Effects of *Chaoborus* kairomone: resource allocation in *Daphnia pulex* and factors influencing the inducible morphological defense

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Zusammenfassung

In vielen stehenden Gewässern spielen Organismen der Gattung *Daphnia* eine zentrale Rolle im trophischen Transfer. Als Primärkonsumenten und gleichzeitig Beute vieler vertebraten und invertebraten Räuber tragen sie maßgeblich zum Transfer von Nährstoffen und Energie durch das aquatische Nahrungsnetz bei. Durch ihre parthenogenetische Reproduktion ist die Kultivierung von klonalen Linien im Labor unkompliziert, weshalb Daphnien beliebte und wichtige Modellorganismen sowohl für Studien zu futterbezogenen Effekten als auch für Studien zu räuberbezogenen Effekten geworden sind. Viele Studien zu räuberbezogenen Effekten beschäftigen sich mit Verteidigungen, die durch räuberbürtige Signalstoffe, so genannte Kairomone, induziert werden. Diese induzierbaren Verteidigungen umfassen Veränderungen der Morphologie, des Verhaltens und Veränderungen des Lebenszyklus von Daphnien. Im Allgemeinen wird angenommen, dass alle Verteidigungen konzeptionell mit Kosten verbunden sind, da sie nicht konstitutiv sind. Futterbezogene Effekte werden häufig mit Hilfe von Wachstumsversuchen untersucht. Generell wird das Wachstum von Daphnien von der Futterquantität sowie –qualität beeinflusst. Die Futterqualität besteht dabei aus verschiedenen Aspekten wie der biochemischen Futterqualität, z. B. den Gehalt an essentiellen Fettsäuren, der stöchiometrischen Futterqualität, der Morphologie der Algen und dem Gehalt an toxischen oder schädlichen Sekundärmetaboliten, z. B. von Cyanobakterien. Diese Aspekte spielen eine große Rolle in der Ernährung von Daphnien, da sie als unselektive Filtrierer ihre Nahrungspartikel nicht gezielt wählen können. Somit ist das Wachstum von Daphnien stark abhängig von der vorhandenen Phytoplankton-Gemeinschaft, die in Gewässern saisonal jedoch enorm variieren kann. Des Weiteren wird das Wachstum von Daphnien von der Temperatur beeinflusst, da sie ektotherme Tiere sind.

In der Natur sind Daphnien häufig gleichzeitig Bedingungen, die das Wachstum limitieren können, und Prädation ausgesetzt. Bisher ist jedoch das Wissen darüber, wie diese beiden Faktoren interagieren und im Zusammenspiel Daphnien, und insbesondere ihre morphologische Verteidigungen, beeinflussen können, noch wenig fundiert. Innerhalb der Studien in dieser Dissertation habe ich den Effekt verschiedener potentiell wachstumslimitierender Faktoren auf die morphologische Verteidigung von *Daphnia pulex* untersucht. *D. pulex* bilden in ihren juvenilen Stadien in Anwesenheit der aquatisch lebenden Mückenlarve *Chaoborus* so genannte

Nackenzähne aus. Nackenzähne sind kleine Protuberanzen in der Nackenregion der Daphnien, die die Wahrscheinlichkeit erhöhen, dass die Daphnien, nachdem sie gefangen wurden, wieder entkommen können. Aufgrund der Morphologie des Fangapparates von *Chaoborus*, sind die Larven in der Beutegröße limitiert, und sie fressen überwiegend kleine Daphnien bis Daphnien mittlerer Größe. Wenn juvenile *D. pulex* allerdings langsamer wachsen, verlängert sich die Zeit, die sie in den gefährdeten Stadien verbringen, und die Wahrscheinlichkeit, dass sie gefressen werden können, steigt an. Demnach müssten sie sich stärker verteidigen um das erhöhte Prädationsrisiko auszugleichen.

Ich zeige innerhalb dieser Dissertation, dass die Stärke der Nackenzahninduktion bei *D. pulex* tatsächlich nicht von dem erhöhten individuellen Prädationsrisiko beeinflusst wird, das durch verlängerte Entwicklungszeiten bei langsamerem Wachstum entsteht. Diesen Zusammenhang habe ich für den Temperatureinfluss auf das Wachstum und für den Gehalt an Cyanobakterien im Futter untersucht. Obwohl dieser Zusammenhang für den Temperatureinfluss durch eine signifikante Korrelation zwischen Nackenzahninduktion und Entwicklungszeit gegeben war, ist es kein genereller Mechanismus, da die Korrelation für den Gehalt an Cyanobakterien nicht signifikant war.

Ich habe außerdem den Einfluss von mehrfach ungesättigten Fettsäuren, die essentiell für das Wachstum der Daphnien sind, auf die Nackenzahninduktion untersucht, indem ich verschiedene Futteralgen genutzt habe, die in ihrem Gehalt an mehrfach ungesättigten Fettsäuren variieren. Diesen Versuch habe ich mit mehreren Klonen von *D. pulex* durchgeführt, um eine allgemeingültigere Aussage treffen zu können, da Klone in ihren Reaktionsnormen zu verschiedenen Umweltveränderungen stark variieren können. Die Nackenzahninduktion wurde in keinem der Klone von dem Gehalt an mehrfach ungesättigten Fettsäuren beeinflusst. Auch die Supplementierung von Eicosapentaensäure, die eine ausschlaggebende Rolle im Wachstum von Daphnien spielt und deren Mangel eine induzierbare Verteidigung als Anpassung des Verhaltens unterdrücken kann, hatte keinen Einfluss auf die Nackenzahninduktion.

Der Effekt von Futterquantität auf Nackenzahninduktion wurde zwar schon mehrfach untersucht, allerdings sind die bisher berichteten Ergebnisse widersprüchlich. Durch Reduzierung des bakteriellen Abbaus des Kairomons, der höchst wahrscheinlich während dem Experiment stattfindet, konnte ich den Effekt der Futterquantität eindeutig untersuchen und zeigen, dass die

Nackenzahninduktion nicht von der Futterquantität beeinflusst wird, sondern von der Abundanz der Bakterien, die häufig in den Algenkulturen enthalten sind, in den jeweiligen Treatments.

Des Weiteren habe ich die Ressourcenallokation von Fettsäuren unter niedriger und hoher Futterkonzentration kombiniert mit der Ab- und Anwesenheit von *Chaoborus* untersucht. Dafür habe ich die Fettsäuregehalte der Gewebe der Eier und Mütter separat mit Hilfe von Gaschromatographie analysiert. Durch Anpassung der Ressourcenallokation und eventuelle Veränderungen des Lebenszyklus können Daphnien bei niedriger Futterkonzentration die Weitergabe von Fettsäuren an ihre Nachkommen erhöhen. Dadurch werden die Nachkommen resistenter gegen Verhungern. Diesen Effekt konnte ich bestätigen. Die Anwesenheit von *Chaoborus* unterdrückte allerdings diese vermehrte Weitergabe von Fettsäuren an die Nachkommen unter niedriger Futterkonzentration, aber erhöhte sie unter saturierten Futterbedingungen. Zusätzlich lässt eine erhöhte Speicherung von Eicosapentaensäure, die eine wichtige Rolle bei der Produktion von Eiern spielt, im Gewebe der Mütter darauf schließen, dass *D. pulex* in Anwesenheit von *Chaoborus* Fettsäuren eher in eine höhere Anzahl an zukünftigen Reproduktionen als in die aktuelle Reproduktion investiert.

In der vorliegenden Dissertation werden neue Erkenntnisse über die morphologische Verteidigung von *D. pulex* gegen *Chaoborus* sowie über physiologische Anpassungen innerhalb des Lebenszyklus gezeigt. Dafür wurden Methoden der klassischen Ökologie mit modernen Methoden der instrumentellen Analytik kombiniert. Diese neuen Erkenntnisse geben Aufschluss über Mechanismen und mögliche Kosten von morphologischen Veränderungen, sowie über Lebenszyklen-Strategien von Beutetieren.

Abstract

Individuals of the genus *Daphnia* play a central role in the trophic transfer in many standing freshwater bodies. As primary consumer and at the same time prey of many vertebrate and invertebrate predators, *Daphnia* contribute greatly to the transfer of nutrients and energy through the aquatic food web. Their parthenogenetical reproduction facilitates the cultivation of clonal lineages in laboratories, and makes *Daphnia* popular and important model organisms for studies about food related effects as well as predator related effects. Many studies about predator related effects deal with defenses that are inducible by predator-borne chemicals, so-called kairomones. Inducible defenses comprise changes in morphology, behavior, or in life history. All of these changes typically entail some kind of costs, as these changes are not constitutive. Food related effects are often investigated by means of growth experiments. The growth of *Daphnia* can be affected by food quantity and food quality, whereas food quality is composed of different aspects: biochemical food quality, e. g. the content of essential fatty acids, stoichiometric food quality, the morphology of the algae, and the content of toxic or harmful secondary metabolites, e. g. of cyanobacteria. Those aspects play an important role in *Daphnia*'s nutrition, because *Daphnia* are unselective filter feeders and cannot discriminate between food particles. Therefore, *Daphnia*'s growth is highly dependent on the phytoplankton community, which can be vastly variable throughout the year. Additionally, the growth of *Daphnia*, as ectothermic organisms, is affected by temperature.

Daphnia frequently experience growth limiting conditions and predation at the same time. However, knowledge about how these factors interact and how they jointly affect *Daphnia*, and especially their morphological defenses, is still scarce. Within the studies of this dissertation, I investigated the effect of several potentially growth limiting factors on the morphological defense of *Daphnia pulex*. Juveniles of *D. pulex* form so-called neckteeth in the presence of the aquatic phantom midge larvae *Chaoborus*. Neckteeth are small protuberances in the neck region of *D. pulex* that increase the escape efficiency after capture by *Chaoborus*. Due to the morphology of the catching basket of *Chaoborus*, the larvae are gape-limited, and they mainly consume small and intermediate-sized *Daphnia*. However, if *D. pulex* is growth limited and develops slower, the time that it spends in vulnerable instars increases, and the overall probability to get caught

increases as well. Therefore, *D. pulex* might need to increase the strength of its defense to counterbalance the increased predation risk.

Within this dissertation, I report that the strength of neckteeth induction in *D. pulex* is, actually, not affected by the increased individual predation risk emerging from longer developmental times at slower growth. I investigated this relation for the factors temperature and the content of dietary cyanobacteria. Although the correlation of neckteeth induction and developmental time was significant in case of the factor temperature, this is not a general relation, as the correlation in case of the content of dietary cyanobacteria was not significant.

Furthermore, I investigated the effect of polyunsaturated fatty acids, which are essential for *Daphnia* growth, on neckteeth induction by using three different food algae that differ in their content of polyunsaturated fatty acids. I performed this experiment with three clones of *D. pulex* to get a more general conclusion, as clones are known to vary in their reaction norms to changing environmental conditions. Neckteeth induction was affected by the availability of polyunsaturated fatty acids in neither of the clones. Moreover, the supplementation of eicosapentaenoic acid, which plays a crucial role in *Daphnia* growth, and was reported to be essential for an induced behavioral defense, did not affect neckteeth induction.

The effect of food quantity on neckteeth induction was already investigated before, but the results were not conclusive so far. By reducing the effect of bacterial degradation of the kairomone, which most probably takes places during the experiment, I was able to solely investigate the effect of food quantity, and I report that food quantity does not affect neckteeth induction. Instead, the strength of neckteeth induction was correlated to the abundance of bacteria in the respective treatments.

Moreover, I investigated the resource allocation with respect to fatty acids at low and high food quantity crossed with the absence or presence of *Chaoborus*. In order to do so, I separately analyzed the fatty acid contents in the tissues of eggs and bodies of the mothers by means of gas chromatography. At low food quantities, *Daphnia* increase the transfer of fatty acids to their offspring by altering the resource allocation or life history. Thus, the offspring are more starvation resistant. I was able to corroborate this effect. However, the presence of *Chaoborus* suppressed this increase in fatty acid transfer to the offspring at low food quantities, whereas the fatty acid transfer increased at high food quantities. Additionally, the increased retention of eicosapen-

taenoic acid, which is important for egg production, in the tissue of mothers suggests that, in the presence of *Chaoborus*, *D. pulex* invests fatty acids rather in an increased number of future reproductive events than in the current reproduction.

Within this dissertation, new insights about the morphological defense of *D. pulex* against *Chaoborus* as well as physiological alterations in the life history are presented. In order to investigate these topics, approaches of classical ecology were combined with modern techniques of instrumental chemistry. The new insights shed some light on mechanisms and potential costs of morphological defenses and on life history strategies of prey organisms.

General introduction and aim of the study

Trophic transfer of nutrients and energy is an important function of ecosystems (Persson et al., 2007). In aquatic ecosystems, phytoplankton, which takes up nutrients and energy, builds up biomass, and forms the base of the trophic levels. The key role in trophic transfer is played by herbivorous zooplankton, because it makes these nutrients and energy accessible to higher trophic levels. Major herbivores in standing freshwater systems are the crustaceans *Daphnia* spp. *Daphnia* reproduce parthenogenetically for long periods throughout the year and have short generation times (Lampert, 2006). This makes *Daphnia* easy to culture, and *Daphnia* have become popular and important model organisms for studies about diverse aspects of standing freshwater systems.

Due to one of their characteristics, unselective filter feeding, *Daphnia* cannot discriminate between food particles. Therefore, *Daphnia* are heavily dependent on the composition of the phytoplankton community, which actually is highly variable throughout the year (Sommer et al., 1986). While cryptophytes and small diatoms, which are considered good quality food for *Daphnia*, are dominating the community in spring, cyanobacteria and filamentous green algae are dominating during summer, which are of inferior food quality to *Daphnia* or just inedible because of their morphology. Good food quality is often associated with the content of polyunsaturated fatty acids (PUFAs) and sterols in the algae: PUFAs, especially eicosapentaenoic acid (EPA), were often reported to be essential for *Daphnia*'s growth and reproduction (Martin-Creuzburg et al., 2009), as *Daphnia* cannot synthesize PUFAs de novo (Harrison, 1990). Moreover, the absence of sterols is as well potentially limiting to *Daphnia* (Martin-Creuzburg et al., 2009). Algal taxa groups have been shown to differ in their contents of PUFAs and sterols: Cryptophytes and diatoms contain high amounts of EPA and other PUFAs, whereas cyanobacteria completely lack PUFAs and sterols (Ahlgren et al., 1990). Additionally to being of inferior biochemical food quality, many cyanobacterial strains are filamentous or produce toxic or harmful secondary metabolites (Paerl & Huisman, 2009) that inhibit the grazer's gut proteases (von Elert et al., 2004) or phosphatases (DeMott & Dhawale, 1995). Exposure to such metabolites negatively affects various growth and fitness parameters of *Daphnia* (DeMott, 1999; Lürling & van der Grinten, 2003).

However, food quality is not the only potentially growth determining factor that fluctuates throughout the year: *Daphnia* become growth limited by food quantity in early summer, and this limitation remains moderate during summer and fall (Müller-Navarra & Lampert, 1996). Besides obvious effects of food limitation, like decreased growth and, therefore, smaller body sizes, *Daphnia* change physiological processes of energy storage and resource allocation (Lynch, 1989). Under food limitation, *Daphnia* increase the allocation of lipids and proteins to their eggs, which increases the offspring's total energy reserves and, therefore, its starvation resistance (Gliwicz & Guisande, 1992; Tessier et al., 1983). In most cases, changes in life history are associated with those changes in resource allocation: maternal animals exhibit decreased growth, mature later and at a smaller body size, and they produce smaller clutch sizes with increased egg volumes, so that neonate body sizes are bigger (Gliwicz & Guisande, 1992).

Moreover, as ectothermic organisms, *Daphnia*'s growth rates increase with the ambient temperature up to a temperature optimum, after which growth rates decline sharply (Mitchell et al., 2004). The temperature optimum, however, can vary greatly depending on the environment that the *Daphnia* species originates from: while the arctic *D. middendorffiana* is adapted to temperature ranges about 5 °C lower than temperate zone *Daphnia* species (optimum around 20 °C) (Yurista, 1999), the temperature optimum of the tropic and sub-tropic *D. lumholtzi* is even exceeding 25 °C (Lennon et al., 2001).

Daphnia, as major herbivores, are important prey organisms for vertebrate predators, like planktivorous fish, as well as for invertebrate predators, like insect larvae. As a crucial link between primary producers and higher trophic levels, *Daphnia* can become not only bottom-up limited by food, but as well become top-down limited by predation (Balseiro et al., 2007). Indeed, predation can impact *Daphnia* populations to a great extent (Balseiro et al., 2007; Elser et al., 1987). Due to its high phenotypic plasticity and cosmopolitan distribution, *Daphnia* occur in temporary rock pools, small ponds or lakes, and reservoirs (Lampert, 2006). In these diverse ecosystems, *Daphnia* are subjected to various predators and have evolved a huge variety of inducible defenses against those predators (Diel et al., 2020), which can greatly reduce predation rates. The expression of inducible defenses is caused by semiochemicals released by the predator, so-called kairomones (Pohnert et al., 2007). Upon recognition, *Daphnia* change their morphology, behavior, or life history, depending on the *Daphnia* species and the predator (Diel et al., 2020).

Morphological changes comprise highly diverse alterations of the carapace as well as, in some cases, an overall thickening of the carapace. The alterations can result in an elongation of the tail spine (Dodson, 1988a), or in the formation of helmets (Dodson, 1988a), huge crests (Laforsch & Tollrian, 2004), neckteeth (Havel & Dodson, 1984), or crowns (Petrusek et al., 2009). Morphological changes can even result in torsion of the entire body (Herzog et al., 2016). Although the exact mechanism of how most morphological changes alter the interaction with the predator is still unknown, it had been hypothesized already more than three decades ago that these morphological changes act as an 'antilock and key'-mechanism at some point during the prey capture, handling, or feeding process (Dodson, 1974). These interferences, thereby, increase the escape and survival probability of *Daphnia* (Havel & Dodson, 1984).

Changes in behavior commonly result in diel vertical or horizontal migration. *D. magna*, one of the biggest *Daphnia* species, migrates in the presence of fish to deeper and darker strata during the day to avoid the visually hunting predator (Stich & Lampert, 1981). The smaller species *D. pulex*, however, performs 'inverse' diel vertical migration to avoid another predator, the phantom midge larvae *Chaoborus* sp., which perceives its prey by mechanoreception, and is as well migrating in response to fish (Dodson, 1988b). Furthermore, it had been reported that *D. longispina* migrates horizontally to the littoral during the day in a lake inhabited by *Chaoborus* (Kvam & Kleiven, 1995).

The induction of life history changes results in a reduced time period that *Daphnia* spend in the developmental stages that are most vulnerable to predation. Changes in life history are accompanied by changes in resource allocation from somatic growth to reproduction or vice versa. In the presence of predators that select for big individuals, like fish, *Daphnia* start reproducing earlier (von Elert & Stibor, 2006) leading to a smaller size at first reproduction. The smaller size at first reproduction increases the probability of successful reproduction before being ingested by fish. In the presence of predators that select for small sized prey, like the phantom midge larvae *Chaoborus*, the first reproduction is delayed (Tollrian, 1995b). The resources are allocated to somatic growth and *Daphnia* mature at a bigger size. Furthermore, the clutch size can be altered to more but smaller offspring in the presence of fish or to fewer but bigger offspring in the presence of predators selecting for small sized prey.

The fact that those kinds of defenses are inducible, and not constitutive, suggests that the expression of inducible defenses is associated to some kind of costs (Havel & Dodson, 1987). So far, however, many conclusions about the costs of inducible defenses, especially morphological and life history changes (Diel et al., 2020), remain partly controversial, while for some defenses, e.g. diel vertical migration, the costs are unambiguous (Dawidowicz & Loose, 1992). Additionally, the concept of associated costs is supported by the fact that most inducible defenses are reversible. The induced phenotype, therefore, seems to be maladapted in absence of the predator compared to the typical phenotype. Furthermore, although many *Daphnia* species can induce several defenses against the same predator, the defenses are never expressed simultaneously, and only one defense is expressed at a certain time point during *Daphnia*'s development.

Considering that *Daphnia* contemporarily experience predation and potentially growth limiting conditions, it is crucial to know how these factors might interact. However, knowledge about how those factors might, especially, affect inducible defenses is still scarce. The aim of this dissertation was to further elucidate how inducible morphological defenses of *Daphnia* are affected by potentially growth limiting factors. Moreover, the combined effects of food quantity and the presence of *Chaoborus* with respect to resource allocation were investigated, and experiments deciphering the effect of the kairomone degradation were conducted.

During this dissertation, all experiments were exclusively conducted using the predator-prey-system of the phantom midge larvae *Chaoborus* feeding on *D. pulex*. Due to the characteristics of *Chaoborus'* feeding appendages, its prey size range is restricted to small and intermediate sized *Daphnia* (Pastorok, 1981). In the chemical presence of *Chaoborus*, juvenile *D. pulex* form neckteeth as a morphological defense, which are small spike-like structures in its neck region (Tollrian, 1993). Additionally to the formation of teeth, the general form of the neck region can change to an angular form or even an angular form with a protuberance. Neckteeth induction can be quantified by applying a scoring system to these structures. It has been shown to be increasing with kairomone concentration, and it is most pronounced during the second juvenile instar (Tollrian, 1993). Furthermore, *D. pulex* is able to adjust the neckteeth induction to the density of conspecifics: at low densities of conspecifics, when the individual predation risk is higher, neckteeth induction is as well higher than at high densities of conspecifics (Tollrian et al., 2015). Moreover, neckteeth induction has been reported to be higher at low food concentrations (Parejko & Dodson, 1991). The reason for this might be decreased growth at low food concentrations, and

thus, increased developmental times. The increased developmental times of *D. pulex* would lead to longer durations of instars that are most vulnerable to predation, which would increase the individual overall predation risk. An increase in neckteeth induction might, therefore, counterbalance this increased predation risk.

During the study presented in Chapter 1, I investigated if *D. pulex* is able to adjust its neckteeth induction to increased developmental times that lead to an increased individual predation risk. I created a gradient of growth conditions by exposing *D. pulex* to different temperature conditions and, in a separate experiment, to increasing concentrations of dietary cyanobacteria. I quantified the neckteeth induction, determined the developmental time, and correlated both parameters. In case of a significant correlation, I further investigated if the differences in neckteeth induction that contributed to the significance of the correlation were caused by physiological restrictions of *D. pulex* or if there is another cause, e.g. altered predation rates. Physiological restrictions might become apparent from decreased values of maximum induction or a general shift of dose-response curves at the different growth conditions. In Chapter 1, I report for the first time how dietary cyanobacteria affect neckteeth induction. Furthermore, I elucidate the effect of temperature on neckteeth induction in individual *D. pulex*, and I determine temperature-specific predation rates of *Chaoborus* on juveniles of *D. pulex*.

However, as explained above, temperature and dietary cyanobacteria are not the only factors possibly affecting *Daphnia*'s growth, and therefore, possibly its individual predation risk. During the study presented in Chapter 2, I investigated the effect of food quality with respect to PUFA availability on neckteeth induction in three clones of *D. pulex*. Due to its parthenogenetical reproduction, clonal lineages of the same *Daphnia* species co-exist in nature. Clones have been reported to differ in their reaction norms, which describes the pattern of phenotypic variability in a reaction to various environmental conditions. Clones of the same *Daphnia* species can differ in their reaction norm to food quality (Brzeziński & von Elert, 2007), toxins of cyanobacteria (Hietala et al., 1995), or in their reaction norm of their inducible defenses (Dennis et al., 2011). In order to get more comprehensive information on how food quality might affect neckteeth induction, I used different clones that have been shown to differ in their sensitivity to the *Chaoborus* kairomone during this study. I created a gradient of PUFA availability by using three different algae as food for *Daphnia* that have been reported to differ in their fatty acid composition

(Ahlgren et al., 1990). In Chapter 2, the effect of PUFA availability on a morphological defense is reported for the first time.

Predation as well as quantitative food limitation can lead to changes in resource allocation and life history in *Daphnia*. In a study on the interaction of those two factors, *D. magna* has been reported to allocate less triacylglycerides to the eggs in the presence of fish, both at high and low food quantity (Stibor & Müller-Navarra, 2000). As triacylglycerides are major energy storage molecules in *Daphnia* (Goulden & Place, 1993), this means that the offspring are less resistant to starvation in the presence of fish. However, the effect of *Chaoborus* kairomone, originating from a gape-limited invertebrate, crossed with food quantity on resource allocation in *D. pulex* is still unknown. During the study presented in Chapter 3, I investigated for the first time if, and how, *D. pulex* changes its resource allocation with respect to fatty acids in response to *Chaoborus*. I performed growth experiments, and, after the animals had reached maturity, dissected the eggs from their mothers' brood chambers to analyze the fatty acid composition in the separate tissues. Therefor I extracted the fatty acids from the tissues and analyzed the samples via gas chromatography.

During the study presented in Chapter 3, I as well quantified the neckteeth induction at the two food concentrations during the growth experiments, and I was able to corroborate a previous finding that neckteeth induction is higher at lower food concentrations (Parejko & Dodson, 1991). However, since inducible defenses are associated to some kind of costs (Havel & Dodson, 1987), it seems maladaptive that neckteeth induction decreases with increasing food availability under the assumption that the cost of the formation of neckteeth per se is independent of food availability. Considering this conceptual discrepancy about the costs of neckteeth induction, I hypothesized that those differences in neckteeth induction had been caused by differences in bacterial degradation rates of the kairomone, since with larger volumes of non-axenic algae cultures also more bacteria are added. The kairomone of *Chaoborus* was recently identified as a group of fatty acid-glutamine-conjugates (Weiss et al., 2018), which consist of components commonly used by all organisms, and even glutamine lipids themselves can be found in bacterial membranes (Zhang et al., 2009). In order to reinvestigate the effect of food quantity on neckteeth induction more precisely, I expanded the experimental design of Chapter 3 (food quantity × *Chaoborus* kairomone) by additional treatments, in which I treated the food algae with antibiotics to reduce degradation rates during the experiment (Chapter 4). During the study presented in

Chapter 4, I quantified the neckteeth induction at two different food concentrations crossed with the antibiotics treatment. Additionally, I determined the abundance of bacteria in the treatments, and I correlated the neckteeth induction to the bacterial abundances.

In summary, during the studies presented within this dissertation, I investigated the effect of several biotic and abiotic factors on the morphological defense of *D. pulex* against *Chaoborus*. Although the interaction of *Chaoborus* preying on *D. pulex* is a well-studied system, the effects of most of these factors were examined for the first time during the present dissertation. I as well present novel insights about factors that have been investigated before. Furthermore, I investigated for the first time, if *D. pulex* undergoes physiological changes that result in an altered resource allocation under *Chaoborus* predation.

Chapter 1

No general effect of developmental time on *Chaoborus*-induced phenotypic plasticity

Abstract

Due to *Daphnia´*s cosmopolitan distribution and the co-occurrence with various predators, *Daphnia* has evolved highly diverse anti-predator defenses. In response to chemical cues of *Chaoborus* larvae, a major predator, neckteeth are induced in vulnerable juvenile instars of *Daphnia pulex*. As only early juvenile instars of *D. pulex* are vulnerable to predation by *Chaoborus* sp., increased developmental time extends the time span that *D. pulex* is in the vulnerable size and thus increases the risk of being preyed upon. Here, we hypothesize that increased developmental time leads to a higher degree of neckteeth formation in vulnerable *D. pulex* instars.

In order to test this, we created a gradient of growth conditions for *Daphnia* that would cause an increase in developmental time by means of decreasing the temperature or the proportion of dietary cyanobacteria in separate experiments. Increasing shares of dietary cyanobacteria increased developmental time, but neckteeth induction was not correlated with the developmental time.

Increasing temperatures resulted in decreasing developmental time and in decreasing neckteeth induction, and developmental time and neckteeth induction were significantly positively correlated. Predation experiments and dose-response curves revealed that neither predation risk nor sensitivity to the kairomone or maximum neckteeth induction in *D. pulex* were reduced at elevated temperature. Therefore, it is reasonable to assume that decreased developmental time in response to increased temperature caused the decreased neckteeth formation. However, we cannot exclude that increased bacterial degradation of the kairomone at elevated temperatures has produced this relationship. Since we found a significant positive relationship between developmental time and neckteeth induction in response to temperature and not to increasing shares of dietary cyanobacteria, we conclude that *Daphnia* in general is not able to adjust neckteeth induction to altered predation risk that is mediated by differences in developmental time.

Introduction

Inducible defenses against predators are widespread across taxa and ecosystems (Adler & Harvell, 1990). In aquatic systems, *Daphnia* sp. has become a popular model organism to investigate inducible defenses (Miner et al., 2012). Due to its cosmopolitan distribution and the co-occurrence of various vertebrate and invertebrate predators (Lampert, 2006), *Daphnia* has evolved highly diverse anti-predator defenses, which comprise behavioral, physiological and morphological defenses (Lass & Spaak, 2003). Behavioral defenses mostly result in diel vertical migration to avoid predator contact (Dawidowicz & Loose, 1992; Dodson, 1988; Hahn et al., 2019). Physiological defenses involve changes of life history (Effertz & von Elert, 2014; Spitze, 1992; Stibor, 1992), and morphological defenses comprise a huge variety of alterations in carapace shape that include helmets, crests, neckteeth or elongated tail spines (Hebert, 1978; Lampert, 2006; Tollrian, 1990, 1993).

One intensively investigated model system, which involves morphological defenses, is *Chaoborus* sp. preying on juvenile instars of *D. pulex*. The aquatic larvae of the phantom midge *Chaoborus* are restricted in prey size by their mouth gape. Pastorok (1981) developed a model describing the relation of prey size and successful capture by including the encounter probability of predator and prey and the strike efficiency of *Chaoborus*. Since the encounter probability increases with increasing prey size and the strike efficiency decreases with increasing prey size, the probability of a successful capture is highest for intermediate prey sizes.

However, *D. pulex* is able to reduce the rates of successful capture by up to 50% (Tollrian, 1995a) by inducing a morphological defense in the presence of chemicals, so called kairomones, released by the larvae (Krueger & Dodson, 1981). Upon recognition of the kairomone neckteeth are induced, which are small protuberances in the neck region of *Daphnia*. The induction of neckteeth is not only dependent on the density of the predator but also dependent on the density of the prey (Tollrian, Duggen et al., 2015). Neckteeth induction was also found to be dependent on the food concentration: it decreased at higher food concentrations (Parejko & Dodson, 1991), and also Riessen (1992) argued, based on a model, that neckteeth induction would increase at limiting food concentrations. The reason for the increased neckteeth induction at lower food concentrations might be the fact that *Daphnia* grow slower at low food concentrations than at

high ones (Giebelhausen & Lampert, 2001; Lampert, 2006). Reduced growth would lead to increased developmental times and *Daphnia* would reach the size at which it can no more be captured by *Chaoborus* at a later time point during its life. The overall predation risk would therefore be higher for slow growing *Daphnia*. There are several other stressors besides low food concentrations that reduce *Daphnia* growth for example inferior food quality (Ahlgren et al., 1990; Sterner et al., 1993), lower temperature (Giebelhausen & Lampert, 2001; Lampert, 2006) or dietary toxins (DeMott, 1999; Schwarzenberger et al., 2010).

As ectothermic animals *Daphnia´*s metabolic rates, development, and growth rates are highly dependent on ambient temperature (Giebelhausen & Lampert, 2001; Lampert, 2006). Mitchell et al. (2004) showed that *Daphnia´*s juvenile growth rates increased with increasing temperature ranging from 17 to around 26 °C. Declining growth rates have also been reported for *Daphnia* growing on food containing cyanobacteria (Jungmann, 1992; Küster et al., 2013). Many cyanobacterial strains contain secondary metabolites that inhibit proteases (von Elert et al., 2004) or phosphatases (DeMott & Dhawale, 1995) in *Daphnia*, which affects somatic growth (DeMott, 1999), survival (Semyalo et al., 2009), fecundity (Lürling & van der Grinten, 2003), and population growth of *Daphnia* (Küster et al., 2013; Lürling & van der Grinten, 2003). Due to reduced somatic growth rates the developmental times of the juvenile stages of *Daphnia* might increase, so that *Daphnia* remains longer in the size spectrum of *Chaoborus* prey, and therefore, the overall individual predation risk of *Daphnia* increases.

As Tollrian et al. (2015) showed that *Daphnia* is able to adjust its defense to its individual predation risk in dependence of the density, *Daphnia* might also be able to adjust their neckteeth induction to the elevated predation risk caused by elongated developmental time. Here, we tested if *D. pulex* is able to adjust its neckteeth induction to their predation risk resulting from elongated developmental time in the chemical presence of *Chaoborus*. We created a gradient of growth conditions for *Daphnia* that caused an increase in developmental time by means of increasing temperature or the proportion of dietary cyanobacteria in separate experiments. We expected higher neckteeth induction at lower temperature and less neckteeth at higher temperature. Accordingly, we expected neckteeth induction to increase with the percentage of dietary cyanobacteria. We further investigated if the altered neckteeth induction was caused by physiological restrictions of *Daphnia*.

Methods

Cultivation of animals

D. pulex clone TCO was cultivated in aged and aerated tap water (19.2 ± 0.3 °C, 16:8 light:dark cycle) at a density of 10-12 individuals per 800 mL. The animals were transferred every second day into fresh water containing at least 1mg C/L of *Chlamydomonas klinobasis* strain #56 (Limnological Institute, University of Constance).

Cultivation of food

C. klinobasis was grown in 5L semi-continuous batch cultures in cyanophyceae medium (von Elert & Jüttner, 1997) modified with vitamins. *Microcystis aeruginosa* strain PCC 7806mut (Pasteur Culture Collection, Paris) was cultivated in cyanophyceae medium without vitamins in chemostats (dilution rate of 0.1 d^{-1}, 20 °C, constant light). *M. aeruginosa* strain PCC7806mut produces the same secondary metabolites as the wild type, except for microcystins (Dittmann et al., 1997). A calibration curve relating the content of particulate carbon (POC) to the optical density at 470 nm was used to photometrically determine POC content of the respective algal or cyanobacterial suspension.

Preparation of *Chaoborus* incubation water extract

The *Chaoborus* incubation water extract was prepared as according to Klintworth and von Elert (2020). Around 300–350 fourth instar larvae (ordered from www.interaquaristik.de) of *C. flavicans* were fed daily with neonates of *D. pulex*. After feeding, the larvae were transferred into 1L of fresh aged and aerated tap water without any food. After an incubation time of 24 h the larvae were removed from the water using a 250 µm gauze. The water was then filtered through a glass fiber filter (Whatman, MN 85/220, 0.4 µm, Macherey-Nagel). Afterwards, a solid-phase extraction was performed to enrich the kairomone (VARIAN, Bond Elut-C18, 10 g of sorbent, volume 60 mL, Agilent Technologies) as according to Christjani, Fink, and von Elert (2016). The methanolic eluates of the columns were pooled and evaporated to dryness. The dried residues of 20 L of incubation water were dissolved in 1mL methanol and stored at −20 °C until use. In this

way different batches of the extract were prepared. Prior to each experiment a dose-response experiment for each batch was performed at 20 °C with *C. klinobasis* as food. The volume of extract that was used in the following experiments was chosen based on those dose-response curves and corresponded always to an intermediate neckteeth induction of 50%.

Effects of cyanobacteria on neckteeth induction

The aim was to determine the effect of dietary cyanobacteria on the degree of neckteeth induction and the developmental time of juveniles until they reach their third instar. Animals of a cohort of 30 synchronous animals that had produced their first clutch were randomly distributed to the different food treatments. These food treatments consisted of either 100% *C. klinobasis*, 90% *C. klinobasis* and 10% *M. aeruginosa*, or 80% *C. klinobasis* and 20% *M. aeruginosa* (referred to as 0%, 10%, and 20% as according to their share of cyanobacteria, 2 mg POC/L in all treatments). After the animals had produced their third clutch, they were further distributed to the control and the Chaoborus treatments within the respective food treatments. For the Chaoborus treatments 3 µL of *Chaoborus* incubation water extract were pipetted onto the bottom of the jars and, after the methanol had evaporated, 100 mL water and the food suspensions were added. Control extract, which was prepared in the same way as the incubation water extract but without any *Chaoborus* larvae, did not induce any neckteeth (Klintworth & von Elert, 2020). The animals were kept individually and transferred to new jars daily. Each treatment was replicated five times. The jars were inspected hourly to determine the time when the third clutch had hatched from the brood chambers. When this hatching had occurred, mothers were removed, and only six neonates were kept in each jar. All other neonates were pooled per treatment and the dry mass (w_0) of subsamples of two times ten neonates per treatment was determined. Further on, the jars were inspected hourly for exuviae that would indicate that the animals had molted to the next instar. When more than half of the animals had molted, all of the animals in the replicate were considered to be in the second instar. During the second instar the animal´s neckteeth were scored as according to Tollrian (1993) with the modification that each tooth was scored with 10%. The neckteeth of five animals per replicate were scored and the average was calculated. Further on, the jars were inspected hourly until the animals had reached the third instar in order to determine the developmental time needed after having left the brood chamber to reach the third instar. When the animals had produced their first clutch, three animals per replicate were taken for the

determination of dry mass (w_t). Somatic growth rates (g) were calculated: $g = (\ln(w_t) - \ln(w_0)) / t$, with w_t being the individual weight at day t and w_0 being the individual weight at day 0. Subsequently, the neonates of the first to third clutch were counted daily to calculate the intrinsic rates of increase (r) according to the formula: $r = \sum l_x \times m_x \times e^{-rx}$, with l_x being the survival rate at day x and m_x being the average number of offspring per surviving individual at day x.

Effects of temperature on neckteeth induction

When a cohort of 30 synchronous animals had just produced their first clutch, the animals were randomly distributed to the different temperature treatments, i.e. 16.3 ± 0.7 °C, 19.8 ± 0.2 °C and 24.1 ± 0.2 °C (referred to as 16 °C, 20 °C and 24 °C). The animals were fed with at least 2.5 mg C/L *C. klinobasis*. The further experimental protocol was identical to the one described above, except that the batch of *Chaoborus* incubation water extract used here differed from the one used above, and 1µL of this extract was used per 100 mL.

Dose-response experiments

When a cohort of synchronous *D. pulex* had produced their first clutch, the animals were transferred to the respective temperature (16 °C, 20 °C and 24 °C). After they had produced their third clutch, the animals were transferred individually to jars containing 0, 0.5, 1, 2, 3, 5 or 8 µL of *Chaoborus* incubation water extract in 100 mL water. The jars were prepared as described above and each concentration was replicated three fold. The animals were fed with *C. klinobasis* (2 mg POC/L) and they were transferred daily into new jars. After the third clutch had hatched from the brood chambers, mothers were removed from the jars and 6-8 neonates were kept per replicate. The jars were inspected three times a day for exuviae that would indicate that the animals had reached the second instar. During the second instar the neckteeth were scored as described above.

Predation experiments

For the predation experiments fourth instar larvae (ordered from www.interaquaristik.de) of *C. flavicans* were cultured at 16 °C, 20 °C and 24 °C for at least two days before the experiment.

They were fed daily with low numbers of neonates of *D. pulex*. At the start of the experiment two larvae and fifty neonates of *D. pulex* that had hatched within 24 h were placed in 900 mL water (1 mg C/L of *C. klinobasis*, 16:8 light:dark cycle). Each treatment was replicated three fold. The experiment ran for four days and every 24 h the remaining animals were counted. The ingested or dead ones were replaced by live neonates to keep the prey density constant. Only the animals that had been ingested by the larvae were considered for the calculation of the predation rates. Since it is known that ingestion rates of *Chaoborus* larvae can vary from one day to another, the average predation rate of the four days was calculated per jar.

Statistical analyses

In case a parameter was determined on more than one animal per replicate, the average of those values per replicate was calculated and used for statistics. All statistical analyses were performed in R Studio version 1.1.423 using R version 3.6.1. The data of the correlation experiment were analyzed using a type 3 ANVOVA followed by a Games-Howell-test due to unbalanced design. For the correlation of the neckteeth induction and the developmental time the data were checked for normal distribution and homoscedasticity. In case the data were normally distributed and variances were homogenous, Pearson's product-moment correlation was used; otherwise Spearman's rank correlation was applied. In order to derive dose-response curves, a Michaelis-Menten-model was fitted to the data. From the fitted model the maximum induction and the concentration at which half of the maximum induction is reached and also their standard errors were obtained for each temperature. Those values were tested for differences using a one-way ANOVA. The data of the predation experiments were checked for normal distribution and homoscedasticity and, since the assumptions were met, analyzed using a type 2 ANOVA. The significance level for all analyses was $p<0.05$.

Results

The stressor temperature significantly affected the developmental time (Fig. 1a). Developmental time decreased from around 75 h at 16 °C to around 60 h at 20 °C and further to around 35 h at 24 °C. Developmental times in the Chaoborus treatments did not differ from their respective Control treatments. In none of the Control treatments were neckteeth observed (Fig. 1c). Neckteeth induction in the Chaoborus treatments ranged from 51 ± 27 % at 16 °C to 43 ± 14 % at 20 °C and was significantly lower at 24 °C (3 ± 7 %).

Dietary cyanobacteria resulted in a significant increase in developmental time in the Chaoborus treatment, but not in the Control (Fig. 1b). Developmental times increased significantly from around 60 h in the treatments containing 0% cyanobacteria to around 70 h in the treatments containing 10% cyanobacteria and further to 80 h in the treatment containing 20% cyanobacteria in the food. Again, developmental times in the Chaoborus treatments did not differ from their respective Control treatments. *Daphnia* in the Control treatments did not show any neckteeth (Fig. 1d). In the Chaoborus treatments containing 0% and 10% cyanobacteria a neckteeth induction of 65 ± 3 % and 58 ± 7 %, respectively, was observed, and in the presence of 20% cyanobacteria significantly more neckteeth induction (76 ± 5 %) occurred.

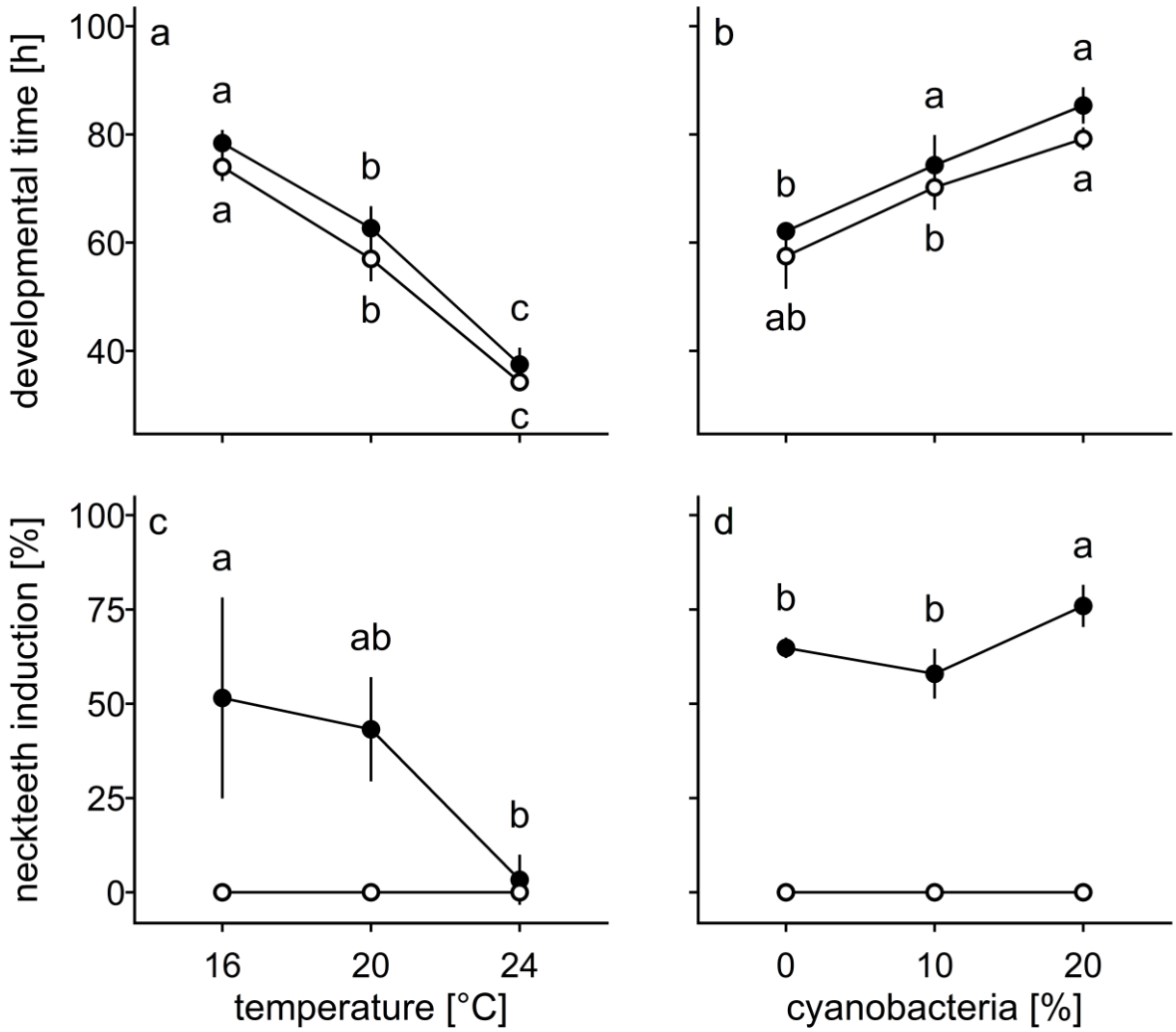

Figure 1: mean ± SD of developmental time [h] and neckteeth induction [%] of *D. pulex* at 16 °C, 20 °C or 24 °C (a,c) and at 0%, 10% or 20% shares of dietary cyanobacteria (b, d) under either control conditions (○) or in the chemical presence of *Chaoborus* (●). Different letters indicate significant differences between different treatments of either the control conditions or in the chemical presence of *Chaoborus* (type 3 ANOVA followed by Games-Howell-test; $p<0.05$). There was no difference in developmental time between Control and Chaoborus treatments. Neckteeth induction in the Chaoborus treatments differed from the Control treatments, except for the neckteeth induction at 24 °C.

Juvenile somatic growth rates (Fig. S1 c, d) as well as intrinsic rates of increase (Fig. S1 a, b) increased with temperature and decreased with increasing shares of dietary cyanobacteria. Chaoborus treatments did not differ significantly from their respective Control.

We correlated the neckteeth induction to the developmental time in order to check if neckteeth induction would increase with developmental time. In the case of dietary cyanobacteria, neckteeth induction was not related to developmental time (Pearson's product-moment correlation, p=0.2; Fig. 2), whereas neckteeth induction significantly increased with developmental time, when temperature was the experimental factor (Spearman's rank correlation, p=0.016, rho=0.68; Fig. 2).

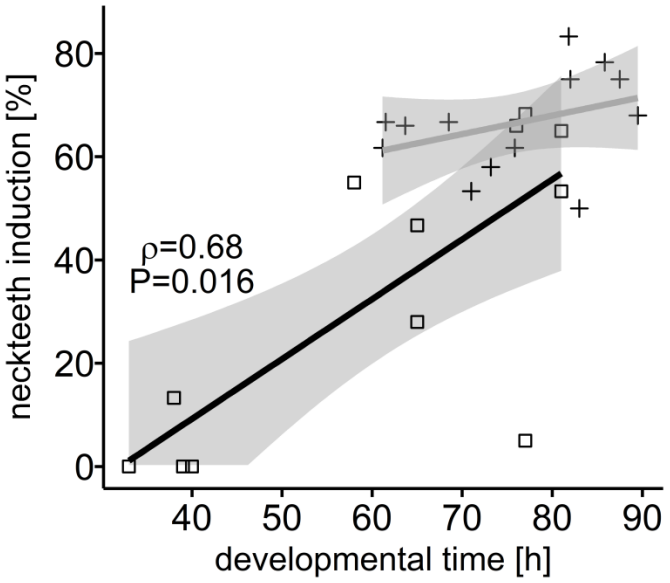

Figure 2: Neckteeth induction in *D. pulex* as a function of developmental time to reach juvenile instar 3. Data were obtained at different temperatures (□; 16 °C, 20 °C, 24 °C) or in the presence of different concentrations of dietary cyanobacteria (+; 0%, 10%, 20%). The black line indicates a linear regression with 95% confidence interval for the effects of temperature, which is significant (rho=0.68; p=0.016), and the grey line indicates a linear regression with 95% confidence interval for the effects of dietary cyanobacteria, which is not significant (p=0.2).

In order to test if the observed decline in neckteeth induction with temperature reflects a physiological constrain, we performed dose-response experiments of neckteeth induction at all three temperatures. A Michaelis-Menten-model was fitted to the data obtained a 16 °C, 20 °C and 24 °C (Fig. 3). From each fitted function we derived values for the maximum neckteeth induction and for the concentration of *Chaoborus* incubation water extract at which half of the maximum induction was reached ($Conc_{0.5max}$, Table 1).

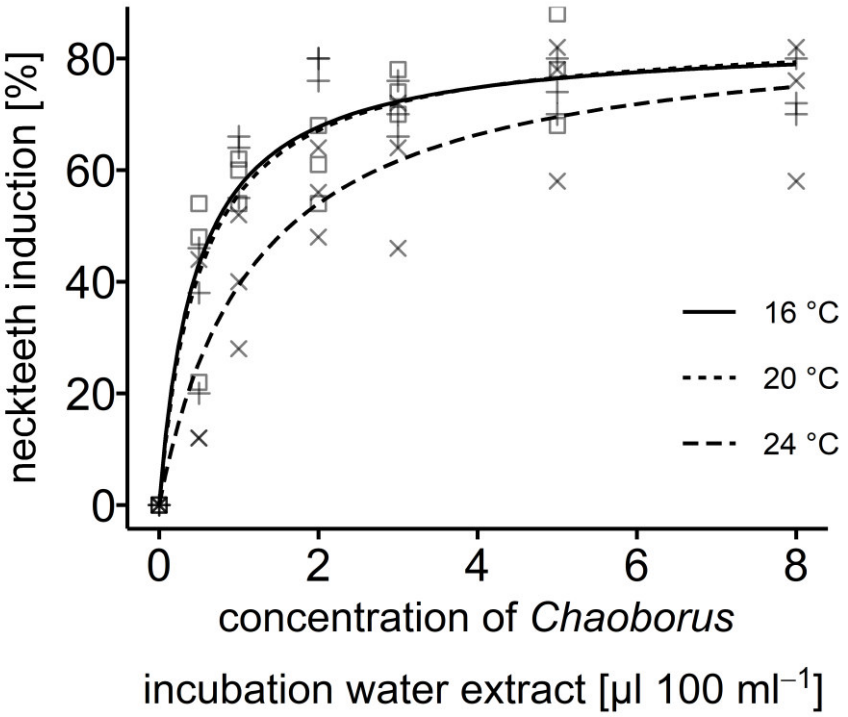

Figure 3: Neckteeth induction in *D. pulex* at 16 °C, 20 °C and 24 °C as a function of the concentration of *Chaoborus* incubation water extract (+: 16 °C; □: 20 °C; ×: 24 °C). The lines represent the results of Michael-Menten-models fitted to the respective data sets (solid: 16 °C; dashed: 20 °C; long-dashed: 24 °C). Details on the models and statistics are presented in Table 1 and Table 2.

Table 1: Maximum neckteeth induction and concentration of *Chaoborus* incubation water extract needed for induction of 50% of neckteeth maximum ($Conc_{0.5max}$). Depicted are mean values ± SE and the corresponding p-values of the model fit. Both parameters were derived from the fit of a Michaelis-Menten-model to the data obtained at 16 °C, 20 °C or 24 °C (see Fig. 3).

	16 °C		20 °C		24 °C	
	Mean ± SE	p-value	Mean ± SE	p-value	Mean ± SE	p-value
Max. induction [%]	83.6 ± 3.9	<0.001	84.5 ± 5.6	<0.001	86.1 ± 7.8	<0.001
Conc. at half max. [µl 100 ml^{-1}]	0.47 ± 0.1	<0.001	0.52 ± 0.1	0.002	1.19 ± 0.4	0.003

$Conc_{0.5max}$ did not differ with temperature (Table 2), which indicated that experimental temperature did not affect sensitivity of *D. pulex* to the *Chaoborus* kairomone. Maximum neckteeth induction was not affected by temperature (Table 2), which demonstrated that

maximum neckteeth induction in *D. pulex* was not physiologically constrained at 24 °C. Nevertheless, neckteeth induction at the concentration that we used during the first experiment, namely 1 µl, was significantly lower at 24 °C (40 ± 12 %) than at 16 °C (62 ± 6 %), but not lower than at 20 °C (59 ± 4 %). Neckteeth induction at 16 °C was not significantly higher than at 20 °C (Table 2).

Table 2: Results of the three one-way ANOVAs for the analysis of parameters obtained from the dose-response experiment (see Fig. 3). The values of maximum neckteeth induction and concentration of *Chaoborus* incubation water extract needed for induction of 50% of neckteeth maximum ($Conc_{0.5max}$) were derived from the fit of a Michaelis-Menten-model to the data obtained at 16 °C, 20 °C or 24 °C (see Table 1) and analyzed in separate ANOVAs. Another ANOVA was performed using the values of neckteeth induction at a concentration of 1 µl of *Chaoborus* incubation water extract obtained during the experiment. Significant p-values are indicated in bold ($p<0.05$).

	dF (group; residual)	F	p-value
Max. induction	2; 6	0.04	0.96
Conc. at half max.	2; 6	3.10	0.12
Induction at 1µl extract	2; 6	6.34	**0.03**

In order to check if the differences in neckteeth induction between the different temperatures might have an ecological cause, i.e. different predation risks at different temperatures, we performed predation experiments at all three temperatures with *D. pulex* without neckteeth. The experiment ran for four days, and the average predation rate per day was calculated. The predation rates were 6.4 ± 3.1 *Daphnia* per day, 9.7 ± 0.8 *Daphnia* per day and 5.6 ± 0.8 *Daphnia* per day at 16 °C, 20 °C and 24 °C, respectively. We found no significant difference between the predation rates at the different temperatures ($p=0.08$) (Fig. 4).

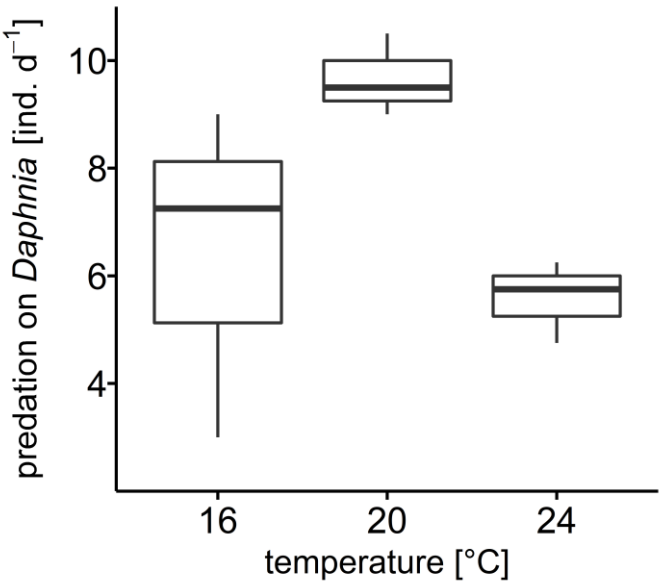

Figure 4: Predation rates [ind. d^{-1}] of larvae of *C. flavicans* on *D. pulex* at 16 °C, 20 °C and 24 °C. Horizontal lines represent the medians, boxes represent 25% and 75% quartiles, and whiskers represent the extremes. No significant differences were found between temperatures (p=0.08).

Discussion

The two chosen stressors, temperature and increasing shares of dietary cyanobacteria, affected developmental time: According to our expectations, developmental time decreased with increasing temperature and increased with increasing shares of cyanobacteria in the food. Further, according to our expectations, neckteeth induction of *D. pulex* decreased with increasing temperature and increased with increasing shares of toxic cyanobacteria in the food. Still, the correlation of neckteeth induction and developmental time was only significant for the data set of the temperature experiment.

Theoretically, the here observed positive correlation of developmental time and neckteeth induction in a temperature gradient may be due to physiological constraints of elevated temperature on neckteeth induction in *D. pulex*. However, neither maximum neckteeth induction nor the concentration at which half of the maximum neckteeth induction is reached differed with temperature. This demonstrates that neither *Daphnia*'s sensitivity to the kairomone nor the degree of phenotypic plasticity with respect to neckteeth formation were restricted at elevated temperatures. Hence, decreased neckteeth induction at elevated temperatures is not caused by physiological constraints in *Daphnia* but may, instead, be caused by shorter developmental time. Alternatively, this decrease in neckteeth induction with temperature might be caused by bacterial degradation of the kairomone as has been discussed before relating to the way of preparing the *Chaoborus* incubation water extract or the medium for *Daphnia* (Parejko & Dodson, 1991; Tollrian, 1993). The metabolic rates of bacteria increase with temperature (Ingraham & Bailey, 1959; Price & Sowers, 2004) and therefore, most probably, bacterial degradation of the kairomone is higher at elevated temperatures. The kairomone of *Chaoborus* is a fatty acid-amino acid conjugate (Weiss et al., 2018), and thus, might be a profitable source of nutrients for bacteria. During the temperature experiment we used 1 µL of *Chaoborus* incubation water extract and found significantly lower neckteeth induction at higher temperature. Those differences were also found in the dose-response experiments between the different temperatures at a concentration of 1 µL of extract. As we did not use axenic *Chlamydomonas* cultures, addition of different volumes of food suspension resulted in addition of different numbers of bacteria to the experimental jars. Since we fed 2.5 mg POC/L in the temperature experiment and only 2 mg POC/L in the dose-

response experiments, this putatively has caused higher bacterial degradation rates in the temperature experiment than in the dose-response experiment at identical experimental temperatures.

Contrasting to our results, in nature the frequency of *Daphnia* individuals with neckteeth was found to increase with water temperature (Havel, 1985). Havel (1985) reported higher percentages of induced *Daphnia* morphs during summer and no induced morphs during winter in two *D. pulex* populations that coexisted with *Chaoborus*. In order to rule out that these seasonal differences in percentage of neckteeth-bearing *D. pulex* were only caused by increased abundances of *Chaoborus* larvae during summer (Voss & Mumm, 1999), Havel (1985) used a single *D. pulex* clone and identical density of *Chaoborus* larvae in a laboratory experiment, and found increased frequencies of neckteeth-bearing individuals with increasing experimental temperature. Although these experiments suggest a positive effect of temperature on neckteeth expression in populations, our study is the first that investigates temperature effects on the *degree* of neckteeth induction, i.e. on the individual extent of morphological defense. In another laboratory experiment on the effect of temperature and the presence of *Chaoborus* on life history and neckteeth induction of *D. pulex*, during which identical concentrations of *Chaoborus* incubation water were used, temperature did not affect neckteeth induction (Walls & Ventelä, 1998). This might contradict our results and those of Havel (1985), but considering that Walls and Ventelä (1998) only fed 0.2 mg POC/L during their study and we fed at least 2 mg POC/L, bacterial degradation was probably lower during their study. Furthermore, they performed their study at 16 °C and 20 °C, which corresponds to two temperature treatments of our study, in which neckteeth induction was as well not significantly different.

To the best of our knowledge, there are no studies on the effect of dietary cyanobacteria on morphological defenses of *D. pulex* against predators. However, *D. lumholtzi* in the absence of *Chaoborus* produced longer head and tail spines at high shares of cyanobacteria in the food, whereas this trend was suppressed in the presence of *Chaoborus* (Whittington & Walsh, 2015).

We here report that predation rates of *C. flavicans* did not increase with temperature. Previous studies have shown that predation rates of *C. americanus* on *D. pulex* were increasing with temperature (Spitze, 1985), possibly because of increasing encounter rates due to increased swimming speed of *Daphnia* at higher temperatures. However, *Daphnia* swimming speed does not change with temperature (Gorski & Dodson, 1996; Swift & Fedorenko, 1975). Furthermore, the temperature dependency of predation rates can vary with season and also with age of the

larval instar (Fedorenko, 1975): In line with our results, predation rates of fourth instar larvae were largely lacking temperature dependency, except for old fourth instar larvae (reached fourth instar the year before) that were captured during early summer. Our finding that predation rates of *C. flavicans* did not increase with temperature means that the risk for *D. pulex* of being preyed upon by a given abundance of *Chaoborus* larvae does not increase with temperature. This is in line with our results of the dose-response experiments that *Daphnia*'s sensitivity for neckteeth induction is not affected by temperature.

In our study, we found no costs related to the induction of neckteeth, neither with respect to juvenile somatic growth nor to intrinsic population growth. Costs of neckteeth induction have been reported as a delay of reproduction or reduced fitness measured as the intrinsic rate of increase (Black & Dodson, 1990; Havel & Dodson, 1987). Here, mean developmental times of *Daphnia* in Chaoborus treatments were indeed longer than those in the respective Control treatments, but not significantly different. Similarly, somatic growth rates or the intrinsic rates of increase were not affected by *Chaoborus* kairomones. This absence of detectable costs of neckteeth induction is corroborated by numerous studies (Riessen, 2012; Tollrian, 1995b; Walls et al., 1997; Whittington & Walsh, 2015). These contradicting results that can be found in literature may most probably be attributed to the fact that different *D. pulex* clones were used and that clones can vary greatly in their responses to predators (Dennis et al., 2011; Lüning, 1992; Parejko & Dodson, 1991).

We here report that neckteeth induction in *D. pulex* decreases with developmental time in response to temperature, but is not affected by developmental time in response to dietary cyanobacteria. Hence, we conclude that developmental time is not a general cue that *D. pulex* uses to account for increased predation risk resulting from extended developmental time. We cannot exclude that the temperature-dependent differences in neckteeth induction that we observed were caused by different rates of bacterial degradation of the kairomone in the different treatments. We further show that *D. pulex* is neither physiologically restricted in its ability to express neckteeth at different temperatures nor that there is an ecological need to adjust the neckteeth induction with temperature as the risk of being preyed upon by *Chaoborus* larvae did not depend on temperature.

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Supplements

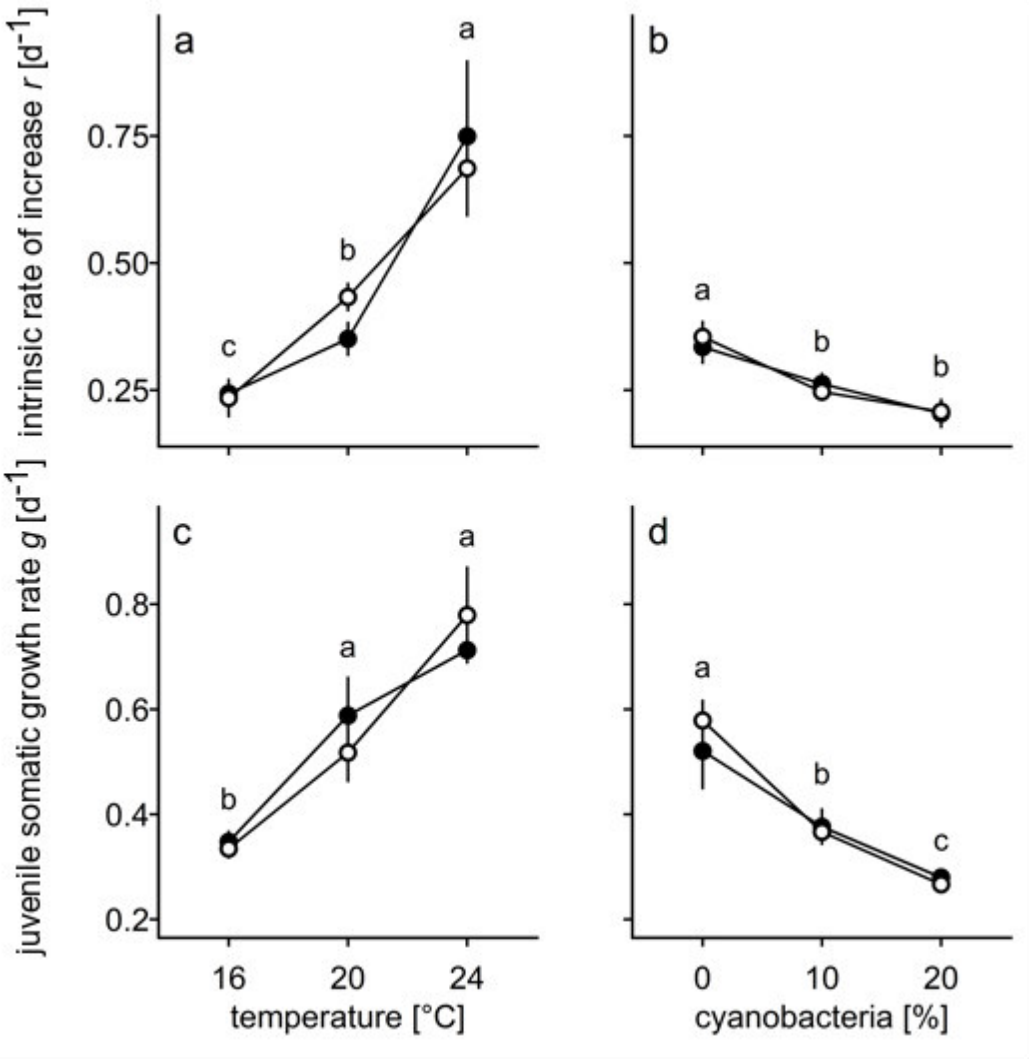

Figure S1: mean ± SD of the intrinsic rate of increase r [d^{-1}] (a, b) and the juvenile somatic growth rate g [d^{-1}] (c, d) of *D. pulex* at 16 °C, 20 °C or 24 °C (a, c) and at a concentration of dietary cyanobacteria of 0%, 10% or 20% (b, d) under either control conditions (○) or in the chemical presence of *Chaoborus* (●). Different letters indicate significant differences between different treatments of either the control conditions or in the chemical presence of *Chaoborus* (type 3 ANOVA followed by Games-Howell-test; $p<0.05$). There were no differences found between Control and the respective Chaoborus treatments. In Fig. S1 a, b and d the significance codes for Control and Chaoborus treatments are the same. In Fig. S1 c the significance codes above the error bars represent the Chaoborus treatments and the significance codes below the error bars represent the Control.

Chapter 2

No food quality effect on morphological defense in *Daphnia pulex*

Abstract

1. In lakes, a crucial link between trophic levels is herbivorous zooplankton, which can be bottom-up limited by food quality. For the genus *Daphnia*, food quality has often been reported to be determined by the content of polyunsaturated fatty acids (PUFAs). The PUFA content of phytoplankton can be highly fluctuating throughout the year, and can even become limiting to *Daphnia* growth. Another factor that can restrict *Daphnia* populations is predation. Since predation pressure is as well highly fluctuating throughout the year, *Daphnia* has evolved inducible defenses against various predators that are only expressed when predators are present. However, knowledge about how these two factors, biochemical food quality and the presence of predators, interact is still scarce.
2. Here, we crossed biochemical food quality with respect to fatty acid composition with the chemical presence of the predacious phantom midge larvae, *Chaoborus*, which preys on juvenile *Daphnia pulex*. *D. pulex* forms neckteeth during its juvenile instars as a morphological defense in the presence of *Chaoborus*. We used three clones of *D. pulex* and performed dose-response experiments, which showed that the clones differed in sensitivity to *Chaoborus* incubation water extract with respect to neckteeth induction. We created a gradient of food quality with increasing PUFA availability using *Acutodesmus obliquus*, *Chlamydomonas klinobasis*, and *Cryptomonas* sp. as food for *D. pulex* and quantified the neckteeth induction during the second juvenile instar of three different clones of *D. pulex*.
3. Food quality did not affect neckteeth induction, but the presence of *Chaoborus* and the identity of the clone did. The only significant interaction of factors was detected for the presence of *Chaoborus* and the identity of the clone.
4. We can, therefore, conclude that neckteeth induction is not affected by biochemical food quality.
5. *D. pulex* seems to be able to protect itself against persistent predation throughout the seasons to the same extend and independently of food quality. In turn, *D. pulex* seems to not be able to adjust its defenses to inferior food quality although its growth rates are reduced.

Introduction

Trophic transfer of energy and nutrients is an important function of ecosystems (Schmitz et al., 2008). In lakes, zooplankton that feeds on phytoplankton is a crucial link between primary producers and higher trophic levels. Many of those zooplankton species belong to the genus *Daphnia* spp., and they are unselective filter feeders. For *Daphnia* it has repeatedly been reported that the transfer efficiency can be highly dependent on food quantity and food quality (Müller-Navarra et al., 2000; Persson et al., 2007; Sterner & Hessen, 1994). When determining food quality for *Daphnia*, different aspects have to be considered: edibility (Vanni & Lampert, 1992), morphology (Gliwicz & Siedlar, 1980; Hartmann & Kunkel, 1991; Wejnerowski et al., 2015), stoichiometric quality (Lürling & van Donk, 1997), or biochemical quality like the content of fatty acids or sterols (Ahlgren et al., 1990; Brett & Müller-Navarra, 1997; Martin-Creuzburg & von Elert, 2009).

First indications that the availability of fatty acids potentially limits the growth of *Daphnia* were provided by Ahlgren et al. (1990), who measured the contents of single fatty acids of several phytoplankton taxa and determined growth rates of *Daphnia* feeding on those phytoplankton taxa. Ahlgren et al. (1990) reported higher growth rates for *Daphnia* feeding on taxa with high contents of long-chained (≥ 20 C-atoms) polyunsaturated fatty acids (PUFA), especially eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), whereas *Daphnia* feeding on taxa containing mainly short-chained saturated and monounsaturated fatty acids exhibited lower growth rates. Further evidence that specific fatty acids potentially limit the growth of zooplankton was provided by a study during which several sestonic parameters were related to *Daphnia* growth rates over a whole season (Müller-Navarra, 1995b). The strongest correlation was found for EPA. A similar approach in a study on a different lake suggested that not EPA is the limiting fatty acid, but α-Linolenic acid (ALA) (Wacker & von Elert, 2001). Ultimately, supplementation on the same basal food with single fatty acids proved that food quality may be limited by a low content of specific fatty acids. One of the first studies indicated that *Daphnia* convert ALA into EPA, and that actually EPA is the limiting fatty acid (von Elert, 2002). Further studies corroborated the limiting role of EPA and additionally demonstrated a co-limitation by EPA and sterols (Martin-Creuzburg et al., 2009; Martin-Creuzburg & von Elert, 2009), while

others found groups of PUFAs or several single PUFAs to be increasing the somatic and population growth of *Daphnia* (Ilić et al., 2019; Martin-Creuzburg et al., 2010; Ravet et al., 2003).

Since a low phosphorus (P) content can also be a constraint of phytoplankton food quality for *Daphnia* (Elser et al., 2001), and since P may affect the content of PUFAs in algae (Müller-Navarra et al., 2004), stoichiometric and biochemical food quality may interact. In fact, several studies reported that growth of *Daphnia* is P-limited at C:P ratios higher than 350 and limited by fatty acids at C:P ratios lower than 350, but no interaction was detected (Becker & Boersma, 2003; Ravet & Brett, 2006; Sundbom & Vrede, 1997). Overall, it was concluded that in lakes with phytoplankton with C:P < 350 food quality will be constrained by the availability of fatty acids (Müller-Navarra, 1995a; Ravet & Brett, 2006), and single or groups of fatty acids are better predictors of *Daphnia* growth than the C:P ratio (Park et al., 2002).

The abundance of *Daphnia* may not only be limited by the bottom-up parameter food quality, but as well by the top-down parameter predation (Balseiro et al., 2007; Burns & Dodds, 1999; Wu & Culver, 1994). Predators can impact *Daphnia* abundances to a great extend (Elser et al., 1987; Mills & Forney, 1983), and predator abundances can vary largely during the year (Burns & Dodds, 1999; Fedorenko & Swift, 1972), resulting in highly fluctuating predation risk. *Daphnia* has evolved various inducible defenses, which are only expressed in the presence of those predators, comprising changes in behavior, shifts in life history, or alterations of the phenotype (Diel et al., 2020). The strength of expression of many inducible defenses is influenced by predator abundance (Tollrian, 1993; von Elert & Pohnert, 2000; von Elert & Stibor, 2006), food quantity or the density of conspecifics (Tollrian et al., 2015). High food quantity was often reported to enhance inducible defenses, whereas quantitative food limitation suppressed some inducible defenses (Dodson, 1988; Dodson & Havel, 1988; Hanazato, 1991; Johnsen & Jakobsen, 1987; Loose & Dawidowicz, 1994).

Several studies have correlated growth of *Daphnia* on seston over a whole season with seston parameters (Müller-Navarra, 1995b; Müller-Navarra et al., 2004; Wacker & von Elert, 2001). The fact that in all of them particulate organic carbon (POC) and sestonic C:P explained substantially less of the variation of *Daphnia* growth rates over the whole season than particulate fatty acids, indicates that for *Daphnia* growth in nature low food quantity is of minor relevance than low dietary content of particulate fatty acids. We were, therefore, interested in potential effects of fatty-acid related food quality on inducible anti-predator defenses in *Daphnia*.

However, insight into how biochemical food quality affects the expression of inducible defenses is still scarce. To the best of our knowledge, there is only one study investigating the effect of PUFA availability on inducible defenses of *Daphnia*. Diel vertical migration (DVM), a behavioral inducible defense in *Daphnia* as a response to fish, was suppressed, when PUFAs were limiting (Brzeziński & von Elert, 2015), because DVM-performing *Daphnia* are exposed to a lower mean temperature (Stich & Lampert, 1981) and the demand for PUFAs increases to maintain membrane fluidity (Hahn & von Elert, 2020; Hazel, 1995; Sperfeld & Wacker, 2012).

In order to further elucidate the effects of PUFA availability on inducible defenses, we crossed food quality with respect to fatty acid composition with the chemical presence of *Chaoborus* sp. We created a gradient of food quality providing three different algae as food for *D. pulex*, which are known to differ in their fatty acid composition and known to result in different somatic growth rates (Ahlgren et al., 1990). We chose *Acutodesmus obliquus*, *Chlamydomonas klinobasis* and *Cryptomonas* sp. *Cryptomonas* contains high concentrations of stearidonic acid (SDA), and, more importantly, it contains the long-chained PUFAs EPA, docosapentaenoic acid (DPA) and DHA, whereas both *Acutodesmus* and *Chlamydomonas* lack any long-chained fatty acids. In comparison to *Chlamydomonas*, *Acutodesmus* contains more saturated fatty acids (SFAs) and monounsaturated fatty acids (MUFAs), most of them short-chained, and *Chlamydomonas* contains more short-chained PUFAs (Ahlgren et al., 1990; Taipale et al., 2013; von Elert, 2002; von Elert & Stampfl, 2000). We investigated if the morphological inducible defense of *D. pulex* is affected by the availability of dietary fatty acids. In the chemical presence of *Chaoborus D. pulex* forms neckteeth during its juvenile stages, which are small protuberances in its neck region that have been reported to reduce predation rates of *Chaoborus* to a great extend (Tollrian, 1995a). We quantified the strength of expression of this defense by scoring the neckteeth during the second juvenile instar, during which the neckteeth are known to be most pronounced (Tollrian, 1993). We used three clones of *D. pulex* that slightly differed in their sensitivity to *Chaoborus* to achieve a generalization of our results. Since it has been reported before that EPA affected another inducible defense in *Daphnia*, we further tested the effect of EPA-supplementation on neckteeth induction.

Methods

Study species

D. pulex clones Gerstel (Koch et al., 2009), clone TCO (Colbourne et al., 2011), and clone SGus (pers. comm. S. Gustafsson, Sweden) were cultured in aged and aerated tap water at 19.2 ± 0.3 °C and a 16:8 light:dark cycle at a density of 10-12 individuals per 800 mL. The animals were transferred into fresh medium containing at least 1 mg C/L of *Chlamydomonas klinobasis* strain # 56 (Limnological Institute, University of Constance) every second day.

Acutodesmus obliquus strain SAG 276-3a (Culture Collection of Algae, Göttingen University), *C. klinobasis*, and *Cryptomonas* sp. strain SAG 26.80 (Culture Collection of Algae, Göttingen University) were grown in 5 L semi-continuous batch-cultures. *C. klinobasis* and *Cryptomonas* were grown in cyanophyceae medium (von Elert & Jüttner, 1997) modified with vitamins, whereas *A. obliquus* was grown in Z/4 medium (Zehnder & Gorham, 1960). Every second day, 1 L of the cultures was replaced by fresh medium. The food suspensions were screened using a 30 µm gauze, and the volume of the food suspension that was needed was determined photometrically at a wavelength of 470 nm by using a respective calibration curve relating the carbon content to the optical density. The algae *A. obliquus*, *C. klinobasis*, and *Cryptomonas* sp. are subsequently referred to as *Acutodesmus*, *Chlamydomonas*, and *Cryptomonas*, respectively.

Preparation of *Chaoborus* incubation water extract

The extract of *Chaoborus* incubation water was prepared as according to Klintworth and von Elert (2020). Approximately 300-350 fourth instar larvae (ordered from www.interaquaristik.de) of *Chaoborus flavicans* were fed with 1-2 neonates of *D. pulex* per larva. After 1-2 hours of feeding, the larvae were transferred into 1 L of fresh aged and aerated tap water without any food. After 24 hours the larvae were removed from the water using a 250 µm gauze, and the water was filtered through a glass fiber filter (Whatman, MN 85/220, 0.4 µm). Subsequently, the kairomone was enriched by solid phase extraction (VARIAN, Bond Elut-C18, 10 g of sorbent, volume 60 mL, Agilent Technologies, CA, USA) as according to Christjani et al. (2016). All eluates were pooled and evaporated to dryness in a rotary evaporator and a vacuum centrifuge. The pooled and

dried residues of 20 L were dissolved in 1 mL methanol and stored at -20 °C until use. In previous experiments a control extract, which was prepared in exactly the same way but without any animals in the water, did not induce neckteeth in *D. pulex* (Klintworth & von Elert, 2020).

Study design

Dose-response experiments

During the experiment different batches of *Chaoborus* incubation water extract were used for each clone. In order to maintain comparability between batches, dose-response experiments were performed beforehand for each batch and the respective *D. pulex* clone on *Chlamydomonas*. Animals that had released their third or fourth clutch into the brood chamber were divided into the different treatments containing 0, 0.2, 0.5, 1, 2, 3, 5, 6, or 7 µl of *Chaoborus* incubation water in 100 ml of aged and aerated tap water. The treatments containing 0.2 and 6 µl of *Chaoborus* incubation water extract were only prepared for clone SGus. The treatment containing 7 µl of *Chaoborus* incubation water extract was only prepared for clone Gerstel. *Chlamydomonas* was provided at a concentration of 2 mg C/L. Animals were kept individually and each treatment was replicated threefold. The animals were transferred daily to freshly prepared jars. After the neonates had hatched from the brood chambers, the mothers and neonates were removed from the jars so that no more than six neonates remained in the jars. During their second juvenile instar, neckteeth induction of five randomly chosen animals per jar was determined using the method developed by Tollrian (1993) with the slight modification that no differentiation was made between small and big teeth and that each tooth was scored with 10%. Dose-response curves were fitted as Michaelis-Menten models on the data for each clone. From the models, the maximum induction and the concentration of *Chaoborus* incubation water extract needed for the induction of 50% of neckteeth induction maximum ($Conc_{0.5max}$) were derived for each clone. A volume slightly higher than the volume of $Conc_{0.5max}$ was used during the next experiment. For the subsequent experiment with clone Gerstel, TCO, and SGus 1.5 µL, 1 µL, and 0.2 µL of the *Chaoborus* incubation water extract were used, respectively.

Experimental setup

Animals that had released their first clutch into the brood chamber were divided into the different food treatments, containing 2 mg C/L of either *Acutodesmus*, *Chlamydomonas*, or *Cryptomonas*. When the animals had released their second clutch into the brood chamber, they were further divided into a control treatment and a kairomone treatment. The kairomone treatment contained the respective volume of *Chaoborus* incubation water extract for each clone. For clone SGus each treatment was replicated threefold, and for the clones Gerstel and TCO each treatment was replicated fivefold.

The animals carrying their second clutch in the brood chamber were kept individually in 100 mL water containing the respective food and either the *Chaoborus* incubation water extract or no extract. After the neonates of the second clutch had been released from the brood chambers, the mothers were removed from the jars. Six experimental neonates remained in the jars. All neonates that were removed were pooled per treatment and their dry mass was measured in subsamples of two times ten neonates per treatment. These dry masses were later on used as w_0 for the calculation of the somatic growth rates. The animals were transferred to freshly prepared jars every day. During the second juvenile instar, neckteeth induction was determined as described above. When the animals had deposited their first clutch into their brood chambers, the time to maturity was determined, and three animals per jar were taken randomly for the determination of their dry mass (w_t). The somatic growth rates (g) were calculated according to the following formula: $g = (\ln(w_t) - \ln(w_0)) / t$, with w_t being the individual weight at day t and w_0 being the individual weight at day 0 (Rothhaupt & Lampert, 1992).

Supplementation experiment

EPA was supplemented via liposomes that were prepared as according to Martin-Creuzburg and von Elert (2009). The final concentration of EPA in the liposome suspension was 150 µg/mL. The volume of EPA liposomes that were supplemented was adjusted to the concentration of EPA in 2 mg C of *Cryptomonas*. The same volume of control liposomes was added to the control treatment.

The supplementation experiments were conducted using *D. pulex* clone TCO as described under 'Experimental setup'. *Acutodesmus* was used as basal food in all four treatments. The control was

pure *Acutodesmus* with no *Chaoborus* incubation water extract. The second treatment was *Acutodesmus* with 1 µl of *Chaoborus* incubation water extract. The second treatment was further supplemented with 13.3 µL of either control liposomes or EPA liposomes, resulting in the third and fourth treatment, respectively. The liposomes as well as the incubation water extract were pipetted on the bottom of the jars, and after the solvents had evaporated, 100 mL water and 2 mg C/L of *Acutodesmus* were added. All treatments were replicated fivefold.

Data analysis

All statistical analyses were performed in RStudio version 1.1.423 (R version x64 3.6.1). In case a parameter was determined on more than one animal per replicate, the average of those values per replicate was calculated and used for statistics. For the analysis of the data derived from the Michaelis-Menten models that were fitted to the dose-response data of each clone, one-way ANOVAs type 2 followed by Tukey´s HSD test were performed. Data were always checked for homoscedasticity, and, if given, an ANOVA type 3 followed by a Games-Howell test was performed due to unequal sample sizes. The data of the supplementation experiment were homoscedastic and analyzed by performing a one-way ANOVA. The significance level for all analyses was $p<0.05$.

Results

Dose-response experiments revealed that mean values of maximum neckteeth induction among the *D. pulex* clones ranged from 84% in clone TCO over 93% in clone SGus to 104% in clone Gerstel (Fig. 1, Table 1). The concentration of *Chaoborus* incubation water extract needed for the induction of 50% of neckteeth induction maximum ranged from 0.04 µl in clone SGus over 0.5 µl in clone TCO to 1.1 µl in clone Gerstel (Fig. 1, Table 1). The values of the maximum neckteeth induction did not differ (one-way ANOVA, $F_{2;6}$=2.47, p=0.17) (Fig. 1), but there was a significant difference between clones revealed by the analysis of the concentration of *Chaoborus* incubation water extract needed for induction of 50% of neckteeth induction maximum ($Conc_{0.5max}$) (one-way ANOVA, $F_{2;6}$=8.28, p=0.02) (Fig. 1).

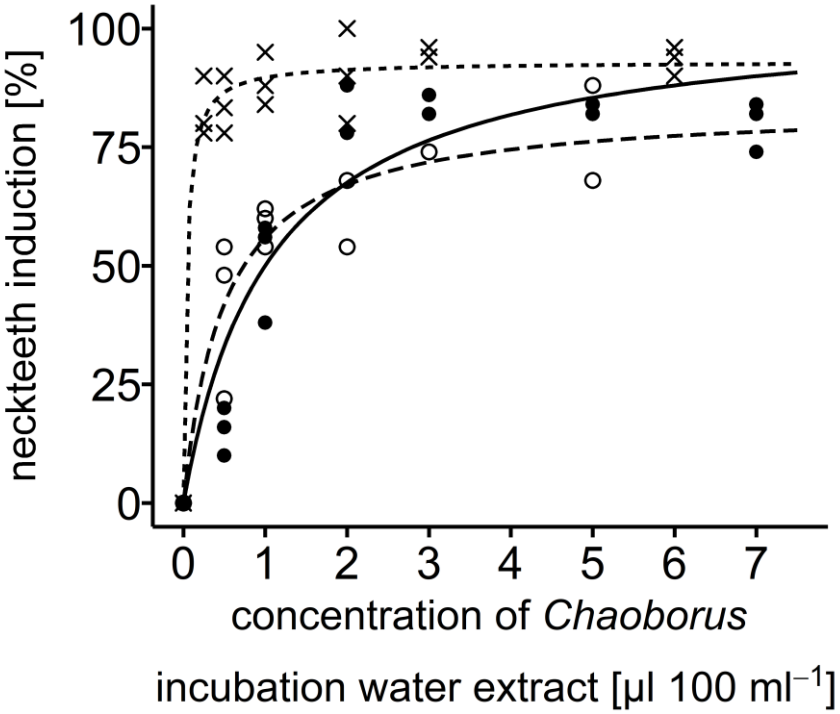

Figure 1: Neckteeth induction of *D. pulex* clones Gerstel (●), SGus (×), and TCO (○), growing on *C. klinobasis* as a function of the concentration of *Chaoborus* incubation water extract. The lines represent the results of Michael-Menten-models fitted to the respective data sets (solid: Gerstel; dashed: SGus; long-dashed: TCO). Details on the models are presented in Table 1. Details on the statistical analysis are given in the text.

For clone SGus a significantly lower concentration than for clone Gerstel was needed (Tukey's HSD test, SGus vs. Gerstel p=0.016). $Conc_{0.5max}$ for clone TCO was not significantly different from either of the other clones (Tukey's HSD test, TCO vs. Gerstel p=0.14, TCO vs. SGus p=0.24).

Table 1: Maximum neckteeth induction and concentration of *Chaoborus* incubation water extract needed for induction of 50% of neckteeth induction maximum ($Conc_{0.5max}$). Both parameters were derived from the fit of a Michaelis-Menten-model to the data obtained for *D. pulex* clones Gerstel, SGus, and TCO (see Fig. 1). Presented are mean values ± SE and the respective p-values of the model fit.

	Gerstel		SGus		TCO	
	Mean ± SE	p-value	Mean ± SE	p-value	Mean ± SE	p-value
Max. induction [%]	103.7 ± 7.7	<0.001	93.0 ± 1.9	<0.001	83.8 ± 7.6	<0.001
Conc. at half max. [µl 100 ml^{-1}]	1.07 ± 0.3	<0.001	0.04 ± 0.0	0.011	0.50 ± 0.2	0.012

Neckteeth induction in the Chaoborus treatments on the different food algae ranged between 59% and 83% for all clones (Fig. 2). No neckteeth were observed in the Control treatments. A three-way ANOVA for the analysis of the neckteeth induction revealed that food algae did not affect the neckteeth induction, whereas the presence of the kairomone and the identity of the clone did (Table 2). There was no significant interaction with the factor food, but the presence of the kairomone and the identity of the clone interacted significantly. There was neither a significant difference between different food treatments within the same clone nor was there a significant difference between clones within the same food treatment (Fig. 2).

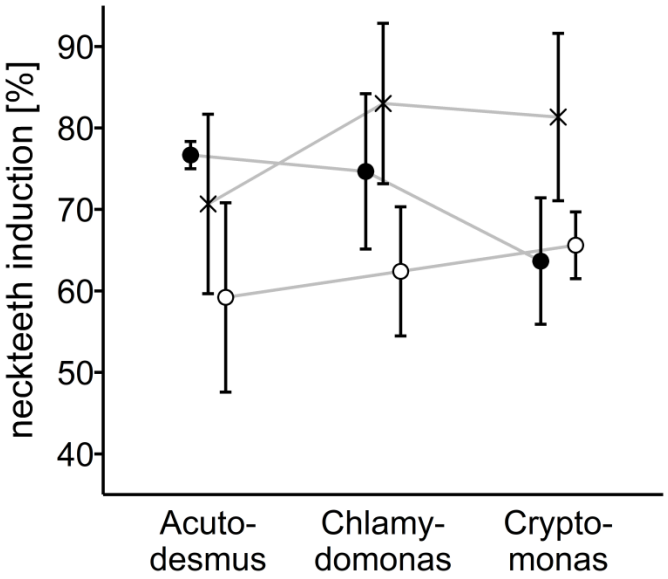

Figure 2: Mean ± SD neckteeth induction [%] of *D. pulex* clones Gerstel (●), SGus (×), and TCO (○), growing either on *Acutodesmus*, *Chlamydomonas*, or *Cryptomonas*. Only data of the Chaoborus treatments are depicted. No neckteeth were observed in the Control treatments. For details of the statistical analysis see Table 2. For reasons of clarity symbols referring to the same clone were connected by lines.

Table 2: Results of a three-way ANOVA type 3 for the analysis of the neckteeth induction (see Fig. 2). Only data of the treatments containing *Chaoborus* incubation water extract were included in the analysis. Significant p-values are indicated in bold ($p<0.05$).

	Df	Sum Sq	F-value	p-value
(Intercept)	1	87005	2302.01	**<0.001**
Food (F)	2	62	0.82	0.45
Kairomone (K)	1	87005	2302.01	**<0.001**
Clone (C)	2	758	10.03	**<0.001**
F × K	2	62	0.82	0.45
F × C	4	334	2.21	0.08
K × C	2	758	10.03	**<0.001**
F × K × C	4	334	2.21	0.08
Residuals	56	2117		

Somatic growth rates ranged from 0.3 to 0.7 d^{-1} in all clones (Fig. 3). Clone Gerstel exhibited a growth rate of 0.29 d^{-1} on *Acutodesmus*, which increased significantly on *Chlamydomonas* and *Cryptomonas* to 0.48 d^{-1} and 0.52 d^{-1}, respectively. The growth rates of clone SGus were 0.33 d^{-1} and 0.4 d^{-1} on *Acutodesmus* and *Chlamydomonas*, respectively, and increased significantly on

Cryptomonas to 0.51 d^{-1}. Clone TCO grew at a rate of 0.41 d^{-1} on *Acutodesmus*, and its growth rates on *Chlamydomonas* and *Cryptomonas* increased significantly to 0.62 d^{-1} and 0.68 d^{-1}, respectively. A two-way ANOVA for the analysis of the somatic growth rates revealed that both factors, food algae and the identity of the clone, affected somatic growth (Table 3). No significant interaction of both factors was detected.

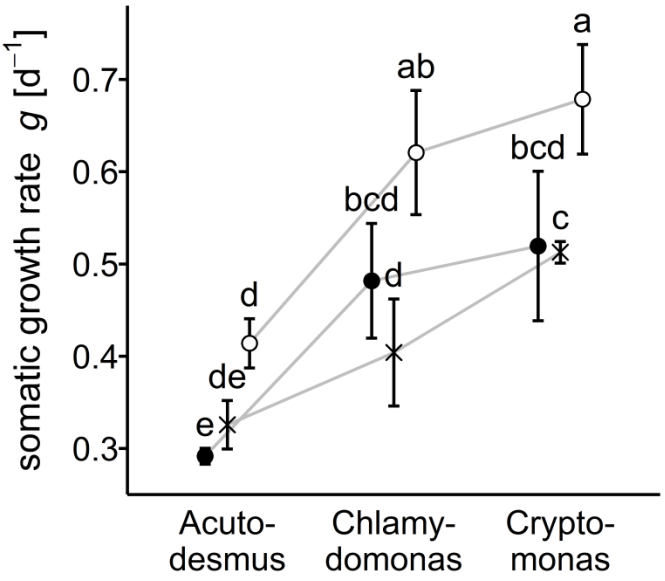

Figure 3: Mean ± SD somatic growth rate g [d^{-1}] of *D. pulex* clones Gerstel (●), SGus (×), and TCO (○) growing either on *Acutodesmus*, *Chlamydomonas*, or *Cryptomonas*. Treatments with the same letters are not significantly different (two-way ANOVA type 3 followed by Games-Howell test; $p<0.05$). For details of the statistical analysis see Table 3. For reasons of clarity symbols referring to the same clone were connected by lines.

Table 3: Results of a two-way ANOVA type 3 for the analysis of the somatic growth rates in the absence of *Chaoborus* (see Fig. 3). Significant p-values are indicated in bold ($p<0.05$).

	Df	Sum Sq	F-value	p-value
(Intercept)	1	7.22	2379.04	**<0.001**
Food (F)	2	0.30	48.99	**<0.001**
Clone (C)	2	0.19	30.58	**<0.001**
F × C	4	0.02	1.30	0.30
Residuals	27	0.08		

Time to maturity ranged from 4.4 d to 9 d in all clones (Fig. 4). Clone Gerstel needed 9 d to reach maturity when growing on *Acutodesmus*, whereas it took significantly less time to reach maturity when growing on *Chlamydomonas* and *Cryptomonas* (5.8 d and 5.6 d, respectively). Although Clone SGus showed a similar pattern with decreasing time to maturity with increasing food quality (from 8.7 d to 6 d), none of those treatments were significantly different due to high variances. Clone TCO growing on *Acutodesmus* needed only 6.2 d to reach maturity and needed significantly less time when growing on *Chlamydomonas* and *Cryptomonas* (4.8 d and 4.4 d, respectively). A two-way ANOVA for the analysis of the time to maturity revealed that the two factors, food algae and the identity of the clone, as well as the interaction of the two factors affected the time to maturity (Table 4).

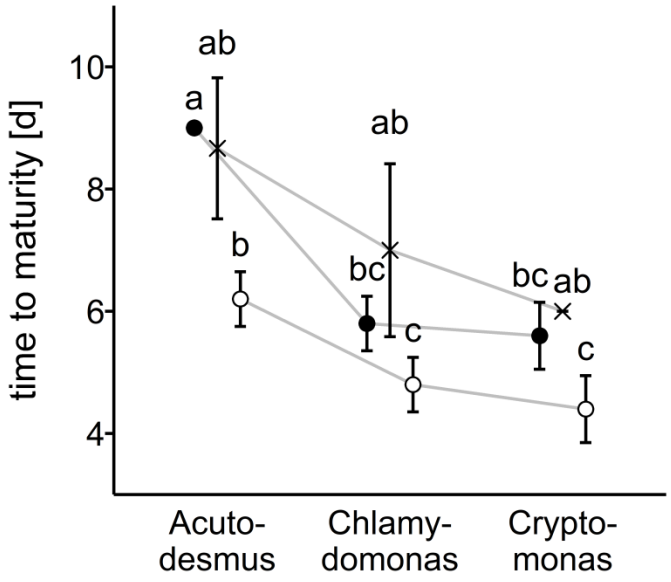

Figure 4: Mean ± SD time to maturity [d] of *D. pulex* clones Gerstel (●), SGus (×), and TCO (○) growing either on *Acutodesmus*, *Chlamydomonas*, or *Cryptomonas*. Treatments with the same letters are not significantly different (two-way ANOVA type 3 followed by Games-Howell test; $p<0.05$). For details of the statistical analysis see Table 4. For reasons of clarity symbols referring to the same clone were connected by lines.

Table 4: Results of a two-way ANOVA type 3 for the analysis of the time to maturity in the absence of *Chaoborus* (see Fig. 4). Significant p-values are indicated in bold ($p<0.05$).

	Df	Sum Sq	F-value	p-value
(Intercept)	1	1320.97	3767.55	**<0.001**
Food (F)	2	41.78	59.58	**<0.001**
Clone (C)	2	29.53	42.11	**<0.001**
F × C	4	4.47	3.19	**0.029**
Residuals	27	9.47		

Neckteeth induction in the supplementation experiments was 59% on *Acutodesmus*, and 47% and 58% in the treatments containing the control liposomes and EPA liposomes, respectively (Fig. 5). No neckteeth were observed in the control treatment. There was no significant difference between the treatments containing *Chaoborus* incubation water extract (one-way ANOVA, $F_{2;12}=0.904$, $p=0.43$).

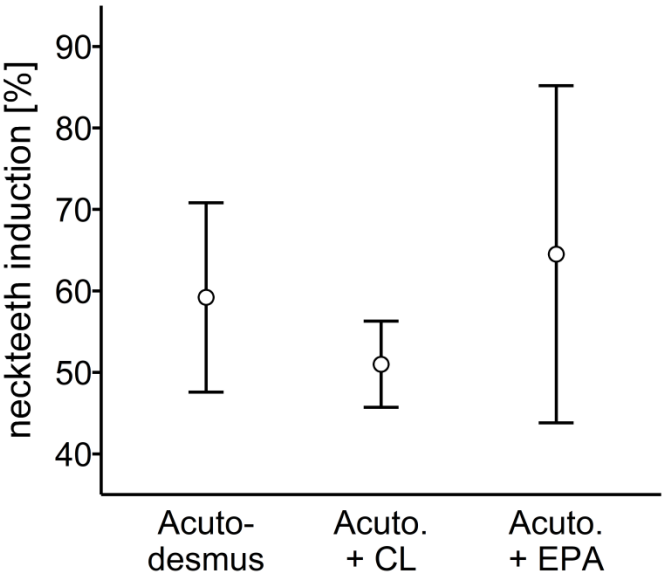

Figure 5: Mean ± SD neckteeth induction [%] of *D. pulex* clone TCO, growing either on *Acutodesmus*, *Acutodesmus* and control liposomes (Acuto. + CL), or *Acutodesmus* and EPA liposomes (Acuto. + EPA). Only data of the Chaoborus treatments are depicted. No neckteeth were observed in the Control treatment.

Discussion

We crossed biochemical food quality in terms of fatty acid composition with the chemical presence of the predator *Chaoborus* to investigate if neckteeth induction in *D. pulex* as a morphological defense is affected by food quality. In order to create a gradient of food quality, we provided three algae that are known to differ in their fatty acid composition and in their quality as food for *Daphnia* (Ahlgren et al., 1990). Food quality affected somatic growth and we observed the expected gradient with growth rates increasing with PUFA availability, which confirms that the three different algal food sources were of different quality. Furthermore, identity of the *D. pulex* clone affected the somatic growth response to the different food qualities, which is in line with an earlier report of clonal variability in response to biochemical food quality (Brzeziński et al., 2010). Accordingly, the time to maturity was affected by food quality as well as the identity of the clone and the interaction of both factors, resulting in the expected gradient with time to maturity decreasing with increasing PUFA availability.

Food quality did not affect neckteeth induction, and we found no significant differences between the food treatments in neither of the investigated *D. pulex* clones. However, the identity of the clone affected neckteeth induction, and we also detected differences in the clones' sensitivity to the kairomone. By creating dose-response curves and fitting a model to these curves, we were able to derive values for maximum neckteeth induction and the concentration of the *Chaoborus* incubation water extract, which is needed to induce 50% of the maximum neckteeth induction ($Conc_{0.5max}$). The clones did not differ in their possible maximum neckteeth induction, but $Conc_{0.5max}$ differed significantly between *D. pulex* clone Gerstel and clone SGus. *D. pulex* clone SGus needed significantly less volume of the *Chaoborus* incubation water extract than clone Gerstel to induce 50% of its maximum neckteeth induction. These differences reflect different sensitivities of the *D. pulex* clones to *Chaoborus* kairomone, and such clonal variability in response to the *Chaoborus* kairomone is well known (Boeing et al., 2006; Hammill et al., 2008) and reported also for other predators or *Daphnia* species (Boeing et al., 2006; Weber & Declerck, 1997).

D. pulex clone SGus seems to be generally inferior in comparison to the other clones with lower somatic growth rates and later maturity. In lakes, under *Chaoborus* predation, *D. pulex* clone

SGus might still be able to compete due to its higher sensitivity to the kairomone, and therefore, potentially higher neckteeth induction. Since the gain in inducible protection is correlated with the degree of neckteeth induction (Tollrian, 1995a), this potentially higher neckteeth induction might result in lower predation losses of *D. pulex* clone SGus. However, due to the fact, that we adjusted the volume of *Chaoborus* incubation water extract to $Conc_{0.5max}$ for each clone, neckteeth induction between clones within the same food treatment is not comparable.

We found no effect of food quality on the inducible defense and no interaction of food quality and kairomone, which is notwithstanding Brzeziński and von Elert (2015), who reported that fish-induced DVM in *Daphnia* is suppressed when PUFAs are limiting. This effect of food quality on DVM might be due to the reduced temperatures *Daphnia* is exposed to during DVM (Stich & Lampert, 1981). In order to cope with the cost of reduced temperatures associated with this defense, the share of saturated fatty acids in *Daphnia* decreases and the relative PUFA content as well as the allocation of PUFAs to the offspring increases under simulated DVM conditions (Hahn & von Elert, 2020). These effects were most pronounced for EPA. Accordingly, *Daphnia* grows faster and exhibits higher population growth rates on EPA-sufficient food during simulated DVM (Isanta Navarro et al., 2019). Thus, DVM associated costs increase the demand for PUFAs in *Daphnia*. However, it is controversial if costs of neckteeth induction are detectable (Riessen, 2012; Tollrian, 1995b); and in those studies where costs were shown (Black & Dodson, 1990; Riessen & Sprules, 1990) it remains unclear if these costs are caused by changes in morphology or in life history. The fact that we did not find effects of PUFA-related food quality strongly suggests that induction of neckteeth is not associated with a higher PUFA-demand. This is further corroborated by the fact that the supplementation of EPA had no effect on neckteeth induction.

As there is only a limited number of studies on the effect of biochemical food quality crossed with the chemical presence of a predator on inducible defenses, it is merely possible to draw conclusions from studies on stoichiometric food quality and the presence of predators. P-availability was reported to affect life history responses of *D. pulex* to *Chaoborus* (Jeyasingh & Weider, 2005). Low P-availability (LP) led to reduced growth and smaller size at first reproduction and an increased age at first reproduction. In the presence of *Chaoborus, D. pulex* increased its size and age at first reproduction, its somatic growth and also its neonate size, both at low P- (LP) and high P-availability (HP). All those changes in life history are well-known

responses of *D. pulex* to *Chaoborus* (Diel et al., 2020). In Jeyasingh and Weider (2005) life history changes were affected by P-availability and the predator, however, an effect of food-quality on the induction of the defense was only found for size at first reproduction and fecundity (Jeyasingh & Weider, 2005). The study was performed with two clones differing in their P-susceptibility: clone 1 performed better under HP, whereas clone 2 performed better under LP. The response in life history changes to *Chaoborus* was bigger under the non-favorable conditions for each clone. Predation experiments revealed that independent of P-availability fewer individuals of clone 1 were consumed. While the authors suggested that differences in behavioral defenses might be a reason for different predation rates, differences in sensitivity to the kairomone as shown here might also be the cause. This clonal variability in responses to food quality and predators very likely allows for the co-existence of clones in nature. However, when *D. pulex* was exposed to natural lake seston sampled from two different lakes, which differed in their phytoplankton composition and trophic state, and *Chaoborus* was allowed to prey upon *D. pulex* during night to create conditions closely resembling the ones in nature, no interaction of food quality and predator presence was detected (MacKay & Elser, 1998).

Jeyasingh and Weider (2005) reported that predation rates of *Chaoborus* preying on *Daphnia* that grow on LP food were higher. This might be caused by decreased growth under P-limitation, which would lead to longer periods under predation in case of a gape-limited predator like *Chaoborus*. Considering the increase in time to maturity with decreasing PUFA availability that we report here, PUFA-related food limitation might as well result in increased predation rates by *Chaoborus*. This finding of Jeyasingh and Weider (2005), however, contradicts the finding of MacKay and Elser (1998), who detected under more natural conditions no effect of P-related food quality on *Daphnia* survival under *Chaoborus* predation. This discrepancy on possibly increased predation rates under nutrient limitation, and therefore altered trophic transfer, stresses the importance of studies on trophic transfer efficiency using multiple trophic levels.

In contrast to the results presented here, we reported in a previous study a significant effect of dietary cyanobacteria on neckteeth induction (Klintworth & von Elert, submitted) such that an increased share of the cyanobacterium in the diet resulted in increased neckteeth induction. During that study we used *D. pulex* clone TCO, for which we here report no effect of fatty acid availability on induction of neckteeth. As cyanobacteria lack long-chained PUFAs and sterols (Martin-Creuzburg & von Elert, 2009) and can contain toxic secondary metabolites (Sadler et al.,

2014; Wiegand & Pflugmacher, 2005), we can now conclude that this effect of cyanobacteria on neckteeth induction was probably caused by either the lack of sterols or the presence of harmful secondary metabolites contained in *M. aeruginosa* PCC7806 mut, which we used for those experiments.

To the best of our knowledge, we here investigated for the first time the effect of food quality on an inducible morphological defense. During the year, *Daphnia* is exposed to seasonally varying food quality and quantity (Müller-Navarra & Lampert, 1996; Wacker & von Elert, 2001), as well as varying predation pressure (Fedorenko & Swift, 1972). We here report that the morphological defense in *D. pulex* is not affected by food quality in terms of fatty acid availability. Especially, the availability of EPA does not affect neckteeth induction in *D. pulex*. *D. pulex* seems to be able to protect itself against persistent predation throughout the seasons to the same extend and independently of food quality. In turn, *D. pulex* seems to not be able to adjust its defenses to inferior food quality although its growth rates are reduced.

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Chapter 3

Risk of predation alters resource allocation in *Daphnia* under food limitation

Abstract

Life history theory predicts that animals adjust their resource allocation to somatic growth or to reproduction to maximize fitness. Resource allocation in *Daphnia* is known to respond to quantitative food limitation as well as to kairomones released from predators. Here we investigated in a full-factorial design how kairomone from larvae of *Chaoborus flavicans*, a gape limited predator, and food quantity (0.5 mg C/L versus 1.5 mg C/L) affect the fatty acid allocation of *D. pulex*. Low food diminished somatic growth, clutch size and clutch biomass and increased neckteeth formation in response to the kairomone. Low food further led to increased fatty acid amounts per individual egg as well as to increased fatty acid content in eggs and to increased relative fatty acid allocation to reproduction. The latter effect was suppressed by kairomone of *Chaoborus*, whereas on high food the provision of eggs was further enhanced. We also found that more eicosapentaenoic acid was retained in the body of mothers in the presence of the predator at low food concentrations. These findings indicate that under food limitation and in the presence of kairomone from *Chaoborus* larvae, *Daphnia* switches from allocation into current reproduction to investment into future reproductive events.

Introduction

Since lakes are known to be ecosystems that are rapidly and continually changing over seasons (Sommer et al., 1986), zooplankton of the genus *Daphnia* needs to be able to adjust its life history to highly fluctuating food availability and predation pressure. In spring high nutrient and increasing light availability lead to a phytoplankton bloom, which causes herbivorous zooplankton to grow exponentially. At the point, at which grazing rates are higher than the growth rates of the phytoplankton, the phytoplankton biomass decreases rapidly, which results in a clear water phase. Afterwards, constant grazing and nutrient recycling by herbivorous zooplankton supports a more complex phytoplankton community comprising high shares of grazing resistant, filamentous or colonial taxa and only a low share of phytoplankton that is edible for *Daphnia*. Therefore, *Daphnia* is limited by food quantity most of the growing season (Müller-Navarra & Lampert, 1996).

During these times of low food quantity, *Daphnia* increase the allocation of lipids to their eggs to increase the offspring´s total energy reserves and thus its resistance to starvation during postembryonic development (Tessier et al., 1983). These changes in resource allocation result in changes in *Daphnia´*s life history such that the maternal animals exhibit decreased somatic growth with smaller body and clutch sizes at later maturation (Lynch, 1989). The decreased clutch sizes correspond with increased egg volumes, so that at low food concentrations large neonates are released whereas at high food concentrations small neonates are released (Gliwicz & Guisande, 1992). The same study revealed that larger eggs contain relatively more carbon, proteins and lipids, which makes the neonates of these clutches more resistant to starvation (Gliwicz & Guisande, 1992).

Changes of life history, comparable to those induced by different food concentrations, can be found in *Daphnia* when predators are present. *Daphnia* perceive the presence of predators by chemical cues derived from the predator itself, so-called kairomones (Hahn et al., 2019; Parejko & Dodson, 1990; Stibor, 1992). In the chemical presence of visually hunting predators that select for large bodied *Daphnia*, e.g. fish, *Daphnia* can change its life history by reallocating resources from somatic growth to reproduction so that *Daphnia* reaches maturity earlier, but at a smaller size, and produces bigger clutches with smaller eggs (Macháček, 1991; Rinke et al., 2008; Stibor,

1992). These changes are coupled to plasticity in metabolic processes in a way that in the presence of fish *D. magna* starts producing yolk proteins about one instar earlier, which results in an earlier onset of reproduction (Stibor, 2002). Production of yolk proteins proceeds at the same rate and to the same maximum level as in the absence of fish, but *D. magna* allocates earlier and relatively more of the produced yolk protein to the eggs (Stibor, 2002). Stibor (2002) also investigated the effect of larvae of *Chaoborus* on yolk production. The larvae of the phantom midge *Chaoborus* are gape limited ambush predators that prefer small, juvenile *Daphnia* (Pastorok, 1981). In the presence of *Chaoborus* kairomone, *Daphnia* started producing yolk proteins at the same developmental time point as *Daphnia* did in the absence of *Chaoborus*. The production proceeded at a slower rate, which led to a delayed reproduction with the same amount of yolk proteins passed on to the neonates so that *Daphnia* reproduce at a bigger size and produce fewer but bigger neonates (Havel & Dodson, 1987; Lüning, 1992; Tollrian, 1995). This delayed reproduction is a typical life history change in *Daphnia* in response to larvae of *Chaoborus*.

Taking into account that *Daphnia* is food limited most of the growing season (Müller-Navarra & Lampert, 1996) and that metabolic processes in *Daphnia* are adjusted as depicted above, the question arises to what extent *Daphnia* changes its resource allocation when it experiences food limitation and predation at the same time. This issue of effects of quantitative food limitation and predator-born kairomones on resource allocation has been addressed in a seminal paper by Stibor and Müller-Navarra (2000), in which they investigated if the content of triacylglycerides in eggs of *D. magna* changes in the presence of fish. Triacylglycerides are known to be major energy storage molecules in *Daphnia* (Goulden & Place, 1993). Stibor and Müller-Navarra (2000) found that the content of triacylglycerides in eggs decreased in the presence of kairomones from fish both at high and at low food concentrations, which means that, in the presence of fish kairomones, *Daphnia* produces neonates that are less resistant to starvation. These findings were supported by another study (Pauwels et al., 2010).

In line with their function as major energy storage molecules, triacylglycerides mainly contain saturated fatty acids. However, other types of fatty acids serve different functions. This is in particular the case for polyunsaturated fatty acids, which are needed for reproduction and somatic growth (Martin-Creuzburg & von Elert, 2009; von Elert, 2002), and which repeatedly have been shown to be a potentially limiting resource for *Daphnia* (Becker & Boersma, 2003; von Elert, 2002; Wacker & von Elert, 2001). Polyunsaturated fatty acids are as well allocated to the eggs,

and thus, affect the fitness of the offspring. Offspring of *Daphnia* that were raised on food supplemented with the polyunsaturated fatty acid eicosapentaenoic acid exhibited higher growth rates and produced bigger clutches than offspring of *Daphnia* raised on food lacking eicosapentaenoic acid (Sperfeld & Wacker, 2012). It is, therefore, of interest to investigate the effect of predator-born kairomones not only on the allocation of energy as in Stibor and Müller-Navarra (2000) but as well on the allocation of polyunsaturated fatty acids to the offspring. Hahn and von Elert (2020) have recently assessed effects of fish kairomone on the allocation of fatty acids to *Daphnia* offspring under saturating food concentrations. However, effects of kairomones from invertebrate predators have not been investigated yet.

Here we investigated how kairomone from larvae of *Chaoborus flavicans*, a gape-limited invertebrate predator, and food concentration affected the fatty acid allocation of *D. pulex*. In a full factorial design, we crossed low (0.5 mg C/L) and high (1.5 mg C/L) food concentration with the absence or presence of the kairomone. We used a clone of *D. pulex* that forms neckteeth during its juvenile instars as an inducible morphological defense in response to *Chaoborus* kairomone. We investigated functional groups of fatty acids as well as single fatty acids. Functional groups were categorized by their level of saturation leading to following groups: saturated fatty acids possess no double bonds in their fatty acid chain, monounsaturated fatty acids possess one double bond, and polyunsaturated fatty acids possess two or more double bonds in their fatty acid chain. In *Daphnia* saturated fatty acids are energy storage molecules that are the major energy reserve in *Daphnia* eggs where they may protect offspring after hatching against starvation (Stibor & Müller-Navarra, 2000), whereas unsaturated fatty acids, especially polyunsaturated fatty acids, are needed for growth, reproduction, and homeoviscous adaptation of membranes (Brzeziński & von Elert, 2015; Hazel, 1995; Müller-Navarra et al., 2000; von Elert & Fink, 2018; Wacker & von Elert, 2001).

It has been shown before that *Daphnia* increases the resource provision of eggs on low food concentration (Guisande & Gliwicz, 1992; Tessier et al., 1983). So we expected to find higher concentrations of fatty acids in the eggs in the low food treatment. Crossed with the chemical presence of *Chaoborus*, there were two possible outcomes: first, *Daphnia* further increases the allocation of fatty acids to the eggs to provide its neonates with sufficient resources to outgrow the most vulnerable instars faster; or second, *Daphnia* shifts its allocation of fatty acids from investment into current reproduction to investment into an increased lifespan with increased

numbers of reproductive events in the future. Taylor and Gabriel (1992) used a discrete-time model for growth and reproduction of *Daphnia*. In order to investigate resource allocation patterns, they solved the model to maximize the intrinsic rate of increase *r* under various size dependencies of predation risk. For this scenario the model predicts an intermediate allocation to reproduction during the first three adult instars and increasing allocation to reproduction for later adult instars. Therefore, the investment into further clutches is more probable in the presence of *Chaoborus* kairomone (Law, 1979; Taylor & Gabriel, 1992), because adult *D. pulex* are not vulnerable to predation of *Chaoborus* anymore and can produce future clutches without the risk of being preyed upon by larvae of *Chaoborus*. This outcome should hold especially for low food concentrations, as *Daphnia* has limited resources and should not invest all of the available resources in the current reproduction but allocate it to several reproductive events.

Methods

Cultivation of animals

D. pulex clone TCO (Colbourne et al., 2011) originated from a pond in the Siuslaw National Forest, near the Pacific coast in Oregon, USA. Prior to the experiments the animals were kept in aged and aerated tap water. The animals were kept in 800 mL with 10-12 individuals per jar and they were transferred into fresh medium every second day containing at least 1 mg C/L of *Chlamydomonas klinobasis* strain #56 (Limnological Institute, University of Constance). The cultures were kept at dim light at a constant temperature of 19.2 ± 0.3 °C and a 16:8 light dark cycle. *C. klinobasis* was grown in 5 L semi-continuous batch cultures in cyanophycea medium (von Elert & Jüttner, 1997) modified with vitamins. The volume of the food suspension that was needed was determined photometrically at a wavelength of 470 nm by using a calibration curve relating the carbon content to the optical density.

Preparation of *Chaoborus* incubation water extract

Approximately 300-350 fourth instar larvae (ordered from www.interaquaristik.de) of *Chaoborus flavicans* were fed with 1-2 neonates of *D. pulex* clone TCO per larva. After 1-2 hours of feeding, the larvae were transferred into 1 L of fresh aged and aerated tap water without any food. After 24 hours the larvae were removed from the water using a 250 µm gauze, and the water was filtered through a glass fiber filter (Whatman, MN 85/220, 0.4 µm, Macherey-Nagel, Germany). Subsequently, the kairomone was enriched by solid phase extraction (VARIAN, Bond Elut-C18, 10 g of sorbent, volume 60 mL, Agilent Technologies) as according to Christjani, Fink, and von Elert (2016).The eluates were pooled and evaporated to dryness in a rotary evaporator and a vacuum centrifuge. The dried residues were dissolved in methanol to a final concentration of 1:17,250 and stored at -20 °C until use. In order to exclude that processing the incubation water affects the fatty acid allocation, a control extract was prepared in exactly the same way but without any animals in the water.

Experimental setup

Forty animals from the same culture that had just released their first eggs into the brood chamber were divided randomly into the different food treatments, containing either 0.5 mg C/L or 1.5 mg C/L. The treatments are subsequently referred to as 'low food' and 'high food'. After the animals had deposited the eggs of their second clutch into the brood chamber, the animals were further divided randomly into control treatment ('Control') and kairomone treatment ('Chaoborus'). The kairomone treatment contained 1.5 µL of the *Chaoborus* incubation water extract. This volume of extract was chosen based on a dose response curve of *D. pulex* clone TCO (same conditions as for the cultivation) in which this volume had induced an intermediate degree of neckteeth induction. The control treatment contained the same volume of control extract.

The animals carrying their third clutch in the brood chamber were kept individually in 150 mL aged and aerated tap water containing either 0.5 mg C/L or 1.5 mg C/L of *C. klinobasis* combined with either the control extract or the extract of the *Chaoborus* incubation water extract. Each treatment was replicated ten times. The animals were transferred daily to freshly prepared jars as described above. Mothers were removed from the jars after the third clutch neonates had hatched, and eight experimental neonates remained in the jars. Neonates that were removed were pooled per treatment, and their dry mass was measured in subsamples of two times ten neonates per treatment. Later on, the dry mass of the neonates was used as w_0 to calculate the somatic growth rates. The jars were inspected every few hours for exuviae of the neonates, indicating that the animals reached their second juvenile instar. As the animals within the same jar developed synchronously, all of them molted within one or two hours. Neckteeth induction of the experimental animals was determined in their second juvenile instar using the method developed by Tollrian (1993) with the slight modification that each tooth was scored with 10 % and no differentiation was made between big and small teeth as described in Schwarzenberger, Christjani, and Wacker (2014). For all statistical analyses the mean per replicate was calculated. Further, the time to maturity and somatic growth rate were determined, and the size at first reproduction, the clutch size and biomass, as well as the size of the neonates were measured in the experimental animals. Sizes of the animals were measured using the open source image processing program ImageJ. When the animals had deposited their first clutch into their brood chambers, two animals per jar were taken for the determination of dry mass. The somatic growth rates (g) were calculated

according to the following formula: $g = (\ln(w_t) - \ln(w_0)) / t$, with w_t being the individual weight at day t and w_0 being the individual weight at day 0 (Rothhaupt & Lampert, 1992).

Six animals remained in the jar, and the neonates of the first clutch were counted after hatching for the calculation of the intrinsic rate of increase (r) using the following formula: $r = \sum l_x * m_x * e^{-rx}$, with l_x being the survival rate at day x and m_x the average number of offspring per surviving individual at day x (Lotka, 1907). Within 4 hours after the mothers had deposited the second clutch into the brood chamber, the eggs were removed from the brood chambers, and mothers and eggs were analyzed separately. In either low food treatment the eggs of some replicates had to be pooled to obtain sufficient material for the fatty acid analysis. Three replicates per treatment were processed for the analysis of fatty acids.

Fatty acid extraction

Fatty acid extraction and analysis were performed as according to von Elert and Fink (2018). Briefly, mothers or eggs were incubated in 5 mL of dichloromethane-methanol (2:1, v:v) and stored at -20 °C until extraction. Prior to extraction, 5 µg of C23:0 methyl ester were added as internal standard. Samples were vortexed and put into an ultrasonic bath for 3 minutes. Extraction solvents were transferred to new reagent tubes and 3 ml fresh extraction solvents were added to the remaining tissues. Samples were vortexed again and incubated for one minute in an ultrasonic bath. The extraction solvents were pooled, centrifuged, and supernatants were transferred to new reagent tubes and evaporated to dryness at 40 °C under a stream of nitrogen. Fatty acids were then transesterified at 70 °C for 20 minutes in 5 mL of 3 M methanolic HCl. After letting the samples cool to room temperature, the fatty acid methyl esters were extracted three times with approximately 2 mL isohexane, respectively. The pooled isohexane phases were evaporated to dryness at 40 °C under a stream of nitrogen, and the remaining fatty acid methyl esters were dissolved in 50 µL of isohexane. The samples were stored at -20 °C until measuring. One microliter per sample was injected splitlessly on a 6890-N GC System (Agilent Technologies, Waldbronn, Germany) equipped with a DB-225 capillary column (27.5 m, 0.25 mm i.d., 0.25 µm film thickness, J&W Scientific, Folsom, CA, USA) and an FID detector. The GC conditions were as follows: injector and FID temperatures 220 °C; initial oven temperature 60 °C for 1 min, followed by a 120 °C/min temperature ramp to 180 °C, then a ramp of 50 °C/min to 200 °C

followed by 10.5 min at 200 °C, followed by a ramp of 120 °C/min to 220 °C, which were held for 7.5 min; helium with a flow rate of 1.5 mL/min was used as a carrier gas. The fatty acid methyl esters were identified by comparison of retention times with those of reference compounds and quantified via the internal standard and previously established calibration curves.

Statistical analyses

For all statistical analyses of the life history parameters the mean per jar was calculated, resulting in n=10 per treatment. All statistical analyses were performed in RStudio version 1.1.423 (R version x64 3.4.3). Data were checked for homoscedasticity, and, if given, a two-way ANOVA type 2 followed by Tukey´s HSD test was performed. In cases of unequal sample sizes, a two-way ANOVA type 3 followed by a Games-Howell test or Tukey´s HSD test was performed. If homoscedasticity was not given, a Kruskal-Wallis test followed by a pairwise Wilcoxon test applying Bonferroni-correction was performed. Fatty acid profiles were analyzed by performing a PerMANOVA. As the results might have been biased by a significant effect of the non-interacting factor 'tissue', the dataset was split, and the fatty acid profiles of the eggs and mothers were analyzed separately. Fatty acids that were found to be most contributing (at least 70% of cumulative contribution) to the differences between treatments were further analyzed by performing ANOVAs on the single fatty acids as described above.

Results

Somatic growth rates of animals growing on low food concentration were significantly decreased compared to the growth rates of animals on high food concentration (Table 1, Fig. S1). On high food, the mean (± SD) somatic growth rates were 0.57 (± 0.06) d^{-1} (H Co) and 0.59 (± 0.07) d^{-1} (H Ch). The presence of the kairomone only affected the growth rates on low food, which were significantly lower (0.41 ± 0.01 d^{-1} (L Ch)) than those in the control (0.47 ± 0.02 d^{-1} (L Co)).

The mean neckteeth induction of animals growing in the presence of the kairomone on low food was 74.4 (± 8.6) % and significantly higher than on high food, which was 30.6 (± 16.8) % (Table 1, Fig. S1). There were no neckteeth found in the control treatments.

Only the food concentration and not the presence of the kairomone had an effect on clutch size and also on clutch biomass (Table 1, Fig. S1). High food led to twice as many eggs as on low food (11.2 ± 0.9 (H Co), 12.4 ± 1.1 (H Ch), 5.5 ± 1.2 (L Co), 4.9 ± 1.2 (L Ch)), which resulted also in twofold higher masses of the clutches (27.3 ± 5.3 ng (H Co), 30.1 ± 5.1 ng (H Ch), 14.9 ± 4.3 ng (L Co), 14.9 ± 2.4 ng (L Ch)) (Table 1, Fig. S1).

Table 1: Mean (± SD) life history parameters of *D. pulex* grown on low (0.5 mg C/L; L) or high (1.5 mg C/L; H) food concentrations and either in the absence (Control, Co) or presence of *Chaoborus* kairomone (Ch). Different superscript letters indicate significant ($p<0.05$) differences. The type of statistical test is indicated by symbols (×: two-way ANOVA type 2 and Tukey´s HSD test; Δ: Kruskal-Wallis and pairwise Wilcoxon test). For details on statistical analyses see Table S1. Reaction norm plots are given in Fig. S1.

Parameter	L Co	H Co	L Ch	H Ch
Clutch size ×	5.5 (± 1.2)^{b}	11.2 (± 0.9)^{a}	4.9 (± 1.2)^{b}	12.4 (± 1.1)^{a}
Population growth [d^{-1}] ×	0.179 (± 0.032)^{b}	0.270 (± 0.036)^{a}	0.154 (± 0.024)^{b}	0.293 (± 0.030)^{a}
SFR [µm] ×	1758.0 (± 49.0)^{b}	1868.5 (± 85.6)^{a}	1746.6 (± 19.5)^{b}	1902.3 (± 48.8)^{a}
Size of neonates [µm] Δ	623.7 (± 21.7)^{b}	610.7 (± 24.9)^{b}	674.0 (± 12.7)^{a}	612.0 (± 24.6)^{b}
Somatic growth [d^{-1}] Δ	0.47 (± 0.02)^{b}	0.57 (± 0.06)^{a}	0.41 (± 0.01)^{c}	0.59 (± 0.07)^{a}
Neckteeth induction [%] Δ	0^{c}	0^{c}	74.4 (± 8.6)^{a}	30.6 (± 16.8)^{b}
Time to maturity [d] ×	5.7 (± 0.5)^{ab}	5.3 (± 0.5)^{b}	6.0 (± 0)^{a}	5.6 (± 0.5)^{ab}
Dry mass body + clutch [ng] ×	47.4 (± 7.5)^{b}	74.3 (± 10.6)^{a}	46.5 (± 4.0)^{b}	79.7 (± 9.8)^{a}
Clutch dry mass [ng] ×	14.9 (± 4.3)^{b}	27.3 (± 5.3)^{a}	14.9 (± 2.4)^{b}	30.1 (± 5.1)^{a}

Fatty acid amount was normalized either to the dry mass of the mothers or of the clutch resulting in fatty acid content. Under control conditions low food compared to high food led to an increase of the fatty acid content of eggs (Fig. 1) (2.7 ± 0.7 % (L Co eggs), 1.33 ± 0.1 % (H Co eggs)). This increased fatty acid content of eggs corresponded with a decrease of the maternal fatty acid content (0.99 ± 0.1 % (L Co mother), 1.37 ± 0.1 % (H Co mother) (Fig. 1), which indicated that low food resulted in increased fatty acid allocation into offspring.

However, in the presence of the kairomone there was no such effect on fatty acid allocation: low food did not lead to an increase of fatty acid content in the eggs and resulted in a marginal increase in maternal fatty acid content, so that the contents of eggs and mothers were in a similar range (1.54 ± 0.02 % (L Ch eggs), 1.48 ± 0.1 % (L Ch mother), 1.56 ± 0.1 % (H Ch eggs), 1.22 ± 0.1 % (H Ch mother)) (Fig. 1).

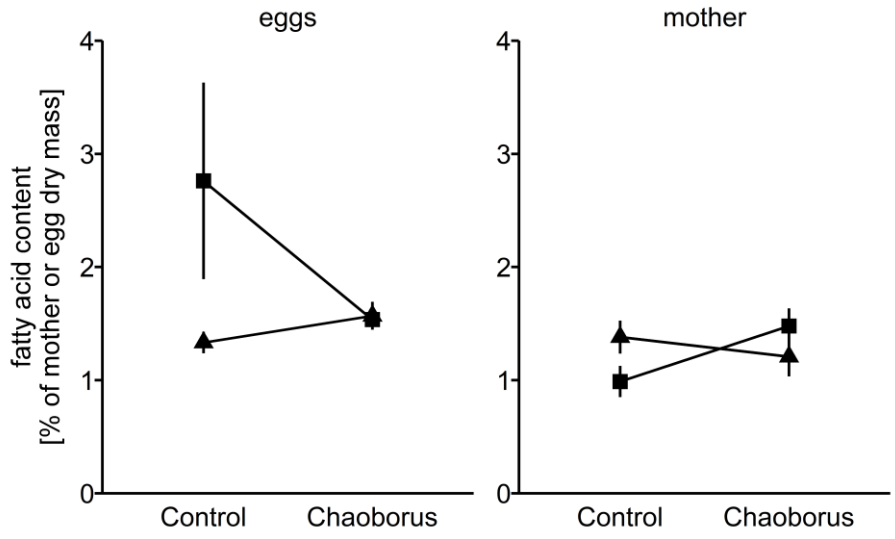

Figure 1: Mean (± SD) total fatty acid content [% of mother or egg dry mass] of *D. pulex* grown on low (0.5 mg C/L; squares) or high (1.5 mg C/L; triangles) food concentrations and either in the absence (Control) or presence of *Chaoborus* kairomone (Chaoborus). Asterisks indicate significant ($p<0.05$) differences either among eggs (Kruskal-Wallis and pairwise Wilcoxon test) or among mothers (two-way ANOVA type 2 and Tukey´s HSD test). Asterisks above means indicate significant differences between food treatments and asterisks next to the lines between means indicate significant differences between treatments of the same food level. For details on statistical analyses see Table S2.

This pattern is also visible in the fatty acid investment into eggs depicted as the percentage of the total fatty acid content, i.e. of the pool of fatty acids from eggs and maternal tissue (Fig. 2). Under control conditions, low food led to 54.1 (± 8) % of fatty acids being allocated into eggs,

which was considerably higher than under high food (35.9 ± 4 %) (Fig. 2). However, in the presence of kairomone, low food even diminished fatty acid allocation into eggs (32.9 ± 5 %) compared to high food (43.9 ± 6 %) (Fig. 2). We found a significant effect of the factor kairomone on the fatty acid investment into eggs (two-way ANOVA and Tukey´s HSD test, $F_{(1,36)} = 12.01$, $p<0.01$, Table S2) as well as a significant interaction of both factors food and kairomone ($F_{(1,36)} = 58.13$, $p<0.001$, Table S2).

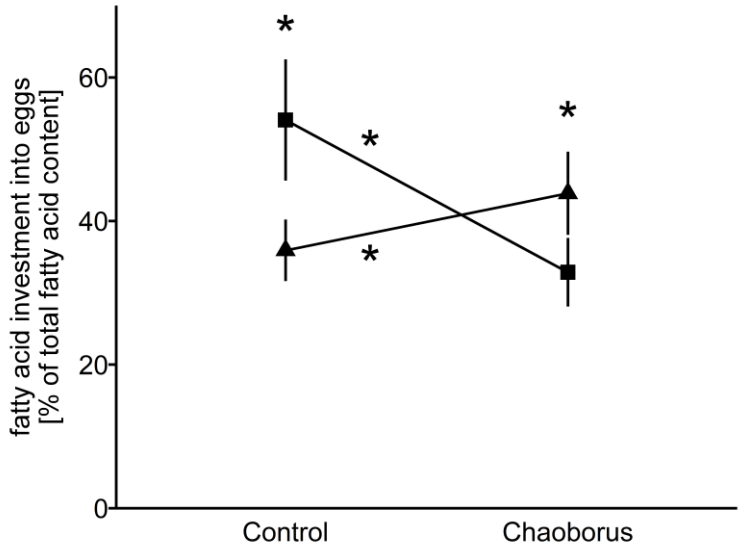

Figure 2: Mean (± SD) fatty acid investment [% of total fatty acid content] of *D. pulex* grown on low (0.5 mg C/L; squares) or high (1.5 mg C/L; triangles) food and either in the absence (Control) or presence of *Chaoborus* kairomone (Chaoborus). Asterisks indicate significant ($p<0.05$) differences (two-way ANOVA type 2 and Tukey´s HSD test). Asterisks above means indicate significant differences between food treatments and asterisks next to the lines between means indicate significant differences between treatments of the same food level. For details on statistical analyses see Table S2.

Fatty acids were grouped into different functional groups (saturated fatty acids, monounsaturated fatty acids, and polyunsaturated fatty acids), and their content was considered as percentages of the total fatty acids. This analysis revealed that the percentages of these functional fatty acid groups were only affected by food concentration and not by the presence of the kairomone (Fig. 3). Low food, in comparison to high food, led to increased contents of saturated fatty acids in eggs (12.5 ± 0.4 % (L Co), 10.0 ± 0.3 % (H Co), 12.3 ± 0.2 % (L Ch), 10.1 ± 0.1 % (H Ch)) (Fig. 3, Table S2), whereas the content of monounsaturated fatty acids decreased (17.1 ± 0.7 % (L Co), 20.5 ± 0.3 % (H Co), 16.7 ± 0.6 % (L Ch), 21.5 ± 0.6 % (H Ch)) (Fig. 3, Table S2).

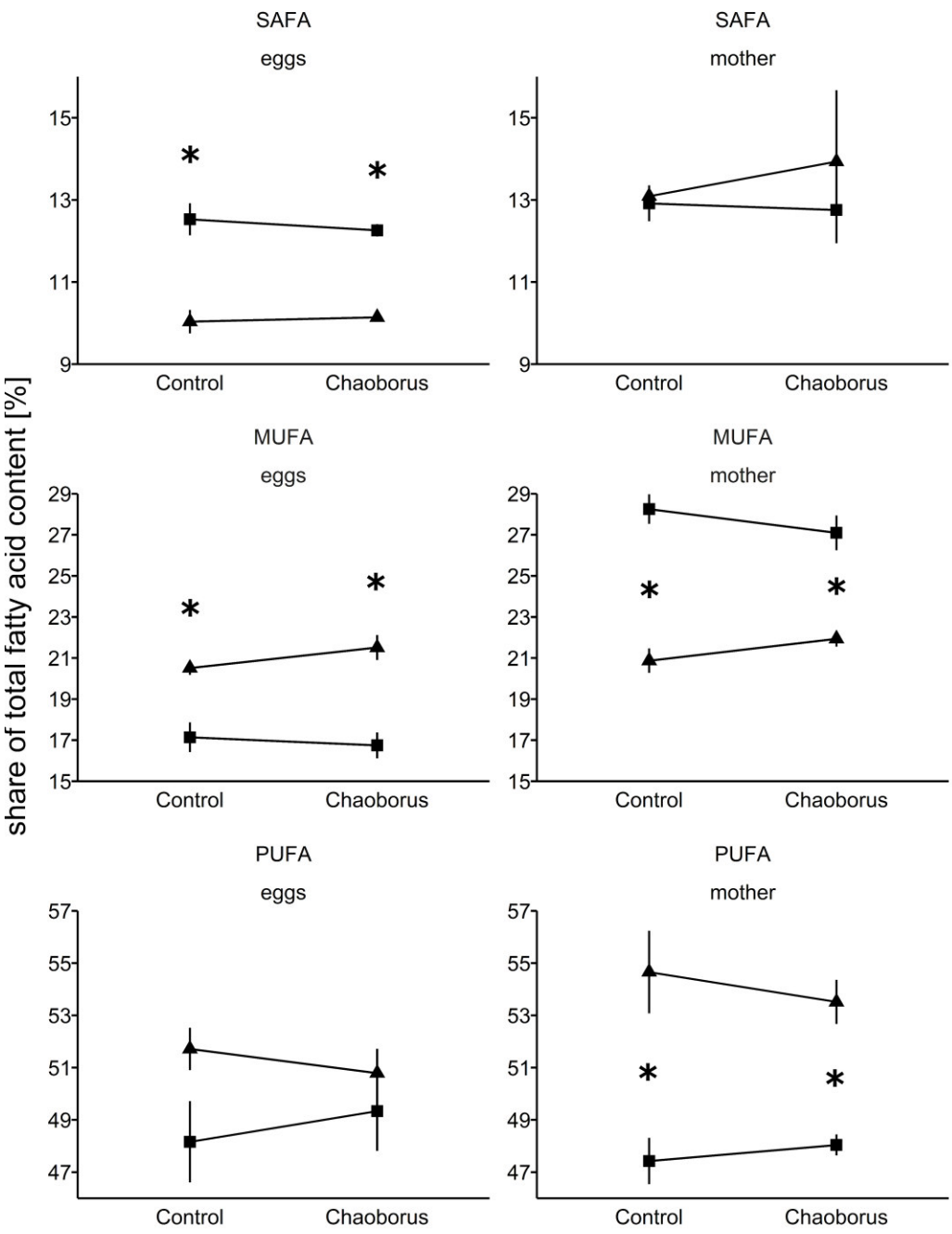

Figure 3: Mean (± SD) share of saturated (SAFA), mono-unsaturated (MUFA), and polyunsaturated fatty acids (PUFA) [% of total fatty acids] of *D. pulex* grown on low (0.5 mg C/L; squares) or high (1.5 mg C/L; triangles) food and either in the absence (Control) or presence of *Chaoborus* kairomone (Chaoborus). Asterisks indicate significant ($p<0.05$) differences either among eggs (two-way ANOVA type 3 and Games-Howell test) or among mothers (two-way ANOVA type 2 and Tukey´s HSD test). Asterisks above or below means indicate significant differences between food treatments. For details on statistical analyses see Table S2.

Accordingly, low food resulted in increased percentages of monounsaturated fatty acids in maternal tissue (28.3 ± 0.7 % (L Co), 20.9 ± 0.6 % (H Co), 27.1 ± 0.8 % (L Ch), 21.9 ± 0.4 % (H Ch)). Furthermore, low food led to a lower percentage of polyunsaturated fatty acids in maternal tissue (42.2 ± 0.5 % (L Co), 50.8 ± 1.2 % (H Co), 42.6 ± 1.1 % (L Ch), 48.9 ± 2.1 % (H Ch)) (Fig. 3, Table S2).

In order to inspect the differences in fatty acid profiles between the different treatments a PerMANOVA was performed. Due to a highly significant effect of the non-interacting factor 'tissue' in a first analysis on the dataset comprising the fatty acid profiles of eggs and mothers, the dataset was split and the fatty acid profiles of the eggs and mothers were analyzed separately. For both eggs and mothers, we found only a significant effect of the food concentration (Table 2) and for all single fatty acids that were found to be most contributing (at least 70% of cumulative contribution) two-way ANOVAs were performed (Table 3, Table S3). Except for C20:5(n-3) (eicosapentaenoic acid), the content of single fatty acids was, if at all, only affected by food concentration and not by the presence of the kairomone (Table 3, Table S3). The relative content of eicosapentaenoic acid as percentages of the total fatty acids in the eggs was not different between treatments, but in the presence of the kairomone mothers on low food retained significantly more eicosapentaenoic acid in their tissue than mothers in the other treatments (1.6 ± 0.3 % (L Co), 1.3 ± 0.2 % (H Co), 2.5 ± 0.3 % (L Ch), 1.5 ± 1.2 % (H Ch)).

Table 2: Results of PerMANOVAs on the fatty acid profiles of the eggs and mothers of *D. pulex* grown on low (0.5 mg C/L) or high (1.5 mg C/L) food and either in the absence (Control) or presence of *Chaoborus* kairomone. Due to a possible bias by a significant effect of the non-interacting factor 'tissue' in an analysis on the full dataset, the dataset was split by the type of tissue into 'eggs' and 'mothers', and the fatty acid profiles of eggs and mothers were analyzed separately. Significant effects ($p<0.05$) are indicated by bold p-values.

Factor tested	Df	F	R^2	p-value
Eggs and mothers				
Food	1	6.73	0.14	**0.003**
Kairomone	1	0.74	0.01	0.5
Tissue	1	23.40	0.47	**< 0.001**
Food:kairomone	1	0.62	0.01	0.6
Residuals	18		0.36	
Total	22		1.0	
Eggs				
Food	1	29.26	0.76	**0.002**
Kairomone	1	0.58	0.02	0.5
Food:kairomone	1	1.67	0.04	0.2
Residuals	7		0.18	
Total	10		1.0	
Mothers				
Food	1	19.64	0.67	**0.001**
Kairomone	1	0.68	0.02	0.5
Food:kairomone	1	1.02	0.03	0.3
Residuals	8		0.27	
Total	11		1.0	

Table 3: Mean (± SD) share of single fatty acids [%] in the tissue of *D. pulex* grown on low (0.5 mg C/L; L) or high (1.5 mg C/L; H) food and either in the absence (Control, Co) or presence of *Chaoborus* kairomone (Ch). Only fatty acids are given that were found to be most contributing (at least 70 % cumulative contribution) during PerMANOVA analysis. Different superscript letters indicate significant ($p<0.05$) differences either among eggs (two-way ANOVA type 3 and Games-Howell test) or among mothers (two-way ANOVA type 2 and Tukey´s HSD test). For details on statistical analyses see Table S3.

Fatty acid	Tissue	L Co	H Co	L Ch	H Ch
C 16:0	eggs	12.4 (± 0.6)^{a}	9.5 (± 0.2)^{b}	12.3 (± 0.7)^{a}	9.6 (± 0.07)^{b}
C 16:0	mothers	10.6 (± 0.2)	10.2 (± 0.1)	10.5 (± 0.6)	10.7 (± 0.8)
C 16:1 n-7	eggs	4.6 (± 0.9)^{b}	10.5 (± 0.7)^{a}	4.0 (± 0.02)^{b}	12.0 (± 0.8)^{a}
C 16:1 n-7	mothers	16.8 (± 2.1)	7.6 (± 1.3)	16.2 (± 0.7)	9.0 (± 0.3)
C 18:0	eggs	3.2 (± 0.5)^{ab}	2.7 (± 0.1)^{ab}	3.1 (± 0.05)^{a}	2.7 (± 0.05)^{b}
C 18:0	mothers	4.0 (± 0.2)	4.5 (± 0.1)	4.0 (± 0.3)	4.8 (± 0.9)
C 18:1 n-9 c	eggs	13.2 (± 1.5)^{ab}	10.1 (± 0.3)^{b}	12.4 (± 0.1)^{a}	9.8 (± 0.4)^{b}
C 18:1 n-9 c	mothers	9.5 (± 0.9)^{bc}	10.9 (± 0.3)^{a}	9.1 (± 0.1)^{c}	10.5 (± 0.3)^{ab}
C 18:3 n-3	eggs	50.9 (± 1.7)	50.8 (± 0.5)	51.6 (± 1.1)	50.9 (± 1.1)
C 18:3 n-3	mothers	42.4 (± 0.8)^{b}	49.3 (± 1.4)^{a}	42.8 (± 0.7)^{b}	47.6 (± 2.2)^{a}
C 18:4 n-3	eggs	1.13 (± 0.56)	2.36 (± 0.45)	1.25 (± 0.31)	2.37 (± 0.15)
C 18:4 n-3	mothers	0.83 (± 1.45)	3.71 (± 1.21)	1.42 (± 1.23)	3.05 (± 0.88)
C 20:3 n-3	eggs	2.7 (± 1.04)	2.3 (± 0.2)	2.0 (± 0.9)	2.1 (± 0.1)
C 20:3 n-3	mothers	4.3 (± 0.3)	3.0 (± 0.2)	3.7 (± 0.6)	3.6 (± 1.2)
C 20:5 n-3	eggs	1.05 (± 0.5)	0.77 (± 0.06)	1.38 (± 0.4)	0.82 (± 0.04)
C 20:5 n-3	mothers	1.63 (± 0.3)^{b}	1.30 (± 0.17)^{b}	2.49 (± 0.3)^{a}	1.50 (± 0.16)^{b}
C 22:0	eggs	0.426 (± 0.255)	0.041 (± 0.071)	0.306 (± 0.166)	n.d.
C 22:0	mothers	n.d.	n.d.	n.d.	n.d.
C 22:1 n-9	eggs	0.608 (± 0.324)	n.d.	0.74 (± 0.054)	n.d.
C 22:1 n-9	mothers	n.d.	n.d.	n.d.	n.d.
C 22:2 n-6	eggs	0.370 (± 0.219)	0.534 (± 0.070)	0.548 (± 0.266)	0.033 (± 0.057)
C 22:2 n-6	mothers	n.d.	n.d.	n.d.	n.d.
C 22:6 n-3	eggs	0.807 (± 0.278)	2.481 (± 0.113)	1.020 (± 0.26)	1.765 (± 1.850)
C 22:6 n-3	mothers	n.d.	n.d.	n.d.	n.d.

For the purpose of investigating the effect of altered fatty acid investment on individual offspring, we calculated the amounts of total fatty acids, saturated, monounsaturated, and polyunsaturated fatty acids per individual egg (Fig. 4). In the absence of the *Chaoborus* kairomone the increase of the amount of total fatty acids on low food was significant (131.8 ± 41.3 ng (L Co), 43.9 ± 3.1 ng (H Co)), whereas this significance could not be detected in the presence of *Chaoborus* kairomone (108.2 ± 2.1 ng (L Ch), 43.4 ± 3.4 ng (H Ch)). The amount of polyunsaturated fatty acids increased on low food both in the absence as well as in the presence

of *Chaoborus* kairomone (81.4 ± 24.3 % (L Co), 27.6 ± 1.9 % (H Co), 68.3 ± 1.0 % (L Ch), 26.8 ± 2.5 % (H Ch)). The amount of monounsaturated fatty acids increased on low food in the absence of *Chaoborus* kairomone (29.3 ± 10.7 ng (L Co), 10.9 ± 1.0 ng (H Co)), but in the presence of *Chaoborus* kairomone this increase was not significant (23.1 ± 0.2 ng (L Ch), 11.3 ± 0.5 ng (H Ch)). The amount of saturated fatty acids increased on low food both in the absence as well as in the presence of *Chaoborus* kairomone (21.1 ± 6.3 % (L Co), 5.3 ± 0.3 % (H Co), 16.9 ± 1.0 % (L Ch), 5.3 ± 0.4 % (H Ch)).

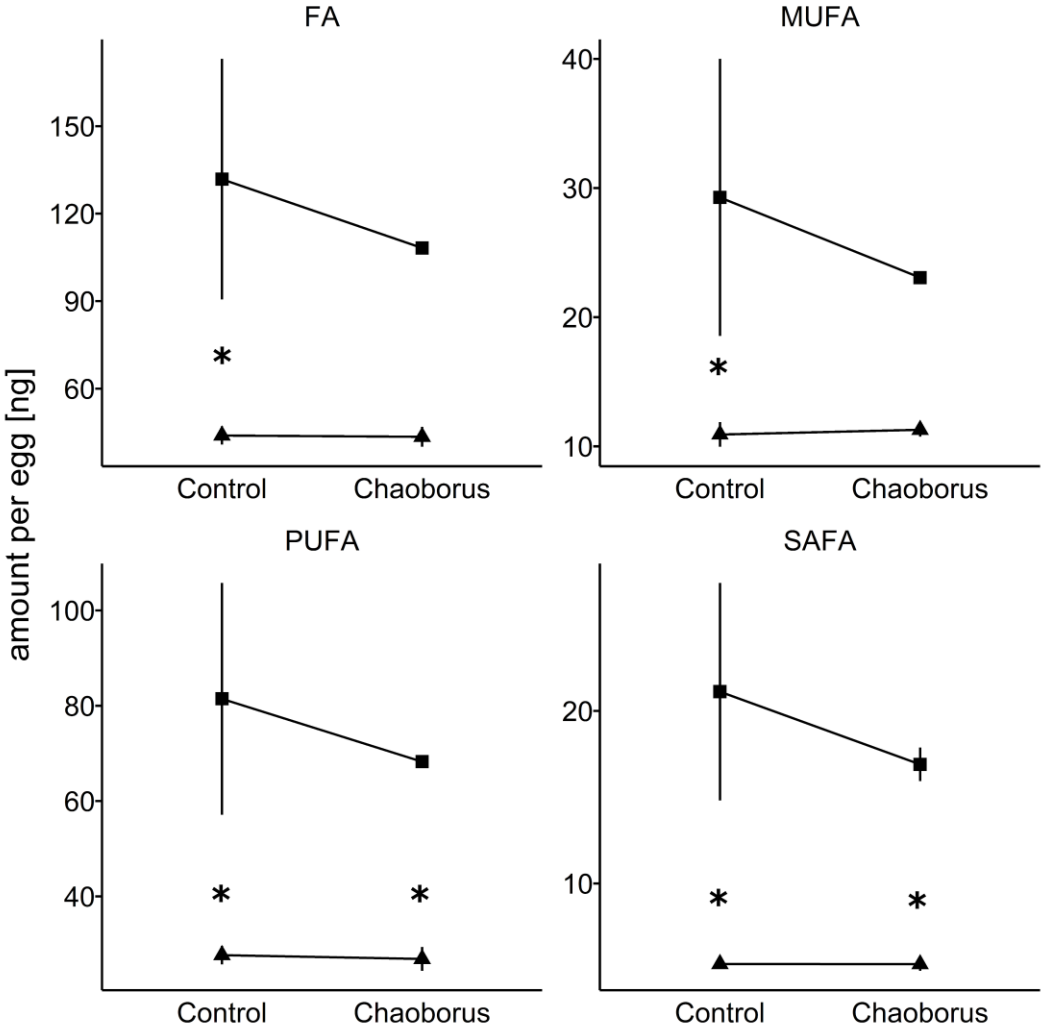

Figure 4: Mean (± SD) amount [ng] of total fatty acids (FA), monounsaturated (MUFA), polyunsaturated (PUFA), and saturated fatty acids (SAFA) per individual egg of *D. pulex* grown on low (0.5 mg C/L; squares) or high (1.5 mg C/L; triangles) food and either in the absence (Control) or presence of *Chaoborus* kairomone (Chaoborus). Asterisks above or below means indicate significant ($p<0.05$) differences between food treatments (two-way ANOVA type 3 and Tukey´s HSD test). For details on statistical analyses see Table S2.

Discussion

*Daphnia´*s life history was strongly affected by food concentration. At low food *Daphnia* matured later, at a smaller body size and with smaller clutch sizes compared to the animals exposed to high food. All of this resulted in decreased fitness measured as the intrinsic rate of population increase (*r*). Furthermore, some life history parameters, namely somatic growth rates and time to maturity, were not only affected by the food concentration but also by the presence of *Chaoborus* kairomone. *Daphnia* in kairomone treatments matured slightly delayed compared to the respective control. This delay in maturation has been reported frequently and has often been interpreted as costs associated with morphological defenses in response to predators (Black & Dodson, 1990; Havel & Dodson, 1987). The neckteeth induction was significantly higher on low food, which confirmed earlier findings by Parejko and Dodson (1991). Riessen (1992) concluded that enhanced neckteeth expression under low food conditions is advantageous, because otherwise the impact of *Chaoborus* predation on *Daphnia* populations would be stronger due to the diminished fitness of *Daphnia* under food limitation.

The changes in the investment of fatty acids into eggs as percentage of the total fatty acid content are consistent with both scenarios that could have been expected for this experiment. In the first scenario a further increase in the fatty acid investment in the presence of the kairomone was expected to support faster juvenile growth rates in the offspring. This was found on high food when *Daphnia* was not resource-limited. However, on low food the provision of resources to the offspring was suppressed by the presence of the kairomone as resources were limiting. We speculate that under these conditions *Daphnia* did not invest into the current reproduction, but shifted the resource allocation to the investment into future reproductive events.

The changes in the fatty acid investment can affect the performance of the offspring, as it has been shown before that neonates with an increased content of lipids or triacylglycerides are more resistant to starvation (Guisande & Gliwicz, 1992; Tessier et al., 1983). For the purpose of investigating the effect of altered fatty acid investment on individual offspring, we calculated the amounts of total fatty acids, saturated fatty acids, monounsaturated fatty acids, and polyunsaturated fatty acids per individual egg. We observed an increase in the content of all fatty acid groups per individual egg on low food concentrations, which corroborates the above

mentioned studies. Guisande and Gliwicz (1992) determined the total lipid content of neonates, and under the assumption that total lipid content correlates with total fatty acids, we can support their findings. Tessier et al. (1983) found the same effect for the triacylglyceride contents, which can be compared to the group of saturated fatty acids in our study. However, as we found the same pattern for all of the functional groups, it can be concluded that *Daphnia* does not allocate specific fatty acids to the eggs on low food but all of them to the same extent.

In the presence of *Chaoborus* kairomone, however, the elevated investment of fatty acids into offspring was suppressed. A possible explanation is that, due to predator-mediated mortality in *Daphnia,* the risk of starvation has become relatively low since *Daphnia* densities will remain low due to predation, so that increased resource allocation into eggs has become mal-adaptive (Stibor & Müller-Navarra, 2000). This is in accordance with Cleuvers et al. (1997), who reported that resource allocation to the offspring depends on *Daphnia* densities: Despite of constant food concentrations, *D. magna* in groups produced less but bigger offspring with higher lipid contents than *Daphnia* growing individually, because the risk of starvation is higher for *Daphnia* growing in higher densities than for those growing singly.

We here report percentages of functional fatty acid groups in eggs and mothers of *D. pulex* that are comparable to other studies (Brett et al., 2006; Schlechtriem et al., 2006). Despite differences in *D. pulex* genotypes and food algae, the *D. pulex* clone used here seems representative for *D. pulex* with respect to fatty acid composition. It is well established that *D. pulex* genotypes differ in sensitivity to *Chaoborus* kairomone with respect to induction of morphological defenses (Lüning, 1995; Parejko & Dodson, 1991; Spitze, 1992) and induction of life history changes (Lüning, 1995; Scheiner & Berrigan, 1998; Spitze, 1992). Allocation of fatty acids to offspring is part of life history changes in response to predator-borne kairomones (Hahn & von Elert, 2020; Stibor & Müller-Navarra, 2000). However, this is the first study investigating the effect of *Chaoborus* kairomones on fatty acid allocation, and it remains to be tested if *D. pulex* genotypes that differ in life history responses to *Chaoborus* kairomone also differ in their inducible changes in fatty acid allocation to offspring.

Although there was no difference in the allocation of specific fatty acids to the eggs in our study, we found a difference in the retention of fatty acids by the mothers. The only fatty acid for which a significant difference was found was eicosapentaenoic acid. *Daphnia* on low food retained more eicosapentaenoic acid in their body in the presence of the kairomone. Since it is known that

eicosapentaenoic acid is needed for somatic growth (Becker & Boersma, 2003; Sperfeld & Wacker, 2012; von Elert, 2002), reproduction (Martin-Creuzburg et al., 2008; Ravet et al., 2003), and also ultimately supports high population growth rates (Martin-Creuzburg et al., 2010), *Daphnia* on low food in the presence of the kairomone indeed seem to retain more resources for possible future reproductive events instead of investing into the current reproduction.

In contrast to our study using a gape-limited predator, Stibor and Müller-Navarra (2000) crossed low and high food concentration with the absence or presence of kairomones from fish, a non-gape limited predator, and investigated the effect on the triacylglyceride content of the eggs. They found that the chemical presence of a non-gape limited predator led to a decreased triacylglyceride content of the eggs. In our study we found no differences between the allocations of different functional fatty acid groups to the eggs, so we can assume that the investment of saturated fatty acids shows the same pattern as the investment of total fatty acids with decreased investment at low food and increased investment on high food in the presence of the predator, respectively. Different from the response to *Chaoborus* kairomone reported here, the triacylglycerides investment normalized to the somatic dry mass was not influenced by the presence of fish (Stibor & Müller-Navarra, 2000). This underlines that in addition to predator-specific inducible life history changes (von Elert, 2012) *Daphnia* also shows predator-specific changes in allocation of maternal fatty acids to reproduction.

Conclusions

Life history theory states that individuals adjust their resource allocation according to environmental conditions in a way that fitness is optimized. Especially *Daphnia* has been shown numerous times to adjust its resource allocation to factors like food availability or predation. In this study we demonstrated that *D. pulex* optimizes its resource allocation into offspring not only to single factors like food quantity or predation but also to both of them concurrently. At low food the elevated resource provision to eggs, which is typically found during limitation by food quantity, was suppressed by the chemical presence of a predator, *C. flavicans*, whereas on high food the provision of eggs was further enhanced. We also found that more eicosapentaenoic acid was retained in the body of mothers in the presence of the predator at low food concentrations. This suggests that under food limitation and in the presence of kairomone from *Chaoborus*

larvae, *Daphnia* switches from investment into current reproduction to investment into future reproductive events.

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Supplements

Table S1: Statistical analyses of the life history parameters as depicted in Table 1. Different statistical tests are indicated by different letters (test A2: two-way ANOVA type 2 and Tukey´s HSD test; test KW: Kruskal-Wallis test and pairwise Wilcoxon test). Significant effects ($p<0.05$) are indicated by bold p-values.

parameter	test	factor	df	F/χ2	p-value	L Co	H Co	L Ch	H Ch
clutch size	A2	Chaoborus	1	0.383	0.54	b	a	b	a
		food	1	358.468	**< 0.001**				
		Ch × food	1	7.293	**0.011**				
population growth	A2	Chaoborus	1	0.025	0.875	b	a	b	a
		food	1	140.926	**< 0.001**				
		Ch × food	1	6.051	**0.019**				
size at first reproduction	A2	Chaoborus	1	0.403	0.53	b	a	b	a
		food	1	56.802	**< 0.001**				
		Ch × food	1	1.638	0.209				
size of neonates	KW		3	45.049	**< 0.001**	b	b	a	b
somatic growth	KW		3	31.832	**< 0.001**	b	a	c	a
neckteeth induction	KW		3	37.549	**< 0.001**	c	c	a	b
time to maturity	A2	Chaoborus	1	4.909	**0.033**	ab	b	a	ab
		food	1	8.727	**0.006**				
		Ch × food	1	0	1				
dry mass mother + eggs	A2	Chaoborus	1	0.704	0.407	b	a	b	a
		food	1	128.124	**< 0.001**				
		Ch × food	1	1.388	0.246				
clutch dry mass	A2	Chaoborus	1	1	0.324	b	a	b	a
		food	1	97.184	**< 0.001**				
		Ch × food	1	1.073	0.307				

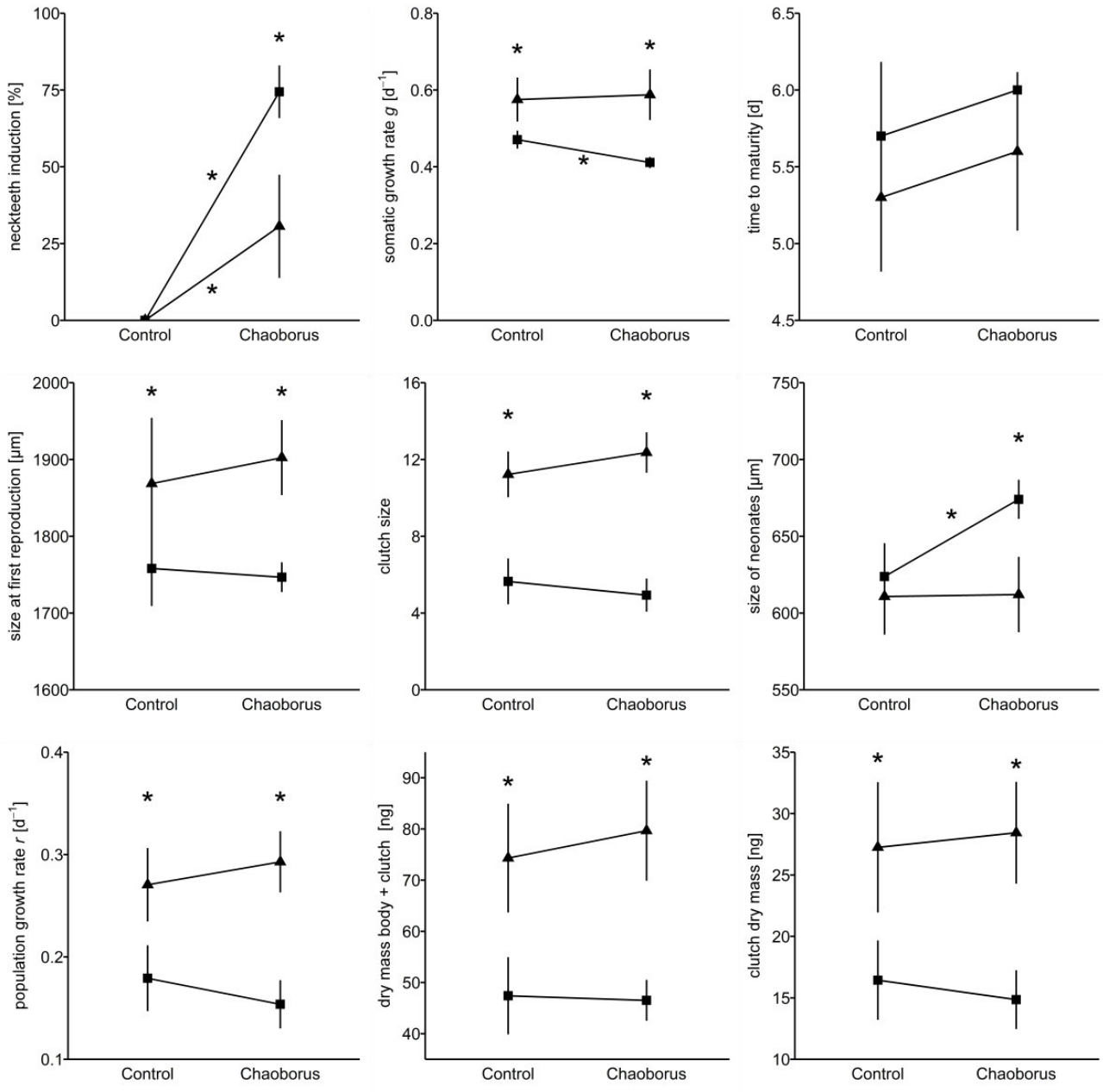

Figure S1: Mean (± SD) life history parameters of *D. pulex* grown on low (0.5 mg C/L; squares) or high (1.5 mg C/L; triangles) food concentrations and either in the absence (Control) or presence of *Chaoborus* kairomone (Chaoborus). Asterisks indicate significant ($p<0.05$) differences. Asterisks above means indicate significant differences between food treatments and asterisks next to the lines between means indicate significant differences between treatments of the same food level. For details on statistical analyses see Table S1.

Table S2: Statistical analyses of the fatty acid analyses depicted in Fig. 1, Fig. 2, Fig. 3, and Fig. 4. Different statistical tests are indicated by different letters (test A2: two-way ANOVA type 2 and Tukey´s HSD test; test A3: two-way ANOVA type 3 and Games-Howell test (Fig. 3) or Tukey´s HSD test (Fig. 4); test KW: Kruskal-Wallis test and pairwise Wilcoxon test). Significant effects ($p<0.05$) are indicated by bold p-values. FA: total fatty acids, SFA: saturated fatty acids, MUFA: mono-unsaturated fatty acids, PUFA: polyunsaturated fatty acids.

figure	parameter	test	factor	df	F/χ2	p-value	L Co	H Co	L Ch	H Ch
1	FA content eggs	KW		3	33.22	**<0.001**	a	c	b	b
1	FA cont. mother	A2	Chaoborus	1	17.851	**<0.001**	c	ab	a	b
			food	1	2.283	0.139				
			Ch × food	1	62.825	**<0.001**				
2	FA investment	A2	Chaoborus	1	12.012	**0.001**	a	c	c	b
			food	1	3.496	0.069				
			Ch × food	1	58.13	**<0.001**				
3	SFA eggs	A3	Chaoborus	1	0.249	0.633	a	b	a	b
			food	1	197.662	**<0.001**				
			Ch × food	1	1.305	0.291				
3	SFA mother	A2	Chaoborus	1	0.358	0.566				
			food	1	1.409	0.269				
			Ch × food	1	0.775	0.404				
3	MUFA eggs	A3	Chaoborus	1	0.724	0.423	b	a	b	a
			food	1	128.103	**<0.001**				
			Ch × food	1	3.755	0.094				
3	MUFA mother	A2	Chaoborus	1	0.013	0.911	a	b	a	b
			food	1	270.231	**<0.001**				
			Ch × food	1	8.503	**0.019**				
3	PUFA eggs	A3	Chaoborus	1	0.029	0.87				
			food	1	11.468	**0.01**				
			Ch × food	1	2.009	0.199				
3	PUFA mother	A2	Chaoborus	1	0.202	0.665	b	a	b	a
			food	1	116.263	**<0.001**				
			Ch × food	1	2.222	0.174				
4	FA	A3	Chaoborus	1	0.782	0.406	a	b	ab	b
			food	1	31.484	**<0.001**				
			Ch × food	1	0.725	0.423				
4	PUFA	A3	Chaoborus	1	0.758	0.413	a	b	a	b
			food	1	35.118	**<0.001**				
			Ch × food	1	0.595	0.466				
4	MUFA	A3	Chaoborus	1	0.687	0.435	a	b	ab	b
			food	1	18.204	**0.004**				
			Ch × food	1	0.866	0.383				
4	SFA	A3	Chaoborus	1	1.031	0.344	a	b	a	b
			food	1	43.208	**<0.001**				
			Ch × food	1	1.023	0.346				

Table S3: Statistical analyses of the share of single fatty acids as depicted in Table 2. Different statistical tests are indicated by different letters (test A2: two-way ANOVA type 2 and Tukey´s HSD test; test A3: two-way ANOVA type 3 and Games-Howell test). Significant effects ($p<0.05$) are indicated by bold p-values.

fatty acid	tissue	test	factor	df	F	p-value	L Co	H Co	L Ch	H Ch
C 16:0	eggs	A3	Chaoborus	1	0.009	0.928	a	b	a	b
			food	1	105.11	**< 0.001**				
			Ch × food	1	0.191	0.675				
C 16:0	mother	A2	Chaoborus	1	0.486	0.506				
			food	1	0.007	0.937				
			Ch × food	1	0.87	0.378				
C 16:1 n-7	eggs	A3	Chaoborus	1	0.962	0.359	b	a	b	a
			food	1	228.23	**< 0.001**				
			Ch × food	1	5.328	0.054				
C 16:1 n-7	mother	A2	Chaoborus	1	0.304	0.597	a	b	a	b
			food	1	127.28	**< 0.001**				
			Ch × food	1	1.867	0.209				
C 18:0	eggs	A3	Chaoborus	1	0.175	0.688	ab	ab	a	b
			food	1	7.643	**0.028**				
			Ch × food	1	0.495	0.504				
C 18:0	mother	A2	Chaoborus	1	0.309	0.594				
			food	1	5.303	**0.05**				
			Ch × food	1	0.297	0.601				
C 18:1 n-7	eggs	A3	Chaoborus	1	0.732	0.421				
			food	1	1.425	0.272				
			Ch × food	1	1.379	0.279				
C 18:1 n-7	mother	A2	Chaoborus	1	0.04	0.847	a	a	a	a
			food	1	11.161	**0.01**				
			Ch × food	1	0.096	0.765				
C 18:1 n-9 c	eggs	A3	Chaoborus	1	1.127	0.324	ab	b	a	b
			food	1	30.67	**< 0.001**				
			Ch × food	1	0.341	0.578				
C 18:1 n-9 c	mother	A2	Chaoborus	1	2.122	0.183	bc	a	c	ab
			food	1	22.591	**0.001**				
			Ch × food	1	0.001	0.971				
C 18:2 n-6 c	eggs	A3	Chaoborus	1	0.293	0.605	ab	bc	a	c
			food	1	99.918	**< 0.001**				
			Ch × food	1	0.981	0.355				
C 18:2 n-6 c	mother	A2	Chaoborus	1	4.004	**0.08**				
			food	1	0.608	0.458				
			Ch × food	1	0.824	0.391				
C 18:3 n-3	eggs	A3	Chaoborus	1	0.431	0.532				
			food	1	0.31	0.595				
			Ch × food	1	0.198	0.669				

C 18:3 n-3	mother	A2	Chaoborus	1	0.523	0.49	b	a	b	a
			food	1	51.344	**< 0.001**				
			Ch × food	1	1.689	0.23				
C 18:3 n-6	eggs	A3	Chaoborus	1	1.432	0.27				
			food	1	15.7	**0.005**				
			Ch × food	1	0.088	0.776				
C 18:3 n-6	mother	A2	Chaoborus	1	0.027	0.875	a	a	a	a
			food	1	6.505	**0.034**				
			Ch × food	1	0.976	0.352				
C 18:4 n-3	eggs	A3	Chaoborus	1	0.073	0.795				
			food	1	21.926	**0.002**				
			Ch × food	1	0.054	0.823				
C 18:4 n-3	mother	A2	Chaoborus	1	0.003	0.96	a	a	a	a
			food	1	10.356	**0.012**				
			Ch × food	1	0.792	0.399				
C 20:3 n-3	eggs	A3	Chaoborus	1	1.018	0.347				
			food	1	0.058	0.817				
			Ch × food	1	0.39	0.552				
C 20:3 n-3	mother	A2	Chaoborus	1	0	0.987				
			food	1	2.634	0.143				
			Ch × food	1	2.027	0.192				
C 20:5 n-3	eggs	A3	Chaoborus	1	0.932	0.367				
			food	1	4.566	0.07				
			Ch × food	1	0.475	0.513				
C 20:5 n-3	mother	A2	Chaoborus	1	11.414	**0.01**	b	b	a	b
			food	1	17.848	**0.003**				
			Ch × food	1	4.428	0.068				
C 22:0	eggs	A3	Chaoborus	1	0.717	0.425				
			food	1	13.282	**0.008**				
			Ch × food	1	0.174	0.689				
C 22:0	mother	n.d.								
C 22:1 n-9	eggs	A3	Chaoborus	1	0.387	0.553				
			food	1	39.956	**< 0.001**				
			Ch × food	1	0.387	0.553				
C 22:1 n-9	mother	n.d.								
C 22:2 n-6	eggs	A3	Chaoborus	1	2.666	0.147				
			food	1	3.14	0.12				
			Ch × food	1	11.77	**0.011**				
C 22:2 n-6	mother	n.d.								
C 22:6 n-3	eggs	A3	Chaoborus	1	0.166	0.696	b	a	ab	ab
			food	1	3.852	0.09				
			Ch × food	1	0.569	0.475				
C 22:6 n-3	mother	n.d.								

Chapter 4

Inducible morphological defense in *Daphnia pulex*: food quantity effects revised

Abstract

In aquatic systems, organisms largely rely on chemical cues to perceive information about the presence of predators or prey. *Daphnia* recognize the presence of the predatory larvae of *Chaoborus* via a chemical cue, emitted by the larvae, a so-called kairomone. Upon recognition, neckteeth, an alteration of the carapace, are induced in *Daphnia* that reduce predation rates of *Chaoborus*. Neckteeth induction was often reported to entail costs. In a previous study, food quantity affected the level of neckteeth induction, with stronger neckteeth induction at low food concentrations and weak induction at high food concentrations. However, reducing neckteeth induction at high food quantities seems to be maladaptive and not in accordance with the concept that inducible defenses are associated with costs.

Here, we hypothesized that weaker neckteeth induction at high food concentrations is caused by increased bacterial degradation of the kairomone. More specifically, we assume that higher algal food concentration is associated with higher bacterial abundances, which degrade the kairomone during the experiment. We tested our hypothesis by treating food algae with antibiotics before providing them as food to *Daphnia*. Antibiotics reduced bacterial abundances at high and low food concentrations. Reduced bacterial abundances at high food concentrations led to the same level of neckteeth induction as at low food concentrations. A linear regression revealed a significant correlation of neckteeth induction to bacterial abundances. We, therefore, conclude that differences in neckteeth induction at different food concentrations are not caused by the food quantity but by differences in bacterial degradation of the kairomone.

Introduction

In freshwater systems, communication is mostly reduced to chemical cues due to conditions like high turbidity or poor light transmission, which make it difficult for animals to rely on visual cues (Brönmark & Hansson, 2000). Chemical cues may transmit information about the presence of prey or predators, food or mating partners (Brönmark & Hansson, 2000). One well investigated model organism in this field of research is *Daphnia* sp., a herbivorous filter-feeder and important link between trophic levels (Lampert, 2006). *Daphnia* recognizes the presence of predators via chemical cues that are emitted by the predator, so-called kairomones (Diel et al., 2020). Kairomones are semiochemicals that induce responses which are advantageous for the receiver and disadvantageous for the emitter. Although many cases of information conveyance by kairomones are known, the chemical identity of kairomones themselves has been uncovered in only a few of those cases (Hahn et al., 2019; Weiss et al., 2018). Upon recognition of the kairomone, a wide range of inducible defenses can be expressed, which comprise physiological, behavioral, or morphological changes (Lass & Spaak, 2003). The expression of those inducible defenses is associated with costs, which trade-off the benefits of a defense in terms of fitness (Hammill et al., 2008). The associated costs are regarded as the cause why those defenses are inducible, because constitutive expression would lead to reduced fitness in the absence of the predator.

In many cases physiological changes in response to kairomones lead to changes in life history (Dodson & Havel, 1988; Tollrian, 1995b). The effect of food quantity on kairomone-mediated physiological changes might be the most difficult to investigate, because changes in food quantity per se can lead to life history changes (Vanni & Lampert, 1992). Variability introduced by genetic variance (Reznick et al., 2000) as well as the dependence of reaction norms on intrinsic values of traits (Wolinska et al., 2007) further complicates this relation (Cressler et al., 2010). This might be the explanation for the fact that in the presence of predators that select for big prey, like fish or *Notonecta* sp., some *Daphnia* only respond to the kairomone at high food concentrations (Dodson, 1988; Dodson & Havel, 1988), whereas others show enhanced responses under food stress (Pauwels et al., 2010). For predators that select for small prey, like *Chaoborus* sp., this relation with food quantity gets even more complex, and responses of *Daphnia* are highly variable (Dodson, 1988; Tollrian, 1995b). Due to the fact that costs of inducible defenses are

measured as life history traits, it is hardly possible to differentiate life history change as part of the defense from those that represent the cost of the inducible defense.

Behavioral changes as inducible defenses can result in diel vertical or horizontal migration (Dawidowicz & Loose, 1992; Kvam & Kleiven, 1995; von Elert & Loose, 1996). Vertically migrating *Daphnia* experience lower temperatures in deeper strata, so that the associated costs of migration are reduced somatic growth and reproduction rates (Dawidowicz & Loose, 1992). As in nature food availability is decreasing in deeper strata, lower food quantity in the epilimnion leads to a slight decrease in diel vertical migration of *D. magna* in the presence of fish (Loose & Dawidowicz, 1994) or even to a suppression of migration when food quantity was limiting (Beklioglu et al., 2008; Johnsen & Jakobsen, 1987).

Morphological defenses comprise a huge variety of alterations in carapace shape like the elongation of head or tail spines, formation of head crests, neckteeth, or helmets (Dodson, 1988; Spaak & Boersma, 1997; Tollrian, 1993). The effect of food quantity on morphological defenses was investigated in various predator-prey-systems of *Daphnia*. *D. ambigua* increased its helmet size with increasing food concentration in the presence of *Chaoborus* (Hanazato, 1991). The same response of increased defense at high food concentrations was observed in *D. retrocurva* for its increase in head length in the presence of *Chaoborus* or *Notonecta* (Dodson, 1988). Furthermore, the relative tail spine length of individuals of the *D. galeata* × *cucullata* × *hyalina* complex increased significantly at high food concentrations compared to low ones in the presence of fish (Spaak & Boersma, 1997).

In our study, we focused on the morphological defense of *D. pulex* against larvae of *Chaoborus* sp. *D. pulex* changes its morphology during the juvenile instars, which are most vulnerable to *Chaoborus* predation (Pastorok, 1981; Tollrian, 1995a). The formation of neckteeth, which are small protuberances in the neck region of *D. pulex*, reduces predation rates to a great extent (Tollrian, 1995a). Interestingly, neckteeth induction is, so far, the only morphological defense reported to be decreasing at high food concentrations (Parejko & Dodson, 1991). Associated costs of neckteeth induction were often detected as a delay of reproduction, shorter body lengths at first reproduction or reduced fitness measured as the intrinsic rate of increase (Black & Dodson, 1990; Havel & Dodson, 1987; Riessen & Sprules, 1990). Under the assumption that costs of neckteeth induction per se are independent of food availability, neckteeth induction should not be suppressed when food quantity is saturating. Hence, the observation of a reduced

neckteeth induction at high food levels (Parejko & Dodson, 1991) seems not consistent with the concept of costs associated with the induction of these morphological changes.

Considering this conceptual discrepancy, we hypothesized that the effect of food quantity on neckteeth induction is actually caused by differing bacterial abundances: we assumed that with increasing food concentrations higher abundances of (accompanying) bacteria were added, which lead to higher degradation rates of the *Chaoborus* kairomone at high food concentrations. We tested this hypothesis by treating the food alga *Chlamydomonas klinobasis* with antibiotics before adding the alga as food for *Daphnia pulex*. We counted bacterial abundances in the experimental jars, scored neckteeth, which were induced by the *Chaoborus* kairomone, during the second instar of *D. pulex*, and performed a linear regression with the two parameters.

Methods

Cultivation of animals

Prior to the experiments *D. pulex* clone TCO (Colbourne et al., 2011) was kept in aged and aerated tap water at a density of 10-12 individuals per 800 mL at 19.2 ± 0.3 °C and a 16:8 light:dark cycle. Every second day the animals were transferred into fresh medium containing at least 1 mg C/L of *Chlamydomonas klinobasis* strain #56 (Limnological Institute, University of Constance).

Cultivation of food

C. klinobasis was grown in 5 L semi-continuous batch cultures in cyanophyceae medium (von Elert & Jüttner, 1997) modified with vitamins. For the experiment *Chlamydomonas* was incubated for different treatments. The control treatment ('Control') contained *Chlamydomonas* suspension without any further treatment. For the antibiotics treatment ('Antibiotics') two times 45 mL of *Chlamydomonas* suspension were centrifuged at 3,214 g for 5 minutes and the supernatant was discarded. The pellets were each resuspended in 100 mL cyanophyceae medium containing 500 µg/mL ampicillin and 50 µg/mL tetracycline, and incubated in sterile Erlenmeyer flasks for 22 hours on a rotary shaker set to 80 rpm at constant light. After incubation, the cultures were centrifuged as above. The pellets were resuspended in fresh cyanophyceae medium without antibiotics and centrifuged again to wash the cells. Afterwards, the pellets were resuspended in 100 mL fresh cyanophyceae medium and stored in Erlenmeyer flasks for subsequent use. In order to account for effects of processing the cultures, an additional control treatment for centrifugation and incubation on the rotary shaker was established ('Shaker'). After screening the respective food suspensions using a 30 µm gauze, the volume of the food suspension that was needed was determined photometrically at a wavelength of 470 nm by using a calibration curve relating the carbon content to the optical density.

Preparation of *Chaoborus* incubation water extract

The extract of *Chaoborus* incubation water was prepared as according to Klintworth and von Elert (2020). Approximately 300-350 fourth instar larvae (ordered from www.interaquaristik.de) of *Chaoborus flavicans* were fed with 1-2 neonates of *D. pulex* clone TCO per larva. After 1-2 hours of feeding, the larvae were transferred into 1 L of fresh aged and aerated tap water without any food. After 24 hours the larvae were removed from the water using a 250 µm gauze, and the water was filtered through a glass fiber filter (Whatman, MN 85/220, 0.4 µm). Subsequently, the kairomone was enriched by solid phase extraction (VARIAN, Bond Elut-C18, 10 g of sorbent, volume 60 mL, Agilent Technologies) as according to Christjani et al. (2016). The eluates were pooled and evaporated to dryness in a rotary evaporator and a vacuum centrifuge. The dried residues were dissolved in 58 µL methanol and stored at -20 °C until use. A control extract was prepared in exactly the same way but without any animals in the water.

Experimental setup

A cohort of synchronized animals that had just released their first eggs into the brood chamber was distributed to the different food level treatments, containing either 0.5 mg C/L or 1.5 mg C/L of *C. klinobasis*. Subsequently, the treatments are referred to as 'low food' and 'high food'. When the animals had deposited their second clutch into the brood chamber, they were divided into the food treatments control ('Control'), control of the rotary shaker ('Shaker') and the antibiotics treatment ('Antibiotics'). For details on the preparation of those treatments see 'Cultivation of food'. For each of these food treatments, there was a control treatment and a kairomone treatment. The kairomone treatment contained 1.5 µL of the *Chaoborus* incubation water extract per 150 mL. This volume of extract had induced an intermediate degree of neckteeth induction during a dose response experiment of *D. pulex* clone TCO (same conditions as for the cultivation). The control treatment contained the same volume of control extract. Each treatment was replicated fivefold.

The animals carrying their second clutch in the brood chamber were kept individually in 150 mL aged and aerated tap water containing either 0.5 mg C/L or 1.5 mg C/L of *C. klinobasis* of the respective food treatment combined with either the control extract or the extract of the *Chaoborus* incubation water extract. After the second clutch had hatched, mothers were removed

from the jars, and neonates were removed so that no more than six experimental neonates remained in the jars. Neonates that were removed were pooled per treatment, and their dry mass was determined in subsamples of two times ten neonates per treatment. Those dry masses were later on used as w_0 for the calculation of the somatic growth rates. The experimental animals were transferred daily to freshly prepared jars. After transferring the animals, a sample of 8 mL was taken from each jar that had contained animals in their first instar to quantify the abundance of bacteria. For details on fixing, staining, and counting the bacteria see 'Bacteria counting'. Neckteeth induction of five experimental animals per jar was determined during their second juvenile instar using the method developed by Tollrian (1993) with the slight modification that each tooth was scored with 10 % and no differentiation was made between big and small teeth as described in Schwarzenberger et al. (2014). After the animals had deposited their first clutch into their brood chambers, the clutch size was determined under a binocular and three animals per jar were taken for the determination of their dry mass. The time until maturity was determined and the somatic growth rates (g) were calculated according to the following formula: $g = (\ln(w_t) - \ln(w_0)) / t$, with w_t being the individual weight at day t and w_0 being the individual weight at day 0 (Rothhaupt & Lampert, 1992).

Bacteria counting

The samples were immediately fixed in 4 % sugar-formol. The bacteria in the samples were stained using DAPI (4′,6-Diamidin-2-phenylindol). The filtration device was rinsed with deionized water and the required volume of the sample was filtered (0.2 µm pore size, 25 mm ø, Whatman Nuclepore Polycarbonate Membrane) to reach the appropriate density of bacteria on the filter. The filter with the sample on it was rinsed with deionized water and 300 µL of 5 µg/mL DAPI were put on the filter. During the incubation time of 5 minutes the filters were covered. Subsequently, the DAPI was removed and the filters were rinsed with deionized water again. Bacteria were counted immediately after staining under a fluorescence microscope (ZEISS Axioskop equipped with Shutter HXP 120) using 100x magnification. The abundance of bacteria on the filer was calculated by counting 20 randomly selected fields per filter (1 field ≙ 15,625 μm^2). One filter per experimental replicate was prepared (n=5 per treatment).

Statistical analyses

All statistical analyses were performed in RStudio version 1.1.423 (R version x64 3.4.3). In case a parameter was determined on more than one animal per replicate, the average of those values per replicate was calculated and used for statistics. Data were checked for homoscedasticity, and, if given, an ANOVA type 2 followed by Tukey´s HSD test was performed. If not given, a Kruskal-Wallis rank sum test was performed. In cases of unequal sample sizes, an ANOVA type 3 followed by a Games-Howell test was performed. Spearman´s rank correlation was used for the correlation of neckteeth induction and bacteria abundances. The significance level for all analyses was $p<0.05$.

Results

Neckteeth induction was affected by the food concentration and the treatment of the food (Table 1). Neckteeth induction in the Control treatment was significantly different at the two food concentrations (Fig. 1). On low food neckteeth induction was 63 (± 4) % (mean ± SD), whereas on high food neckteeth induction was only 42 (± 11) %. This reduction in neckteeth induction on high food was not found in the Shaker or the Antibiotics treatment. In the Shaker treatment neckteeth induction was 62 (± 9) % on low food and 52 (± 7) % on high food. In the Antibiotics treatment neckteeth induction was 75 (± 6) % on low food and 71 (± 6) % on high food.

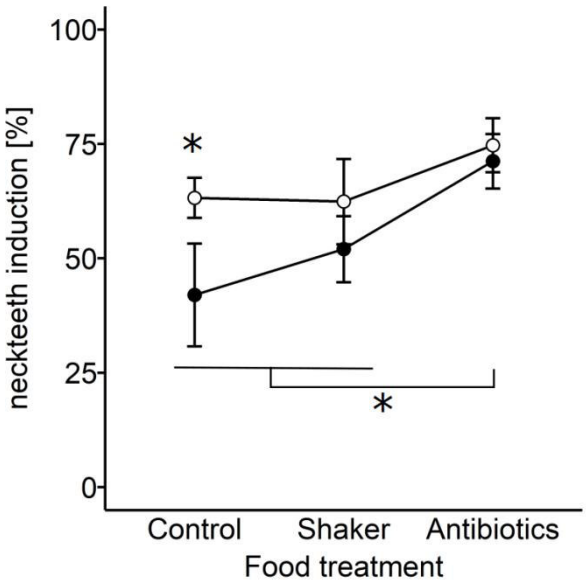

Figure 1: Mean ± SD of neckteeth induction [%] of *D. pulex* growing on *C. klinobasis* that had either been cultured under control conditions (Control) or incubated on a rotary shaker for 22 h (Shaker) or incubated on a rotary shaker in antibiotic-containing medium (Antibiotics). *D. pulex* was either grown under high food conditions (●; 1.5 mg POC / L) or low food conditions (○; 0.5 mg POC / L). Only data from *D. pulex* grown in the chemical presence of *Chaoborus* are presented. Neckteeth induction in the chemical absence of *Chaoborus* was 0% in all treatments. Asterisks associated to brackets represent significant differences within the same food concentration, whereas single asterisks indicate significant differences within the same food treatment (two way ANOVA type 2 followed by Tukey´s HSD test). Details on the statistical analysis can be found in Table 1.

Neckteeth induction on high food in the Antibiotics treatment was significantly higher than in the Control and the Shaker treatment and was not significantly different from the neckteeth induction

on low food. All *Daphnia* that grew in the chemical absence of *Chaoborus* did not express any neckteeth and were excluded from statistical analyses. No interaction between treatment of the food and food concentration was found (Table 1).

Table 1: Results of a two-way ANOVA type 2 for the analysis of the neckteeth induction (see Fig. 1). Only data of the treatments containing *Chaoborus* incubation water extract were included in the analysis. Significant p-values are indicated in bold.

	Df	Sum Sq	Mean Sq	F-value	p-value
Food concentration	1	1027	1027	17.39	**0.0003**
Treatment	2	2278	1139	19.29	**1.0e-05**
Food conc. × Treatment	2	398	199	3.37	0.051
Residuals	24	1417	59		

Bacterial abundances were also affected by both the food concentration and the treatment of the food (Table 2). A significant difference in bacterial abundances between food concentrations was only found in the Control treatment (Fig. 2). At low food only 2.2 (± 0.8) $\cdot 10^5$ cells/mL were counted, whereas at high food 3.5 (± 0.3) $\cdot 10^5$ cells/mL were counted. In the Shaker treatment bacterial abundances were slightly but not significantly reduced to 1.8 (± 0.6) $\cdot 10^5$ cells/mL at low food compared to 2.6 (± 0.7) $\cdot 10^5$ cells/mL at high food. Antibiotics significantly decreased the abundance of bacteria to 0.5 (± 0.1) $\cdot 10^5$ cells/mL and 0.8 (± 0.2) $\cdot 10^5$ cells/mL at low food and high food, respectively.

Table 2: Results of a two-way ANOVA type 2 for the analysis of the bacterial abundance (see Fig. 2). Only data of the treatments containing *Chaoborus* incubation water extract were included in the analysis. Significant p-values are indicated in bold.

	Df	Sum Sq	Mean Sq	F-value	p-value
Food concentration	1	4.67e+10	4.67e+10	18.11	**0.0002**
Treatment	2	2.62e+11	1.31e+11	50.80	**2.3e-09**
Food conc. × Treatment	2	1.29e+10	6.45e+09	2.50	0.10
Residuals	24	6.18e+10	2.57e+09		

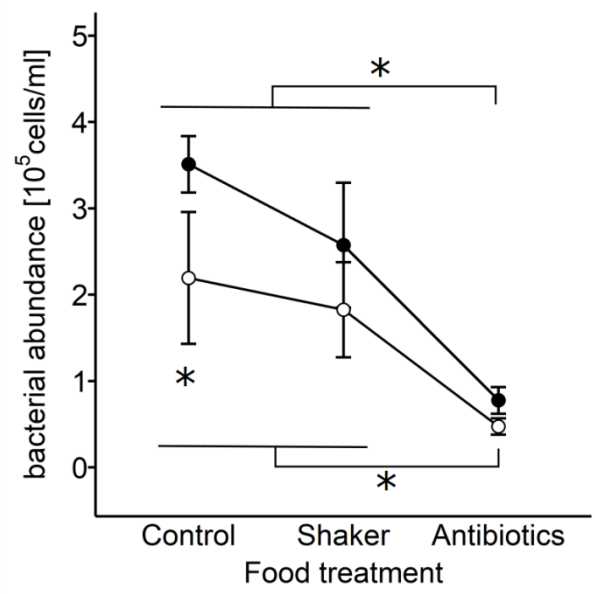

Figure 2: Mean ± SD of bacterial abundances [10^5 cells / ml] in the jars of *D. pulex* growing on *C. klinobasis* after 22 h. *C. klinobasis* had either been cultured under control conditions (Control) or incubated on a rotary shaker for 22 h (Shaker) or incubated on a rotary shaker in antibiotic-containing medium (Antibiotics). *D. pulex* was either grown under high food conditions (●; 1.5 mg POC / L) or low food conditions (○; 0.5 mg POC / L). Only data from jars containing *D. pulex* grown in the chemical presence of *Chaoborus* are presented. Asterisks associated to brackets represent significant differences within the same food concentration, whereas single asterisks indicate significant differences within the same food treatment (two way ANOVA type 2 followed by Tukey´s HSD test). Details on the statistical analysis can be found in Table 2.

In order to test if neckteeth induction would decrease with the abundance of bacteria, we correlated the neckteeth induction to the bacterial abundances and performed a linear regression (Fig. 3). Spearman´s rank correlation revealed a significant negative correlation (rho = -0.78; $p = 3.6*10^{-7}$) with neckteeth induction decreasing with increasing bacterial abundances.

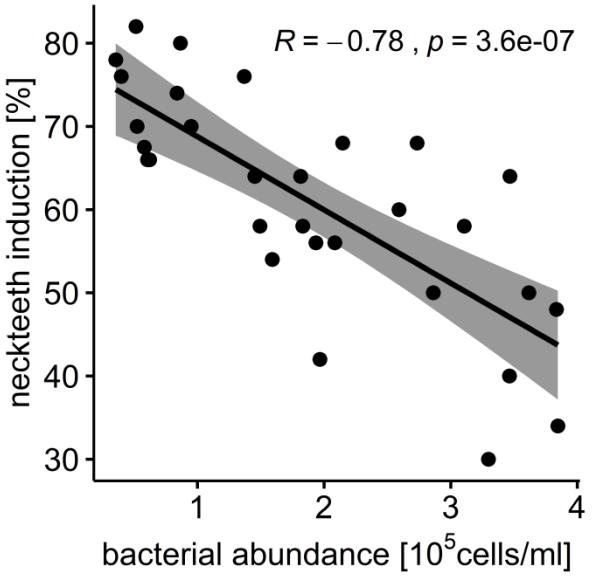

Figure 3: Neckteeth induction [%] in *D. pulex* as a function of bacterial abundance [10^5 cells/ml] after 22 h. Points represent single replicates. Only data from jars containing *D. pulex* grown in the chemical presence of *Chaoborus* are presented. The black line indicates a linear regression with 95% confidence interval (Spearman´s rank correlation: rho = -0.78; p = $3.6*10^{-7}$).

The analysis of clutch size and somatic growth rates revealed that a lower food concentration reduced both parameters, whereas the presence neither of antibiotics nor of the kairomone had an effect (Fig. S1, S2, Table S1, S2). The analysis of the time to maturity revealed that there were no significant differences between treatments (Fig. S3, Table S3).

Discussion

We found that neckteeth induction decreased with increasing abundances of bacteria. Bacterial abundances were reduced by treating the algae suspensions with antibiotics before the algae were provided as food for *Daphnia*. The reduction of bacteria was significant both at low and high food concentrations and reduced bacterial abundances at high food led to the same level of neckteeth induction as at low food. A linear regression of neckteeth induction to bacterial abundances revealed a significant negative correlation: neckteeth induction decreased with increasing bacterial abundances.

Parejko and Dodson (1991) reported high neckteeth induction at low food and less neckteeth induction at high food. Thus, they had concluded that neckteeth induction is influenced by food quantity. We found less neckteeth induction at high food in the Control and therewith corroborate the findings of Parejko and Dodson (1991). Due to an additional treatment of the food suspension with antibiotics we here revealed that this decrease in neckteeth induction at high food is related to the high abundance of bacteria. In our experiments increasing experimental food concentrations were obtained by diluting increasing aliquots of the same stock food suspension. Since the food suspension was not bacteria free, addition of different aliquots of this suspension did not only result in low and high algal concentrations but as well in low and high bacterial abundances. Although no bacterial abundances are reported by Parejko and Dodson (1991) it is reasonable to assume that in their experiments high and low food concentrations as well were related to high and low bacterial abundances, since their food alga was not explicitly cultured axenically, and since the different food concentrations supposedly were as well prepared from the same stock suspension.

We hypothesized that higher bacterial abundances result in higher degradation rates. By treating the food suspensions with antibiotics, this treatment contained reduced bacterial abundances and initially identical kairomone concentrations. Due to the fact that at high food concentrations the neckteeth induction was significantly lower in the Antibiotics than in the Control treatment, we conclude that kairomone concentrations, in line with higher bacterial abundances, most probably decreased at higher rates in the Control than in the Antibiotics treatment. Therefore, our results clearly demonstrate that food quantity does not affect neckteeth induction. This conclusion is in

line with a study, in which a flow-through system was used to determine food quantity effects on neckteeth induction (Tollrian, 1995b). In that setup a continuous input of the kairomone, and thus, a constant concentration of the kairomone, was assured. Therefore, no difference in neck-teeth induction on different food concentrations was observed.

The kairomone produced by *Chaoborus* was identified as a group of fatty acid-amino acid conjugates, which are made up of a glutamine head group and long chain fatty acids conjugated to the α-amino group of glutamine (Weiss et al., 2018). The fact that glutamine lipids themselves can be incorporated in bacterial cell membranes (Zhang et al., 2009) makes it very likely that the kairomone is degradable by bacteria and that bacteria use such conjugates as sources for those molecules.

The expression of inducible defenses can be considered adaptive as long as the degree of expression of the defense is linked to the real predation risk. Since inducible defenses are associated with costs, expression of such defenses in the absence of predators would bear costs without any benefits. It is, therefore, highly adaptive to link the expression of inducible defenses to chemical cues that reliably indicate the risk of predation. Hence, from an evolutionary perspective, kairomones must reliably signal not only the presence of a predator but as well predator abundances or predator activities. Accordingly, higher predator densities result in stronger expression of defenses, for example in increased amplitudes of diel vertical migration (von Elert & Pohnert, 2000), in a decreased size at first reproduction (von Elert & Stibor, 2006) or stronger expression of a morphological defense (Tollrian, 1993).

In order to be considered reliable cues for predator densities, kairomones dissolved in water should be subjected to a certain turnover, so prey can also perceive declining predation risk. One way of turnover or removal is bacterial degradation. The effect of bacterial degradation of a kairomone on an inducible defense of *Daphnia* was already shown for the kairomone produced by fish (Loose et al., 1993). After incubating the non-sterile fish incubation water for 24 h at 37 °C, *D. magna* did not respond to the fish incubation water by performing diel vertical migration, whereas fish incubation water that was incubated in the absence of bacteria remained active. Furthermore, incubation of fish incubation water at 4 °C under otherwise identical conditions did not lead to a loss of activity and it still induced diel vertical migration in *D. magna*. The kairomone produced by fish is the bile salt 5α-cyprinol-sulphate (Hahn et al., 2019). Bacteria are

known to be involved in synthesis pathways of bile salts (Hofmann & Hagey, 1998), and also in the degradation of bile salts (Philipp, 2011). Another study on the bacterial degradation of the fish kairomone provided further evidence that the amplitude of diel vertical migration of *D. magna* is dependent on bacterial abundances (Beklioglu et al., 2006).

We here assured that *Daphnia* would not be affected by the antibiotics treatment of the food algae by washing the algae with medium free of antibiotics prior to feeding them to the daphnids. It has been reported that antibiotics may negatively affect growth rates or clutch sizes in *Daphnia* (Kim et al., 2012; Wollenberger et al., 2000). However, we detected neither of those effects, which makes it highly improbable that in the antibiotics treatment any direct effect of the antibiotics on *Daphnia* occurred. Accordingly, the effects of food quantity on somatic growth rate or clutch size that we observed here are in accordance with the well-known effects of food quantity on these parameters (Lampert & Trubetskova, 1996; Vanni & Lampert, 1992).

Furthermore, we detected no apparent costs of neckteeth induction in our study. Clutch sizes, somatic growth rates, and the time until maturity were not affected by the kairomone treatment. Especially the time until maturity was considered a cost of neckteeth induction (Black & Dodson, 1990; Havel & Dodson, 1987), which could be also caused by changes in life history. More recent studies, however, found no costs of neckteeth induction (Riessen, 2012; Tollrian, 1995b). Tollrian (1995b) disentangled the effects of life history change and costs of morphological defense by restricting the kairomone exposure of *Daphnia* to the developmental times when the respective defense is expressed and found no life history shifts in the treatments, in which neckteeth were induced. Riessen (2012) performed life history experiments with two clones that differed in their degree of neckteeth formation, but found the same degree of life history shifts, which, assuming neckteeth induction would entail costs, should have also differed. As neckteeth induction does not seem to have any apparent costs that would affect life history or fitness of *Daphnia*, it might be maladaptive that this morphological defense would be affected by the availability of resources.

From our study we conclude that the food quantity effects on neckteeth induction that have been reported before (Parejko & Dodson, 1991) were most probably caused by differences in bacterial abundances and bacterial degradation rates of the kairomone in the experimental setup. We largely excluded the effect of bacterial degradation of the kairomone in our study, and we were

able to show that differences in neckteeth induction between food concentrations, as they were detected in the Control, were not detected in the treatment, in which bacterial degradation was reduced. The inducible morphological defense of *D. pulex* against *Chaoborus* does not only include the formation of neckteeth, but comprises further morphological changes as a thickening of the carapace (Laforsch et al., 2004). This has been corroborated by the finding that *Chaoborus* incubation water extract induces an upregulation of chitin deacetylase genes in *D. pulex* (Christjani et al., 2016). Although we focused solely on neckteeth induction in this study, we assume that further morphological changes like the thickening of the carapace are as well not affected by food concentration in *D. pulex*.

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Supplements

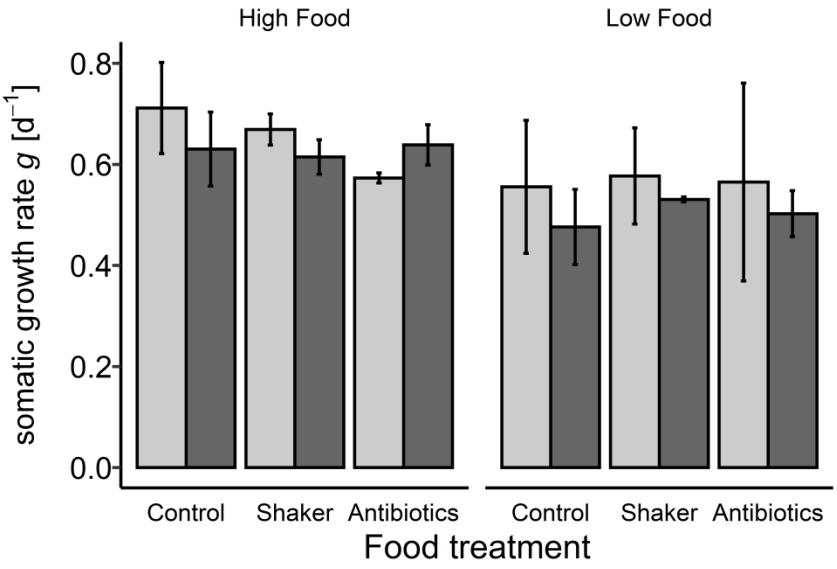

Figure S1: Mean ± SD juvenile somatic growth rate g [d^{-1}] of *D. pulex* growing on *C. klinobasis* that had either been cultured under control conditions (Control) or incubated on a rotary shaker for 24 h (Shaker) or incubated on a rotary shaker in antibiotic-containing medium (Antibiotics). Light grey bars indicate the chemical absence of *Chaoborus*, and dark grey bars indicate the chemical presence of *Chaoborus*. Details on the statistical analysis can be found in Table S1.

Table S1: Results of a three-way ANOVA type 2 for the analysis of the somatic growth rates g (see Fig. S1). Significant p-values are indicated in bold.

	Df	Sum Sq	Mean Sq	F-value	p-value
Food concentration	1	0.1655	0.16548	22.11	**2.2e-05**
Treatment	2	0.0091	0.00454	0.60	0.548
Kairomone	1	0.0280	0.02795	3.73	0.059
Food conc. × Treatment	2	0.0194	0.00970	1.29	0.282
Food conc. × Kairomone	1	0.0058	0.00584	0.78	0.381
Treatment × Kairomone	2	0.0172	0.00858	1.14	0.326
Food conc. × Treatment × Kairomone	2	0.0148	0.00740	0.98	0.379
Residuals	48	0.3591	0.00748		

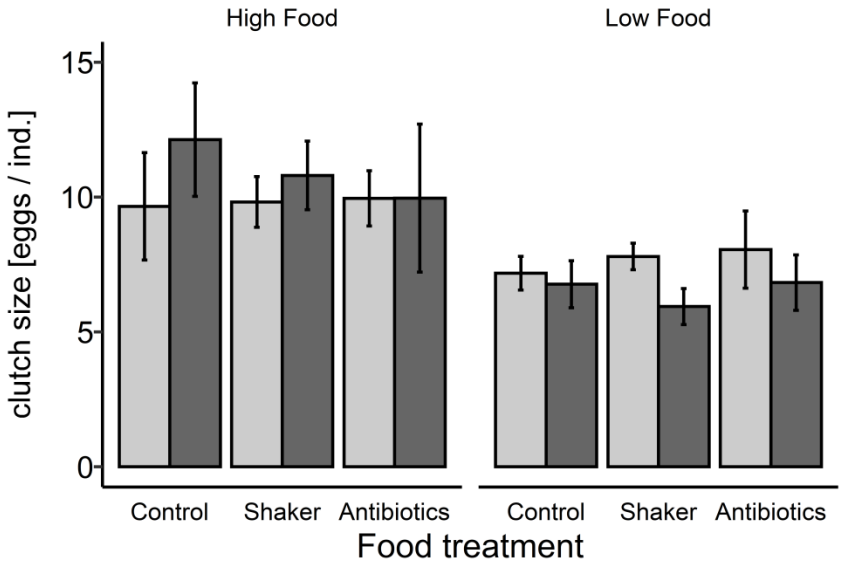

Figure S2: Mean ± SD of the size of the first clutch [nr. of eggs] of *D. pulex* growing on *C. klinobasis* that had either been cultured under control conditions (Control) or incubated on a rotary shaker for 24 h (Shaker) or incubated on a rotary shaker in antibiotic-containing medium (Antibiotics). Light grey bars indicate the chemical absence of *Chaoborus* and dark grey bars indicate the chemical presence of *Chaoborus*. Details on the statistical analysis can be found in Table S2.

Table S2: Results of a three-way ANOVA type 3 for the analysis of the clutch size (see Fig. S2). Significant p-values are indicated in bold.

	Sum Sq	Df	F-value	p-value
(Intercept)	4176.2	1	1964.15	**< 2.2e-16**
Food concentration	148.2	1	69.67	**1.286e-10**
Treatment	1.1	2	0.25	0.776
Kairomone	0.0	1	0.00	0.994
Food conc. × Treatment	4.5	2	1.06	0.351
Food conc. × Kairomone	18.4	1	8.63	**0.005**
Treatment × Kairomone	7.0	2	1.64	0.205
Food conc. × Treatment × Kairomone	2.1	2	0.48	0.619
Residuals	93.6	44		

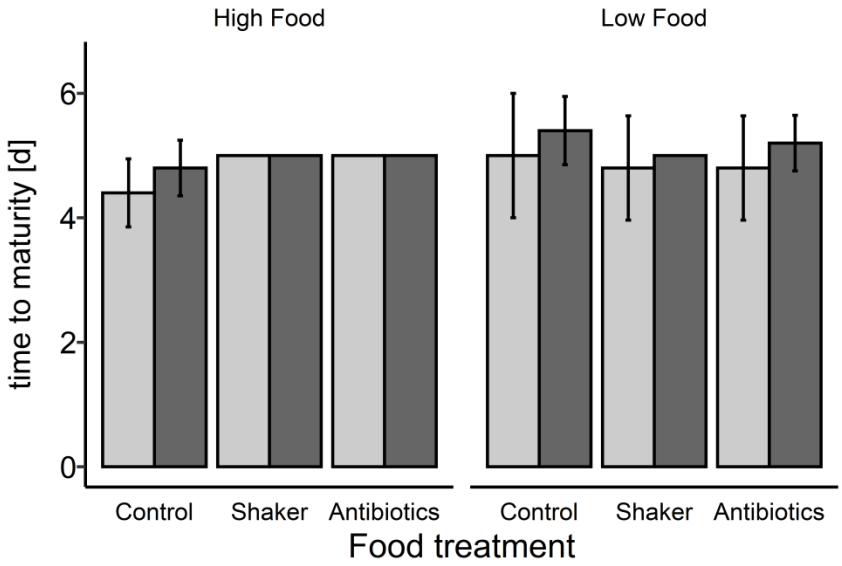

Figure S3: Mean ± SD of the time to maturity [d] of *D. pulex* growing on *C. klinobasis* that had either been cultured under control conditions (Control) or incubated on a rotary shaker for 24 h (Shaker) or incubated on a rotary shaker in antibiotic-containing medium (Antibiotics). Light grey bars indicate the chemical absence of *Chaoborus* and dark grey bars indicate the chemical presence of *Chaoborus*. Details on the statistical analysis can be found in Table S3. Missing error bars indicate SD = 0.

Table S3: Results of a Kruskal-Wallis rank sum test for the analysis of the time until maturity (see Fig. S3).

χ^2	df	p-value
11.577	11	0.3963

Concluding remarks and perspectives

Predation is one of the most important drivers of natural selection. While effects of direct consumption can have huge impacts on prey populations, non-lethal trait-mediated effects have been repeatedly reported to impact populations even stronger, because these induced effects on trait phenotype commonly entail costs, or the induction of trait alteration result in decreased competitive abilities and fitness of the prey (Preisser et al., 2005; Werner & Peacor, 2003). Inducible defenses, which comprise any trait alteration in response to the presence of a predator, are wide spread across taxa and ecosystems (Adler & Harvell, 1990).

In standing freshwater systems, the popular model organisms *Daphnia* have been the subject of countless studies on inducible defenses. This dissertation covers the interaction of *D. pulex* and the predacious insect larvae *Chaoborus*. The presence of *Chaoborus* can induce alterations in morphological, behavioral, as well as life history traits of *D. pulex* (Black & Dodson, 1990; Dodson, 1988b; Tollrian, 1995b). During this dissertation, I focused on the morphological defense: *D. pulex* forms neckteeth during its juvenile stages, which experience the highest predation risk (Pastorok, 1981; Tollrian, 1995a). Since the expression of inducible defenses entails costs by concept, and some studies reported costs associated with neckteeth induction (Black & Dodson, 1990; Havel & Dodson, 1987; Riessen & Sprules, 1990), it is crucial for *Daphnia* to accurately assess the predation risk, so the costly defense is only expressed when required. Neckteeth induction in *D. pulex* has been extensively studied over the past decades, and even the origin and identity of the *Chaoborus* kairomone was uncovered recently (Weiss et al., 2018). Nevertheless, knowledge about the environmental conditions and factors that might affect neckteeth induction is still scarce. During this dissertation, I investigated how several factors that *Daphnia* are exposed to in nature at varying conditions affect neckteeth induction in *D. pulex*.

In times of global warming, eutrophication of lakes due to anthropogenic impacts has become a rising issue for water resource management. Elevated temperatures and nutrient intake facilitate cyanobacterial blooms during summer (Paerl et al., 2001). These blooms often contain cyanobacterial strains that produce secondary metabolites, which are toxic or harmful not only to human health but especially to grazers like *Daphnia* (Paerl & Huisman, 2009). During the study presented in Chapter 1, I investigated if neckteeth induction in *D. pulex* is affected by the ambient

temperature or dietary cyanobacteria that have been reported to be harmful to *Daphnia*. Furthermore, I hypothesized that the neckteeth induction is correlating with the developmental time of the juvenile instars. This hypothesis is based on the assumption that the longer the juveniles take to outgrow the favorable predation size range of *Chaoborus*, the stronger the juveniles would need to defend themselves, as the individual overall predation risk increases. Neckteeth induction was significantly increased at the highest proportion of dietary cyanobacteria, and it decreased with temperature. The correlation of neckteeth induction and developmental time, however, was only significant in case of the factor temperature. In order to further examine the effect of temperature on neckteeth induction, I performed dose-response experiments at the different temperatures, as well as predation experiments. Due to the fact that the dose-response curves did not differ between temperatures, I concluded that *D. pulex* is not physiologically restricted in their ability to fully develop neckteeth at all investigated temperatures. Predation rates of *Chaoborus* were as well not affected by temperature. Thus, the reason for decreasing neckteeth induction with temperature was neither a physiological restriction at higher temperatures, nor was it caused by differences in predation risk. Instead, the differences in neckteeth induction presumably had been caused by different rates of bacterial degradation of the kairomone at the different temperatures, as the metabolic rates of bacteria increase with temperature (Ingraham & Bailey, 1959; Price & Sowers, 2004).

While it was reported in many field studies that inducible morphological defenses are positively correlated with temperature (cyclomorphosis) (Dodson, 1988a; Havel, 1985), the results from controlled laboratory studies presented here suggest that temperature does not directly affect neckteeth induction. Instead, it rather affects neckteeth induction indirectly in the opposite way via bacterial degradation, thus, the metabolic rate of bacteria. Corresponding to this, the density of *Chaoborus* and the temperature alone had been found to be poor predictors for the percentage of neckteeth-bearing *D. pulex* over several years in a temperate lake (Luecke & Litt, 1987). The best predictors had been found to be the metabolic rate and the feeding rate of *Chaoborus*, both calculated from the density of the larvae and the respective temperature. This finding suggests that neckteeth induction might be largely affected solely by the concentration of the kairomone, which, as a metabolic product of digestive processes (Parejko & Dodson, 1990; Weiss et al., 2018), is coupled to both the metabolic rate as well as the feeding rate of *Chaoborus*. However, all studies concerning the temperature effect on neckteeth induction so far have focused on the

percentages of individuals bearing neckteeth in a population, and not on the strength of induction in individuals. This study (Chapter 1) presents for the first time the effect of temperature on the strength of the morphological defense of *D. pulex* in individuals.

During the study presented in Chapter 2, I investigated for the first time the effect of food quality with respect to PUFA availability on neckteeth induction in three clones of *D. pulex*. The gradient of PUFA availability was established by using three different algal species that had been shown to differ in their PUFA contents (Ahlgren et al., 1990). According to the hypothesis of Chapter 1, I hypothesized that neckteeth induction decreases with PUFA availability, as the individual predation risk decreases due to faster growth and shorter developmental times. However, neckteeth induction did not change with PUFA availability, although PUFA availability affected *Daphnia*'s somatic growth and time to maturity as expected. I further investigated the role of one particular PUFA, EPA, for which an effect on a behavioral inducible defense had been reported before (Brzeziński & von Elert, 2015). In order to do so, I supplemented liposomes containing EPA to the same basal food. While the absence of EPA suppressed diel vertical migration in *D. magna*, it did not affect neckteeth induction in *D. pulex*. The reason for this might be that neckteeth induction is not accompanied by a higher EPA demand, in contrast to diel vertical migration, during which *Daphnia* experience colder temperatures that require *Daphnia* to incorporate more PUFA to the membranes to maintain membrane fluidity (Hazel, 1995; Sperfeld & Wacker, 2012). Since it is known that *Daphnia* clones can differ in their reaction norms to various environmental changes (Spitze, 1992), the usage of three clones provides more comprehensive results, and enables to make a more generally valid statement. Although two clones differed in their sensitivity to the kairomone, neckteeth induction was not affected in either of the clones. The clones differed in their reaction norms to the increasing PUFA availability with respect to growth rate and time to maturity. This result supports the finding of the study presented in Chapter 1, during which no general correlation of neckteeth induction to developmental times, i.e. growth rates, was found. In Chapter 2, I report for the first time that biochemical food quality did not affect a morphological defense in *Daphnia*.

The effect of food quantity on neckteeth induction had been studied before, but the results have not been conclusive so far: while neckteeth induction decreased with food concentration in one study (Parejko & Dodson, 1991), it was not affected by food quantity in another (Tollrian,

1995b). In association with the result of Chapter 1 that *D. pulex* is able to induce neckteeth to the same extent at different temperatures, and that differences in neckteeth induction might have been caused by different degradation rates, I hypothesized in Chapter 4 that the differences in neckteeth induction found at different food concentrations were as well caused by different rates of bacterial degradation. As most algal cultures are not axenic, bacterial abundances presumably increase with food quantity in experiments. By greatly reducing bacterial degradation during my experiment using antibiotics, I observed the same degree of neckteeth induction at high food concentrations as at low food concentrations. I as well determined the abundance of bacteria in the treatments, and I found neckteeth induction to be significantly correlated with the bacterial abundance. In this study (Chapter 4), I was able to revise a previous report on the effect of food quantity on neckteeth induction, which stated that neckteeth induction would be decreasing with food concentration (Parejko & Dodson, 1991). In doing so, I corroborated the finding of Tollrian (1995b), who reported no effect of food quantity on neckteeth induction. The result of Chapter 4 expressly underlines the importance of an appropriate experimental design that excludes all effects inherent in the system, as otherwise results might not be reproducible or just misleading. Furthermore, the results presented in Chapter 4 support the assumption of temperature correlated bacterial degradation rates of the kairomone, which might cause differences in neckteeth induction at different temperatures, made in Chapter 1.

Taken together, *D. pulex* seems not to be able to adjust its morphological defense to the individual predation risk caused by decreased growth, and thus, an increased duration of vulnerable instars. Although I reported a significant correlation between neckteeth induction and the developmental time for the factor temperature, this is not a general relationship, as this correlation was not significant when dietary cyanobacteria were supplemented. Considering the results presented in Chapter 4, which clearly show that bacterial degradation of the kairomone can affect neckteeth induction to a great extent, the correlation of neckteeth induction and developmental time should be considered cautiously, as bacterial degradation rates are most probably increasing with temperature, just as their metabolic rates (Ingraham & Bailey, 1959; Price & Sowers, 2004).

Predator induced trait alterations, or inducible defenses, were often reported to entail costs (Black & Dodson, 1990; Havel & Dodson, 1987; Riessen & Sprules, 1990). In order to maximize its fitness, prey needs to balance the costs and benefits of trait alterations, because the alteration of one trait, which increases fitness, might lead to an alteration of another trait, which decreases fitness

(Stearns, 1989). Trait alteration might also lead to increasing demands of certain resources, which then is the reason for decreased fitness, as these demands cannot be met. Neckteeth induction is affected by the calcium concentration of the water with neckteeth induction increasing with calcium concentrations at the same kairomone concentration (Riessen et al., 2012). One can conclude, therefore, that *Daphnia* have higher demands for calcium when inducing morphological defenses, since calcium is required to strengthen the carapace. Accordingly, the fact that food quality, with respect to PUFA availability, and food quantity did not affect neckteeth induction suggests that morphological defenses might not entail costs that emerge from higher PUFA or energy demands.

Models developed to predict the relative fitness of neckteeth-bearing *Daphnia* can be very informative with regard to the effects of environmental conditions (Riessen, 2015; Riessen & Young, 2005). These models integrate among other parameters the size of *Daphnia*, density of *Chaoborus*, and the resultant instar specific probability of survival for *Daphnia*. A model on the effect of water temperature revealed that the predation risk of *Daphnia* increases with temperature (Riessen, 2015). In order to compensate for the increased predation risk, *Daphnia* would require a stronger defense at higher temperatures. In contrast to the prediction of the model and its implication, I reported within this dissertation that neckteeth induction has been decreasing with temperature. This discrepancy might stem from the fact that the model is solely based on a theory, which describes higher encounter rates with increasing temperature due to an increased swimming speed of *Daphnia*, and thus, higher predation risk for *Daphnia*. However, there are two other theories described by Riessen (2015): the first one states that temperature is used by *Daphnia* as an indirect seasonal signal that indicates times of increased predator abundances, and therefore, higher predation risk. The second theory states that higher metabolic rates of the predator lead to higher kairomone concentrations in the water. The results presented within this dissertation suggest that the effect of temperature on neckteeth induction cannot be described by only one theory, but metabolic rates and activity might also play a crucial role. This seems reasonable, as the kairomone needs to accurately mediate the predation risk for *Daphnia*, and the theory that Riessen's model is based on is purely mechanistic. Furthermore, some of the models' assumptions are not based upon experimentally collected data. Cyclomorphosis has been, so far, only reported for the percentages of neckteeth-bearing *Daphnia* in a population, and not for the individual degree of neckteeth induction (Havel, 1985). The developers did not

integrate the degree of neckteeth induction, and presumably concomitant with this, altered predation rates (Altwegg et al., 2006; Tollrian, 1995a). Instead, they assumed a general reduction in *Chaoborus*' strike efficiency of 50% (Riessen & Young, 2005). Integrating the degree of neckteeth induction might strengthen the explanatory power of those kinds of models.

Although many of the here investigated factors did not directly affect the morphological defense of *D. pulex*, it is possible that physiological processes in *D. pulex* change, which are not detectable by classical ecological approaches. In Chapter 3, I present results of a life table experiment crossing food quantity and the absence/presence of *Chaoborus*. At the end of the experiment, I examined the fatty acid allocation to the eggs of the experimental animals by separately measuring the fatty acid concentrations in the eggs and the body tissue of the mothers via gas chromatography. The results corroborated earlier findings about the effect of food limitation on resource allocation: *D. pulex* allocated relatively more fatty acids to the eggs under food limitation (Gliwicz & Guisande, 1992; Lynch, 1989; Tessier et al., 1983). The presence of *Chaoborus*, however, suppressed this increased provision of eggs under food limitation, but enhanced fatty acid allocation to eggs under saturating food availability. Furthermore, I detected an increased retention of EPA, which is crucial for the production of eggs (Ravet & Brett, 2006), in the tissue of mothers under food limitation. These results suggest a shift in resource allocation from an investment into current reproduction to an investment into greater numbers of future reproductive events. This shift would be in accordance with the general life history strategy (Law, 1979), which states that organisms should invest their resources in the stages that are less vulnerable to predation when resources are limiting. In case of *Chaoborus* predation on *D. pulex*, this means that resources should be invested in adult instars, instead of juvenile instars, as the adult animals are not vulnerable to predation anymore. This shift in investment leads to an increased number of reproductive events, and thus, an increased number of overall offspring, and counteracts predation losses of juveniles. In Chapter 3, I report for the first time that *D. pulex* changes its fatty acid allocation in response to *Chaoborus*. Interestingly, these changes in resource allocation occurred independently from classical life history changes (Tollrian, 1995b), as the size at first reproduction and time to maturity did not change in *D. pulex* clone TCO during the life table experiment. This emphasizes the importance of integrating methods of instrumental chemistry or physiological measurements in ecological studies. Additionally, this study

emphasizes the impact of non-lethal trait-mediated effects on food webs and ecosystems (Preisser et al., 2005; Werner & Peacor, 2003).

During the studies presented within this dissertation, I investigated several yet unknown effects of *Chaoborus* kairomone on an otherwise well-studied inducible anti-predator defense in *D. pulex*. I used classical ecological approaches to investigate the morphological defense of *D. pulex*, as well as modern methods of instrumental chemistry like gas chromatography to investigate physiological changes and resource allocation in response to *Chaoborus*. The results obtained via gas chromatography suggest striking shifts in the life history strategy of *D. pulex* in the presence of *Chaoborus* dependent on the availability of food. Additionally, the effects of dietary cyanobacteria, temperature, and food quality on neckteeth induction in *D. pulex* were investigated for the first time. Within these experiments, I was able to show that *D. pulex* is not able to adjust its morphological defense to the individual predation risk emerging from increased developmental times under gape-limited predation. Furthermore, the effect of bacterial degradation of the kairomone was examined, and I revised previously reported effects of food quantity on neckteeth induction. This finding emphasizes the importance of an appropriate experimental design of studies in chemical ecology. This dissertation presents new insights about the morphological defense of *D. pulex* against *Chaoborus* as well as physiological alterations in the life history. These new insights shed some light on mechanisms and potential costs of morphological defenses and on life history strategies of prey organisms.

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Record of achievement

Chapter 1: No general effect of developmental time on *Chaoborus*-induced phenotypic plasticity

Experiments were performed by me or under my direct supervision. Data analyses were performed exclusively by me. Eric von Elert was involved in technical discussions and has critically read the manuscript.

Chapter 2: No food quality effect on morphological defense in *Daphnia pulex*

Experiments were performed by me or under my direct supervision. Data analyses were performed exclusively by me. Eric von Elert was involved in technical discussions and has critically read the manuscript.

Chapter 3: Risk of predation alters resource allocation in *Daphnia* under food limitation

Experiments and data analyses were performed exclusively by me. Eric von Elert was involved in technical discussions and has critically read the manuscript.

Chapter 4: Inducible morphological defense in *Daphnia pulex*: food quantity effects revised

Experiments and data analyses were performed exclusively by me. Eric von Elert was involved in technical discussions and has critically read the manuscript.

Erklärung (gemäß § 7 Absatz 8)

Hiermit versichere ich an Eides statt, dass ich die vorliegende Dissertation selbstständig und ohne die Benutzung anderer als der angegebenen Hilfsmittel und Literatur angefertigt habe. Alle Stellen, die wörtlich oder sinngemäß aus veröffentlichten und nicht veröffentlichten Werken dem Wortlaut oder dem Sinn nach entnommen wurden, sind als solche kenntlich gemacht. Ich versichere an Eides statt, dass diese Dissertation noch keiner anderen Fakultät oder Universität zur Prüfung vorgelegen hat; dass sie - abgesehen von unten angegebenen Teilpublikationen und eingebundenen Artikeln und Manuskripten - noch nicht veröffentlicht worden ist sowie, dass ich eine Veröffentlichung der Dissertation vor Abschluss der Promotion nicht ohne Genehmigung des Promotionsausschusses vornehmen werde. Die Bestimmungen der Promotionsordnung sind mir bekannt. Darüber hinaus erkläre ich hiermit, dass ich die Ordnung zur Sicherung guter wissenschaftlicher Praxis und zum Umgang mit wissenschaftlichem Fehlverhalten der Universität zu Köln gelesen und sie bei der Durchführung der Dissertation zugrundeliegenden Arbeiten und der schriftlich verfassten Dissertation beachtet habe und verpflichte mich hiermit, die dort genannten Vorgaben bei allen wissenschaftlichen Tätigkeiten zu beachten und umzusetzen. Ich versichere, dass die eingereichte elektronische Fassung der eingereichten Druckfassung vollständig entspricht.

Köln, 7. April 2021

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Sandra Klintworth

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[1] entspricht Chapter 3

[2] entspricht Chapter 1, (bisher nur online) veröffentlicht in überarbeiteter Version

[3] entspricht Chapter 4, veröffentlicht in überarbeiteter Version

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