed to kairomones in the presence of light and a lower, albeit not significant, increase in the absence of light (Fig. 1).

Here we conducted the proteomics with daphnids from the above discussed life-history experiments with the aim to identify proteins involved in the observed LHC. The large intestine of Daphnids might lead to high proteolytic activity during protein extraction prior to the proteome analysis. However, we could demonstrate that no negative effect of proteolysis occurred during our extraction of *Daphnia* proteins (supplementary Fig. 1). The largest group of identified kairomone-responsive proteins

in the presence of light that were differentially higher expressed belonged to the group of ribosomal proteins (Tab. 1). It is known, that the insect fat body plays a central role in energy storage and utilization and is also crucial for oogenesis (Arrese and Soulages 2010). In *Daphnia* the carbon assimilation is not affected by fish kairomones (Stibor and Machacek 1998), but an earlier allocation of assimilated resources into yolk protein synthesis has been observed (Stibor 2002). Since the fat body as central storage depot has to respond to metabolic requirements, it is involved in the regulation of protein synthesis (Arrese and Soulages 2010) and it is obvious, that the observed upregulation of ribosomal proteins in *Daphnia* is related to an increased protein synthesis, which is needed for the production of yolk proteins in the fat body. For *Drosophila* it was shown, that protein synthesis in the fat body is influenced by heat shock conditions (Johnson et al. 2009), and our data indicate that the fish kairomone as environmental stressor as well affects protein synthesis in the *Daphnia* fat body.

In line with the findings that fish kairomones lead to an earlier onset of yolk protein synthesis in *Daphnia* (Stibor 2002), we could show a higher differential expression of proteins (vitellogenin and lipoprotein) related to the vitellogenesis (Tab. 1) in daphnids that were exposed to fish kairomones in the presence of light. This effect was smaller in the absence of light, which corresponds to the results of the life-history experiments, as animals exposed to kairomones in the absence of light showed a smaller and not significant increase in clutch size (Fig. 1). Vitellogenins as major yolk proteins are synthesized in the fat body and are precursors of the egg storage protein vitellin (Tufail and Takeda 2008). Most of the studies on vitellogenins (Vgs) were done in insects (for a review see Tufail and Takeda (2008)). It is known that in hemimetabolous insects, after synthesis, the Vgs are cleaved into several peptides to facilitate the export from the fat body to the oocytes. This post-translational proteolytic cleavage of Vgs differs between insect groups and might also be different in *Daphnia*, which would explain, why we were not able to detect any known vitellin or vitellin-related protein. Although we were not able to identify vitellin or vitellin-related proteins in our sets we could identify one peptidase (Tab. 1), which might play a role in the post-translational cleavage of Vgs in Daphnia. The other identified protein involved in vitellogenesis is a lipoprotein (Tab. 1), that showed the

same differential expression pattern as vitellogenin in the presence and absence of light. Lipoproteins are involved in the lipid transport in insects and play an important role for the transport of lipids out of the fat body to the oocytes (van der Horst et al. 2002; Soulages and Wells 1994). However, Otte *et al.* (2014) observed the presence of different vitellogenin-related proteins in *D. magna* embryos exposed to *Triops* kairomones, and two of them showed a higher abundance indicating as well an influence of the invertebrate kairomone on Vgs. Since two groups of fish kairomone-responsive proteins in *Daphnia* that were differentially expressed (proteins involved in the protein synthesis and vitellogenesis) are involved in metabolic pathways, which were shown to be connected to the well-studied fat body in insects (Tufail and Takeda 2008), it is reasonable to assume that the role of the fat body as central tissue for protein synthesis and vitellogenesis in *Daphnia* might be comparable. Nevertheless, this the first study that reveals the involvement of these proteins in *Daphnia* as response to fish kairomones.

Pijanowska & Kloc (2004) have shown that the protein concentration of actin in *Daphnia* decreased upon exposure to fish kairomones. This finding is corroborated by the proteome analyses here as actin was exclusively lower expressed in animals exposed to fish kairomones compared to control extract in the presence of light (Tab 1). The integrity and the remodelling of the cytoskeleton were shown to be affected by environmental stressors and to be affected by physiological processes in different animal systems (Furukawa et al. 1995; Houle et al. 2003; Kelley et al. 1997; Liang and Macrae 1997). Actins were also found to be affected in *Daphnia* that were exposed to *Triops* kairomones, an invertebrate predator (Otte et al. 2014). Based on these results we hypothesized that actin as highly dynamic component of the cytoskeleton may play a major role in the observed decrease of SFR (Weider and Pijanowska 1993) and that may hence be regulated in response to fish-born kairomones in *Daphnia*. The observed lower differential expression of this known target protein for fish kairomones in this proteomics study in line with earlier immunological findings that did not distinguish between the different isoforms of actin (Pijanowska and Kloc 2004) validates our state-of-the art analyses. We could observe a kairomone-responsive higher differential expression of globin/myoglobin (Tab. 1) and it was shown, that the proteome of *Daphnia* responded to hypoxia with a

strong up-regulation of hemoglobin leading to enhanced oxygen affinity (Zeis et al. 2009), even during short episodes of tissue hypoxia. It was suggested that this proteome response in *Daphnia* due to hypoxic conditions leads to a maintenance of ATP production (Zeis et al. 2009) and the up-regulation of globin/myoglobin described here may point towards a high energy demand of the metabolism for the protein synthesis during the response to fish kairomones. More regulated proteins (Tab. 1) were indentified (e.g. peptidases, proteases). Overall twenty proteins were found to respond to fish kairomones and 16 out of twenty differentially expressed functional proteins of the responsive protein set could lead to a more detailed view of the kairomone-induced response in *Daphnia*.

Our findings indicate that in *Daphnia* fish kairomones lead to an increase of the protein synthesis. This leads to an earlier and higher extent of vitellogenesis, which goes along with an increased allocation of resources into reproduction. This allocation of resources into vitellogenesis happens at the expense of allocation to somatic growth and thus results in smaller SFR (Fig. 1), which is reflected at the protein level by the upregulation of vitellogenesis related proteins and ribosomal proteins as well as by the lower expression of the structural protein actin (Tab. 1). We could identify sets of kairomone-responsive proteins which are differentially expressed to different extents (Tab. 1). One set of kairomone-responsive proteins showed a higher average change ratio in the presence of light, and an overlapping set of these proteins showed a lower average change ratio in the absence of light. The observed differential expression of the protein set in the absence of light reveals that, even in the absence of light, *Daphnia* responds physiologically to fish kairomones (e.g. by upregulation of vitellogenesis), although the daphnids are not endangered by visually hunting fish and do not decrease in SFR. It seems reasonable to assume that this modest higher differential expression observed in the dark is further increased in light, so that a certain threshold is exceeded that is necessary to lead to significant changes in life history parameters (i.e. reduced SFR, increased clutch size) observed in the presence of light only (Effertz and Von Elert 2014).

The results obtained here demonstrate that proteomics contributes to better understanding of underlying molecular mechanisms in plasticity of the model organism *Daphnia*. For the first time we have combined established life-history experiments with high throughput proteomics in an ecologically relevant predator-prey system, and identified groups of functional proteins, which are involved in phenotypic changes in *Daphnia* induced by fish kairomones. This is a promising approach to identify the underlying physiology of inducible defences in the model organism *Daphnia*, which might pave the road for similar proteomic approaches in other inducible anti-predator defences in *Daphnia* to understand the physiology and evolutionary background of these evolved strategies (Miner et al. 2012).

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Concluding remarks and perspectives

Inducible defences are widespread in plants and animals. *Daphnia magna*, a well-established model organism in ecology, ecotoxicology, and evolutionary research has become a textbook example for inducible defences in response to chemical signals released by predators (Lampert 2006). *Daphnia* as major prey organism for planktivorous fish show a multitude of defences against predators including changes in life-history (LHC), morphology and diel vertical migration (DVM, Lampert 1994), which have been shown to be induced or enhanced by chemical cues released by predators, so-called kairomones (Larsson and Dodson 1993). In *Daphnia*, the DVM is initiated by a relative rather than by an absolute change in light intensity (Ringelberg 1991), and the velocity of upward and or downward migration in DVM is linearly related to a decrease or increase of this relative rate of change in light intensity (Ringelberg 1991; van Gool and Ringelberg 1997; van Gool and Ringelberg 2003). If fish are present in the system, then the kairomones are dispersed over the epilimnion all the time. Thereby, the kairomones indicate predator presence, but provide neither a cue for the timing nor for the direction of the migration. However, it was shown that the presence of fish kairomones leads to an increase in vertical displacement velocity (van Gool and Ringelberg 1998a; van Gool and Ringelberg 1998b) and thus alters the amplitude of DVM (van Gool and Ringelberg 1997; van Gool and Ringelberg 2003). Thus, the fish kairomone only does not induce DVM behavior in *Daphnia*, but also enhances the amplitude of migration caused by changes in the relative light intensity. Despite the well-known relevance of light for the induction of DVM, investigations of the induction of LHC have been confined to fish kairomones only (Weider and Pijanowska 1993; Stibor and Lüning 1994; Von Elert and Stibor 2006). It was demonstrated that fish kairomones do not affect carbon assimilation (Stibor and Machacek 1998) but lead to earlier allocation of assimilated resources into vitellogenin (Stibor 2002). This earlier allocation of resources into vitellogenin synthesis happens at the expense of allocation to somatic growth and thus results in smaller size at first reproduction (SFR). The role of light as environmental factor for LHC in response to predator kairomones might easily have been overlooked in the aforementioned studies, as all these investigations were performed in the presence of light.

It is hypothesized in **chapter 1** that the light level a given *Daphnia* genotype is exposed to during a large part of the day determines the degree of LHC: low light levels would lead to no or very low LHC, and high light levels would lead to stronger LHC in response to kairomones from fish. The results of the life-history experiments in **chapter 1** show for the first time that only in the presence of both light and fish kairomones a reduction of SFR in *Daphnia* was observed, demonstrating the interactive effect of the two cues. These results suggest that LHC in *D. magna* occurred only in the presence of light suggests that a coupling between fish kairomone and LHC is modulated by light. When *Daphnia* clones capable of using both defences were prevented from migration, they showed a decrease in SFR (Slusarczyk and Pinel-Alloul 2010), indicating that a higher light intensity leads to a higher degree of life-history shifts. The observations described in **chapter 1** point at a plastic coupling of anti-predator defences modulated by light intensity in *Daphnia*. I argued that the light intensity an individual *Daphnia* is exposed to results from its daytime residence depth, i.e. its DVM amplitude, which thereby determines the degree of LHC. The experimental animals exposed to fish kairomones in the presence of light were supposed to simulate weak migrators with a daytime residence in the epilimnion (`light`), whereas the experimental animals in the absence of light simulate strong migrators with a daytime residence in the hypolimnion (`dark`). Based upon the findings in **chapter 1** I assumed that in a scenario in which *Daphnia* are forced to descent deep under heavy predation pressure by fish, the amplitude of DVM alters the light level that a given *Daphnia* genotype is exposed to. This assumption proposes an inverse coupling of LHC and DVM, which would be especially adaptive in habitats that allow for DVM only during the parts of the season, i.e. spring; later in the season, when a deep-water refuge is no longer available due to anoxia in the hypolimnion, alternative defences such as a decrease in SFR may be required.

In **chapter 1**, I elucidated the effect of presence and absence of light on LHC in *D. magna* under constant light conditions, whereas in the field animals are subjected to alternating periods of light and dark with different light intensities under non-migrating conditions. In line with **chapter 1** it was reasonable to assume that these findings, light is crucial for the induction of LHC in *Daphnia*, could also be applied in a more

ecological context concerning the different light conditions animals are exposed to in the field. In **chapter 2**, the daphnids were exposed to five different light intensities, which were supposed to be comparable with light intensities measured during a season in an oligotrophic lake in central europe (Tilzer *et al.* 1995). The five light intensities used in **chapter 2** correspond approximately to light regimes of this lake in a range from the surface to the depth of 20 m in February and from the surface to a depth of 8 m in May (Tilzer *et al.* 1995). In addition, the intermediate light intensity used in the experiments of **chapter 2** corresponds to the light intensity of the treatment `light`, to which the animals were exposed in **chapter 1**. I addressed the question, whether a gradient of light intensity would influence the extent of LHC in *Daphnia* exposed to fish kairomones; in particular, if increasing light intensities will lead to stronger LHC or if a certain light intensity threshold for the induction of LHC in *Daphnia* exposed to fish kairomones exists. **Chapter 2** demonstrates that LHC in *Daphnia* exposed to fish kairomones were only affected in the intermediate and the two higher light intensities and reveals a light intensity threshold, which is crucial for the induction of LHC in *Daphnia*. The results described in **chapter 1** and **2** corroborate with observations that *Daphnia* clones that were collected at daytime from the epilimnion showed a smaller SFR (De Meester and Weider 1999). These observations provide evidence for the assumption that the amplitude of DVM alters the light intensity a given Daphnia genotype is exposed to, which allows for a plastic coupling of both DVM and LHC modulated by the light intensity. The potential to deploy more than one defence is widespread in *Daphnia* (Boersma et al. 1998; Slusarczyk and Pinel-Alloul 2010), and the mechanistic explanation suggested in this study describes for the first time a new mode of possible adaptation of multiple alternative deployable defences in *Daphnia* as response to fish kairomones. It was shown that the DVM amplitude differed between *Daphnia* genotypes (De Meester et al. 1994) and it remains to be seen if genotypes differ with respect to light intensity thresholds for the induction of LHC. Furthermore, animals in the field are subjected to alternating periods of light and dark under non-migrating conditions and it deserves further investigation on which extent the induction of LHC in *Daphnia* is controlled by a certain light period.

Despite these known phenotypic responses against predator kairomones and the new mechanistic explanation for a possible plastic coupling of defences modulated by the light intensity in *Daphnia* described here (**chapter 1** and **2**), the underlying molecular biology of inducible defences against fish has not been investigated. In order to elucidate the underlying molecular mechanisms, which might explain the fish-kairomone mediated phenotypic changes in *Daphnia* I combined the established life-history experiments in **chapter 1** and **2** with investigations on the gene level. I conducted the approach for fish kairomones effects on the gene expression of *Daphnia* with genes that were shown to be involved in the LHC response (Schwarzenberger et al. 2009; Pijanowska and Kloc 2004; Pauwels et al. 2007). These genes were announced to be candidate genes and expected to show a specific response in the time series (2h and 4h) after exposure to fish kairomones in our experiments. The final qPCR results in **chapter 1** revealed a strong response to fish kairomones and light on candidate gene expression in *Daphnia* after 2 h of exposure for one *actin* and *cyclophilin*. It has been shown that the mRNA level of HSP genes in the arthropod *Tribolium* exposed to UV radiation was strongly affected after 2 h and the mRNA level decreased after 3.5 h (Sang et al. 2012), supporting our observed gene response over time in *Daphnia*. The actin protein concentration in *Daphnia* was shown to be decreased after exposure to fish kairomones (Pijanowska and Kloc 2004) and as a dynamic component of the cytoskeleton, actin is supposed to play a major role in the decrease of SFR (LHC, Weider and Pijanowska 1993). *Cyclophilin*, the other gene that was significantly affected by fish kairomones and light, has well-known functions in the *Drosophila* system. In *Drosophila*, cyclophilin was shown to be also involved in the biogenesis of G-protein coupled receptors (GPCRs) influencing photoconversion and signal transduction in the optical system of the flies (Ferreira and Orry 2012). I demonstrated that the presence of light is crucial for the induction of LHC, and therefore the response of a gene that is putatively involved in the perception of light intensities differences in the optical system of *Daphnia* deserves further investigation. **Chapter 1** describes for the first time that the fish kairomone-mediated expression of candidate genes in *Daphnia* is affected by the light intensity, similar to the observed effect for the LHC.

In **chapter 2** I continued the approach for the effects on the gene expression of *Daphnia* with the *actin* gene that was shown to be highly responsive to fish kairomones in the presence of light (**chapter 1**) and might therefore serve as an indicator for the response on the gene level under exposure to the light intensity gradient. The qPCR data in **chapter 2** shows a significant increase of *actin* expression between two groups of light intensities indicating that the gene expression affected by fish kairomones showed the same pattern of light intensity dependency like the LHC did (**chapter 2**). My results suggest, that the established LHC and the expression of kairomone-responsive genes is differentially affected by different light intensities in the same way with the same light intensity threshold, as I could observe the strongest LHC and strongest gene expression at the high light intensities and no LHC responses and weaker gene expression at the two decreasing light intensities.

It is known that the actual phenotype of an organism is reflected in its proteome and a holistic proteomics approach could elucidate the role of unpredicted functional proteins that might explain phenotypic plasticity in *Daphnia*. **Chapter 3** describes for the first time a LC-MS/MS based proteomics approach that I conducted with the aim to identify functional proteins involved in the phenotypic changes on the protein level for the fish kairomone-induced LHC in *Daphnia*. In line with the findings in **chapter 1** the experimental animals exposed to fish kairomones showed the expected significant decrease in SFR in the presence of light and this LHC response was not observed in the absence of light. Interestingly, I observed a significant increase in the clutch size of daphnids that were exposed to kairomones in the presence of light and a lower, albeit not significant increase in the absence of light. This egg number increase with a concomitant decrease of SFR in daphnids exposed to fish kairomones might be explained by a very strong response to fish kairomones and might be due to the highly enriched fish kairomone extract, which I used in the life-history experiments in **chapter 3**. The daphnids from the above discussed life-history experiments were used for the high-throughput proteomics. The largest group of identified kairomone-responsive proteins in the presence of light that were differentially higher expressed belonged to the group of ribosomal proteins. In *Daphnia* the carbon assimilation is not affected by fish kairomones (Stibor and Machacek 1998), but an earlier allocation of assimilated resources into yolk protein

synthesis has been observed (Stibor 2002). Since it is known that the insect fat body is involved in the protein synthesis and is crucial for the oogenesis (Arrese and Soulages 2010), it might be obvious that the observed upregulation of ribosomal proteins in *Daphnia* is related to an increased protein synthesis, which is needed for the production of yolk proteins in the fat body. In line with the findings that fish kairomones lead to an earlier onset of yolk protein synthesis in *Daphnia* (Stibor 2002), I could show a higher differential expression of proteins (vitellogenin and lipoprotein) related to the vitellogenesis in daphnids that were exposed to fish kairomones in the presence of light. This effect was smaller in the absence of light, which corresponds to the results of the life-history experiments, as animals exposed to fish kairomones in the absence of light showed a smaller and not significant increase in clutch size. Vitellogenins (Vgs) as major yolk proteins are synthesized in the fat body and are precursors of the egg storage protein vitellin (Tufail and Takeda 2008). The other identified protein involved in the vitellogenesis is a lipoprotein. Lipoproteins are involved in the lipid transport out of the fat body to the oocytes (van der Horst et al. 2002; Soulages and Wells 1994). Since two groups of proteins in *Daphnia* that were differentially expressed (proteins involved in the protein synthesis and vitellogenesis) are involved in metabolic pathways, which were shown to be connected to the well-studied fat body in insects (Arrese and Soulages 2010), it is reasonable to assume that the role of the fat body as central tissue for protein synthesis and vitellogenesis in *Daphnia* might be comparable. Nevertheless, this is the first study that reveals the involvement of these proteins in *Daphnia* as response to fish kairomones. The finding that the protein concentration of actin in *Daphnia* decreased upon exposure to fish kairomones (Pijanowska and Kloc 2004) is corroborated by the proteome analyses in **chapter 3**, as actin was exclusively lower expressed in animals exposed to fish kairomones compared to the control extract in the presence of light. These results on the protein level for actin, in line with the results on the gene expression of *actin* (**chapter 1** and **2**), give even more evidence that actin as highly dynamic component of the cytoskeleton plays a major role in the observed decrease of SFR (Weider and Pijanowska 1993). In sum, twenty differentially expressed functional proteins were identified in **chapter 3** and further research concerning these proteins is deserved for an even more detailed view of the kairomone-induced response in *Daphnia*.

My findings in **chapter 3** indicate that in *Daphnia*, fish kairomones might lead to an increase of the protein synthesis. The increase of protein synthesis leads to an earlier and higher extent of vitellogenesis, which goes along with an increased allocation of resources into reproduction. This allocation of resources into vitellogenesis happens at the expense of allocation to somatic growth and thus results in smaller SFR (LHC), which is reflected at the protein level by the upregulation of vitellogenesis related proteins and ribosomal proteins as well as by the lower expression of the structural protein actin. I identified sets of proteins, which are differentially expressed at different extents. One set of kairomone responsive proteins showed a stronger response in the presence of light, and an overlapping set of these proteins showed a lower response in the absence of light. Even in the absence of light, *Daphnia* respond physiologically to fish kairomones (e.g. upregulation of vitellogenesis), although the daphnids are not endangered by visually hunting fish and do not significantly respond with LHC. I assumed that this modestly higher differential expression observed in the dark is further increased in light, so that a certain light intensity threshold is exceeded that is necessary to lead to significant LHC (i.e. reduced SFR, increased clutch size), which is suggested by the results in **chapter 1** and **2**.

In addition to the findings described above, this study demonstrates progress in the enrichment and characterization of the fish kairomone. In **chapter 1** and **2** the experiments were conducted with fish kairomones of *Perca fluviatilis*, extracted and enriched out of fish incubation water (Von Elert and Stibor 2006), which contains all types of fish excretion products and might therefore have many effects on *Daphnia* than only those related to inducible defences. This fish kairomone extract was purified in a long series of experiments (e.g. fractionation via LC-MS/MS) and I was able to confine the biological activity to one highly specific fraction containing the fish kairomones (see **chapter 3**), which was then used in the further life-history experiments for the proteomics approach.

The present study covers investigations on the mechanisms underlying a well-known anti-predator response in *Daphnia* on a broad range and includes classical ecological approaches (e.g. life-history experiments), qPCR analyses, LC-MS analyses and a

holistic LC-MS/MS proteomics approach. The study reveals for the first time the role of the environmental factor light as a mediator between defensive strategies in *Daphnia* and its influence on the mechanisms of the molecular level of these anti-fish defences. These findings provide strong evidence for a light-mediated coupling of migration behavior (DVM) and life-history changes (LHC) that allows for plastic adjustment of inducible defences to fluctuating environments of standing freshwater ecosystems. The state-of-the art proteomics approach led to the identification of functional proteins that are affected in *Daphnia* by fish kairomones and light intensity. These results reveal for the first time a metabolic pathway that yields a mechanistic understanding of the observed phenotypic changes.

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Abstract

Planktonic crustaceans of the genus *Daphnia* play an important role as keystone species in almost all standing freshwater ecosystems. As unselective filter feeders, *Daphnia* are typically the major herbivorous grazers of phytoplankton and are major prey for predators at higher trophic levels. *Daphnia magna* has become a textbook example for inducible defences in response to chemical signals released by predators. *Daphnia* as important prey organism for both invertebrate predators and vertebrate predators like planktivorous fish, show a multitude of defences against these predators including changes in life-history (LHC), morphology and diel vertical migration (DVM), which have been shown to be induced or enhanced by chemical cues released by predators, so-called kairomones. Inducible anti-fish defences like LHC and DVM are associated with substantial costs. There is evidence for a coadaptation of both defences (LHC and DVM) in *Daphnia* that hints to a plastic coupling of LHC and DVM and due to the substantial costs it has been argued that it would be maladaptive to exhibit all available defences at the same time, as selecting one or the most efficient defences against a predator. *Daphnia* appear to be plastic with respect to the degree of response with either defence and it is still an open question, which additional factor besides the fish kairomones might control a possible coupling of anti-predator defences in *Daphnia.* Furthermore, although the *Daphnia* genome and the molecular tools are available, there is not much known about the molecular mechanisms that may underlie the anti-predator defences induced by fish kairomones in *Daphnia*.

The present study covers presents novel and deep insights into the mechanisms underlying a well-known inducible anti-predator response in *Daphnia* on a broad range: besides classical ecological approaches I conducted qPCR analyses, LC-MS analyses and a holistic LC-MS/MS based proteomics approach. I showed for the first time that the presence of light is crucial for the induction of LHC in *Daphnia* exposed to fish kairomones and the same effect was observed for the fish kairomone-mediated expression of candidate genes. These observations point at a plastic coupling of anti-predator defences modulated by the environmental factor light in *Daphnia*. Under natural conditions animals are subjected to alternating periods of

light and dark and different light intensities. I further explored the role of the ambient light intensity for the induction of LHC in *Daphnia* and the animals were exposed to a gradient of light intensities that represents naturally occurring light intensities in the water column of a lake. The results revealed that LHC in *Daphnia* are inversely coupled to the ambient light intensity and that a certain thresholds for the induction of LHC exists. A similar light intensity dependence was found for the fish kairomone-mediated expression of a candidate gene in *Daphnia*. I argue that the DVM amplitude alters the ambient light intensity an individual is exposed to and thus inversely determines the degree of LHC. My findings provide strong evidence for a light intensity-mediated plastic coupling of migration behaviour (DVM) and life-history changes (LHC) in *Daphnia*, which allows for plastic adjustment to fluctuating environments and simultaneously minimizes the associated costs of multiple deployable defences. Such a proposed inverse coupling of DVM and LHC would be especially adaptive in habitats that allow for DVM only during parts in the season, i.e. spring; later in the season, when a deep-water refuge is no longer available owing to hypolimnetic anoxia, alternative defences like LHC (as is frequently observed in eutrophic shallow systems) may be required.

The last part of my thesis describes for the first time a LC-MS/MS based proteomics approach with the aim to identify functional proteins involved in the phenotypic changes on the protein level for the fish kairomone-induced LHC in *Daphnia*. In line with my findings described above, the experimental animals exposed to fish kairomones showed the expected LHC in the presence of light and this LHC response was not observed in the absence of light. In addition, due to a long series of experiments to purify the fish kairomone extract (e.g. fractionation via LC-MS) I was able to confine the biological activity to one highly specific fraction, which was then used in the life-history experiments for the proteomics approach. Only daphnids that showed the expected LHC response were used for the high throughput proteomics. The state-of-the-art proteomics approach leads to the identification of functional proteins in *Daphnia* that are affected by fish kairomones and light intensity. My results revealed for the first time a metabolic pathway that yields a mechanistic understanding of the observed phenotypic changes.

Zusammenfassung

Daphnien sind in nahezu jedem stehenden limnischen Ökosystem abundant und besitzen als häufig dominierende herbivore Zooplankter eine Schlüsselrolle im Nahrungsnetz eines Gewässers. Daphnien dienen als Modellorganismen für Verteidigungsmechanismen, die durch räuberbürtige chemische Signalstoffe, sogenannte Kairomone, induzierbar sind. Als Hauptnahrungsquelle für invertebrate und vertebrate Räuber wie planktivore Fische haben Daphnien eine Vielzahl von induzierbaren Verteidigungsmechanismen evolviert, um die räuberbedingte Mortalität zu senken. Zu diesen Verteidigungsmechanismen gehören Veränderungen des Lebenszyklus (Veränderungen der Life-History: LHC), morphologische Strukturen und Anpassungen des Verhaltens (Tagesperiodische Vertikalwanderung: DVM). Induzierbare Verteidigungen wie LHC und DVM gegen planktivore Fische sind mit erheblichen Kosten für Daphnien verbunden und es gibt Hinweise auf eine mögliche Koadaptation dieser beiden Verteidigungsmechanismen in Daphnien, die auf eine plastische Kopplung von LHC und DVM hindeuten. Basierend auf den substanziellen energetischen und demographischen Kosten dieser induzierbaren Verteidigungen wurde außerdem die Hypothese aufgestellt, dass es für Daphnien nicht adaptiv wäre, alle dem Individuum möglichen Verteidigungen zur selben Zeit auszuprägen, sofern eine oder die effizienteste Verteidigung gegen den Räuber ausreichen würde. Sollte also eine plastische Kopplung dieser beiden durch fischbürtige Kairomone induzierbaren Verteidigungsmechanismen (LHC und DVM) in Daphnien vorliegen, so muss es einen zusätzlichen Faktor neben den Kairomonen geben, der diese Kopplung kontrolliert. Des Weiteren ist die molekulare Ebene dieser Verteidigungsmechanismen wenig untersucht, obwohl das Daphnien Genom entschlüsselt worden ist und die dazu notwendigen Methoden der Molekularbiologie verfügbar sind.

Die vorliegende Arbeit zeigt neuartige und umfassende Einblicke in die Mechanismen einer bekannten induzierbaren Verteidigung von Daphnien gegen planktivore Fische: Neben klassischen ökologischen Experimenten wurden qPCR Analysen, LC-MS Analysen und eine umfassende Proteomanalyse (basierend auf LC-MS/MS) durchgeführt. Ich konnte zum ersten Mal zeigen, dass die Anwesenheit von Licht ausschlaggebend für die Induktion von LHC in Daphnien in Anwesenheit

von fischbürtigen Kairomonen ist. Den gleichen Effekt konnte ich für die relative Expression von Zielgenen für fischbürtige Kairomone nachweisen. Diese Ergebnisse deuteten auf eine Kopplung der Verteidigungsmechanismen (LHC und DVM) hin, die über den abiotischen Faktor Licht reguliert wird. Unter natürlichen Bedingungen sind Daphnien allerdings einer Lichtperiode und unterschiedlichen Lichtintensitäten ausgesetzt. Demzufolge habe ich die Rolle von unterschiedlichen Lichtintensitäten bei der Induktion von LHC in Daphnien weiter untersucht. Die Daphnien wurden einem Lichtintensitätsgradienten ausgesetzt, welcher auf Lichtintensitäten basiert, die in der Wassersäule eines natürlichen Sees vorkommen. Ich konnte zeigen, dass die LHC in Daphnien gegensätzlich an die umgebende Lichtintensität gekoppelt sind und dass ein Schwellenwert für eine Lichtintensität zur Induktion von LHC existiert. Eine gleiche Abhängigkeit von der Lichtintensität konnte ich für die Expression eines Zielgens für fischbürtige Kairomone in Daphnien nachweisen. Basierend auf diesen Beobachtungen lautet meine Hypothese, dass die Amplitude der tagesperiodischen Vertikalwanderung (DVM) Einfluss auf die Lichtintensität nimmt die ein Individuum umgibt und somit, den Grad der Ausprägung der Veränderungen in der Life-History bestimmt (LHC). Meine Ergebnisse deuten auf eine plastische Kopplung von LHC und DVM in Daphnien hin, die über die Lichtintensität der Umgebung reguliert wird. Solch eine Kopplung würde eine variable Anpassung an sich verändernde Umweltbedingungen ermöglichen und gleichzeitig die assoziierten Kosten senken, die mit dem gleichzeitigen Einsetzen von beiden Verteidigungsmechanismen verbunden sind. Diese plastische Kopplung wäre vor allem in solchen Habitaten für Daphnien adaptiv, die eine Vertikalwanderung nur zu bestimmten Zeiten in der Saison (z.B. dem Frühling) ermöglichen. Wenn im späteren Jahresverlauf das Hypolimnion eines Sees für Daphnien bedingt durch Anoxia nicht mehr verfügbar ist, würden eine alternative Verteidigung wie die LHC notwendig werden. Dieser Sachverhalt, dass LHC als alternative Verteidigungsmechanismen zu DVM in Daphnien auftreten können, konnte in Studien schon beobachtet werden.

Der letzte Abschnitt meiner Dissertation beschreibt erstmalig eine Proteomanalyse (basierend LC-MS/MS) mit dem Ziel funktionelle Proteine zu identifizieren, die in den von fischbürtigen Kairomonen induzierten LHC von Daphnien involviert sind, um die phänotypischen Veränderungen auf Proteinebene zu erklären. In Übereinstimmung

mit den oben beschrieben Ergebnissen konnte ich wiederum zeigen, dass die Daphnien nur in Anwesenheit von Licht mit LHC auf die Anwesenheit von fischbürtigen Kairomonen reagieren. Zusätzlich konnte ich den Extrakt, der die fischbürtigen Kairomone enthält und für die Experimente in Kapitel 1 und 2 dieser Arbeit benutzt wurde, in einer langen Serie von Experimenten (z.B. Fraktionierung mittels LC-MS) weiter aufreinigen und anreichern. Die biologische Aktivität konnte einer spezischen und hochaufgereinigten Fraktion zugeordnet werden, welche für Life-History Experimente der Proteomanalyse benutzt wurde. Es wurden ausschließlich Daphnien für die Proteomanalysen verwendet, die die erwarteten LHC aufzeigten. Die Proteomanalysen führten zu der Identifizierung von funktionellen Proteinen, die von fischbürtigen Kairomonen und Lichtintensität beeinflusst werden. Die Analyse dieser Daten führte zu einem Stoffwechselweg, der erstmalig eine mechanistische Erklärung für die beobachteten phänotypischen Veranderungen von Daphnien in Anwesenheit von fischbürtigen Kairomonen liefert.

Record of achievement

Chapter 1: Light intensity controls anti-predator defences in *Daphnia*: the suppression of life-history changes

Results described in this chapter were exclusively performed by me.

Chapter 2: Coupling of anti-predator defences in *Daphnia*: the importance of light

Results described in this chapter were exclusively performed by me or under my direct supervision.

Chapter 3: Differential peptide labeling (iTRAQ) in LC-MS/MS based proteomics in *Daphnia* reveal mechanisms of an anti-predator response

The experiments were exclusively performed by me. The iTRAQ labelling reaction, the LC-MS/MS analysis and the statistics were performed in the lab of Dr. Stefan Müller in the Central Bioanalytics facility at the University of Cologne. I contributed to the bioinformatical analyses and exclusively analyzed the data.

Bisherige Publikationen im peer-review Verfahren

[1]**Effertz, C.**, von Elert, E. (2014) Light intensity controls anti-predator defences in *Daphnia*: the suppression of life-history changes. *Proceedings of the Royal Society: Biological Sciences 281:20133250* (doi: 10.1098/rspb.2013.3250).

[2]**Effertz, C.**, von Elert, E. (2014) Coupling of anti-predator defences in *Daphnia*: the importance of light. *Limnology & Oceanography,* under review

[3]**Effertz, C.**, Müller, Stefan, von Elert, E. (2014) Differential peptide labeling (iTRAQ) in LC-MS/MS based proteomics in *Daphnia* reveal mechanisms of an anti-predator response. *Proteomics,* under review

[1]entspricht chapter 1

[2]entspricht chapter 2

[3]entspricht chapter 3

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Erklärung

Ich versichere, dass ich die von mir vorgelegte Dissertation selbstständig angefertigt, die benutzten Quellen und Hilfsmittel vollständig angegeben und die Stellen der Arbeit – einschließlich Tabellen und Abbildungen -, die anderen Werken in Wortlaut oder dem Sinn nach entnommen sind, in jedem Einzelfall als Entlehnung kenntlich gemacht habe; dass diese Dissertation noch keiner anderen Fakultät oder Universität zur Prüfung vorgelegen hat; dass sie – abgesehen von unten angegebenen Teilpublikationen – noch nicht veröffentlicht worden ist sowie, dass ich eine solche Veröffentlichung vor Abschluss des Promotionsverfahrens nicht vornehmen werde.

Die Bestimmungen der Promotionsordnung sind mir bekannt. Die von mir vorgelegte Dissertation ist von Prof. Dr. Eric von Elert betreut worden.

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Curriculum vitae

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