Pheromone communication in the model organism *Nasonia vitripennis* (Hymenoptera: Pteromalidae)
Pheromone communication in the model organism

*Nasonia vitripennis*

(Hymenoptera: Pteromalidae)
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SUMMARY

In insects chemical communication is an important channel to exchange information between and within species. Pheromones serve as transmitter for communication within species and are involved in the whole repertoire of insect behaviour. The focus of this doctoral thesis is on sex pheromones in the model organism *Nasonia vitripennis* (Hymenoptera: Pteromalidae), a haplodiploid parasitoid wasp. Males attract virgin females by deposits of a male sex pheromone: a mixture of (4R,5R)- and (4R,5S)-5-hydroxy-4-decanolide (HDL) and 4-methylquinazoline (4-MeQ) as a minor synergetic component. It has been shown that the HDL amount correlates with the functional fertility of males and works as a quality indicator for the females. During courtship males elicit females’ receptivity by the release of a second pheromone, a so-called “aphrodisiac” that is not identified yet.

In this doctoral thesis the influence of nutritional fatty acids on the male HDL titre was investigated and in the course of these experiments two new steps of HDL biosynthesis were identified. Furthermore the influence of the male body size on the HDL titre and on the mating success was analysed. Finally a new effect of the aphrodisiac in precopulatory courtship was revealed.

Two fatty acids, namely linoleic acid (LA) and oleic acid (OA) could be identified as precursors for HDL biosynthesis. It was shown that *N. vitripennis*, as the first insect in the order Hymenoptera, is able to convert OA into LA, an ability known only from a few other animals before. LA is commonly biosynthesized from OA under involvement of a \( \Delta^{12} \)-desaturase. Two putative *Nasonia* genes, with the highest sequence homology to other insect \( \Delta^{12} \)-desaturase genes were expressed successfully in competent yeast cells. However none of the two genes could be determined as \( \Delta^{12} \)-desaturase gene.

*Nasonia* females preferred hosts artificially enriched in OA and LA for oviposition. The male offspring developing in these hosts had higher sperm amounts and produced and released a higher amount of HDL and were able to attract more virgin females than males from hosts poor in OA and LA.

Females of haplodiploid species are not able to produce sons of high reproductive success by choosing an attractive male. The results of this doctoral thesis demonstrate that females of haplodiploid parasitoids can increase the reproductive success by choosing hosts with high amounts of sex pheromone precursors for oviposition.

Hosts were fed with different tissues of beef in order to simulate the variability of host quality on a cadaver, a natural habitat of *Nasonia*. The fatty acid compositions of the hosts were
different and very similar to the one of their diet. Therefore parasitoid females supposedly also have the choice between hosts of different nutritional value in the field.

Male body size was supposed to be a further factor influencing the HDL titre. Chemical analyses showed that large males are able to produce and release higher pheromone deposits than small males. However, in direct competition for a female the mating success of a large male and a small male did not differ, because of the high agility of small males.

During courtship of *Nasonia* a second pheromone becomes important. When performing precopulatory courtship, males release a so-called “aphrodisiac” from an oral gland that elicits females’ receptivity. It has been shown that simultaneously the “aphrodisiac” causes a behavioural switch in the females. A few minutes after coming in contact with the aphrodisiac, females’ attraction to HDL was switched off, even without copulation and sperm transfer. This shows that a pheromone can inactivate the attractive effect of another pheromone.

The results of this doctoral thesis provide new, exciting insights into pheromone communication of insects. Results showed that the diet of the hosts can influence the reproductive success of the male offspring. Hosts ingest primary nutrients from their diet that function as pheromone precursors of the parasitoids. Females are able to choose hosts with a high amount of sex pheromone precursors with the effect that their sons developing in these hosts have a high sex pheromone titre. Further results have shown that the body size can be positively correlated with sexual signalling; however mating success can be independent of body size in direct mate competition. Finally a new example of interaction of two sex pheromones was demonstrated: the inactivation of a pheromone with another pheromone.


Weibchen von *N. vitripennis* bevorzugten zur Eiablage Wirte, die experimentell mit OA und LA angereichert wurden. Der männliche Nachwuchs aus diesen Wirten zeichnete sich durch eine größere Anzahl an Spermien aus, produzierte eine größere HDL-Menge und wirkte auf virginelle Weibchen attraktiver als Männchen, die aus LA- und OA-armen Wirten stammten.

Weibchen haplodiploider Arten fehlt die Möglichkeit, Söhne mit hohem reproduktiven Erfolg allein durch die Wahl eines attraktiven Partners zu produzieren. Jedoch zeigen die
Ergebnisse dieser Doktorarbeit, dass Weibchen einer haplodiploiden Art den reproduktiven Erfolg ihrer Söhne positiv beeinflussen können, indem sie Wirte mit einem hohen Gehalt an Vorläufersubstanzen des Sexualpheromons wählen.


LIST OF PUBLICATIONS

This thesis incorporates the following manuscripts


Ruther, J., Thal, K., Blaul, B. & Steiner, S. 2010: Behavioural switch in the sex pheromone response of *Nasonia vitripennis* females is linked to receptivity signaling. *Animal Behaviour*, 80, 1035-1040 (chapter 7).
CHAPTER 1

General Introduction

Chemical communication between insects

Chemical communication is a highly efficient and important channel of insect communication. In addition to acoustic and visual cues, insects can exchange a wide variety of information with chemical cues. Insects live in a world of odours. So-called infochemicals are secreted from exocrine glands of the sender, are detected highly effectively by specialized sensillae of the receivers and evoke a behavioural or physiological response from the receiver (Tegoni et al., 2004; Leal, 2005). Infochemicals can be used for communication between species and for communication within species. For communication between species, infochemicals are divided into synomones, allomones and kairomones depending on the costs and benefits of the sender and the receiver (Vet & Dicke, 1992). Synomones are favourable for the sender and for the receiver. A widespread example is the interaction between insects and plants for pollination. Plants release synomones to attract pollinators and insects obtain food from the plants in exchange. Allomones are only favourable for the sender, as in chemical defence against parasitoids or predators. Kairomones, however, are only beneficial for the receiver. Examples are volatiles used to locate food or hosts of parasitoids (Wyatt, 2003; Ruther et al., 2002).

Chemical substances used for communication within species are called pheromones (Godfray, 1994; Wyatt, 2003). Low concentrations of pheromones are often sufficient to cause a behavioural response in the receiver, a fact that complicated the identification of the first pheromones. Insects have remarkable abilities to detect pheromones. Some insects are able to detect pheromones in a concentration of few molecules per millilitre air (Harborne, 1993) or to discriminate highly selective substances of different stereochemistry (Leal, 2005). Pheromones are involved in the whole repertoire of insect behaviour, especially for social insects, whose pheromones are important for social communication, e.g. the multi-functional queen pheromones (Karlson & Butenandt, 1959; Billen & Morgan, 1998). Pheromones further function, for instance, as alarm pheromones, warning conspecifics against predators or as trail pheromones leading conspecifics to a food source. When the chemical signal has to disperse fast, for instance in alarm situations, usually small molecules are used; large molecules are typically used when pheromones should be presented prolonged, for instance in mate attraction with so-called sex pheromones (Tumlinson & Teal, 1987). Solitary insects
also use sex pheromones, in particular to attract and recognize potential mates (Wyatt, 2003).

**Sex pheromones**

Sex pheromones are released either by males or by females to attract mating partners of the other sex. First, sex pheromones are used for species and mate recognition. The receivers are able to recognize potential mating partners on the basis of sex- and species-specific odours. Additionally, sex pheromones can be combined with other courtship signals, such as visual signals or motion signals (Harborne, 1993; Andersson, 1994; Johansson & Jones, 2007). However, sex pheromones can also provide additional information; for instance, they reveal the reproductive status of a potential mate over a long distance. Hence, they can be used economically during the search for a receptive mating partner (Johansson & Jones, 2007). A further function of sex pheromones is mate assessment. Mostly, the males compete with each other for a mate and the intention, especially of the females, is to copulate with a male of sufficient quality to produce offspring of high quality (Andersson, 1994). Male sex pheromones can be decisive for the acceptance of a female (Harborne, 1993). Sex pheromones that are unsusceptible to cheating or costly in production or maintenance represent “honest signals” indicating the potential of the sender. Pheromone titres correlate for instance with food availability, level of health or other environmental factors of the sender and work as indicators of indirect or direct benefits (Johansson & Jones, 2007). “Indirect benefits” means that males are genetically compatible and have “good genes” that lead to a high fitness of the offspring. “Direct benefits” are potential resources, such as gifts or territories that allow conclusions concerning the strength and assertiveness of the male in finding gifts or defending a territory. A further benefit is fertility; the amount of pheromone can correlate with the amount of sperm of a male (Johansson & Jones, 2007).

**Biosynthesis of pheromones**

Pheromone biosynthesis is investigated mainly in the orders Lepidoptera, Diptera, Coleoptera and Blattodea (Tillman et al., 1999). Pheromones have been identified in more than 500 species of Lepidoptera (Roelofs & Wolf, 1988; Harborne, 1993) and the biosynthetic pathways of some of these pheromones have been analysed in detail (Roelofs & Bjostad, 1984). In insects no new set of enzymes for pheromone biosynthesis has evolved. Instead, basic biosynthetic pathways were modified and enzymes of the primary and secondary metabolism were used such as elongases, dehydrogenases or desaturases.
(Knipple & Roelofs, 2003). These enzymes convert metabolites of the daily metabolic pathway into specific pheromones. Primary nutrients, traced through dietary intake, for instance fatty acids often function as precursors of pheromone biosynthesis (Jurenka, 2004; Tillman et al., 1999; Blomquist & Vogt, 2003). The regulation of pheromone biosynthesis and pheromone release is important for effective pheromone communication. Currently three different hormonal messengers for endocrine pheromone regulation have been identified in insects: juvenile hormone, the steroidal hormone 20-hydroxyecdysone and the pheromone-biosynthesis-activating neuropeptide (PBAN) (Tillman et al., 1999).

Parasitoids

The insects whose chemical communication is studied in this doctoral thesis are parasitoid wasps. Parasitoids lay their eggs directly in or on a host and the larvae develop in (endoparasitoids) or on (ectoparasitoids) the body of the host. The hosts are usually other arthropods, mostly insects (Godfray & Cook, 1997). Parasitoids that feed alone on a host are so-called solitary parasitoids. In contrast, in gregarious parasitoids up to several thousand individuals can develop on a single host (Godfray, 1994). A feature common to all parasitoids is that they kill their host during parasitoid development. Especially parasitoids that have specialized on only one host species live in co-evolutionary arms races with their hosts (Loxdale et al., 2011). Parasitoids regulate the natural balance of their hosts and can play an important role as biological control agents (Godfray & Cook, 1997). Parasitoid imagos sometimes waive nutrition or can have a diet different from their larvae. Many visit flowers to obtain nectar. Some females feed from the haemolymph of the hosts after oviposition. Most parasitoids belong to the order Hymenoptera, in particular to the taxa Ichneumonidae, Braconidae and Chalcidoidea (Godfray, 1994).

Pheromones in parasitoids

Chemical communication in parasitoids is mainly host-associated, which means that chemical cues are used to locate their hosts and chemical signals to mark their hosts after parasitisation (Vinson, 1976; Kainoh, 1999). Furthermore, aggregation pheromones and mainly sex pheromones have been found in parasitoids. Most isolated parasitoid sex pheromones are volatile others work as contact sex pheromones and act by contact behaviour like mounting or antennation (van den Assem, 1986; Kainoh, 1999). In the most parasitoid species females attract males by their pheromones; in a few species, however,
male sex pheromones attract females (Kainoh, 1999). The attracting effect of parasitoid sex pheromones is used in traps to monitor parasitoid populations and to control host populations, especially in species that parasitize pest insects (Karlson & Butenandt, 1959; Powell, 1986). The focus of this doctoral thesis is on sex pheromones of the parasitic wasp *Nasonia vitripennis*.

**The genus *Nasonia***

The genus *Nasonia* are small gregarious parasitoid wasps (Hymenoptera: Chalcidoidea: Pteromalidae). The hosts of *Nasonia* are pupae of various cyclorrhaphous flies. *Nasonia* wasps are haplodiploid, fertilized eggs becoming females and unfertilized eggs becoming males. *Nasonia vitripennis* (Walker) can be found worldwide in contrast to its three closely related species, namely *N. longicornis* (Darling) which occurs in western North America, *N. giraulti* (Darling), which is found in eastern North America, and *N. oneida* (Raychoudhury & Desjardins), which has only been found in upstate New York so far (Darling & Werren, 1990; Raychoudhury et al., 2010; Figure 1.1). Normally, no hybrids occur in crosses between the species. The intracellular bacterium *Wolbachia* is responsible for reproductive incompatibility between species in a variety of insects. In *Nasonia*, its cytogenetic mechanism of incompatibility is known; a condensation of the paternal chromosomes in fertilized eggs is the reason that no hybrids can develop (Breeuwer & Werren, 1990). *Nasonia* wasps can be cured from *Wolbachia* with antibiotics and thus the genetic isolation is cancelled (Werren, 1997).

![Figure 1.1: distribution map of the genus Nasonia](http://www.rochester.edu/college/bio/labs/WerrenLab/WerrenLab-NasoniaDistribution.html)
**Nasonia vitripennis**

*Nasonia vitripennis* naturally occurs as two ecotypes: one lives in nests of cavity-breeding birds and parasitizes hosts that feed on the blood of nestlings (Peters & Abraham, 2010) and the second ecotype parasitizes hosts that feed on the tissues of carrions (Voss et al., 2009). The parasitic wasp lays up to 50 eggs in a fly pupa, depending on the host species (Whiting, 1967). When a female has encountered a pupa, egg-laying behaviour occurs as follows (Edwards, 1954): the *Nasonia* female climbs onto the pupa and starts drumming the host with its antennae. The female begins to tap, with the tip of the abdomen, on the surface of the pupa. In a next step, the female exposes the ovipositor and begins drilling and piercing the pupa (Figure 1.2a). If the host is considered as suitable, the female lays eggs but, otherwise, the female searches for a new host. The number of offspring is dependent on the size of the host and therefore the host species. After oviposition and removal of the ovipositor, the female feeds on the haemolymph of the host leaking from the injection site, a behaviour called “host feeding” (Whiting, 1967). The development time of the offspring is dependent on the temperature; at 25°C, the offspring emerge after two weeks (Figure 1.2b). When females have encountered their first host and drilled their ovipositor into it, they are able to learn host-associated odours, to remember the odour for few days and to use this memory for searching for further hosts (Schurmann et al., 2009; Steidle & van Loon, 2003). By drilling and subsequent host feeding, a protein-synthesis-dependent long-term memory (LTM) is established (Schurmann et al., 2012).

![Figure 1.2(a): Oviposition behaviour of a *Nasonia vitripennis* female. 2(b): Pupae of *Nasonia vitripennis* excised from host puparia one day before eclosion.](image-url)
The mating system of *N. vitripennis* is characterized by local mate competition (LMC). The offspring sex ratio is female-biased (Hamilton, 1967; Werren, 1983). When a host has previously been parasitized, *Nasonia* females either reject the host or lay fewer eggs and more males (Wylie, 1970; Werren, 1983; King & Skinner, 1991; Ivens et al., 2009). This behaviour is regardless of whether a conspecific female or a heterospecific female has parasitized the host (Ivens et al., 2009). Males are flightless, do not disperse and wait directly at the emergence site for their sisters, which emerge a little later, in order to mate with them (van den Assem & Vernel, 1979). Males compete for access to females (Godfray & Cook, 1997).

When a male has encountered a female, he displays stereotypic courtship behaviour (Barrass, 1960; van den Assem & Vernel, 1979; van den Assem et al., 1980b). The male climbs on the female, moves to the front and places its forefeet on the head of the female (Figure 1.3a). In this position, the male starts a series of headnodding movements associated with mouth part extrusions and the discharge of a pheromone (see below) over the antennae of the female (van den Assem et al., 1981). The female shows her receptivity by lowering her antennae and head and exposes the genital pocket. After this signal, the male moves backwards for copulation (Figure 1.3b; Whiting, 1967). Subsequently the male returns to the back of the female and commences postcopulatory courtship, after which females become unreceptive to other males (van den Assem & Visser, 1976). Most females mate only once under natural conditions (Grillenberger et al., 2008). The mated females search for hosts.

Figure 1.3(a): Courtship behaviour of *Nasonia vitripennis*. 3(b): Copulation of a *Nasonia vitripennis* pair.
The \textit{Nasonia} Genome

Their haploid genetics, their short generation time and the comfort of easy breeding are some of the reasons that \textit{Nasonia} has become a good genetic model system for evolutionary and developmental genetics. The whole genome of \textit{N. vitripennis}, \textit{N. longicornis} and \textit{N. oneida} has been sequenced (Werren et al., 2010). \textit{Nasonia} has been the second genus of Hymenoptera after \textit{Apis mellifera} with a whole sequenced genome. Several quantitative trait loci (QTL) of \textit{N. vitripennis} have been mapped, such as wing size, sex ratio and host preferences (see, for instance, Loehlin et al., 2010; Pannebakker et al., 2011; Desjardins et al., 2010). Furthermore, 90 sequences in the \textit{Nasonia} genome have been identified as encoding predicted odorant-binding proteins (OBP). \textit{Nasonia} has the largest family of OBPs ever found in an insect species. OBPs are highly abundant, soluble proteins occurring in the sensillar lymph of chemosensory organs of insects and function as carrier proteins by transporting odorant molecules to olfactory receptors (Meillour & Jacquin-Joly, 2003; Vieira et al., 2012). Additionally more odorant receptor (OR) genes have been identified in \textit{Nasonia} than in the honey bee despite the expanded olfactory repertoire, because of the social communication seen in the honey bee (Robertson et al., 2010). OBPs and ORs contribute to the specificity of the cell response to a chemical signal (Leal, 2005). In vertebrates OR genes have been individually expressed resulting in a highly restricted expression pattern. Therefore, the olfactory is mediated by a diversity of odorants and a distinction between this diversity is possible (Kratz et al., 2002).

Sex pheromones of female \textit{Nasonia vitripennis}

Cuticular hydrocarbons (CHCs) are known to occur in \textit{Nasonia} males and females; most are methyl-branched alkanes with one to four methyl groups. However, the relative abundances of the hydrocarbons differ between males and females. In contrast to males, higher amounts of 9-, 11-, 13- and 15-methylalkanes and 9,x-, 11,x-, 13,x- and 15,x-dimethylalkanes as well as alkanes with three or more methyl branches have been found in females. Sex-specific female cuticular hydrocarbons are assumed to elicit male courtship behaviour (Steiner et al., 2006). Steiner et al. (2006) have shown that the hexan fraction of female extracts can elicit males’ arrestment and courtship behaviour. At a distance of 3 cm males are not attracted by the presence of females. No volatile female sex pheromone is known in \textit{Nasonia} that is effective over a longer distance.
Sex pheromones of male *Nasonia vitripennis*

In *N. vitripennis* males, chemical substances are important for the attraction of females and mating success. During courtship, the *N. vitripennis* male sits on top of the female and licks the antennae of the female with his extruded mouthparts, until the female shows a sign of receptivity. Van den Assem et al. (1980b) have shown that a male whose mouth part is sealed with glue loses its ability to initiate the receptivity signal of the female. However, by air supply from an unsealed courting *Nasonia* pair to the *Nasonia* pair with the sealed male, female’s receptivity is elicited. We have repeated this experiment but could not replicate the results of van den Assem et al. (1980b). No receptivity signal of the females was detectable by air supply of a courting *Nasonia* pair (unpublished data). Van den Assem et al. (1981) cut the abdomen of males and showed that they still show courtship behaviour but are not able to elicit female receptivity, in contrast to males with a dissected abdomen but a plugged cut. The suspicion has arisen that males release a pheromone (“aphrodisiac”) from an oral gland with a hydraulic system. The “aphrodisiac” is necessary for the females to signal receptivity. The chemical composition of this substance is not as yet been identified. We have been able to show (Chapter 7) that the “aphrodisiac” has a further function concerning a behavioural switch of the females after copulation (Ruther et al., 2010).

Males release pheromone deposits that are highly attractive for virgin females. This male sex pheromone has been identified as a mixture of (4\(R\),5\(R\))- and (4\(R\),5\(S\))-5-hydroxy-4-decanolide (HDL) and 4-methylquinazoline (4-MeQ) as a minor component (Ruther et al., 2007; Ruther et al., 2008). 4-MeQ synergizes the attractive effect of HDL but is not attractive to females when offered alone (Ruther et al., 2008). The male sex pheromone is released via the anal orifice by “abdomen dipping” i.e. dabbing movements of the abdominal tip on the ground, especially after mating. The male pheromone is attractive over a radius of at least 4.5 cm and remains attractive for at least 2 h (Steiner & Ruther, 2009b). Males stay at the place of mating to attract further virgin females with their pheromone. However, male pheromone deposits and 4-MeQ alone also attract additional males. Ruther et al., (2011) have shown that males return to scent-marked sites, although they cannot discriminate between their own markings and those released by conspecifics.
Biosynthesis of HDL

HDL is synthesized in the rectal vesicle of males (Figure 1.4a,b). After the application of $^{13}$C-labelled vernolic acid (erythro-12,13-epoxy-octadec-9Z-enoic acid) to the abdominal tip of *N. vitripennis* males, incorporation of $^{13}$C into threo-HDL ((4$^R$,5$^R$)-HDL) but not into erythro-HDL ((4$R$,5$S$)-HDL) was found. Therefore, vernolic acid was initially assumed to work as a precursor of the male pheromone, with the first biosynthesis product being (4$^R$,5$^R$)-HDL (Adel-Latif et al., 2008).

However, *N. vitripennis* is the only species in the genus *Nasonia* whose males biosynthesize (4$R$,5$S$)-HDL and (4$R$,5$R$)-HDL combined with 4-MeQ; the pheromone of the other *Nasonia* species consists only of (4$R$,5$S$)-HDL, combined with 4-MeQ. This leads to the evolutionary question as to whether *N. vitripennis* evolved (4$R$,5$R$)-HDL as the only species or whether the other *Nasonia* species lost (4$R$,5$R$)-HDL during evolution. Niehuis et al., (2012) have shown, in behavioural analyses, that neither of the two species are attracted to (4$R$,5$R$)-HDL alone but that *N. vitripennis* females and *N. giraulti* females are attracted to (4$R$,5$S$)-HDL. *N. giraulti* females are also attracted to (4$R$,5$S$)-HDL combined with (4$R$,5$R$)-HDL, they do not discriminate between (4$R$,5$S$)-HDL combined with (4$R$,5$R$)-HDL and their species-specific male sex pheromone (4$R$,5$S$)-HDL alone. *N. vitripennis* females clearly prefer their species-specific male sex pheromone (4$R$,5$S$)-HDL combined with (4$R$,5$R$)-HDL to (4$R$,5$S$)-HDL alone. Niehuis et al. (2012) suggest from these data that (4$R$,5$S$)-HDL is the
evolutionary older diastereomer and (4R,5R)-HDL evolved as a novel component in the sex pheromone of *N. vitripennis* males resulting in a change of pheromone preference of *N. vitripennis* females. QTL analysis, gene expression analysis and gene knockdown have confirmed this presumption indicating three closely linked short-chain dehydrogenases/reductases (SDRs) that are involved in for the ability of *N. vitripennis* males to synthesize the novel pheromone compound (Niehuis et al., 2012).

Consequently, the putative biosynthetic pathway probably starts with threo-12,13-epoxy-octadec-9Z-enoic acid, which is hydrolyzed under inversion into the respective erythro-diol (Figure 1.5). The involvement of an epoxide hydrolase has been demonstrated by RNAi gene silencing analysis (Adel-Latief et al., 2008). Further steps might involve chain shortening and lactonization to (4R,5S)-HDL. The hydroxyl group at carbon atom five might be oxidized by one of three putative short-chain dehydrogenases/reductases to a ketone that is immediately reduced under inversion of stereochemistry to (4R,5R)-HDL (Niehuis et al., 2012).

The hypothesis that two fatty acids, namely linoleic acid (LA) and oleic acid (OA), work as precursors of HDL is tested by 13C-labelling experiments (*Chapters 2 and 4*) (Blaul & Ruther, 2011). The ability to convert OA into LA is known from a few insects and only a few animals in general. In *chapter 5*, the work on the identification of the necessary \(\Delta^{12}\)-desaturase is presented.

**HDL as a quality indicator**

The amount of HDL released by the male is known to be a signal of male fertility, because the HDL titre correlates with the sperm amount. At the age of 2 days, males achieve their full pheromone capacity and simultaneously have their full sperm amount. The sperm amount decreases synchronously with the pheromone titre after each copulation. After seven copulations, males are sperm-limited correlated with a clearly reduced pheromone titre. Females prefer high HDL amounts to lower ones. Thus, females avoid mating with a sperm-limited partner (Ruther et al., 2009).
Figure 1.5: Putative biosynthetic pathway of the male sex pheromone HDL of *Nasonia vitripennis* (Niehuis et al., 2012). Enzymes and enzymatic steps are accentuated in blue letters. Parts of the biosynthetic pathway that were investigated in this doctoral thesis are accentuated in red.

Open questions - outline of the doctoral thesis

Primary nutrients like fatty acids often function as pheromone precursors (Jurenka, 2004). To test whether fatty acids also are incorporated into the HDL biosynthesis, host quality was manipulated by feeding hosts with diets of various fatty acid compositions and the offspring of *N. vitripennis* reared on these hosts was analysed. It has been shown that LA from the host diet functions as a precursor of the male sex pheromone HDL. Furthermore, females favour LA and OA rich hosts for oviposition (chapter 2).
However, is such a choice between hosts of different fatty acid composition realistic in the field? A natural host habitat, a carcass was simulated by rearing hosts on different tissues of beef. The fatty acid composition of the hosts and of the Nasonia offspring reared on these hosts was analysed (chapter 3).

LA is not the only fatty acid that is involved in HDL biosynthesis. It has been shown that N. vitripennis is one of a few animals that are able to convert OA into LA and, therefore, it has to possess an enzyme Δ12-desaturase (chapter 4). In a next step, two putative Δ12-desaturases of N. vitripennis were expressed in yeast cells (chapter 5).

Another possible criterion influencing male mating success could be the male body size. Does the male body size influence the male HDL titre? Do males of different size have different mating success? The HDL titre of large and small males and their courtship behaviour were analysed (chapter 6).

This doctoral thesis was completed with control experiments to females’ behaviour caused by the “aphrodisiac” released by males during courtship (chapter 7).
How parasitoid females produce sexy sons: a causal link between oviposition preference, dietary lipids and mate choice in *Nasonia*

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2.1 ABSTRACT

Sexual selection theory predicts that phenotypic traits used to choose a mate should reflect honestly the quality of the sender and thus, are often costly. Physiological costs arise if a signal depends on limited nutritional resources. Hence, the nutritional condition of an organism should determine both its quality as a potential mate and its ability to advertise this quality to the choosing sex. In insects, the quality of the offspring’s nutrition is often determined by the ovipositing female. A causal connection, however, between the oviposition decisions of the mother and the mating chances of her offspring has never been shown. Here, we demonstrate that females of the parasitic wasp *Nasonia vitripennis* prefer those hosts for oviposition that have been experimentally enriched in linoleic acid (LA). We show by 13C-labelling that LA from the host diet is a precursor of the male sex pheromone. Consequently, males from LA-rich hosts produce and release higher amounts of the pheromone and attract more virgin females than males from LA-poor hosts. Finally, males from LA-rich hosts possess three times as many spermatozoa as those from LA-poor hosts. Hence, females making the right oviposition decisions may increase both the fertility and the sexual attractiveness of their sons.
2.2 INTRODUCTION

It is well established by sexual selection theory that signals enabling mate finding and recognition can also be used by the choosing sex (typically the female) to assess the quality of the signaler (typically the male) (Andersson, 1994). Benefits for females are highest if the signal reflects honestly the condition of the advertising male. Therefore, reliable signals should be insusceptible to cheating (Maynard Smith & Harper, 2003). This can be achieved, for instance, if the signal is costly as predicted by the handicap hypothesis (Zahavi, 1975). The costs of a sexually selected signal should covary with the signal attractiveness thus enabling males of superior quality to bear the expenses of the signal at lower fitness losses (Grafen, 1990; Cotton et al., 2004; Johansson & Jones 2007).

Direct physiological costs accrue, for instance, if the signal intensity depends on the availability of limited nutritional resources like certain secondary metabolites being accumulated as signals themselves (Kodric-Brown 1989; Eisner et al., 1996; Gray et al., 1996; Hill et al., 2002) or functioning as signal precursors (Conner et al., 1990; Eisner & Meinwald 1995; Landolt & Phillips 1997; Shelly 2000; Shelly & Nishida 2004; Shelly et al., 2007). But also macronutrients may influence the intensity of sexual signals. Studies addressing this aspect focused mainly on protein (Cotton et al., 2004). By contrast, the impact of dietary fats on sexual signaling has hardly ever been studied although chemical signals are often derived from the fatty acid metabolism (Tillman et al., 1999; Jurenka, 2004; Martin & Lopez, 2010) and some polyunsaturated fatty acids (PUFA) are essential nutrients for most animals (Blomquist et al., 1991; Wathes et al., 2007) and thus, like essential amino acids may be a limiting dietary resource. Furthermore, many of the studies addressing the impact of macronutrients on sexual signals are merely correlative and the underlying mechanisms remain poorly understood (Meikle et al., 1995; Cotton et al., 2004; Fisher & Rosenthal, 2006; Giaquinto et al., 2010).

In insects, it is often the ability of the mother to find suitable oviposition sites that controls the availability and quality of food for the offspring because immature insect stages are often restricted in their mobility. Hence, females are predicted by the preference-performance hypothesis (Jaenike, 1978) to choose oviposition sites according to the nutritional and environmental requirements of their offspring. This phenomenon, often referred to as the ‘mother knows best’ principle (Gripenberg et al., 2010), has been demonstrated both in herbivores (Awmack & Leather, 2002) and carnivores (Vinson & Iwantsch, 1980) and is particularly true for parasitic wasps in which host organisms chosen by the mother are the only source of nutrients for the offspring (Godfray, 1994, 2010; Quicke, 1997).
A model organism for the study of parasitic wasp biology is the jewel wasp *Nasonia vitripennis* Walker (Hymenoptera: Pteromalidae) (Godfray, 2010). Females of this species parasitize puparia of numerous cyclorrhaphous fly species (Whiting, 1967). *Nasonia vitripennis* occurs in two ecotypes, one found in the nests of hole-breeding birds, the other living on and near decaying carcasses (Schröder, 1997). Males of *N. vitripennis* attract females by releasing a substrate-borne sex pheromone consisting of a mixture of (4R,5R)- and (4R,5S)-5-hydroxy-4-decanolides (HDL) (Ruther et al., 2007). The response is shown by virgin females only (Ruther et al., 2007, 2010; Steiner & Ruther, 2009a) and is synergized by the trace component 4-methylquinazoline (Ruther et al., 2008). The HDLs are biosynthesized in the rectal vesicle of males and released via the anal orifice by dabbing movements of the abdominal tip (Abdel-Latief et al., 2008). Females have been shown to orient along HDL concentration gradients thereby avoiding the danger of mating with sperm-depleted males that release less of the attractive chemicals than males of sufficient fertility (Ruther et al., 2009).

Vernolic acid (= erythro-12,13-epoxy-octadec-9Z-enoic acid) is a precursor of HDL (Abdel-Latief et al., 2008) suggesting that the pheromone biosynthesis starts from linoleic acid (LA) which is a possible epoxidation substrate of cytochrome P450 enzymes (Moran et al., 2000). LA is an essential nutrient for most animals and is, like other PUFA, important for a number of metabolic processes in animals including sperm production (Wathes et al., 2007).

In the present study we investigated the putative function of LA as a pheromone precursor in *N. vitripennis* males. Furthermore, we tested the hypothesis that the availability of dietary fat rich in LA has an impact on the fertility of *N. vitripennis* males and increases their sexual attractiveness by enabling them to release higher amounts of the abdominal sex pheromone. Finally we tested the prediction of the preference-performance hypothesis that females are able to detect and prefer those hosts for oviposition that contain higher amounts of unsaturated fatty acids including LA.
2.3 MATERIALS & METHODS

Insects

The *N. vitripennis* strain used in this study originated from an inbred strain that was collected from a bird’s nest in Northern Germany and reared on freeze-killed puparia of the green bottle fly *Lucilia caesar* as described elsewhere (Steiner et al., 2006).

Manipulation of host quality

To obtain hosts with high (LA+) and low (LA-) contents in LA, newly emerged *L. caesar* flies of both sexes were provided for 8 days with honey, water and ground meat, until females were ready to oviposit. Subsequently, females were separated into two groups and allowed to oviposit on two oviposition substrates differing in the fatty acid composition. A larval diet relatively rich in LA was prepared by adding 10 % of safflower oil (Goldhand, Düsseldorf, Germany) to lean ground beef fillet and homogenizing it using a scoop. A larval diet poor in linoleic acid was prepared by the same way using coconut oil instead (Palmin™, Peter Köln KGaA, Elmshorn, Germany). The addition of 10 % fat to lean meat ensured that the diets were comparable to natural dietary resources with respect to the total fat content. The fatty acid composition of the two fats was determined as described below (Table 1). Emerging fly larvae fed on the diets for about a week and then pupated. Pupae of either type were collected daily, frozen 2 days after pupation and stored at -20°C until used for parasitoid rearing or chemical analysis (see below).

Fatty acid analysis of LA+ and LA- hosts

The fatty acid composition of the two host types and the fats used to produce them was determined by coupled gas chromatography-mass spectrometry (GC-MS) of the fatty acid methyl esters (FAME). Groups of three 2-day old LA+ and LA- host puparia were extracted for 30 min with 500µl hexane. Prior to the extraction, puparia were homogenized using a scoop. The solvent was removed under a gentle stream of nitrogen and one mg of extracted raw lipids was re-suspended in 100 µl of methanol and subsequently mixed with 10 µl of acetyl chloride (10 % dissolved in methanol). Samples were kept for 1 h at 60°C for transesterification. Then 200 µl of sodium hydrogen carbonate (5% dissolved in H₂O) were added and the FAMEs were extracted for 30 s with 200 µl of pentane. FAMEs were analyzed on a Shimadzu QP2010 Plus GC-MS system equipped with a BPX5 capillary column (30 m x
0.32 mm inner diameter, 0.25 µm film thickness) (SGE, Analytical Science Europe, Milton Keynes, UK). Samples were injected at 300°C with a split ratio of 1:5 using an AOC 20i autosampler. Helium was used as carrier gas at a constant flow rate of 2 ml/min. The initial oven temperature of 50°C (held for 4 min) was increased at 3°C/min to 280°C (held for 15min). The MS was operated in the electron impact (EI) mode at 70 eV. Identification of FAMEs was done by analyzing reference fatty acids (Aldrich, Deisenhofen, Germany) which were derivatized under the same conditions. Relative FAME composition (in %) was determined by relating peak areas of individual compounds to the total peak area of all FAME in the sample.

Production of parasitoid males on LA+ and LA- hosts

Groups of two virgin *N. vitripennis* females were allowed to oviposit for 48 h into LA+ and LA- hosts, respectively. Virgin females were used because they produce all-male broods for the subsequent experiments (haplodiploidy). Parasitoid pupae of both treatments were excised from the hosts 1-2 days before eclosion and kept individually in microcentrifuge tubes until being used for behavioral bioassays and chemical analyses, respectively (see below). Males of either treatment used for comparing experiments were generally of equal size (head width 700 µm) and age (2 days).

13C-labelling experiments

To test the hypothesis that linoleic acid from the host diet is directly incorporated into the male sex attractant we reared *L. caesar* hosts on a diet enriched in [13C18]-linoleic acid (13C-LA). For this purpose, we added 100 mg of 13C-LA (Campro Scientific GmbH, Berlin, Germany) to 2 g of lean ground beef fillet as described above. Groups of ten 5-days old LA+ host larvae (see above) were transferred to the 13C-LA diet and allowed to feed for another day until pupation. Subsequently, they were offered to virgin wasp females for parasitization. Abdomens of 2-days old *N. vitripennis* males reared on these 13C-LA-labelled hosts were extracted with 25 µl of dichloromethane and used for GC-MS analysis using the equipment and conditions described below. Incorporation of 13C-LA into the two HDL stereoisomers was concluded from the appearance of diagnostic ions in the mass spectra as described elsewhere (Abdel-Latief et al., 2008).
Quantification of pheromone deposits released by males from LA+ and LA- hosts

Pheromone deposits were quantified by GC-MS using thermal desorption (TD) sampling. For this purpose, empty 89 mm x 5 mm ID TD glass tubes (Supelco, Bellefonte, PA) were filled at one end with 50 mg of Tenax TA (Supelco). The adsorbent layer of 25 mm was fixed using fine mesh metal screens (Supelco). One µl of an internal standard solution containing 100 ng/µl methyl undecanoate (Sigma-Aldrich, Deisenhofen, Germany) in methanol was applied to the adsorbent and the tube was purged for 5 min (adsorbent upwind) using a nitrogen flow of 100 ml/min to remove the solvent. Subsequently, a 2-days old male (LA+ males n = 25; LA- males n = 24) was transferred together with a virgin female to the empty side of the tube and mating within the tube was observed under a stereo microscope. Then, the female was removed and the male was allowed to mark the inner side of the tube for 10 min. After removal of the male, TD tubes were purged for 3 min with nitrogen at a flow of 60 ml/min (adsorbent upwind) and capped for TD volatile sampling. By this means, both the volatilized proportion of the marking and the substrate borne residues were subjected to quantitative analysis with losses kept to a minimum. TD sampling was done using an automated Shimadzu TD 20 thermal desorber (Shimadzu GmbH, Duisburg, Germany) coupled to a Shimadzu QP2010 Plus GC-MS system. Volatiles were desorbed from the TD tubes for 8 min at 250°C with helium at a flow rate of 60 ml/min and cryofocused at -20°C on an internal Tenax trap. Subsequently, volatiles were injected into the GC-MS by heating the internal trap to 280°C for 5 min at a split ratio of 1:20. The GC was equipped as described above but the temperature programme started at 80°C, increased at 5°C/min to 200°C and then at 15°C to 280°C (held for 15min). Quantification of total HDL deposited by individual males was done by the internal standard method. For this purpose a calibration curve was created by analyzing defined amounts (5 - 200 ng) of synthetic (4R,5R)-HDL in methanol and an internal standard using the method described above. The reference compound was synthesized by Sharpless asymmetric dihydroxylation of ethyl (4E)-decenoate as described by Garbe & Tressl (2004). Pheromone amounts deposited by males from LA+ and LA- hosts were compared by Mann-Whitney U-test.

Quantification of pheromone titers of males from LA+ and LA- hosts

Two-day old males from LA+ (n = 23) and LA- hosts (n = 20) of comparable size were killed by freezing and their abdomens were cut-off and extracted for 30 min with 25µl dichloromethane containing 10 ng/µl methyl undecanoate as an internal standard. Extracts were subjected to GC-MS analysis using the instrumentation and conditions described for
the TD sampling. However, an AOC 20i auto injector operated in splitless mode was used for sampling of the extracts. Quantification of total HDL in individual males was done by the internal standard method. For this purpose a calibration curve was created by analysis of defined amounts (5-200 ng/µl) of synthetic (4R,5R)-HDL dissolved in dichloromethane containing the internal standard at 10 ng/µl. Pheromone titers of males from LA+ and LA- hosts were compared by a Mann-Whitney U-test.

Sperm count of males from LA+ and LA- hosts

Virgin males of equal size and age (2 days) from LA+ and LA- hosts (n = 10 per host type) were killed by freezing. Testes and seminal vesicles were dissected in 10 µl of Ringer solution. One haphazardly chosen testis and seminal vesicle per male was opened and dispersed for 2 min using a fine needle. The solution was diluted with another 10 µl of Ringer solution and dispersed again. The spot was air-dried and fixed in 70% ethanol. The cover slip was put on a square grid (side length 2.5 mm) and sperm numbers of 3 haphazardly chosen squares were counted blind under a microscope. The total sperm count for each male was estimated by extrapolation.

Female response to pheromone deposits released by males from LA+ and LA- hosts

The response of virgin females to pheromone markings deposited by the two groups of males was examined using a static four-cavity olfactometer (Steiner & Ruther, 2009b). This was made of acrylic glass and consisted of a round walking arena (9 cm diameter, 8 mm thick) equipped with four symmetrically arranged spherical cavities (1 cm diameter, 4 mm deep), a low rim (2 mm high), and a glass plate to cover the arena. Males of both groups were allowed to mate simultaneously with a virgin female in opposing cavities of the olfactometer. Subsequently, females were removed and males were allowed to deposit the sex pheromone in their cavity for 10 min. The other two cavities remained empty and functioned as controls. After removal of the males, virgin females were released individually into the center of the olfactometer and the time they spent in the four cavities was recorded for 5 min using The Observer XT observational software (Noldus, Wageningen, the Netherlands). Additionally it was noted which cavity was entered first by the females (first choice). Parasitoids were used only once and pheromone deposits were renewed after every replicate (n = 30). Residence times of females spent in the two marked cavities of the
olfactometer were analyzed by a Wilcoxon matched-pairs tests. First choice of females for the LA+ and LA- cavity, respectively, was compared by a two-tailed binominal test.

**Oviposition preference of parasitoid females**

To test the hypothesis that female parasitoids prefer LA+ hosts over LA- hosts for oviposition, single virgin females \( n = 40 \) were placed into Petri dishes containing a LA+ and a LA- host of equal size for oviposition. To prevent inadvertent rolling of the hosts, these were glued at a distance of 20 mm to the bottom of the dishes using a drop of non-toxic glue (Pentel ER153, Tokyo, Japan). Females were allowed to oviposit for 24 h. After 10 days, male parasitoid pupae were excised from the hosts of either type and counted. In a second experiment we used mated females \( n = 30 \) to study whether females lay a higher proportion of male eggs in LA+ than in LA- hosts. In general, the experiment was performed as described above. But in this case the test lasted 3 days and the females were exposed to new hosts each day. After the third day, parasitized LA+ and LA- hosts were separated and emerging offspring was sexed and counted. Parasitoids from LA+ and LA- hosts were compared by a Wilcoxon matched pairs test.

### 2.4 RESULTS

**Linoleic acid is a precursor of the male sex pheromone**

Our \(^{13}\text{C}\)-labeling experiments revealed that LA from the host diet is directly incorporated into the male sex pheromone of the parasitoid. Incorporation of \(^{13}\text{C}\) was indicated by mass shifts of the four diagnostic ions \( m/z \) 86, 101, 115 and 186 in the mass spectra of \((4R,5R)\)- and \((4R,5S)\)-HDL (Abdel-Latief et al., 2008; Fig. 2.1). The incorporation rate was 36 % each.
Figure 2.1: Mass spectra of HDL from *N. vitripennis* males reared on (A) a normal host (control) and (B) a host reared on a diet enriched in [13C18]-linoleic acid. Arrows indicate diagnostic ions resulting from the incorporation of the labelled precursor. 13C-atoms are represented by black dots.

**Fatty acid composition of the host depends on its dietary fats**

The fatty acid composition of LA+ and LA- hosts differed significantly and was clearly influenced by the fats used for preparing the host diets (Table 2.1). LA- hosts contained mainly saturated and monounsaturated fatty acids with chain lengths between 8 and 18 carbon units whereas LA was a minor component amounting less than 4 % of the total fatty acids. A threefold higher amount of LA was found in the fatty acid profile of LA+ hosts which was dominated by oleic acid, the major component of the safflower oil used to prepare their diet. Despite the differences found in the fatty acid composition of LA+ and LA- hosts in our study, both were within the range reported earlier for potential hosts of *N. vitripennis* (Thompson, 1973).
Table 2.1: Relative fatty acid composition of lipids extracted from LA+ and LA- hosts and the fats used to produce them. Compounds are listed in the elution order of the FAME on the DB-5 stationary phase used for chemical analysis.

<table>
<thead>
<tr>
<th>name</th>
<th>trivial name</th>
<th>LA+ host</th>
<th>safflower oil</th>
<th>LA- host</th>
<th>coconut oil</th>
</tr>
</thead>
<tbody>
<tr>
<td>hexanoic acid</td>
<td>caproic acid</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>0.63</td>
</tr>
<tr>
<td>octanoic acid</td>
<td>caprylic acid</td>
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<td>-</td>
<td>1.16</td>
<td>8.50</td>
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<tr>
<td>decenoic acid*</td>
<td>caprylic acid</td>
<td>-</td>
<td>-</td>
<td>0.11</td>
<td>-</td>
</tr>
<tr>
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<td>capric acid</td>
<td>-</td>
<td>-</td>
<td>1.23</td>
<td>6.25</td>
</tr>
<tr>
<td>dodecanoic acid*</td>
<td>capric acid</td>
<td>-</td>
<td>-</td>
<td>0.67</td>
<td>-</td>
</tr>
<tr>
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<td>lauric acid</td>
<td>-</td>
<td>-</td>
<td>17.51</td>
<td>39.12</td>
</tr>
<tr>
<td>(9Z)-tetradec-9-enoic acid</td>
<td>myristoleic acid</td>
<td>-</td>
<td>-</td>
<td>4.79</td>
<td>-</td>
</tr>
<tr>
<td>tetradecanoic acid</td>
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<td>0.08</td>
<td>11.47</td>
<td>19.36</td>
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<tr>
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<td>0.83</td>
<td>-</td>
</tr>
<tr>
<td>(9Z)-hexadec-9-enoic acid</td>
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<td>0.11</td>
<td>16.28</td>
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<td>6.74</td>
<td>17.38</td>
<td>11.80</td>
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<td>18.24</td>
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<td>0.70</td>
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<td>69.04</td>
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</tr>
<tr>
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<td>1.53</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
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<td>3.32</td>
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</tr>
<tr>
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<td>1.62</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>(5Z,8Z,11Z,14Z)-5,8,11,14-eicosatetraenoic acid</td>
<td>arachidonic acid</td>
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<td>-</td>
<td>0.77</td>
<td>-</td>
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<tr>
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<td>-</td>
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<td>-</td>
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<tr>
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<td>arachidic acid</td>
<td>-</td>
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<td>-</td>
<td>-</td>
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</table>

*Position and configuration of the double bond(s) not determined.

Parasitoid males from LA+ hosts are more fertile

Although the diets used to prepare the two host types were comparable in terms of energetic value, *N. vitripennis* males from LA+ hosts had three times as many spermatozoa in their seminal vesicle as those from LA- hosts (*t*-test: $t = 6.466$, d.f. = 18, $P < 0.001$, Fig. 2.2).
Figure 2.2: Mean sperm number (± SEM) counted in the seminal vesicles of individual *N. vitripennis* males from LA+ and LA- hosts. Asterisks indicate significant differences at $P < 0.001$ ($t$-test).

**Parasitoid males from LA+ hosts produce and release more of the sex pheromone**

Males from LA+ hosts had significantly higher total HDL titres (Mann-Whitney-$U$-test, $U = 141$, $Z = -2.167$, d.f. = 41, $P = 0.030$, Fig. 2.3A) and deposited almost twice as much of the pheromone as those from LA- hosts (Mann-Whitney-$U$-test, $U = 179$, $Z = -2.903$, d.f. = 47, $P = 0.004$, Fig. 2.3B).

Figure 2.3: Total HDL amounts (mean ± SEM) (A) extracted from the abdomens and (B) deposited within an observation time of 10 min by 2-days old *N. vitripennis* males from LA+ and LA- hosts. Asterisk indicates significant differences at $P < 0.01$ (**) and $P < 0.05$ (*) (Mann-Whitney-$U$-test).
Pheromone markings of males from LA+ hosts are more attractive for virgin females

In our olfactometer bioassays, virgin females were attracted more often to cavities marked by males from LA+ hosts (two-tailed binominal test, $P = 0.0075$, Fig. 2.4A) and spent significantly more time in these cavities (Wilcoxon matched pairs test, $Z = -2.306$, $P = 0.021$, Fig. 2.4B) when compared with cavities marked by males from LA- hosts.

![Figure 2.4: Response of virgin N. vitripennis females in the olfactometer bioassay to pheromone deposits released by males from LA+ and LA- hosts, respectively. (A) First choice for and (B) mean residence time ($\pm$ SEM) in the marked cavities within an observation time of 5 min. Asterisks indicate significant differences at $P < 0.01 (**)$ and $P < 0.05 (*)$ (first choice: two-tailed binominal test, residence time: Wilcoxon matched pairs test).](image)

Female parasitoids prefer LA+ hosts for oviposition

In our two-choice oviposition experiments both virgin females (producing only male offspring due to the haplodiploid sex determination of parasitic wasps) and mated females (producing offspring of either sex) laid more eggs into LA+ than in LA- hosts (Wilcoxon matched pairs test: virgin females: $Z = -3.550$, d.f. =40, $P < 0.001$ ; mated females: $Z = 3.079$, d.f. = 30, $P = 0.0021$, Fig. 2.5). However, mated females did not lay a higher proportion of male offspring into LA+ than in LA- hosts (Chi²-test: Chi² = 0.73, d.f.= 1, $P = 0.394$).
2.5 DISCUSSION

The present study clearly demonstrates that LA taken up by the host during feeding is directly incorporated into the abdominal sex pheromone of *N. vitripennis* males, and thus functions as a precursor of the chemical signal. Furthermore, the fatty acid composition of the host is of crucial importance for both the fertility and the pheromone-mediated sexual attractiveness of *N. vitripennis* males. Males developing in hosts containing higher amounts of unsaturated fatty acids including LA possess much higher numbers of spermatozoa and release more of the sex pheromone than males whose dietary lipids contain higher proportions of saturated fatty acids. Since *N. vitripennis* females are monandrous in natural populations (Grillenberger et al., 2008) and typically mate with the first male they encounter, LA+ males have clearly enhanced mating chances by bringing more virgin females to orient toward their pheromone markings. Furthermore, the threefold higher number of spermatozoa found in males from LA+ hosts should translate into significant fitness benefits because of delayed sperm depletion. *Nasonia vitripennis* is a haplodiploid species with male offspring developing from unfertilized eggs only. In a recent study we found a correlation between the mating history of males and the proportion of sons in the offspring they fathered and
significant sperm depletion was measurable already after seven successive copulations (Ruther et al., 2009). Our data suggest that this might occur even earlier in males of poor nutritional condition. Sex ratios in species under local mate competition like *Nasonia* are typically strongly female-biased (Werren, 1980; Shuker & West, 2004). Females mating with males from hosts poor in LA are more likely to run out of sperm and get constrained to produce suboptimal offspring sex ratios. However, females may avoid these costs by using the male sex pheromone to discriminate against these males of lower quality.

The link between dietary lipids and sexual attractiveness on the one hand and fertility on the other makes the abdominal sex pheromone of *N. vitripennis* a reliable signal indicating male mate quality. However the signal might not be completely insusceptible to cheating because males of inferior quality might invest more of the available LA into the pheromone at the cost of decreased sperm production to get mating opportunities at all. Males of superior nutritional condition can afford to produce high amounts of the sexual signal without facing this trade-off. Thus, our study is one of the very few explaining why male signals provide reliable information about direct benefit quality and supports both the handicap hypothesis (Zahavi, 1975) and the phenotype-linked fertility hypothesis (Reynolds & Gross, 1990 and references therein; Sheldon, 1994).

Our results demonstrate furthermore that foraging *N. vitripennis* females prefer those hosts for oviposition that contain higher amounts of nutrients making their sons more fertile and more attractive for sexual mates. This suggests that they are able to evaluate the fatty acid composition of their hosts during or already prior to oviposition. But females did not allocate a higher proportion of sons to LA+ hosts. This indicates that the female offspring also benefits from the increased availability of PUFAs as has been shown for several other animals (Wathes et al., 2007).

An important question is whether the choice situation arranged in our experiments is a realistic one and reflects what female wasps might actually encounter in the field. We are confident that this is the case although data on the variability of fatty acid compositions in field populations of *N. vitripennis* host pupae are missing. However, a comparative investigation of adult insects from seven insect orders suggested a high variability of fatty acid composition in cyclorraphous flies, with LA proportions ranging between 0 and 25 per cent (Thompson, 1973). Our results showed that the fatty acid composition of the host is strongly influenced by its diet. Therefore, variability in the fatty acid composition of the natural host diets should result in variability of host quality as well. As for the *N. vitripennis* birds’ nest ecotype, a food web has been modelled for European populations showing that females parasitized mainly four dipteran hosts either sucking the blood of live nestlings
(Protocalliphora azurea, Protocalliphora falcozi), feeding on dead nestlings (Calliphora vicina) or on faeces and other organic materials in the nests (Potamia littoralis) (Peters & Abraham, 2010). It is reasonable to assume that these diets differ clearly in the availability and composition of dietary lipids and, thus, result in differing host qualities within an individual birds’ nest. The same can be predicted for hosts of the N. vitripennis carcass ecotype because fat is not equally distributed within a dead animal. Rabbit liver, for instance, contains 60 per cent more LA than lean muscle tissue or blood plasma (Tres et al., 2008; 2009). Hence, it is likely that host larvae feeding on different tissues and organs of a carcass differ in their fatty acid composition. Chemical analyses are in progress to test this hypothesis and to determine the natural variability of fatty acid compositions of N. vitripennis hosts from both habitat types.

Our findings add another facet to the ‘mother knows best’ principle by demonstrating a causal link between the oviposition preference of the mother and the mating chances of her sons. This is particularly interesting in the light of Nasonia being a haplodiploid species. Here, the classical ‘sexy son’ or ‘good genes’ scenarios of sexual selection theory (Weatherhead & Robertson, 1979; Qvarnström & Price, 2001) does not work because any trait of sexual attractiveness owned by the father cannot be inherited directly to the male offspring. However, by making the right oviposition decisions, a female may nevertheless influence the reproductive success of her sons.

The results of the present study point emphatically to LA as a crucial dietary resource of juvenile N. vitripennis males influencing both their fertility and their pheromone mediated sexual attractiveness as adults. While the causality of this conclusion was supported for the pheromone biosynthesis in our 13C-labeling experiments, also other differences between safflower and coconut oil (e.g. the oleic acid content) might have been responsible for the different numbers of spermatozoa found in males from LA+ and LA- hosts, respectively. However, there is ample literature demonstrating for many taxa of animals and humans that LA and other PUFAs influence the fertility of both males and females (Wathes et al., 2007). PUFAs are essential components of all cell membranes and particularly male spermatozoa require high PUFA contents to provide the plasma membrane with the essential fluidity for gamete fusion (Wathes et al., 2007). Furthermore, animals including many insects are able to elongate LA to C20-PUFA which can be further metabolized to prostaglandins (Blomquist et al., 1991). This hormonally active class of molecules is also known to have an impact on the reproduction of many animals including insects (Stanley, 2006).
It has long been a paradigm that animals are unable to biosynthesize LA by themselves and thus, depend on the dietary uptake of this nutrient (Blomquist et al., 1991). Today we know that this is not true for all animals because at least some have been shown to possess $\Delta$-12-desaturases enabling them to synthesize LA from oleic acid by introducing a second double bond at position 12 (Blomquist et al., 1991, Borgeson et al., 1991; Weinert et al., 1993). Nothing, however, is known about this ability in Nasonia and other Hymenoptera. This aspect needs further investigation.

2.6. Acknowledgements

This research was funded by the Deutsche Forschungsgemeinschaft (DFG, grant RU-717/10-1). The authors thank Alexandra Schrempf for her help in performing the sperm count, Daniela Pothmann for technical assistance and two anonymous reviewers for helpful comments.

Personal Contribution:

I made all experiments and created all figures of this paper. I wrote the text of the paper as a first version.
CHAPTER 3

Differential nutritional quality of hosts of *Nasonia vitripennis*: fatty acid composition of hosts reared on various beef tissues

3.1 ABSTRACT

By manipulating host quality, females of *Nasonia vitripennis* have been shown to be able to discriminate between hosts of differential fatty acid composition and to prefer, for oviposition, hosts that are rich in linoleic acid. As a result of the oviposition decisions made by females, the mating success of male offspring increases, because linoleic acid is the precursor of the male sex pheromone that is highly attractive to virgin females. In this study, we simulate the conditions of hosts living on a carcass, a natural habitat of *N. vitripennis*, by rearing hosts on various tissues of beef. We have analysed the fatty acid amounts and the fatty acid composition of the different tissues and of the pupae reared on these tissues. We have been able to show that the total fatty acid amounts of the hosts fed on the various tissues are similar. However, the hosts differ significantly in their fatty acid composition, mainly in oleic acid, linoleic acid, palmitic acid and palmitoleic acid. Hosts fed on muscle meat incorporate higher amounts of oleic acid and lower amounts of linoleic acid than hosts fed on entrails. These results suggest that *N. vitripennis* females might choose between hosts of different fatty acid compositions in a natural habitat, i.e. on a carcass.
3.2. INTRODUCTION

One factor that is crucial for good development and high fitness in the offspring of parasitoids is the optimal nutritional value of the host. Host organisms are the only source of nutrition for the offspring and a major task of the parasitoid female is to lay eggs in or on a host of high nutritional quality for its offspring (Vinson, 1976). The preference performance hypothesis, originally developed for herbivorous insects, predicts that females choose hosts that are relevant to the nutritional requirements of the offspring (Jaenike, 1978). The hosts of parasitoids are almost exclusively insects (Godfray, 1994) and these insects can differ in their nutritional value, such as in their phospholipids (Fast, 1966) and fatty acid composition (Fast, 1966; Thompson, 1973). The fatty acid composition of insects can be influenced by their diet (Thompson, 1973; Barlow, 1966). The parasitic wasp *Nasonia vitripennis* parasitizes pupae of cyclorrhaphous flies. Blaul & Ruther (2011) have investigated that females of the parasitic wasp *N. vitripennis* are able to recognize hosts that are artificially enriched with linoleic acid (LA), a polyunsaturated fatty acid, and choose preferably these hosts for oviposition. For the female offspring of *N. vitripennis*, and indeed for all organisms, LA is an indispensable fatty acid for cell function (Cripps et al., 1986). The male offspring of *N. vitripennis* additionally benefit from the oviposition decision of the mother, because LA is the precursor of the male sex pheromone and because *Nasonia* males are able to incorporate LA from the host directly into their sex pheromone, thereby enhancing their attractiveness and mating success. Males raised on LA-rich hosts have large amounts of pheromone and transfer large numbers of sperm constituting an advantage for the female mating partner (Blaul & Ruther, 2011). Most recent data have shown that *N. vitripennis* is able to biosynthesize LA *de novo* from oleic acid (OA) (Blaul & Ruther, unpublished, see chapter 4). Therefore, the amount of OA in a host could be of similar relevance as the amount of LA.

In the experiments of Blaul & Ruther (2011), the host quality was manipulated in the laboratory by presenting LA-rich or LA-poor diets to the host. An open question remained as to whether such a host choice is realistic in the natural habitat of *N. vitripennis*. In the field, two ecotypes of *N. vitripennis* occur: one is parasitizing hosts in nests of cavity-breeding birds (Peters & Abraham, 2010) and one is parasitizing hosts on carrions (Voss et al., 2009). Many insects, with Diptera as the most abundant order, lay their eggs on carrions and their offspring live and feed on carcasses (Horenstein et al., 2012). Even the time of death of the carrion can be concluded from the developmental state of the insects, an often used tool in forensic science (Horenstein et al., 2012). On carrion, insects have the choice between various tissues for oviposition (Archer & Elgar, 2003).
In this study, we have not added fatty acids artificially but have simulated the conditions of hosts living in a natural habitat, i.e. on a carcass, by rearing these hosts on various tissues in the laboratory. We selected five different bovine tissues, namely two muscle types and three types of entrails, and analysed the fatty acid composition of the tissues and of the host *Lucilia caesar* after being reared on these tissues. We hypothesize that, depending on the type of food, the fatty acid composition of the hosts differs and consequently that, in the field, *N. vitripennis* might also make a choice between hosts of differential nutritional value.

### 3.3. MATERIALS & METHODS

**Insects**

*Nasonia vitripennis* were originally collected from bird nests in northern Germany and were reared on freeze-killed puparia of the green bottle fly *Lucilia caesar* (Diptera: Calliphoridae) as described in Steiner et al., (2006).

**Variation of host quality - tissues used for host breeding**

Hosts of *Nasonia vitripennis*, the green bottle fly *Lucilia caesar*, were bought commercially as maggots and were reared in a cage at room temperature. After emergence, flies were provided for 8 days with honey, water and minced meat.

Five hundred grams each of the following five beef tissues were bought from a local butcher: (a) fatty muscle meat, (b) lean muscle meat, (c) heart, (d) kidney, (e) liver. The tissues were homogenized separately with a mixer and stored at -20°C in 40 g portions in plastic tubes (8 cm long, 3 cm diameter). As soon as flies were ready to oviposit, portions were thawed and female flies were allowed to oviposit on the five tissues until one egg packet was laid in each tube. Emerging fly larvae fed on the tissues for about a week and then pupated. Pupae were collected daily and, 2 days after pupation, they were frozen and stored at -20°C until used for chemical analysis.
Fatty acid analysis of tissues and hosts

The fatty acid amount and composition of the five tissues and the relevant host types was determined by coupled gas chromatography-mass spectrometry (GC-MS) of the fatty acid methyl esters (FAMEs).

Samples (40-100 mg) of the five tissues fatty muscle, kidney and liver (each n=3), samples of heart (n=2) and a sample of lean muscle (n=1) were used for analysis. Furthermore fatty acids of hosts (fed with heart and fatty muscle: n=10; fed with lean muscle: n=11, fed with liver and kidney: n=12) were analysed. The hosts were homogenized singly with a scoop.

All samples were first extracted for 30 min in 200 µl (pro 100 mg sample) methanol/dichloromethane (3:1) containing heptadecanoic acid (10 ng/µl) as an internal standard for quantification. The supernatant was transferred to a new glass vial, 200 µl dichloromethane containing heptadecanoic acid (10 ng/µl) was added and the sample was extracted a second time for 30 min. The samples were dried with sodium sulfate and 200 µl solvent was transferred to a new glass vial and solvent was removed under a gentle stream of nitrogen. For trans-esterification, extracted lipids in the residue were resuspended in 100 µl methanol and 10 µl acetyl chloride (10%, dissolved in methanol). Samples were kept for 1 h at 60°C. Subsequently, 200 µl sodium hydrogen carbonate (5%, dissolved in water) was added and the FAMEs were extracted by shaking with 600 µl hexane. A Shimadzu QP2010 Plus GC–MS system equipped with a BPX5 capillary column (30 m x 0.32 mm inner diameter, 0.25 mm film thickness, SGE Analytical Science Europe, Milton Keynes, UK) was used for analysis. Samples were injected at 300°C with a split ratio of 1:5 by using an AOC 20i auto sampler. Helium was used as the carrier gas at a constant flow rate of 2 ml/min. The initial oven temperature of 50°C (held for 4 min) was increased at 3°C/min to 280°C (held for 15 min). The MS was operated in the electron impact mode at 70 eV.

As a reference, a mixture of 37 FAMEs with a chain length from four to twenty-two C-atoms (Aldrich, Deisenhofen, Germany) was injected under the same GC-MS conditions and a custom spectral library of these FAMEs was created. Additionally, a mix of n-alkanes (C10 – C26) was used to calculate the linear retention indices for all the FAMEs in the reference mix and these data were also saved in the custom ms-library. FAMEs in the samples were identified by comparing their mass spectra and retention indices with the mass spectra and retention indices of the FAMEs in the custom library.

Quantification of the FAMEs was carried out by the internal standard method. Calibration curves were created of six fatty acids frequently found in nature: myristic acid, palmitic acid, palmitoleic acid, oleic acid (OA), stearic acid and LA. Defined amounts (5-350 ng) of the six
synthetic fatty acids were dissolved in dichloromethane with heptadecanoic acid as a standard (10 ng/µl). Aliquots of 200 µl from each sample were transesterified in the same manner as described above.
For analysis of those fatty acids that did not have their own calibration curve, the calibration curve of the most similar fatty acid was chosen assuming that these have similar response factors in the MS. The first choice criterion was the number of double bonds and the second the chain length of the fatty acids.

**Statistical analysis**

Because of the low number of samples, no statistical analysis was undertaken for the total fatty acid amounts in the tissues.
The total fatty acid amounts and the levels of LA and OA of the hosts were compared by a Kruskal-Wallis-H test followed by a Mann-Whitney-\(U\) test, if necessary (IBM SPSS Statistics 19).
The amount of the nine most abundant fatty acids (myristic acid, C16:1, palmitic acid, palmitoleic acid, stearic acid, OA, elaidic acid, LA and arachidonic acid) identified in the samples of the five host types (nourished on lean muscle, fatty muscle, kidney, liver and heart) were used as variables for a principal component analysis (PCA) with the program PAST version 2.01 scientific software (Hammer et al., 2001) to analyse differences in the fatty acid composition of the hosts. Similarity percentage analysis (SIMPER), based on Euclidean distances, was used to estimate the contribution of the single fatty acids to the overall difference in the fatty acid profile of the five host types and significances were checked with an analysis of similarities (ANOSIM), based on Euclidean distances.

**3.4. RESULTS**

**Total fatty acid contents of the tissues**

The total fatty acid content of the tissues is illustrated in Figure 3.1. Because of the low number of samples (lean muscle \(n=1\), heart \(n=2\), fatty muscle, kidney and liver \(n=3\)), no
statistical analysis was performed. Thus, only tendencies are identifiable in Figure 3.1; the liver had the highest total fatty acid content and lean muscle had the lowest.

![Figure 3.1: Total content of fatty acids (µg) in 1 mg tissue: fatty muscle, heart, kidney, lean muscle and liver (± s.e.m.). Statistical analysis has not carried out, because of the low number of samples.](image)

**Fatty acid composition of the five tissues**

Eleven fatty acids could be identified in various amounts in the tissues and are listed in table 3.1.

**Total fatty acid contents of the hosts reared on various tissues**

The hosts reared on fatty muscle, heart, kidney, lean muscle and liver did not differ significantly in their total fatty acid content (Kruskal-Wallis-H test: \( \chi^2 = 1.185 \), d.f. = 4, \( P = 0.881 \)). Each of the larvae had between 490 µg and 580 µg fatty acids, independently of the content of fatty acids in the tissues on which the larvae had fed (Figure 3.2).

![Figure 3.2: Total amount of fatty acids (mg per host) in the hosts reared on the five different tissues (± s.e.m.): fatty muscle, heart, kidney, lean muscle and liver. Same letters indicate no significant difference (Kruskal-Wallis-H test, \( P >0.05 \)).](image)
Table 3.1: Fatty acid composition of the five studied tissues. Compounds are listed in the elution order of the fatty acid methyl esters on the DB-5 stationary phase used for chemical analysis. Standard error (SE) is only indicated when a FAME occurs in more than one sample.

<table>
<thead>
<tr>
<th>Fatty acid methyl ester</th>
<th>RI</th>
<th>Fatty muscle ± SE (ng / mg tissue)</th>
<th>Heart ± SE (ng / mg tissue)</th>
<th>Kidney ± SE (ng / mg tissue)</th>
<th>Lean muscle ± SE (ng / mg tissue)</th>
<th>Liver ± SE (ng / mg tissue)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Myristic acid</td>
<td>1731</td>
<td>57.8 ± 5.0</td>
<td>44.4 ± 21.4</td>
<td>72.6 ± 10.1</td>
<td>17.4</td>
<td></td>
</tr>
<tr>
<td>Palmitoleic acid</td>
<td>1909</td>
<td>81.5 ± 9.8</td>
<td>10.1</td>
<td>8.8</td>
<td>30.4</td>
<td></td>
</tr>
<tr>
<td>Palmitic acid</td>
<td>1932</td>
<td>996.6 ± 112.5</td>
<td>879.2 ± 373.4</td>
<td>559.7 ± 156.3</td>
<td>264.8 ± 472.6</td>
<td>472.6 ± 73.9</td>
</tr>
<tr>
<td>Linoleic acid</td>
<td>2100</td>
<td>161.7 ± 11.7</td>
<td>510.0 ± 192.2</td>
<td>261.8 ± 50.1</td>
<td>36.2 ± 529.9</td>
<td>529.9 ± 15.4</td>
</tr>
<tr>
<td>Oleic acid</td>
<td>2106</td>
<td>2681.5 ± 297.8</td>
<td>955.7 ± 470.6</td>
<td>730.6 ± 123.3</td>
<td>622.6 ± 689.8</td>
<td>689.8 ± 4.21</td>
</tr>
<tr>
<td>Elaidic acid</td>
<td>2113</td>
<td>97.8 ± 4.9</td>
<td>35.6 ± 3.6</td>
<td>78.4 ± 8.7</td>
<td>28.1 ± 67.6</td>
<td>67.6 ± 12.2</td>
</tr>
<tr>
<td>C18:1</td>
<td>2115</td>
<td>30.5 ± 3.2</td>
<td>18.9 ± 3.4</td>
<td>22.3 ± 8.7</td>
<td>14.7 ± 56.5</td>
<td>56.5 ± 13.2</td>
</tr>
<tr>
<td>Stearic acid</td>
<td>2133</td>
<td>529.9 ± 33.6</td>
<td>748.7 ± 303.3</td>
<td>857.4 ± 201.2</td>
<td>151.7 ± 3988.1</td>
<td>3988.1 ± 721.5</td>
</tr>
<tr>
<td>Arachidonic acid</td>
<td>2262</td>
<td>45.8 ± 4.8</td>
<td>95.2 ± 6.3</td>
<td>117.8 ± 14.9</td>
<td>21.5 ± 345.6</td>
<td>345.6 ± 10.0</td>
</tr>
<tr>
<td>cis-5, 8,11,14,17-</td>
<td>2268</td>
<td></td>
<td>13.8</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Eicosapentaenoic acid</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>cis-8,11,14-</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Eicosatrienoic acid</td>
<td>2281</td>
<td>18.1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Fatty acid composition of the hosts reared on various tissues

Eighteen fatty acids could be identified in the hosts reared on the five tissues and are listed in Table 3.2.

We used the nine most abundant fatty acids, namely myristic acid, C16:1 (a), palmitic acid, palmitoleic acid, stearic acid, OA, elaidic acid, LA and arachidonic acid, found in the five types of hosts as variables for a PCA analysis (Figure 3.3). The first principal component (component 1) explained over 84 % of the total variance in the variables and the second principal component (component 2) accounted for over 11 % of the total variance in the variables. A SIMPER analysis based on Euclidean distances revealed OA, palmitic acid, palmitoleic acid and LA as being compounds with a major influence on the dissimilarity of the fatty acid composition of hosts fed on the different tissues (Table 3.3). The other fatty acids were negligible with contribution values under 1%. Factor scores (component 2) revealed that the hosts reared on muscle tissue differed in their amount of OA (-0.59) and in the amounts of palmitic acid (0.66), palmitoleic acid (0.30) and LA (0.31) from hosts reared on...
entrails (Figure 3.3). This difference was significant (ANOSIM analysis, based on Euclidean distances, \( P \) values describe sequential Bonferroni significance: fatty muscle - kidney: \( P = 0.0137 \), fatty muscle - heart: \( P = 0.0148 \), fatty muscle - liver: \( P = 0.0172 \); lean muscle - kidney: \( P = 0.0415 \), lean muscle - heart: \( 0.0148 \) with the exception of lean muscle - liver: \( P = 0.0522 \).

Table 3.2: Fatty acid composition of the hosts reared on the five tissues. Compounds are listed in the elution order of the fatty acid methyl esters on the DB-5 stationary phase used for chemical analysis. Standard error (SE) is only indicated when a FAME occurs in more than one sample.

<table>
<thead>
<tr>
<th>Fatty acid methyl ester</th>
<th>RI</th>
<th>Host Fatty muscle ± SE (µg / host)</th>
<th>Host Heart ± SE (µg / host)</th>
<th>Host Kidney ± SE (µg / host)</th>
<th>Host Lean muscle ± SE (µg / host)</th>
<th>Host Liver ± SE (µg / host)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Caproic acid</td>
<td>1362</td>
<td>3.2 ± 1.6</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Myristoleic acid</td>
<td>1718</td>
<td>0.2 ± 0.2</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Myristic acid</td>
<td>1731</td>
<td>8.4 ± 0.9</td>
<td>8.0 ± 0.8</td>
<td>12.1 ± 1.3</td>
<td>9.4 ± 0.9</td>
<td>3.6 ± 0.4</td>
</tr>
<tr>
<td>Pentadecanoic acid</td>
<td>1831</td>
<td>1.6 ± 0.4</td>
<td>2.2 ± 0.2</td>
<td>0.8 ± 0.3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C16:1 (a)</td>
<td>1902</td>
<td>17.8 ± 2.2</td>
<td>8.1 ± 1.0</td>
<td>8.4 ± 0.9</td>
<td>11.4 ± 1.2</td>
<td>3.4 ± 0.3</td>
</tr>
<tr>
<td>Palmitoleic acid</td>
<td>1909</td>
<td>85.5 ± 15.5</td>
<td>85.3 ± 11.4</td>
<td>77.2 ± 12.2</td>
<td>78.4 ± 11.0</td>
<td>72.3 ± 13.4</td>
</tr>
<tr>
<td>C16:1 (b)</td>
<td>1912</td>
<td>1.9 ± 0.3</td>
<td>1.2 ± 0.3</td>
<td>1.2 ± 0.3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Palmitic acid</td>
<td>1932</td>
<td>159.2 ± 17.0</td>
<td>171.2 ± 12.4</td>
<td>163.2 ± 16.7</td>
<td>147.2 ± 12.9</td>
<td>171.0 ± 18.1</td>
</tr>
<tr>
<td>Gamma linolenic acid</td>
<td>2082</td>
<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Linoleic acid</td>
<td>2100</td>
<td>10.9 ± 1.9</td>
<td>40.3 ± 5.8</td>
<td>27.8 ± 3.7</td>
<td>4.7 ± 0.8</td>
<td>18.4 ± 2.4</td>
</tr>
<tr>
<td>Oleic acid</td>
<td>2106</td>
<td>255.6 ± 30.3</td>
<td>172.9 ± 13.9</td>
<td>158.6 ± 18.5</td>
<td>210.4 ± 21.7</td>
<td>163.3 ± 18.6</td>
</tr>
<tr>
<td>Elaidic acid</td>
<td>2113</td>
<td>6.2 ± 0.7</td>
<td>5.2 ± 0.4</td>
<td>5.1 ± 0.5</td>
<td>5.4 ± 0.7</td>
<td>1.8 ± 0.4</td>
</tr>
<tr>
<td>C18:1</td>
<td>2115</td>
<td>1.1 ± 0.4</td>
<td>2.8 ± 0.4</td>
<td>1.6 ± 0.5</td>
<td>1.6 ± 0.5</td>
<td>0.1 ± 0.1</td>
</tr>
<tr>
<td>Stearic acid</td>
<td>2133</td>
<td>12.6 ± 1.6</td>
<td>16.1 ± 1.3</td>
<td>23.5 ± 2.6</td>
<td>12.6 ± 1.4</td>
<td>24.2 ± 3.6</td>
</tr>
<tr>
<td>Arachidonic acid</td>
<td>2262</td>
<td>3.7 ± 0.5</td>
<td>7.1 ± 0.6</td>
<td>11.3 ± 1.3</td>
<td>2.7 ± 0.6</td>
<td>13.2 ± 2.0</td>
</tr>
<tr>
<td>cis-5, 8, 11, 14, 17-Eicosapentaenoic acid</td>
<td>2268</td>
<td>0.8 ± 0.4</td>
<td>4.8 ± 0.5</td>
<td>2.8 ± 0.5</td>
<td>3.6 ± 0.5</td>
<td>6.5 ± 0.8</td>
</tr>
<tr>
<td>cis-8, 11, 14-Eicosatrienoic acid</td>
<td>2281</td>
<td>0.1 ± 0.1</td>
<td></td>
<td></td>
<td></td>
<td>4.0 ± 0.7</td>
</tr>
<tr>
<td>cis-11, 14-Eicosadienoic acid</td>
<td>2300</td>
<td>10.6 ± 4.7</td>
<td>1.7 ± 1.7</td>
<td>1.6 ± 0.2</td>
<td>2.4 ± 1.8</td>
<td>6.0 ± 3.1</td>
</tr>
</tbody>
</table>
Figure 3.3: Principal component analysis of the fatty acid composition in the hosts from the five tissues.

Table 3.3: Similarity Percentage (SIMPER) analysis of the fatty acid composition of the tissues

<table>
<thead>
<tr>
<th>Fatty acid</th>
<th>Average dissimilarity</th>
<th>Contribution (%)</th>
<th>Cumulative (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>oleic acid</td>
<td>12070</td>
<td>55.95</td>
<td>55.95</td>
</tr>
<tr>
<td>palmitic acid</td>
<td>5190</td>
<td>24.05</td>
<td>80</td>
</tr>
<tr>
<td>palmitoleic acid</td>
<td>3332</td>
<td>15.44</td>
<td>95.44</td>
</tr>
<tr>
<td>linoleic acid</td>
<td>596</td>
<td>2.762</td>
<td>98.2</td>
</tr>
<tr>
<td>stearic acid</td>
<td>181.2</td>
<td>0.8396</td>
<td>99.04</td>
</tr>
<tr>
<td>C16:1</td>
<td>84.21</td>
<td>0.3903</td>
<td>99.43</td>
</tr>
<tr>
<td>arachidonic acid</td>
<td>73.78</td>
<td>0.3419</td>
<td>99.77</td>
</tr>
<tr>
<td>myristic acid</td>
<td>36.73</td>
<td>0.1702</td>
<td>99.94</td>
</tr>
<tr>
<td>elaidic acid</td>
<td>12.51</td>
<td>0.058</td>
<td>100</td>
</tr>
</tbody>
</table>

Levels of OA and LA in the hosts fed on various tissues

The amounts of LA and OA differed between the hosts fed on the different tissues. The amount of OA in hosts reared on fatty muscle was significantly higher than the amount of OA in hosts reared on entrails (Kruskal Wallis H test: $\chi^2 = 9.663$, d.f. = 4, $P = 0.046$; Mann-Whitney-U-test: fatty muscle - heart: $U = 19$, $Z = -2.343$ $P = 0.019$, fatty muscle - kidney: $U =$
Chapter 3 - Hosts Reared On Different Tissues

Furthermore, hosts reared on fatty and lean muscle showed a significant lower amount of LA than hosts reared on entrails (Kruskal Wallis H test: $\chi^2 = 37.393$, d.f. = 4, $P < 0.001$; Mann-Whitney-U-test: fatty muscle - heart: $U = 2$, $Z = -3.628$, $P < 0.001$, fatty muscle - kidney: $U = 11$, $Z = -3.231$, $P = 0.001$, fatty muscle - liver: $U = 27$, $Z = -2.176$, $P = 0.030$; lean muscle - heart: $U = 0$, $Z = -3.873$, $P < 0.001$, lean muscle – kidney: $U = 0$, $Z = -4.062$, $P < 0.001$, lean muscle - liver: $U= 2$, $Z = -3.939$, $P < 0.001$; Figure 3.4).

3.5 DISCUSSION

The sample number of the tissues was too low for statistical analysis. However, a tendency is visible indicating that their fatty acid amounts are not equal. The average fatty acid amounts of the five studied tissues are listed in scientific tables. The listed fatty acid values per 100 g is strikingly different between the five tissues: fatty muscle meat (topside) 4.53 g, bovine heart 6.00 g, bovine kidney 5.14 g, lean muscle meat 1.90 g, bovine liver 3.66 g (Souci et al., 2008). We have found approximately one tenth of the fat amount listed in the table of Souci et al. (2008). This difference might be attributable to the method used for measurements or to discrepancies concerning the food intake of the animal (Lee et al., 2008).
Remarkably, in our measurements, the liver is the organ with the highest total fatty acid amount, although the fatty acid amount is listed as the second lowest of the tissues by Souci et al. (2008). The reason for the fatty liver could be the high calorific feed, which is rich in fatty acids, of the bovine (Mouzaki & Allard, 2012; Yüksel et al., 2012).

The total fatty acid amounts of the hosts fed on the different tissues did not differ (Figure 3.2). Obviously, the leanest tissue included sufficient fat for the flies. One pupa consumes approximately one tenth of the fat amount of 1 g tissue (Figure 3.1, 3.2) and we know from breeding that larvae eat even more until pupation. Therefore, larvae are able to enrich their basic fat amount.

However, the fatty acid composition and hence the nutritional quality of the hosts reared on the five tissues clearly differed. In particular, the hosts reared on muscle tissue had a different fatty acid composition from the hosts fed on entrails (Figure 3.3). In the muscle-fed pupae, OA was found at higher concentrations and LA at lower concentrations than in entrails-fed pupae (Figure 3.4). We identified hosts reared on fatty muscle as the pupae with the highest amount of OA and heart pupae as the pupae with the highest amount of LA. These data suggest that pupae with different fatty acid compositions can exist on the same carcass, provided that all the tissues are taken up equally by the larvae. In our studies, flies only laid their eggs in liver when they had no other options for oviposition. Because of the consistency of liver, many flies stuck to it and died before they were able to oviposit. The natural oviposition site of the flies and thus the tissues fed on by the maggots is dependent on many factors, such as the habitat of the cadaver (Hanski, 1976), the cause of death, the humidity and the temperature (Smith, 1986). Flies choose a tissue for oviposition with a low risk of desiccation, predators and competition and a sufficient nutritional value for their offspring. Popular oviposition sites of flies are the mouth or injuries of the carcass (Archer & Elgar, 2003). Maggots are able to move and feed their way through carrion (Archer & Elgar, 2003). To confirm that pupae with differences in their fatty acid composition exist on the same carcass, pupae from several cadavers should be collected and the fatty acid composition of these pupae should be analysed in further studies.

The analysed fatty acid composition of the pupae correlates to the fatty acid composition of the tissues, as found in the literature (Souci et al., 2008): in a comparison of the five tissues, fatty muscle is the tissue with the highest OA level and heart is the tissue with the highest LA level. This confirms the result of Blaul & Ruther (2011) showing that the fatty acid composition of the host diet is incorporated into the host pupae. Blaul & Ruther (2011) could also show that *Nasonia* females favour ovipositing into hosts that are rich in LA and additionally rich in OA. By means of their oviposition decisions, females ensure that a high
proportion of LA is usable as a precursor for the male sex pheromone, which in turn leads to male offspring with a high pheromone titre and high mating success. Therefore, when *Nasonia* females have the choice between pupae of the five different tissues, they might favour hosts reared on heart because of its high LA level. This hypothesis needs to be tested in female choice experiments.

Recent experiments have shown that *N. vitripennis* is able to biosynthesize LA *de novo* from OA (Blaul & Ruther, unpublished data, see chapter 4). Therefore, the OA amount of a host might also play a role in the oviposition decisions of a *Nasonia* female. However, OA is the most commonly found fatty acid in all tissues, regardless of the nutrition of the animal (Lorenzo et al., 2012; Yüksel et al., 2012), and as OA is the most frequent fatty acid in the pupae (table 2), there is presumably no lack of OA for *Nasonia* offspring. Females most probably orient themselves with respect to the LA amounts of hosts for ovipositing and, if the host does not contain sufficient LA, the *Nasonia* offspring use their own ability to biosynthesize LA *de novo* from OA.

There is also presumably no lack of palmitoleic acid for *Nasonia* offspring. Flies such as *Lucilia caesar* are characterized by a high level of palmitoleic acid (Fast, 1966, Table 3.2). Therefore, host choice is probably independent of differences in palmitoleic acid. Two open questions are whether *N. vitripennis* is able to recognize differences between hosts in the amounts of palmitic acid, an abundant fatty acid in carcasses (Lorenzo et al., 2012, Atti & Mahouachi, 2011) and whether differences in palmitic acid between hosts influence the oviposition decision. To investigate the role of palmitic acid in the choice of host for *N. vitripennis*, an initial possible experiment would be to analyse female choice between hosts that are artificially enriched with palmitic acid and those that are untreated.

In conclusion, we have been able to show that pupae reared on various tissues differ in their fatty acid composition. Analysis of hosts in the field and host choice tests made by *N. vitripennis* females should illustrate further differences in nutritional host qualities in a natural habitat and the host preferences of *N. vitripennis* females.
CHAPTER 4

De novo biosynthesis of linoleic acid in *Nasonia vitripennis*

4.1 ABSTRACT

The fatty acid linoleic acid (C18:2\(^\Delta9,12\)) is important for many vital membrane functions of animal and plant cells. It has been a paradigm, valid for a long time that in contrast to plants, algae and fungi, most animals are unable to synthesize linoleic acid *de novo* from oleic acid (C18:2\(^\Delta9\)) because they miss a \(\Delta12\)-desaturase for inserting a double bound at the \(\Delta12\) position. Therefore animals have to take up essential linoleic acid with nutrition. In the parasitic wasp *Nasonia vitripennis* linoleic acid is the precursor of the male sex pheromone component (4\(^R\),5\(^R\))- and (4\(^R\),5\(^S\))-5-hydroxy-4-decanolides (HDL) that is important for the mating success of the males. By \(^{13}\)C labelling experiments we detected that oleic acid of the host diet is absorbed from the host as well as from the parasitoid *N. vitripennis*. We further showed that *N. vitripennis*, in contrast to the host, is able to synthesize linoleic acid *de novo* from oleic acid and *Nasonia* males directly incorporate the new synthesized linoleic acid in their sex pheromone. We identified *N. vitripennis* as the first Hymenoptera that is independent from taking linoleic acid with nutrition.
4.2 INTRODUCTION

Linoleic acid (LA), a polyunsaturated fatty acid (PUFA) (C18:2\text{\ Δ9,12}), and its long chain derivatives are involved in many vital membrane functions of animal and plant cells. It is abundant in phospholipids, controls the maintenance of membrane fluidity and regulates the mobility of embedded proteins (Cripps et al., 1986; Opekarova & Tanner, 2003). In mammals, LA is the precursor of γ-linolenic acid (C18:3\text{\ Δ6,9,12}) and arachidonic acid (C20:4\text{\ Δ5,8,11,14}), which are both substrates in the synthesis of eicosanoids such as prostaglandins and leukotrienes, involved in biological processes of almost every organ, tissue and cell (Funk, 2001). Conjugated LA was reported to show free radical scavenging activity (Ali et al., 2012) and to reduce blood pressure (DeClercq et al., 2012a, 2012b). In most insects LA is accumulated in the phospholipid fraction (de Renobales et al., 1987) and is required for optimal larval growth (Dadd, 1985), sexual maturity of insects (Batcabe et al., 2000), final skin-shed and wing spanning in some Lepidoptera (Levinson, 1955). Insects can accumulate high concentrations of fatty acids as fat reserves for diapauses or oogenesis (Genc, 2006).

In contrast to oleic acid (C18:1\text{\ Δ9}), which can be synthesized by all organisms, the synthesis of LA from oleic acid (OA) is restricted to organisms capable of inserting a double bond at the Δ12 position of OA with the help of a Δ12-desaturase. Algae, plants and fungi are able to synthesize LA de novo from OA (Sperling et al., 2003). In contrast, most animals, especially vertebrates, miss the enzyme Δ12-desaturase and, therefore, lack the ability of LA synthesis. Consequently, most animals have to take up LA through their food (Blomquist et al., 1991), often as triglycerides in fats and oils. However, there are some exceptions within the animal kingdom. In the last years some nematodes (Watts & Browse; 1999; Zhou et al., 2011), protozoa (Sayanova et al., 2006) and insects of four orders (de Renobales et al.; 1987; Zhou et al., 2008) were found to possess a Δ12-desaturase and to be able to synthesize LA from OA.

In *Nasonia vitripennis* (Hymenoptera: Pteromalidae), a parasitoid wasp of several cyclorrhaphous flies, LA has an important role for the males. LA is the precursor of the male sex-pheromone components (4R,5R)- and (4R,5S)-5-hydroxy-4-decanolides (HDL) that are biosynthesized in the rectal vesicle of the males (Abdel-Latief et al., 2008). After mating males release HDL together with 4-methylquinazoline (4-MeQ) as a minor pheromone component via the anal orifice by dabbing movements of the abdominal tip (Steiner & Ruther, 2009b). Females mate only once but the male pheromone is highly attractive for further virgin females. *Nasonia* males developing in fly hosts which are enriched with LA are able to produce and release higher amounts of HDL than males from hosts poor in LA. Females
favour the higher pheromone amounts of males from LA rich hosts (Blaul & Ruther, 2011). Therefore more LA in the host diet means higher mating chances of *N. vitripennis* males. Additionally, males from LA rich hosts have more sperm than males from LA poor hosts. By preference of higher HDL concentrations females avoid the danger of mating with sperm-depleted males because sperm limitation correlated with markedly reduced pheromone titres (Ruther et al., 2009). In summary, a high amount of LA in the host of *Nasonia* males leads to optimal mating conditions (Blaul & Ruther, 2011).

Because of the enormous importance of LA in the life of *N. vitripennis* and especially of *Nasonia* males it would be an advantage for *N. vitripennis*, to be able to synthesize LA themselves. In this study we enriched host food with \(^{13}\text{C}\) labelled OA and subsequently analysed the resulting fatty acids occurring in both, the host and the parasitoid *N. vitripennis*. We demonstrate that *N. vitripennis* is the first known insect of the order Hymenoptera which is capable of synthesizing LA from OA. *N. vitripennis* males are able to use OA as precursor for their sex pheromone synthesis.

### 4.3. MATERIAL & METHODS

**Insects**

*N. vitripennis* were originally collected from bird nests in northern Germany and were reared on freeze-killed puparia of the green bottle fly *Lucilia caesar* (Diptera: Calliphoridae) as described in Steiner et al., 2006.

**Manipulation of host quality (\(^{13}\text{C}\) labelling experiments)**

*Lucilia caesar* flies were fed with honey, water and ground meat until the females were ready to oviposit (after about one week). Afterwards females got lean beef for oviposition. 2 g of lean beef fillet were mixed with 100 mg of \(^{13}\text{C}\) labelled OA (\(^{13}\text{C}\)-OA) (Campro Scientific GmbH, Berlin, Germany) and homogenized using a scoop. Ten 5-days old larvae were transferred to the prepared beef fillet. Fly larvae fed from this beef fillet two further days, until they stopped feeding ready for pupating. Two days after pupation, the pupae were frozen.
and stored at -20°C. Nine of the pupae were used for chemical analysis, the remaining pupae for parasitoid rearing (see below).

**Fatty acid analysis of the manipulated hosts**

The fatty acids of the $^{13}$C-OA hosts were analysed as fatty acid methyl esters (FAMEs) by coupled gas chromatography-mass spectrometry (GC-MS) to determine their composition. Batches of three 2-day-old pupae ($n=3$) were homogenized using a scoop and were extracted in a 1.5 ml glass vial for 30 min with 500 µl of dichloromethane. The solvent was transferred to another glass vial and removed under a gentle stream of nitrogen and the extract was re-suspended in 200 µl of methanol and 20 µl of acetyl chloride (10%, dissolved in methanol). The transesterification was done at 60°C for 1 h. Afterwards 400 µl of sodium hydrogen carbonate (5%, dissolved in H$_2$O) were added and the FAMEs were extracted by shaking the mixture with 400 µl of pentane. For chemical analysis of the FAMEs a Shimadzu QP2010 Plus GC-MS system equipped with a BPX5 capillary column (30 m x 0.32 mm inner diameter, 0.25 µm film thickness, SGE Analytical Science Europe, Milton Keynes, UK) was used. Samples were injected splitless at 300°C using an AOC 20i auto sampler. The initial oven temperature was 50°C, after 4 min the temperature was increased at 3°C/min to 280°C (held for 15 min). The MS was operated in the electron impact mode at 70eV. Helium was used as carrier gas at a constant flow rate of 2 ml/min. Identification of FAMEs was done by analysing reference fatty acids (Sigma-Aldrich, Deisenhofen, Germany) that were derivatized under the same conditions.

**Production of parasitoid males from $^{13}$C-OA enhanced hosts**

Virgin *N. vitripennis* females, producing only male offspring (haplodiploidy) were allowed to oviposit for 48 h into labelled hosts, fed a diet enriched in $^{13}$C labelled OA. Parasitoid pupae were excised from the hosts 1 - 2 days prior to eclosion and kept singly in microcentrifuge tubes. Two days after emergence they were killed by freezing and stored at -20°C until chemical analysis (see below).
Fatty acid analysis of *N. vitripennis* males from $^{13}$C-OA hosts

The abdomens of two-days old *N. vitripennis* males (n=5) were cut off and were extracted for 30 min with 100 µl of dichloromethane and transesterified as above, but with half of the volume of all reagents. Abdomens were analysed by the GC-MS method, described above.

Pheromone analysis of males from $^{13}$C-OA enhanced hosts

To test whether $^{13}$C-OA from the host is incorporated into the male sex attractant, the abdomens of two-days old virgin *N. vitripennis* males from $^{13}$C-OA hosts (n=3) of comparable size were cut off. Each abdomen was extracted for 30 min with 25 µl dichloromethane containing 10 ng/µl methyl undecanoate as an internal standard and analysed by GC-MS. For pheromone analysis, samples were injected splitless at 280°C. The initial oven temperature of 50°C was increased at 5°C/min to 200°C and further increased at 15°C/min to 280°C (held for 15 min).

4.4 RESULTS

Comparison of the mass spectrum from LA of the host and from *Nasonia vitripennis*

Figure 4.1 shows the normal mass spectrum of LA methyl ester (a), the mass spectrum of LA methyl ester resulting from the fatty acid analysis of the host, artificially enriched in $^{13}$C labelled OA (b) and resulting of the fatty acid analysis of *N. vitripennis*, reared on these hosts (c). No difference was noticed between the normal mass spectrum of LA (a) and the mass spectrum of LA from the fatty acid analysis of the manipulated hosts (b). Therefore LA from the host is not influenced by the $^{13}$C labelled OA experiments (b). In contrast, the $^{13}$C labelled
OA of the host diet is incorporated into LA of *N. vitripennis*, because incorporation of $^{13}$C labelled LA was indicated by mass shifts of the diagnostic ions of LA m/z 220, 263 and 294 to m/z 236, 281 and 312 in the mass spectrum (c).

![Mass spectra](image)

Figure 4.1: Mass spectra of linoleic acid methyl ester (a), of linoleic acid methyl ester resulting from the fatty acid analysis from hosts, fed a diet enriched in $^{13}$C oleic acid (b) and from *Nasonia vitripennis* reared on these hosts (c). Arrows indicate diagnostic ions resulting from the incorporation of the labelled oleic acid. $^{13}$C atoms are indicated by black dots.

**Fatty acid analysis of the manipulated hosts**

The molecular mass of OA methyl ester is 296, of LA 294. The chromatogram shows that the $^{13}$C-labelled OA from the host diet is incorporated into the fly pupae, because the mass trace at m/z 314, the molecular mass of $^{13}$C-labelled OA methyl ester (296 + 18) showed a peak at the expected retention time of OA (Figure 4.2a). The mass trace at m/z 312, the molecular
mass of $^{13}$C labelled LA methyl ester (294 + 18) showed no peak at the expected retention time of LA. Therefore, the host *Lucilia caesar* is unable to convert $^{13}$C labelled OA from the diet into $^{13}$C labelled LA (Figure 4.2). In the chromatogram of an untreated host as control the mass traces at m/z 294 and at m/z 296 showed peaks at the expected retention times, no peaks were detectable on the mass traces at m/z 312 and at m/z 314 (Figure 4.2b).

![Figure 4.2: Cut–out of the total ion current (TIC) chromatogram of the fatty acid analysis of the manipulated hosts (a) and of an untreated host (b) and mass traces belonging to m/z 294 and m/z 296, (unlabelled LA methyl ester and unlabelled OA methyl ester) as well as 312 and 314 ($^{13}$C-labelled LA methyl ester and $^{13}$C-labelled OA methyl ester).](image)

**Fatty acid analysis of *Nasonia vitripennis* males from $^{13}$C-OA enhanced hosts**

The chromatogram shows that the $^{13}$C-labelled OA from the host diet was ingested during emerging from the *Nasonia* males, because the mass trace belonging to m/z 314 (the molecular mass of $^{13}$C-labelled OA methyl ester) showed a peak at the expected retention time of OA (Figure 4.3a). The mass trace at m/z 312, the molecular mass of $^{13}$C labelled LA also showed a peak at the expected retention time of LA. Therefore, the parasitoid is able to convert OA into LA (Figure 4.3a). In the chromatogram of 10 *N. vitripennis* from an untreated host as control the mass traces at m/z 294 and at m/z 296 showed peaks at the expected retention times, no peaks were detectable on the mass traces at m/z 312 and at m/z 314 (Figure 4.3b).
Figure 4.3: Cut-out of the total ion current (TIC) chromatogram of the fatty acid analysis of *Nasonia vitripennis* from the manipulated hosts (a) and of 10 *Nasonia vitripennis* from an untreated host (b) and mass traces belonging to m/z 294 and 296, (unlabelled LA methyl ester and OA methyl ester) as well as 312 and 314 (13C-labelled LA methyl ester and 13C labelled OA methyl ester).

**Analysis of the pheromone of males from 13C-OA hosts**

Fragmentation of (4*R*,5*R*)- and (4*R*,5*S*)-(HDL) in the mass spectrometer leads to diagnostic ions at m/z 86, 101 and 115. If the carbon atoms are fully 13C-labelled these masses shift to m/z 90, 107 and 120, respectively (Abdel-Latif et al., 2008, Figure 4.4).

Figure 4.4: Diagnostic ions resulting from the fragmentation of unlabelled (without dots) and fully 13C-labelled (black dots) HDL in the mass spectrometer after electron impact ionization.

In all analyses of the abdomen extracts from *N. vitripennis* males from 13C labelled OA enhanced hosts, the 13C labelled OA has been incorporated into the pheromone molecules as indicated by peaks in the mass traces at m/z 90, 107 and 120 at the expected retention
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times of (4R, 5R)- and (4R, 5S)-HDL, respectively (Figure 4.5a) in contrast to HDL from males reared on untreated hosts (Figure 4.5b).

![Figure 4.5: Cut-out of a total ion current (TIC) chromatogram and diagnostic ion traces resulting from GC-MS analysis of an abdomen extract from *Nasonia vitripennis* males. Wasps originated from hosts experimentally enriched in 13C labelled oleic acid (a) or originated from untreated hosts (b). Peaks at 17.65 and 18.0 min belong to the male sex pheromone components (4R,5R)- and (4R,5S)-5-hydroxy-4-decanolide (HDL).](image)

**4.5 DISCUSSION**

The 13C labelled OA experimentally applied to the host diet was found in the host *L. caesar* and in the parasitoid *N. vitripennis*. The host diet accordingly has an influence on the lipid composition of both the host as well as the parasitoid. In addition, we could show that also LA of the parasitoid *N. vitripennis* was partially 13C-labelled while the LA of the host was unlabelled.

This means that the host *L. caesar* is unable to synthesize LA from OA and, therefore, has to take up LA as essential fatty acid from the nutrition like most other animals (de Renobales et al., 1987). Insects generally have the same nutritional requirements as vertebrates but maintaining the balance of their diet and fatty acid uptake is more important for insects due to
their small body size (Dadd, 1985; Genc, 2006). The host *L. caesar* is living on carrions and fatty meat is a source of LA. As its closely related sister species *Lucilia sericata* is able to accumulate high concentrations of LA as larvae (Barlow, 1966), *L. caesar* is likely to accumulate LA, too.

In contrast to the host *L. caesar*, the parasitoid *N. vitripennis* is able to synthesize LA *de novo* from OA and, therefore, represents a counterexample for the paradigm, valid for a long time, that insects and higher animals are incapable of the *de novo* synthesis of LA seen in plants, fungi and algae. So far, only few insects of four orders have been described to be able to use OA as precursor for the production of LA. With *N. vitripennis* we found the first example for LA synthesis within the order Hymenoptera. LA is generated by inserting a double bond at position $\Delta 12$ of OA. The necessary enzyme, a $\Delta 12$-desaturase, has long been missing in insects and many other theories, such as LA synthesis by associated microorganisms instead of insects, were proposed (de Renobales et al., 1987). However, with modern methods all alternative explanations were invalidated and recently few examples of insect $\Delta 12$-desaturases have been described. Zhou et al. (2008) isolated the $\Delta 12$-desaturase genes of *Acheta domesticus* and *Tribolium castaneum* and functionally characterized them. The fatty acid $\Delta 12$-desaturase genes were cloned in yeast expression vectors and expressed in yeast. It was shown by subsequent GC-MS analysis that the yeast strain expressing the insect $\Delta 12$-desaturase gene synthesized LA from OA.

Also for *N. vitripennis* a $\Delta 12$-desaturase would be required for LA synthesis from OA. A first hint as to which desaturase might be involved could be obtained by comparing the genomic sequences of *N. vitripennis* with sequence databases of existing $\Delta 12$-desaturases and the statistical significance of accordance should be calculated. By this way members of gene families and predicted desaturases in *N. vitripennis* could be found. These possible genes should be isolated and their activity should be tested in future work. The evolutionary processes underlying the ability to synthesize LA *de novo* is still unknown (Sperling et al., 2003). Potentially all insects originally might have had the ability for *de novo* LA synthesis and there might have been a loss of this capability. Alternatively, the ability for LA synthesis could be a new evolutionary step of some insects. Between the species capable for LA synthesis no common reason and no visible physiological or nutritional pattern can be found (de Renobales et al.; 1987; Cripps et al., 1986), which would argue for the latter hypothesis.

Also the abdominal male sex pheromone HDL showed the $^{13}$C label of the OA that had been fed to the host (Figure 4.5a). This suggests that the OA is used by the wasps to synthesize LA as pheromone precursor which is directly incorporated into HDL. Fatty acids as precursors for sex pheromones are well known in many species of Lepidoptera (for instance
Ding et al., 2011; Moto et al., 2004). As full parasitoids, *N. vitripennis* cannot choose its larval food and is dependent on the oviposition decisions of its mother. Immediately after emergence *N. vitripennis* males copulate with their sisters, therefore no time remains to feed and accumulate LA before mating. Therefore the nutritional quality of the host is fundamental for the mating success and if LA of the host is low concentrated, *N. vitripennis* has the possibility to synthesize LA themselves. Jaenike (1978) described a theory for oviposition decisions of mothers, originally developed for herbivorous insects. The model predicts that an insect chooses plants for oviposition that provide optimal conditions for the offspring. The so-called preference-performance theory is transferable to parasitic Hymenoptera. Females should also choose a suitable host for their offspring to ensure their survival.

It has been shown that *N. vitripennis* females take care of good nutritional conditions for their offspring as they prefer hosts that are rich in LA to hosts that are poor in LA (Blaul & Ruther, 2011). Naturally *N. vitripennis* are living in nest of cavity breeding birds and on carcasses. It has been shown, that hosts, fed on different tissues of a carcass differ in their LA concentration therefore under natural circumstances females probably have the choice between hosts with different LA concentrations (Blaul, unpublished data, chapter 3). The ability to synthesize LA themselves represents a big additionally advantage for *N. vitripennis* males. Future work will show whether further fatty acids frequently occurring in host organisms, such as palmitic acid, can also be used for OA synthesis and subsequent linoleate synthesis.

In conclusion, we were able to show that the parasitic wasp *N. vitripennis* is able to take up OA from the host and convert it into LA, the precursor of the male sex pheromone. Thus *N. vitripennis* is to our knowledge the first of the order Hymenoptera that is independent of obtaining LA from the nutrition. Future work will be directed towards the isolation and the characterization of the ∆12-desaturase of *N. vitripennis* presumably involved in this biosynthetic pathway.
CHAPTER 5

Functional expression and activity test of two putative $\Delta^{12}$-desaturase genes of *Nasonia vitripennis*

5.1 ABSTRACT

The parasitic wasp *Nasonia vitripennis* is one of relatively few animals that are able to biosynthesize linoleic acid *de novo*. The enzyme $\Delta^{12}$-desaturase is necessary to create a double bond at the $\Delta^{12}$ position of oleic acid to synthesize linoleic acid. Linoleic acid is the precursor of the male sex pheromone that enables *N. vitripennis* males to attract female mating partners. The aim of this study was the functional characterization of the $\Delta^{12}$-desaturase of *N. vitripennis*. By sequence alignment of the *N. vitripennis* genome with known desaturases of other insects, two candidate genes, called D0 and D1, for a $\Delta^{12}$-desaturase of *N. vitripennis* were selected. These genes were isolated and cloned in competent yeast cells. However, comparative GC-MS analyses of yeast cells expressing the candidate genes did not support the $\Delta^{12}$-desaturase activity of either candidate gene. No appearance of linoleic acid in the transformed yeast cells was detectable, although functionality of the expression system could be demonstrated in control experiments with a known $\Delta^{12}$-desaturase gene from *Acheta domesticus*. Nevertheless, the relative fatty acid composition of yeast cells containing D0 changed in comparison with untransformed yeast cells. The amounts of the fatty acids C16:1$^{\Delta^{7}}$ and C18:0 decreased, whereas the amount of C18:1$^{\Delta^{9}}$ increased, suggesting that at least the gene D0 is involved in fatty acid biosynthesis.
5.2. INTRODUCTION

An important fatty acid for membrane functions of cells, regulating the fluidity and mobility of the phospholipid bilayer and the embedded proteins, is linoleic acid (LA) in all organisms (Uemura, 2012). LA is synthesized by the activation of the enzyme Δ12-desaturase by inserting a double bond at the Δ12 position of oleic acid (OA). Δ12-desaturases are well known in plants, fungi and algae (Sperling et al., 2003). A paradigm that remained valid for a long time was that animals do not have Δ12-desaturases and thus are unable to biosynthesize LA themselves but rather have to take up LA with their nutrition (Blomquist et al., 1991). This paradigm has been invalidated by some counterexamples. To date, protozoa (Sayanova et al., 2006), nematodes (Watts & Browse, 1999; Zhou et al., 2011) and insects of four orders (de Renobales et al., 1987; Zhou et al., 2008) have been shown to possess Δ12-desaturases. The first insect Δ12-desaturase genes were identified and characterized by Zhou et al. (2008). They isolated the putative Δ12-desaturase genes from the house cricket Acheta domestica (AdD12Des) and the red flour beetle Tribolium castaneum (TcD12Des) and functionally expressed them in yeast cells. The fatty acids of transformed yeast cells were analysed as fatty acid methyl esters by coupled gas chromatography-mass spectrometry (GC-MS) and the results showed that AdD12Des and TcD12Des had Δ12-desaturase activity because LA occurred as a new fatty acid.

For Nasonia vitripennis, a small parasitic wasp (Hymenoptera, Pteromalidae), LA is of particular importance, because it is the precursor of the major components of the male sex pheromone, namely (4R,5R)- and (4R,5S)-5-hydroxy-4-decanolide (HDL), which is highly attractive for virgin females (Blaul & Ruther, 2011). Males rich in LA have an above-average amount of sperm and possess and release a high amount of pheromone, thus being preferred by females (Ruther et al., 2009; Blaul & Ruther, 2011).

Visser et al. (2012) have been unable to find an increase either in lipids of N. vitripennis, despite sugar feeding, or in the transcription of genes involved in lipid biosynthesis. They have concluded an absence of lipogenesis in N. vitripennis. However, Blaul & Ruther (2011) have shown that N. vitripennis is not only dependent on taking up LA with its nutrition as must most other animals (unpublished data, see chapter 4). Larvae of Lucilia caesar, a host of N. vitripennis, were fed with 13C-labelled OA; subsequently, a fatty acid methyl ester (FAME) analysis of the host and of N. vitripennis was performed. 13C-labelled OA was found in the host and in N. vitripennis but 13C-labelled LA was found only in N. vitripennis. Therefore, in contrast to the host Lucilia caesar, the parasitoid N. vitripennis was able to biosynthesize LA from OA. Furthermore, GC-MS analysis of HDL showed 13C-labelling of the
pheromone; therefore, *N. vitripennis* males directly incorporated the newly synthesized LA into their sex pheromone.

These results thus suggest that *N. vitripennis* also possesses a Δ12-desaturase. The present study aimed at the identification of the putative Δ12-desaturase of *N. vitripennis* by aligning the *Nasonia* genome with known Δ12-desaturase sequences of other insects. The predicted candidate genes were cloned and expressed in yeast cells and their putative functions were investigated by GC-MS analysis of the FAME.

5.3. MATERIALS & METHODS

The sequences of genes and primers, the composition of media and buffers and the used kits are listed in the appendix without further indication in the text. DNA extraction was performed in the laboratory of Prof. Dr. Schneuwly at the Institute of Developmental Biology and further steps were carried out in the laboratory of Prof. Dr. Tschochner at the Institute of Biochemistry III.

Insects

*Nasonia vitripennis* were originally collected from bird nests in northern Germany and were reared on freeze-killed puparia of the green bottle fly *Lucilia caesar* (Diptera: Calliphoridae) as described in Steiner et al. (2006).

Determination of predicted Δ12-desaturases

The website of the National Center for Biotechnology Information (ncbi) was used to compare nucleotide sequences of the *Nasonia* genome (Werren et al., 2010) with sequence databases, to calculate the statistical significance of any analogies and to find regions of local similarity between sequences. In cooperation with Prof. Dr. Merkl (Biochemistry II), two candidate genes XP_001602565.1 (named D0) and XP_001599665 (named D1) for Δ12-desaturases in *Nasonia* were identified to be closely related to other insect desaturases. This
was predicted with the basic local alignment search tool, based on the protein sequence (see appendix).

The nucleotide sequence of D0 was incomplete. In the middle of the sequence existed a gap that was not sequenced.

**DNA extraction**

The nucleotide sequence of D0 was incomplete. Thus, in a first step, DNA of *N. vitripennis* had to be extracted to resequence the D0 gene. Ninety *N. vitripennis* males were homogenized in 1 ml homogenization buffer by using a pestle and afterwards heated for 30 min at 65°C. Subsequently, 150 µl of 8M potassium acetate was added and the extract was chilled for 45 min on ice. Afterwards, the sample was centrifuged twice for 10 min at 14000 rpm and the supernatant was transferred into a new container.

For DNA precipitation, 1 ml of 100% ethanol (EtOH) pro analysis (p.a.) was added to the supernatant, mixed and chilled on ice for 10 min. After centrifugation for 10 min at 14000 rpm, the supernatant was discarded and the pellet was resuspended in 2 ml 70% EtOH in water. The sample was centrifuged for 2 min at 14000 rpm and, after the supernatant had been discarded, the pellet was dried and resuspended in 50 µl sterile water.

To counteract RNA contamination, 5 µl of RNAse (Invitrogen™) (100 µg/µl) was added for 1 h at 37°C. To stop the reaction, 25 µl of 3M sodium acetate and 125 µl sterile water were added. The DNA was gently shaken for 10 min in 200 µl phenol (100%) and centrifuged for 5 min at 14000 rpm. The upper phase was again gently shaken in 200 µl chloroform:isopentanol (24:1) and centrifuged in the same manner as before. Then, 500 µl EtOH (100%) was added to the upper phase and chilled for at least 2 h at -20°C. The sample was centrifuged for 5 min at 14000 rpm and the supernatant was discarded. Subsequently, 500 µl 70% EtOH in water was added and the centrifugation step was repeated. After being dried, the pellet was resuspended in 15 µl sterile water.

**Determination of DNA concentration**

The concentration of DNA was quantified by UV spectroscopy at 260 nm (NanoDrop N-1000 spectrophotometer). Additionally, cross-contamination with proteins was ruled out by paying attention that the ratio of the optical densities at wavelengths of 260 nm and 280 nm (OD<sub>260</sub>:OD<sub>280</sub>) was between 1.8 and 2.0.
PCR of D0 to complete DNA sequence

To complete the DNA sequence of D0, primers D0-F1 and D0-R1 were degenerated with complementary sequences to the sequences before and after the gap in the sequence. PCR was carried out in 50 µl reactions with Herculase II fusion DNA polymerase (Stratagene) following the instructions of the manufacturer. The PCR program consisted of an initial denaturing step at 95°C for 3 min, followed by 35 cycles of denaturation at 95°C for 20 s, annealing at 52°C for 30 s and elongation at 72°C for 1 min, completed by a final step at 72°C for 10 min.

Gel electrophoresis

DNA fragments were separated according to their length by using gel electrophoresis. A 1% agarose gel was produced with 3,5 g agarose (Invitrogen™), 350 ml 1x TBE and 35 µl 1x SYBR Safe DNA Gel Stain (Invitrogen™). 5 µl of DNA, mixed with 1 µl 5x DNA loading buffer, were applied together with 10 µl DNA standard (100 bp or 1 kb ladder (New England Biolabs)). The gel was run at 120 V for approximately 45 min with 1x TBE as the electrophoresis buffer. DNA bands were visualized on a UV transilluminator (470 nm).

Purification of the PCR fragment

Two PCR reactions (90 µl) were mixed with an equal volume (90 µl) of IRN buffer and to the mix three volumes (540 µl) of EtOH p.a. were added. The solution was centrifuged 20 min at 13000 rpm and 4°C, the supernatant was discarded and the pellet was resuspended in 150 µl of 70% EtOH and centrifuged again under the previously mentioned conditions. The supernatant was discarded and the pellet was resuspended in 30 µl sterile water.

Determination of DNA concentration, sequencing and ordering

The concentration of DNA was measured and sequenced at a commercial company (GeneArt, Life technologies, Germany). Finally, the genes D0 and D1 were synthesized by the same company and then delivered in a standard vector with an Ampicillin (Amp) resistance. All further steps were performed with D0 and D1 synchronously.
Ordering a positive control

With regard to a positive control, Dr. Xue-Rong Zhou from the Commonwealth Scientific and Industrial Research Organisation Plant Industry kindly provided us the $\Delta^{12}$-desaturase gene from *Acheta domesticus* (AdD12Des) from his study (Zhou et al., 2008); the gene was transformed in a yeast expression vector with uracil (Ura) selection for yeast cells and Amp selection for *E.coli* cells. AdD12Des was processed the same way as D0 and D1 in all further steps.

Transformation in *Escherichia coli* cells

a) Production of competent cells

An overnight culture of *E. coli* XL1-blue (Stratagene) in SOB medium was diluted 1:100 in 400 ml SOB medium and the culture was grown under shaking at 37°C until an optical density of 0.4 - 0.6 was obtained at a wavelength of 600 nm (OD$_{600}$). After being chilled on ice for 15 min, the cells were centrifuged three times for 10 min at 6000 rpm at 4°C and pellets were washed with 400 ml and 200 ml cold sterile water and finally in 10 ml cold 10% glycerin (v/v). After a last centrifugation step (10 min, 5000 rpm, 4°C) pellets were resuspended in 1.5 ml sterile glycerol (10%, v/v) and stored in aliquots of 50 µl at -80°C.

b) Transformation

An aliquot of competent cells (50 µl) was thawed on ice, mixed with 1 ng plasmid DNA (D0, D1 and AdD12Des) and chilled on ice for 5 min. The sample was transferred into a cold 0.2 cm electroporation cuvette and cells were electroporated with a pulse (2.5 kV, <8ms) from a MicroPulser electroporation apparatus (Bio-Rad). 1 ml LB medium was added immediately and the sample was incubated for 60 min at 37°C and 500 rpm in a new reaction tube. Aliquots of 100 µl of the cell suspension were plated on LB+Amp and incubated at 37°C overnight. The rest of the sample was centrifuged for 1 min at 2000 rpm at room temperature; about 90% of the supernatant was discarded except for about 100 µl in which the pellet was resuspended, plated on LB+Amp and incubated at 37°C overnight.
Miniprep for plasmid isolation

A single colony was picked with a sterile inoculation loop and inoculated in 5 ml LB+Amp medium. This culture was grown overnight at 37°C under shaking. The plasmid was isolated with the peqGOLD Plasmid Miniprep I kit following the manufacturer’s protocol.

PCR amplification of the miniprep product

To amplify D0, D1 and AdD12Des, a PCR of 100 ng of the DNA template in 50 µl samples was performed with Herculase II fusion DNA polymerase (Stratagene) as suggested by the manufacturer. Primers containing restriction sites were degenerated for D0 (D0-\textit{Bam} HI-F1 and D0-\textit{Hind} III-R1), D1 (D1-\textit{Sal} I-F1 and D1-\textit{Hind} III-R1) and AdD12Des (AdD12Des-\textit{Bam} HI-F1 and AdD12Des-\textit{Pst} I-R1). The PCR program started with a denaturing step at 95°C for 3 min, followed by 5 cycles of denaturing at 95°C for 20 s, annealing at 54°C for 30 s and elongation at 72°C for 1 min, followed by 30 cycles of denaturing at 95°C for 20 s, annealing at 65°C for 30 s and elongation at 72°C for 1 min. The final step of the program was carried out at 72°C for 10 min. PCR fragments were purified, the DNA concentration was determined and a gel electrophoresis was made with 10 µl of the PCR product.

Restriction digest

To obtain defined DNA fragments for cloning, the DNA had to be digested. Suitable restriction enzymes (D0: \textit{Bam} HI and \textit{Hind} III, D1: \textit{Sal} I and \textit{Hind} III, AdD12Des: \textit{Bam} HI and \textit{Pst} I (all New England Biolabs)) were used to digest DNA fragments at the specific restriction sites (50 µl reactions, 5 µg DNA template, concentrations were used as suggested by the manufacturer of the restriction enzymes). The reaction was allowed to run for 3 h at 37°C. In the same way, the vector YCplac111-pGAL-Nt-FLAG was digested with the same restriction enzyme pairs as those used for AdD12Des, D0 and D1. The vector used for cloning is controlled by a galactose promoter, has a leucin (Leu2) marker for selection and a N-terminal FLAG Tag for recognition by an antibody (see vector map, appendix).

Subsequently, Antarctic phosphatase (New England Biolabs) was added to the vector DNA to avoid re-ligation as suggested by the manufacturer.

5 µl of the restriction digest of D0 and D1 and the complete restriction digest of the vector were loaded on a 1% agarose gel.
DNA purification

The band of interest was cut out of the gel and extracted by the QIAquick gel extraction kit (Qiagen, Hilden, Germany). PCR reactions of AdD12Des, D0 and D1 were purified by a PCR purification kit (Invitrogen™). All pellets were resuspended in 30 µl sterile water.

Ligation of DNA

By a ligation reaction the DNA fragments were connected with the vector via their sticky ends, as generated by the restriction enzymes. DNA was ligated at a ratio of vector:insert of 1:5 and a T4 DNA ligase (New England Biolabs) was used as suggested by the manufacturer. The reaction volume was 10 µl. The reaction was carried out overnight at 16°C.

Transformation in E. coli, restriction test and sequencing

2 µl of the ligation product (D0, D1 and AdD12Des) were added to aliquots of competent E. coli cells (see above) and the whole sample was plated on LB-Amp plates.

Positive clones were determined by restriction digest in 10 µl reactions. Clones were sent for sequencing.

Transformation in competent yeast cells

a) Production of competent cells

A 50 ml overnight culture of the yeast strain BY4741 (Euroscarf) in YPD medium was centrifuged twice for 5 min at 4000 rpm at room temperature and the pellet was washed with 25 ml sterile water and 5 ml LitSorb. Afterwards, the pellet was resuspended in 360 µl LitSorb. Finally, 40 µl of carrier DNA (salmon sperm DNA, 10 mg ml⁻¹, Invitrogen™) was added. The sample was mixed and stored in aliquots of 50 µl at -80°C.

b) Transformation

200 ng of plasmid DNA (D0, D1 and AdD12Des) were added to a 50 µl aliquot competent cells. Then, 306 µl (equivalent to 6 volumes of the sample) of LitPEG were added and samples were mixed by rotating for 30 min on a turning wheel. After the addition of 39.6 µl
(equivalent to 1/9 of the current volume) dimethylsulfoxide (DMSO), samples were exposed to a heat-shock at 42°C for 15 min at 400 rpm. Cells were centrifuged for 1 min at 3500 rpm and the supernatant was discarded, except for about 100 µl in which the cellular pellet was resuspended, plated on SDC-Leu plates and incubated at 30°C overnight.

**Denaturing protein extraction**

Single colonies on the plates were picked with a sterile inoculation loop and placed in 5 ml SGC-Leu medium. The liquid colony was grown overnight at 30°C under constant shaking. Samples of 1.5 ml were centrifuged for 3 min at 13000rpm and 4°C and pellets were resuspended in 1 ml cold sterile water. Subsequently, 150 µl pre-treatment solution (1 M ß-mercaptoethanol, 1.85 M sodium hydroxide (NaOH)) was added, mixed and chilled on ice for 15 min. Proteins were precipitated by adding of 150 µl 55% trichloroacetic acid (TCA). After centrifugation for 10 min at 4°C and 13000 rpm, the supernatant was discarded and the pellet was resuspended in 15 µl HU buffer. The pH of the sample was neutralized by NH₃ gas. The sample was incubated for 10 min at 65°C for protein denaturation.

**SDS-polyacrylamide gel electrophoresis (SDS-PAGE)**

Proteins were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Two gels are used for SDS-PAGE: an upper stacking gel (325 µl bisacrylamide (BisAA) stock solution, 625 µl Upper Tris, 1.55 ml H₂O, 25 µl APS, 3 µl tetramethylethylenediamine (Temed)) and a lower separating gel (2 ml BisAA stock solution, 1.25 ml Lower Tris, 1.75 ml H₂O, 35 µl APS, 5 µl Temed). The gels were loaded with 15 µl of the samples and 10 µl protein marker (New England Biolabs) of known molecular weight and run at 180 V for an hour. Proteins move differently through the gel matrix depending on the molecular weight of the proteins.

**Western Blot**

Two stacks of three blotting papers (Millipore) were moistened with blotting buffer. Between the stacks of paper, a polyvinylidene difluoride (PVDF) membrane (Millipore) was laid, moistened with methanol and subsequently blotting buffer and covered with the gel moistened with blotting buffer. Separated proteins were blotted onto the membrane by connecting the equipment to a power source set at 24 V for an hour.
Detection of proteins by chemiluminescence

After blotting, the membrane was transferred for 1 h into 5% milk powder (Sukofin) in 1x phosphate-buffered saline (PBS). The non-related proteins from the milk blocked the membrane and so unspecific binding of the antibodies was avoided. Afterwards, the membrane was washed in 1x PBST (PBS plus Tween 20) and incubated with the first antibody (Sigma) targeting the Nt-FlaqTag, in 5% milk powder in 1x PBST for 1 h under rotation. The membrane was washed in 1x PBST for 10 min, followed by incubation with the relevant second peroxidase-conjugated antibody (Jackson IR/Dianova) in 5% milk powder in 1x PBST for 30 min. The membrane was washed again in 1x PBST.

Finally, 2 ml of BM Chemiluminescence Western Blotting Substrate (Roche) was applied to the membrane to make the specifically bound antibodies visible. The Blotting Substrate includes luminol and hydrogen peroxide, a substrate to the peroxidase of the second antibody. Therefore, the specific proteins could be detected with a LAS-3000 chemiluminescence imager (Fujifilm).

Liquid cultures for analysis of enzyme activity

Four different analyses of enzyme activity were performed. An incubator (Memmert) was used for optimal growth conditions at a temperature of 30°C and shaking. Cultures of the yeast strains D0, D1, AdD12Des and the vector YCplac111-pGAL-Nt-FLAG were grown to stationary phase overnight in minimal medium SCG-Leu. From these pre-cultures, fresh cultures were inoculated to an OD\textsubscript{600} of 0.1.

(a) 25 ml yeast cultures were grown to an OD\textsubscript{600} of 0.8 in yeast minimal medium in the presence of 10% NP-40 as an emulsifying agent at 30°C under shaking. OA was prepared as a 25 mM stock in ethanol and added as a precursor so that the final concentration was 0.5 mM. The cultures were grown at 30°C for 3 days under shaking. Yeast cells were harvested by repeated centrifugation and the pellet was washed first with 1% NP-40, then with 0.5% NP-40 and finally twice with water. Afterwards, the pellet was dried for 2 h in a DNA SpeedVac®.

(b) 25 ml yeast cultures were grown at 30°C for 3 days under shaking. No NP-40 or fatty acids as precursors were added. Yeast cells were harvested by repeated centrifugation and the pellet was washed twice with water. Afterwards, the pellet was dried for 2 h in a DNA SpeedVac®.
(c) 10 ml yeast cultures containing D0 and 10 ml yeast cultures containing only the vector as a control were inoculated to an OD$_{600}$ of 0.2. Stearic acid was prepared as a 25 mM stock in ethanol and was added as a precursor so that its final concentration was 0.5 mM. Yeast cells were grown for 3 days in yeast minimal medium in the presence of 10% NP-40 at 30°C under shaking. Yeast cells were harvested by repeated centrifugation and washed first with 1% NP-40, then 0.5% NP-40 and finally twice with water. Afterwards, the pellet was dried for 2 h in a DNA SpeedVac®.

(d) 4ml yeast cultures containing D0 and 4 ml yeast cultures containing only the vector as the control were inoculated to an OD$_{600}$ of 0.2. $d_5$-labelled palmitic acid (C16:0, 15,15,16,16,16) was prepared as a 25 mM stock in ethanol and was added as a precursor so that the final concentration was 0.5 mM. Yeast cells were grown for 3 days in minimal medium at 30°C under shaking. Yeast cells were harvested by repeated centrifugation and washed twice with water. Afterwards, the pellet was dried for 2 h in a DNA SpeedVac®.

Fatty acid analysis

The fatty acid composition of the yeast pellets of the liquid cultures from chapter 2.24 were analysed by coupled gas chromatography-mass spectrometry (GC-MS) of the FAMEs (n=7) of the yeast strains D0, D1, AdD12Des and the empty vector. Pellets were cut to extremely small pieces by using a scoop and extracted by sonification for 30 min in 400 µl dichlormethane containing heptadecanoic acid as the internal standard (10 ng/µl). Afterwards, the extract was transferred to a new vial and concentrated under a gentle stream of nitrogen to about 5 µl. Then, 300 µl methanol and 30 µl acetyl chloride (10% dissolved in methanol) were added and transesterification was carried out for 4 h at 70°C. Subsequently, 400 ml of sodium hydrogen carbonate (5%, dissolved in sterile water) was added and the FAMEs were extracted under shaking with 200 µl pentane. For analysis, a Shimadzu QP2010 Plus GC-MS system equipped with a BPX5 capillary column (30 m x 0.32 mm inner diameter, 0.25 mm film thickness, SGE Analytical Science Europe, Milton Keynes, UK) was used. Helium served as carrier gas at a constant flow rate of 2 ml/min and the MS was operated in the electron ionization (EI) mode at 70 eV. Samples were injected splitless at 300°C by using an AOC 20i auto sampler. The initial oven temperature of 50°C was held for 5 min and then increased at 3°C min$^{-1}$ to 280°C, which was held for 15 min. Identification of FAMEs was performed by analysing reference fatty acids (Sigma-Aldrich, Deisenhofen, Germany) that were transesterified under the same conditions.
The position of the double bonds was determined with a dimethyl disulfide (DMDS) derivatisation followed by GC-MS analysis (Vincenti et al., 1987). After the transesterification, samples were concentrated under a gentle stream of nitrogen. Then, 100 µl of 0.1 M iodine in tert butyl methyl ether and 50 µl DMDS were added and incubated overnight at room temperature. Afterwards, 150 µl sodium thiosulfate and 250 µl sodium hydrogen carbonate were added and FAMEs were extracted under shaking with 200 µl pentane. The sample was concentrated under a gentle stream of nitrogen to 100 µl and injected into the Shimadzu QP2010 Plus GC-MS system equipped with the same column and the same temperature program but with a solvent delay of 30 min.

5.4. RESULTS

DNA extraction and PCR of D0

DNA extraction gave us two samples with a DNA concentration of 866 ng/µl and 1161 ng/µl, respectively. The latter was chosen for PCR. Figure 5.1 shows a DNA fragment of D0, incorporating the gap after PCR. On sequencing, we detected that the gap consisted of 136 bp.

Translating the gene into amino acids, the open reading frame (ORF), i.e. the part of a gene that actually encodes the protein, was interrupted, so we supposed that an insert was part of the gap. Blasting the protein with other desaturases, we found a high accordance but a disagreement followed some bases before the gap started. The sequence after the gap accorded well with the blasted sequences once again. This was a further hint that an intron lay inside the missing link.
We sent the sequence of D0 to Dr. Oliver Niehuis (Research Museum Koenig, Bonn), one of the members of the *Nasonia* genome consortium, and he confirmed our suspicions with regard to the sequence of the intron. The sequence analysis of D0 is shown in the appendix.

![Figure 5.1: Gel electrophoresis of the DNA fragment of D0 after the polymerase chain reaction (PCR) together with a 1 kb ladder and a 100 bp ladder.](image)

**Transformation in *E. coli* cells, Miniprep for isolation of the plasmid and PCR amplification of the Miniprep product**

After transformation into *E. coli* cells and the following Miniprep, DNA concentrations of the samples were approximately 100 ng/µl. The following PCR amplified the genes D0, D1 (Figure 5.2) and AdD12Des (Figure 5.3). D0 has a fragment length of 1080 bp, D1 has a fragment length of 1125 bp and AdD12Des has a fragment length of 1082 bp.

![5.2: Gel electrophoresis of the PCR of D0 and D1 together with a 1kb and a 100bp ladder.](image)  
![5.3: Gel electrophoresis of the PCR of AdD12Des together with a 1kb and a 100bp ladder.](image)
Identification of positive clones with restriction digestion

After miniprep, plasmid DNA was digested to determine positive clones. Suitable clones could be digested into fragments of 1080 bp (D0), 1125 bp (D1) (Figure 5.4) and 1082 bp (AdD12Des) (Figure 5.5). Positive clones were affirmed by sequencing.

![Restriction digestion of plasmid DNA of positive clones resulted in fragments of D0 and D1. Water samples were used as negative controls. A 1 kb ladder was used as a length marker. Arrows indicate positive clones.](image)

Figure 5.4: Restriction digestion of plasmid DNA of positive clones resulted in fragments of D0 and D1. Water samples were used as negative controls. A 1 kb ladder was used as a length marker. Arrows indicate positive clones.

![Restriction digestion of plasmid DNA of positive clones resulted in fragments of AdD12Des. A 1 kb ladder and a 100 bp ladder were used as length markers. Arrows indicate positive clones.](image)

Figure 5.5: Restriction digestion of plasmid DNA of positive clones resulted in fragments of AdD12Des. A 1 kb ladder and a 100 bp ladder were used as length markers. Arrows indicate positive clones.

Transformation in competent yeast cells and Western blot

After the transformation in competent yeast cells positive clones were identified by Western blot. The size of the protein encoded by D0 was 41.5 kDa, of the protein encoded by D1 was 42.9 kDa (Figure 5.6) and of the protein encoded by AdD12Des was 41.2 kDa (Figure 5.7). Two positive clones of D0 and D1 and one of AdD12Des were chosen for further work.
Figure 5.6: Western blot of proteins encoded by the vector Ycplac-111-pGal-Nt-Flag as negative control and encoded by the predicted $\Delta 12$-desaturase genes D0 and D1. Specific proteins are detected by chemiluminescence. Arrows show chosen clones for further work.

Figure 5.7: Western blot of proteins encoded by AdD12Des. Specific proteins are detected by chemiluminescence. Arrow shows chosen clone for further work.

**Fatty acid analysis**

The application of OA as a precursor with NP-40 did not differ from the application without NP-40. GC-MS analysis demonstrated that yeast cells containing only the vector produce mainly the fatty acids C16:1$^{\Delta 7}$, C16:1$^{\Delta 5}$, palmitic acid (C16:0), OA (C18:1$^{\Delta 9}$), C18:1$^{\Delta 7}$ and stearic acid (C18:0) (Figure 5.8a). Expression of AdD12Des in yeast cells produced a new fatty acid LA (C18:2$^{\Delta 9,12}$) (Figure 5.8d), confirming the result of Zhou et al., 2008. Expression of D0 (Figure 5.8b) and D1 (Figure 5.8c) in yeast cells produced no new fatty acid in comparison with yeast cells containing only the vector.
Conspicuously, the expression of D0 in yeast cells led to a decrease of C18:0 and C16:1$^{\Delta 7}$ and to an increase of C18:1$^{\Delta 9}$ (Figure 5.8b). Therefore, two new hypotheses arose. D0 could function as a $\Delta 9$-desaturase and was able to convert the fatty acid C18:0 to C18:1$^{\Delta 9}$ by inserting a double bond at the $\Delta 9$ position or as elongase, able to convert the fatty acid C16:1$^{\Delta 7}$ to C18:1$^{\Delta 9}$. Following experiments were performed to test the two hypotheses.

Figure 5.8: Cut–out of the total ion current (TIC) chromatograms of fatty acid methyl esters extracted from a) yeast cells containing only the vector, b) yeast cells containing D0, c) yeast cells containing D1, d) yeast cells containing AdD12Des.

Figure 5.9: Cut–out of the total ion current (TIC) chromatogram of fatty acid methyl esters after the addition of labelled C18:0, extracted from a) yeast cells containing only the vector, b) yeast cells containing D0. Mass traces belong to m/z 316 and 314 (13C-labelled stearic acid methyl ester and 13C-labelled oleic methyl ester).
The chromatograms (Figure 5.9) show the $^{13}$C-labelled stearic acid (C18:0) methyl ester applied to the yeast cells, with the mass trace at m/z 316 (the molecular mass of $^{13}$C-labelled stearic acid methyl ester) showing a peak at the expected retention time of stearic acid (Figure 5.9). A $\Delta$9-desaturase would convert $^{13}$C-labelled stearic acid (C18:0) into C18:1$\Delta$9. However, the mass trace at m/z 314 (the molecular mass of $^{13}$C-labelled OA methyl ester) has no peak at the expected retention time, either in the yeast culture containing only the vector or in the yeast culture containing D0.

Figure 5.10: Cut–out of the total ion current (TIC) chromatogram of fatty acid methyl esters after the addition of labelled C16:0, extracted from a) yeast cells containing only the vector, b) yeast cells containing D0. Mass traces belong to m/z 275, 273 and 301 ($d_5$-labelled C16:0, $d_5$-labelled C16:1$\Delta$7 methyl ester and $d_5$-labelled C18:1$\Delta$9).

The molecular mass 275 of $d_5$-labelled palmitic acid (C16:0) methyl ester applied to the yeast cultures is visible in the chromatograms (Figure 5.10). The labelled masses occurred at an earlier retention time than the unlabelled masses. Palmitic acid (C16:0) was converted by the yeast cells into C16:1$\Delta$7, as demonstrated by the occurrence of the molecular mass of labelled C16:1$\Delta$7 methyl ester 273 (268+5) (5.10a). An elongase would convert C16:1$\Delta$7 into OA (C18:1$\Delta$9), detectable by the molecular mass 301 (296+5) of labelled OA (C18:1$\Delta$9). The mass trace at m/z 301 was faintly visible in the chromatogram of yeast cells containing D0 and in the chromatogram of yeast cells containing only the vector (Figure 5.10).
5.5. DISCUSSION

OA is converted into LA by inserting a double bound at the $\Delta 12$ position of OA by a $\Delta 12$-desaturase (Sperling, 2003). The enzyme activity of a $\Delta 12$-desaturase in cells is detectable by GC-MS analysis by a new appearance of LA or by an increase of LA, respectively (see for instance Zhou et al., 2008). In our experiments LA was detectable by GC-MS analysis only in yeast cells containing AdD12Des and there was no LA detectable in yeast cells containing D0 or D1.

Expression of desaturases can be related to different aspects, e.g. to the amount of substrate and a nutrition that is high in fatty acids can increase the activity of desaturases in mammals (Steffen et al., 2008; Ntambi et al., 2002). If this phenomenon is transferable in yeast and if the hypothesis that D0 and D1 are $\Delta 12$-desaturase genes is correct, adding higher concentrated amounts of precursor fatty acids to the yeast cells would be a further possible experiment to intensify the $\Delta 12$-desaturase activity of the genes.

Two different groups of fatty acid desaturases exist. A minor group consists of soluble desaturases that identify fatty acids esterified with an acyl carrier protein (Vanhercke et al., 2011) and has been found so far only in plants (Sperling, 2003). A major group consists of integral membrane proteins identifying acyl chains of fatty acids esterified with either coenzyme A (CoA) or phospholipids. The $\Delta 12$-desaturase of *Acheta domesticus* (AdD12Des) might also be an acyl-CoA desaturase (Zhou et al., 2008) like the $\Delta 9$-desaturase of the same species (Riddervold et al., 2002) or desaturases in the European corn borer moth (Xue et al., 2012). It is likely that CoA is also necessary for the desaturase activity of D0 and D1. To be sure that CoA is really available for D0 and D1 we added oleyl-CoA instead of OA to yeast cells containing D0 and D1 (data not shown). But GC-MS analysis did not reveal the presence of LA in the transformed yeast cells. No new fatty acid was detectable in comparison to yeast cells containing the empty vector.

By generating a growth curve of yeast cells containing D0 and D1, we were able to test transformed yeast cells in both stationary and logarithmic growth phase for their ability to synthesise LA. Therefore we could exclude possible effects of desaturase activity concerning the growth phase of the yeast cells (Ferrante et al., 1983). Ferrante et al. (1983) revealed that the growth phase and the growth temperature of yeast cells can have a strong influence on desaturase activity. Therefore it might be a promising experiment to start test series with different growth temperature of yeast cells containing D0 and D1. Furthermore another yeast strain could be used for cloning of D0 and D1.
The results of all experiments performed in this study did not support the assumption that the two genes D0 and D1 code for \( \Delta 12 \)-desaturases, because there was no increase of LA detectable in the fatty acid composition of transformed yeast cells. D0 and D1 are the genes with the highest sequence homology to desaturase genes of other insects. Niehuis et al., (2011) identified 16 putative desaturase genes of *Nasonia* by sequence alignment with desaturase genes of *Drosophila melanogaster*. Therefore putative desaturase genes remain and might be tested in the same manner like D0 and D1 to for \( \Delta 12 \)-desaturase activity.

We put the focus on identifying the role of D0 and D1. The fatty acid composition of yeast cells containing D1 did not differ substantially from the fatty acid composition of yeast cells containing only the vector, so we are not able to conclude anything on the function of D1. Expression of D0 in yeast cells leads to a decrease of C16:1\( \Delta 7 \) and of stearic acid (C18:0) and to an increase of OA (C18:1\( \Delta 9 \)) (Figure 5.8b). The decrease of C18:0 combined with the increase of C18:1\( \Delta 9 \) could be a sign for a \( \Delta 9 \)-desaturase activity.

In contrast to the \( \Delta 12 \)-desaturases, \( \Delta 9 \)-desaturases are known as abundant enzymes in animals (Knipple & Roelofs, 2003; Howard & Blomquist, 2005). \( \Delta 9 \)-Desaturases are known from plants as well as from animals (see for instance Ridderwald et al., 2002; Boersma et al., 2012; Matoušková et al., 2008). \( \Delta 9 \)-desaturases insert a double bond at the \( \Delta 9 \) position of fatty acids and thereby C18:0 is converted to C18:1\( \Delta 9 \) and palmitic acid (C16:0) to palmitoleic acid (C16:1\( \Delta 9 \)). C16:1\( \Delta 9 \) was neither detectable in the fatty acid composition of yeast cells containing only the vector nor in the fatty acid composition of yeast cells containing D0. However, desaturases can be substrate specific (Sperling, 2003). The \( \Delta 9 \)-desaturase of *Mortierella alpine* showed only a low activity with C16:0 as substrate, but greater activity with C18:0 as substrate (MacKenzie et al., 2002). Similar preferences were also found in \( \Delta 9 \)-desaturases of insects. Matoušková et al. (2008) cloned a \( \Delta 9 \)-desaturase gene from *Bombus lucorum* and expressed the desaturase in yeast cells. By subsequent GC-MS analysis a preference of the \( \Delta 9 \)-desaturase from *B. lucorum* for C18:0 was detected. For yeast cells containing D0, a \( \Delta 9 \)-desaturase with the same preference for C18:0 seemed possible, because a clear increase of C18:1\( \Delta 9 \) but no new appearance of C16:1\( \Delta 9 \) was detectable by GC-MS analysis. By an activity of a \( \Delta 9 \)-desaturase, C18:0 could act as a precursor for C18:1\( \Delta 9 \) in the HDL biosynthesis of *Nasonia*. We made a first test of this new hypothesis by applying \( ^{13} \)C-labelled C18:0 to the yeast cells containing D0, expecting to detect \( ^{13} \)C-labelled C18:1\( \Delta 9 \) in the GC-MS analysis. But no \( ^{13} \)C-labelled C18:1\( \Delta 9 \) occurred in yeast cells containing D0 (Figure 5.9). In yeast cells containing only the vector also no labelled C18:1\( \Delta 9 \) occurred and the new question arose how C18:1\( \Delta 9 \) from the yeast cells is synthesized if not using C18:0 as precursor. It could be that C18:0 as free fatty acid maybe was no adequate
substrate for feeding yeast cells containing D0, the suspected Δ9-desaturase, because desaturases use esterified fatty acids as substrate. However a previous experiment, feeding hosts of *N. vitripennis* with 13C-labelled C18:0 revealed any Δ9-desaturase activity in *Nasonia*. Doubts regarding the assumed Δ9-desaturase arise.

Another possibility is that *Nasonia* might have lost their Δ9-desaturase activity in favour of the Δ12-desaturase. The need of C18:1Δ9 synthesis is less important for *Nasonia* than LA synthesis, because C18:1Δ9 can be taken by nutrition in high amounts and LA is highly important for HDL synthesis and finally the mating success in *Nasonia* males.

Liu et al. (1999) cloned a predicted Δ9-desaturase of the cabbage looper moth *Trichoplusia ni* into desaturase deficient yeast cells (L8-14C) which depend on unsaturated fatty acids (UFA) in the medium for growing. After successful cloning of the predicted Δ9-desaturase in the yeast cells the auxotrophy was stopped and cells grew well in YPD medium without applied UFAs, because now UFAs were produced by the desaturase. In this manner Liu (1999) could demonstrate the Δ9-desaturase activity in *Trichoplusia ni*. Thus, a further experiment should be to clone D0 the same way into desaturase deficient yeast cells and to record if transformed cells are able to grow without UFAs applied.

Another hypothesis explaining the results of the GC-MS analyses of D0 transformed in yeast cells (i.e., an increase of C18:1Δ9 and a decrease of C16:1Δ7) was that D0 has elongase activity. Elongases catalyze an extension of fatty acids by two methylene units. In pheromone synthesis C18 fatty acids often occur together with C16 fatty acids as precursors (Vanderwel et al. 1990; Knipple & Roelofs, 2003) and C16 fatty acids might function as further precursors for HDL synthesis. If D0 functions as an elongase, yeast cells containing D0 should be additionally able to produce C18:1Δ9 from C16:1Δ7 by lengthening the hydrocarbon chain. After adding labelled C16:0, GC-MS analysis showed that C18:1Δ9 was detectable in yeast cells containing D0, but in equal quantities in yeast cells containing the empty vector as well (Figure 5.10). Therefore the yeast cells seem to have an own elongase and are able to convert C16:1Δ7, synthesized from the labelled C16:0, into C18:1Δ9 themselves without the catalysis of D0 and therefore an elongase activity cannot be proved. If D0 functions as elongase, at least C18:1Δ9 of cells containing D0 should increase quantitatively more than C18:1Δ9 in yeast cells containing the empty vector. Elongase activity can be difficult to prove, because of their frequency in different eukaryotic organisms. Additionally the primary products of fatty acid biosynthesis in most organisms are 16- and 18-carbon fatty acids (Uemura, 2012), just as with this study.

In conclusion the successful cloning of the candidate genes was controlled in several steps during the work. The proteins were expressed successfully in the assays as we could see by
the \( \Delta 12 \)-desaturase activity of AdD12Des. However we could not demonstrate a \( \Delta 12 \)-
desaturase activity of the candidate genes. But our analyses suggest that D0 has a function in
the fatty acid metabolism. Further experiments, like those mentioned before, are
necessary for functional characterization of D0. More putative desaturases identified of
Niehuis et al. (2011) should be tested for \( \Delta 12 \)-desaturase activity.
5.6. APPENDIX:

Primer

<table>
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<tr>
<th>Name</th>
<th>Sequence from 5' to 3'</th>
<th>Function</th>
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</tr>
<tr>
<td>D0-R1</td>
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<td>Primer (reverse) for obtaining the amplicon of the missing sequence of D0</td>
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<td>Primer (forward) used for cloning of D0</td>
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</tr>
<tr>
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<td>Primer (reverse) used for cloning of D0</td>
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Media

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<td>LB-amp (luria broth plus ampicillin)</td>
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<tr>
<td>SCD-leu (SCD minus leucine)</td>
<td>2% (w/v) glucose 0.67% (w/v) YNB + nitrogen 0.062% (w/v) CSM-his-leu-trp + 20 mg/l L-histidine + 50 mg/l L-tryptophan</td>
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<tr>
<td>SCG-leu (synthetic complete galactose minus leucine)</td>
<td>2% (w/v) galactose 0.67% (w/v) YNB + nitrogen 0.062% (w/v) CSM-his-leu-trp + 20 mg/l L-histidine + 50 mg/l L-tryptophan</td>
</tr>
<tr>
<td>SOB (super optimal broth)</td>
<td>2% (w/v) tryptone 0.5% (w/v) yeast extract 2.03 g/l MgCl2 · 6 H2O 0.5 g/l NaCl 0.19 g/l KCl pH 7.0 with NaOH</td>
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<tr>
<td>YPD (yeast extract, peptone, dextrose)</td>
<td>1% (w/v) yeast extract 2% (w/v) peptone 2% (w/v) glucose</td>
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## Buffer (solvent: H₂O or listed otherwise)

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<td>0.01% Xylene cyanol</td>
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DNA sequence of D0

DNA sequence of D0. The newly sequenced part is marked in red bold letters, introns appear in green. Black bold sequence has been used for primer design for amplify the missing link of D0. Yellow marked sequence has been used for primer design for cloning of D0.

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  ggtgtcctct tcgaggacga aatggtggag

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121  ctccagatag tctggaggaa cgtcacgatc ttcgtctttc tccacatagg cgcgctctac
181  ggagtctacgcgtcctacac gtcggccaag atagtcacga ccgttacgcg ttcgtctttc
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301  atagactcgt ggccgacagg cgtctcagaa cacccggcgc actacgacat ttgctcttgc
361  ggcggacgct cctctgctttc agccttcctc ctctatcagc tcagcggctt cggaattacc gcgggcgcgc
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DNA sequence of D1
Yellow marked sequence has been used for primer design for cloning of D1.

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601  cgaacggagg accgaagaag ggcaagaaac ttgaagaacc ctaccggctc gaaattgtat
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901  agtgggcgcg cgaccacagc gacgctctcg cggatcagcc cggcgacgac
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1081  gccagcggac gtcgagccag ttgctgctgg gcgcgatcgg ccggttgcgg ctgctgtttg
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DNA sequence of AdD12Des
Yellow marked sequence has been used for primer design for cloning of AdD12Des.

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Vector map of Ycplac111-pGAL-Flag-RC with gal promoter elements, Nt Flag Tag, Amp resistance and Leu marker. PUC ORI and ARS indicate origins of replication for E. coli and yeast cells and CEN4 indicates the centromere of yeast chromosome 4. Restriction sites used for cloning are indicated.

**Nucleotid BLAST of desaturase genes**

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<tr>
<td>delta-11_Des.</td>
<td>HIASY</td>
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82
Chapter 5 - \( \Delta ^{12}\)-Desaturase

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CHAPTER 6

Body size influences male pheromone signals but not the outcome of mating contests in *Nasonia vitripennis*

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6.1 ABSTRACT

Adult body size may reflect the availability of nutrients during the development of insects and often covaries with mate quality parameters influencing the outcome of both inter- and intrasexual selection. But large males are not necessarily advantaged in contests for mating partners, because smaller competitors may compensate for lesser physical strength by an increased mating agility. In mating systems involving sexual signalling before mating, body size might be a crucial parameter given that the signal strength depends on the availability of limited nutritional resources. In the parasitic wasp *Nasonia vitripennis*, the male sex pheromone is produced from the polyunsaturated fatty acid linoleic acid. Here we tested the influence of body size on male sex pheromone titres and the outcome of male-male mating contests. We found that large males had significantly higher pheromone titres and released the sex attractant in higher amounts than small males. In an open arena bioassay, virgin females preferred pheromone doses adapted to the amounts released by large males. When directly competing for a female, however, large and small males had the same mating success. Hence, small *N. vitripennis* males, albeit equally successful in direct competition, are likely to be disadvantaged by being less attractive from a distance.
6.2 INTRODUCTION

Sexual selection theory predicts that animals do not mate haphazardly because potential mating partners may vary in their individual quality (Bateson, 1983; Andersson, 1994). Nonrandom mating may be mediated by intrasexual selection when members of one sex (typically the males) compete directly for mating opportunities. In intrasexual selection, one sex (typically but not exclusively the females) choose mates of sufficient quality. Intra and intersexual selection may operate simultaneously but do not necessarily act in the same direction. Hence, male traits beneficial in malemale competition and those preferably chosen by a female are not inevitably identical (Moore, 1990; Moore & Moore, 1999).

According to Halliday (1983), mate choice is defined as ‘any pattern of behaviour, shown by members of one sex, that leads to their being more likely to mate with certain members of the opposite sex than with others’. Mate choice can be active, if the female discriminates between attributes of individual males and rejects those of insufficient quality. But there are also passive processes that restrict a set of potential mates. According to Parker (1983), ‘active mate choice’ has to be distinguished from ‘passive attraction’ where females find males by attraction to male cues, resulting in male phenotypes emitting the strongest stimulus having better mating chances than their competitors.

Males may advertise their sex and quality to females by visual ornaments, courtship songs or behavioural displays but also by sexual signals invisible for the human observer, via the olfactory sense (Wyatt, 2003). Like visible or audible sexual signals, the sex pheromones involved in this process may not only reveal the presence of a potential mate but also mediate honest information about its individual quality, for instance, if signal strength depends on the availability of limited nutritional resources or covaries with other quality parameters (Johansson & Jones, 2007).

One trait that may influence both intra- and intersexual selection is male body size. The size of a male is often dependent on condition and may affect particular elements of the courtship behaviour (Pellitteri-Rosa et al., 2011; Taylor et al., 2011), such as his ability to establish a territory or to release acoustic (Wong & Candolin, 2005) or chemical (Conner et al., 1990) signals. Larger males are often considered superior to smaller ones with respect to nutritional status, longevity, gamete size and fecundity (Durocher-Granger et al., 2011). Thus, in intersexual selection females often show a preference for larger males over smaller ones, to benefit from these advantages (e.g. Savalli & Fox, 1998; Ritz & Köhler, 2010). Additionally, large males are often more competitive in intrasexual selection because of their superior physical strength (Andersson, 1994). However, a benefit of larger males in male-male
contests cannot be concluded a priori, because small males may compensate for inferior physical strength by being more vigorous when searching for mates (Andersson, 1994) and more agile during courtship (Andersson & Norberg, 1981; Steele & Partridge, 1988). Therefore, under certain circumstances small males may have even better mating opportunities than larger ones.

*Nasonia vitripennis* is a pupal parasitoid of several fly species. The mating system of this parasitic wasp is characterized by local mate competition (LMC; Hamilton, 1967; Werren, 1983); that is, hosts are patchily distributed and the brachypterous males are flightless. Therefore, mating occurs typically immediately after emergence at the natal site. Depending on the number of foundresses having colonized a patch, the offspring sex ratio varies. Single females produce clearly female-biased offspring sex ratios, but with increasing female number, the proportion of males increases (Werren, 1983). Therefore, subject to the foundress number at a given patch, mating partners within the next generation can be, but are not necessarily, siblings (Grillenberger et al., 2008).

Males of *N. vitripennis* are protandrous and compete with each other for females emerging shortly after them. Mating occurs after a stereotypic courtship sequence during which an oral aphrodisiac is released by the male to the female antenna and elicits female receptivity (van den Assem et al., 1980b; Ruther et al., 2011). It has been reported that large *N. vitripennis* males defend the exit holes of host puparia aggressively and try to monopolize the emerging virgin females (van den Assem & Vernel, 1979; Leonard & Boake, 2006). However, females often emerge from a host puparium in rapid succession and thus may not be inseminated immediately by the dominant male (van den Assem et al., 1980a). At this point, a long ignored component of the *N. vitripennis* mating system comes into play, i.e. a male-derived sex attractant (Ruther et al., 2007, 2008). As females typically mate only once with the first male they encounter (van den Assem, 1986), it is crucial for a courting dominant male to get the attention of other virgins in his vicinity. Subordinates may engage in scramble competition (van den Assem, 1986) or wait as satellite males in the vicinity and lure the virgins away from the dominant competitor.

For the attraction of females, *N. vitripennis* males produce a sex pheromone in their abdomen that is released to the substrate via the anal orifice by dabbing movements of the abdominal tip. Only virgin females respond to the pheromone (Ruther et al., 2007; Steiner & Ruther, 2009a). The range of activity is approximately 5 cm (Steiner & Ruther, 2009b) and thus matches well the dimensions of a bird’s nesting hole, which is a typical *Nasonia* habitat in nature. Increased marking activity is shown by males directly after mating or after mere contact with a female (Steiner & Ruther, 2009b). After marking, males stay at their
pheromone deposits and wait for females (Ruther et al., 2011). Hence, the intensity of the pheromone signal might be crucial for male mating success, as there should be some kind of sexual advertisement scramble competition (in sensu Parker, 1983) between the different male pheromone markings. In fact, virgin females have been shown to orient along concentration gradients of the male pheromone and typically prefer the higher dose (Ruther et al., 2009). Therefore, males able to produce higher pheromone amounts should have better mating chances. The sex attractant of *N. vitripennis* consists of a mixture of (4R,5S)- and (4R,5R)-5-hydroxy-4-decanolides (HDL) and 4-methylquinazoline (4-MeQ) as a synergizing minor component (Ruther et al., 2007, 2008). HDL biosynthesis starts from the precursor linoleic acid (LA; Blaul & Ruther, 2011), a polyunsaturated fatty acid essential for most animals (Wathes et al., 2007 and references therein; but see Borgeson et al., 1991; Weinert et al., 1993). Pheromone titres of *N. vitripennis* males correlate with their functional fertility. Hence, females preferring higher pheromone doses over lower ones decrease the risk of mating with sperm-limited males (Ruther et al., 2009). However, it is still unknown whether pheromone titres also correlate with male body size. Body size in *N. vitripennis* depends on the number of parasitoids per host. The more eggs laid into a host, the smaller the emerging parasitoids (Lalonde, 2005), because the available nutrition for the offspring influences the final adult size (Chown & Gaston, 2010).

The reports of males monopolizing the exit holes of host puparia aggressively suggest that large individuals might have better mating chances. However, a previous study addressing the impact of male size on mating success in *N. vitripennis* did not support this assumption (Burton-Chellew et al., 2007). The authors exposed a large male and a small male to 10 females either singly or together and quantified the mating success of either male. No correlation between body size of the male and insemination success was found in these experiments. However, it is an open question whether male body size has an effect in a situation of enforced competition (i.e. when two *N. vitripennis* males of different size compete for a single female at the same time). Males might have the same mating success when they have no contact with each other, but in direct competition one of the males might have clear mating advantages (Nilsson & Nilsson, 2000).

In this study we aimed to investigate the effect of male body size on: (1) courtship behaviour and mating success in a situation with enforced competition; and (2) sex pheromone communication. For this purpose we asked the following questions.

(1) Do large and small males differ in their mating success or in their courtship behaviour?

(2) Is there a correlation between pheromone titres and male body size?
(3) Do large and small males release different amounts of pheromone?

(4) Do virgin females discriminate between pheromone doses released by large and small males, and do they prefer one of them?

6.3 MATERIALS & METHODS

Insects

The *N. vitripennis* strain used was collected in 2004 from bird nests (Parus sp.) in northern Germany shortly after the nestlings had left the nests. Parasitoids were reared on freeze-killed puparia of the greenbottle fly, *Lucilia caesar*, under the conditions described in Steiner et al. (2006). To get unmated males of controlled age for the experiments, parasitoids were excised 1-2 days before emergence from host puparia and kept singly in Eppendorf tubes. To standardize male size, the head widths of fully melanized pupae were measured under a stereomicroscope using a measuring eyepiece. For the experiments, wasps of two size categories were used. Males with a head width ≥700 mm and ≤660 mm were categorized as “large” and “small”, respectively (Ruther et al., 2009).

Male Pheromone Titre

Male pheromone titres were analysed by coupled gas chromatography-mass spectrometry (GC-MS). Two-day-old males of different size and mating histories were killed by freezing and had their abdomen cut off and extracted for 30 min in 25 ml dichloromethane containing 10 ng/ml methyl undecanoate (Sigma-Aldrich, Deisenhofen, Germany) as internal standard. For chemical analysis, a Shimadzu QP2010 Plus GC-MS system was used, equipped with a BPX5 capillary column (30 m x 0.32 mm inner diameter, 0.25 mm film thickness; SGE Analytical Science Europe, Milton Keynes, U.K.). Samples were injected splitless at 300 °C using an AOC 20i auto sampler. Helium was used as carrier gas at a constant flow rate of 2 ml/min. The initial oven temperature of 80 °C was increased at 5 °C/min to 200 °C followed
by 15 °C/min to 280 °C (held for 15 min). The MS was operated in the electron impact (EI) mode at 70 eV.

The amounts of the male pheromone components \((4R,5R)\)- and \((4R,5S)\)-HDL were quantified by an internal standard method as described in detail by Blaul & Ruther (2011). To study size-dependent differences in pheromone amounts and especially in the amounts males release after copulation, four groups of 2-day-old large and small males were compared.

(1) Group 1 contained virgin large (N = 12) and small (N = 9) males.

(2) Group 2 contained large and small (N = 10 each) males that were allowed to copulate with one female; after copulation, the females were removed and the males were allowed to release their pheromone for 10 min in a clean glass dish; immediately afterwards, the remaining pheromone amounts in each abdomen were extracted and quantified as described above.

(3) Group 3 contained large and small (N = 13 each) males that were allowed to copulate with two females; after each copulation, females were removed and males were allowed to release their pheromone for 10 min each before pheromone extraction; a new clean glass dish was used for each mating experiment.

(4) Group 4 contained large (N = 14) and small (N = 12) males that were allowed to copulate with three females, following the procedure described under (3).

Total pheromone amounts of large and small males were compared by a Mann-Whitney U test.

**Female Choice Test**

In this experiment we recorded the response of virgin females to the mean total HDL amounts deposited by a large and a small male, respectively, after the first copulation. We calculated the deposited amounts indirectly by subtracting the mean total amount of HDL remaining after the first mating from the pheromone amount of virgin males. The calculated pheromone amounts were 780 ng for a large male and 260 ng for a small male. The pheromone used for the bioassay was extracted from 2-days-old *N. vitripennis* males to make sure that the synergizing minor component 4-MeQ was also present. For this purpose, two batches of 30 *N. vitripennis* males were freeze-killed, and their abdomen cut off and extracted in 200 µl dichloromethane each. The total HDL amount of the extracts was
determined as described above and subsequently extracts were diluted with dichloromethane to a final total HDL concentration of 780 ng/µl and 260 ng/µl, respectively.

To create a near natural choice situation, we used an observation box made of acrylic glass approximating the size of a bird’s nesting box where *N. vitripennis* can be collected in the field (20 x 20 cm and 9 cm high). One microlitre of each extract was applied to disks of filter paper, which were put in two opposite corners of the box, and a circle of 2 cm was marked around each disk. Virgin females (N = 32) were released singly into the middle of the box and observed for 10 min. We recorded the residence time spent by the female in the two circles using Observer XT 9.0 observational software (Noldus, Wageningen, The Netherlands) and noted additionally which of the two circles was entered first.

The residence time in the two circles was compared using a Wilcoxon signed-ranks test, and the first choice was analysed using a two-sided binomial test available online at http://www.graphpad.com/quickcalcs/binomial1.cfm.

**Courtship Behaviour**

In this experiment, we compared courtship behaviour and mating success of large and small males either singly with a female (no competition) or in a natural competitive situation (i.e. two males competing for one female; competition).

(1) In the no competition test large (N = 23) and small (N = 26) males were released singly with one virgin female into an observation chamber (diameter 1 cm, height 0.3 cm). The courtship sequence of *N. vitripennis* can be subdivided into different periods (Barrass, 1960; van den Assem & Vernel, 1979). The following behavioural elements of the mating sequence were recorded using the Observer XT 9.0 observational software (Noldus, Wageningen, The Netherlands): (a) "searching", defined as the time until the male recognized the female. The moment of recognition can be concluded from the male changing his bearing and immediately starting to follow the female. The next elements recorded were: (b) "following", defined as the time between recognition of the female and mounting; (c) "mounting", defined as the time the male spent on the back of the female; (d) "mating" defined as the copulation duration; and (e) "remounting" defined as the time the male spent on the back of the female after copulation showing postcopulatory behaviour (details as described below), until he walks off. During (c) more complex courtship elements are shown by the male but were not recorded separately: initially, the male drums with his fore tarsi on the female’s eyes and starts a series of vellicating wing movements and head nodding. During head nodding the male extrudes an oral gland and releases an aphrodisiac pheromone (van den Assem et al.,
1980b; Ruther et al., 2010). The female signals receptivity by lowering her head, withdrawing her antennae and opening her genital orifice (Barrass, 1976; van den Assem & Vernel, 1979). Directly after this signal, the male stops courting, backs up and copulates with the female. The duration of the courtship elements of large and small males were compared by a Mann-Whitney U test.

(2) In the competition test, a large and a small male (N =48) were released together in an observation chamber (diameter 1 cm, 0.3 cm height). A virgin female was added and the courtship and mating behaviour of each of the males was recorded with the Observer XT observational software. We recorded for each of the males the same courtship elements as in experiment 1, with one exception. For two males observed simultaneously, it was impossible to discriminate exactly between the “searching” and “following” periods, so we put these two periods together. Additionally we noted which male mounted the female first and which one copulated with the female. The durations of courtship behaviours of large and small males were compared using a Wilcoxon signedranks test; first mounting and copulation were compared using a two-tailed binomial test.

6.4 RESULTS

Male Pheromone Titres

Two-day-old large males of all tested mating histories had significantly more total HDL in their abdomen than the respective group of small males (Fig. 6.1; Mann-Whitney U test: virgin males $U = 7.0$, $N_1 = 9$, $N_2 = 12$, $P < 0.001$; males after one copulation: $U = 15.0$; $N_1 = N_2 = 10$, $P = 0.007$; males after two copulations: $U = 27.5$, $N_1 = N_2 = 13$, $P = 0.002$; males after three copulations: $U = 34.5$, $N_1 =14$, $N_2 = 12$, $P = 0.009$). Under consideration of the total HDL amounts remaining in the males after different numbers of copulations, we were able to assess indirectly the approximate mean amounts of pheromone released by the males. These calculations revealed that large males released three times as much pheromone after the first mating than small males (Fig. 6.1), whereas the deposited doses after two matings were almost equal. After three copulations, however, large males released more than four times as much total HDL than small males again.
Figure 6.1: Mean total amounts of (4R,5R)- and (4R,5S)-5-hydroxy-4-decanolide (HDL) (+ s.e.m.) extracted from the abdomen of large and small males with different mating histories. Numbers next to the columns represent the calculated mean amounts of pheromone deposited by the males between the matings.

Female Choice Test

Virgin females chose significantly more often the calculated pheromone dose of large males after the first mating over the respective dose of small males (Fig. 6.2a; first choice, binomial test, \( P = 0.020 \)). Furthermore, virgin females spent significantly more time within the circle around the paper disk treated with the pheromone dose of a large male (Fig. 6.2b; residence time, Wilcoxon signed-ranks test: \( Z = 2.861, N = 25, P = 0.004 \)). Females that did not decide for any of the pheromone doses were excluded from the statistical analysis.

Figure 6.2: Response of virgin *N. vitripennis* females to calculated pheromone doses of large (780 ng) and small (260 ng) males after the first mating. (a) First choice for females and (b) mean residence time (+ s.e.m.) in the marked circles around the treated paper disks within an observation time of 10 min. Asterisks indicate significant differences at \( P < 0.01 \) (**) and \( P < 0.05 \) (*) (first choice: two-tailed binominal test, residence time: Wilcoxon signed-ranks test).
Courtship Behaviour

(1) In the no competition test, small males recognized the female significantly faster than large males, as indicated by a reduced searching time (Fig. 6.3; Mann-Whitney U test: $U = 165; N1 = 26, N2 = 23, P = 0.007$). All other parameters measured did not differ significantly between large and small males (Fig. 6.3). There was, however, a nonsignificant trend that small males had to follow the female longer before mounting (Mann-Whitney U test: following: $U = 202, N1 = 26, N2 = 23, P = 0.052$; mounting: $U = 273, N1 = 26, N2 = 23, P = 0.602$; mating: $U = 230, N1 = 26, N2 = 23, P = 0.235$; remounting: $U = 235, N1 = 26, N2 = 23, P = 0.279$). After the periods of searching and following were summarized as in the competition experiment (Fig. 6.4), the interval of small males still remained significantly shorter than that of large males ($U = 198, N1 = 26, N2 = 23, P = 0.043$). All males of either size were able to elicit female receptivity.

(2) In the competition experiment, there was no significant difference between the mating success of large and small males. The small males copulated in 28 experiments with the female, the large male in 20 experiments (two-tailed binomial test: $P = 0.312$).

Figure 6.3: Mean duration of behavioural courtship elements of large and small *Nasonia vitripennis* males (+ s.e.m.) in the no competition experiment (one male and one female). Asterisks indicates a significant difference at $P < 0.01$ (**) (Mann-Whitney-U test).

Figure 6.4: Mean duration of courtship elements of large and small males (+ s.e.m.), in the competition experiment (two males and one female; ns = not significant; Wilcoxon signed ranks test).
When analysing the duration of courtship and mating, we found no significant difference between large and small males (Fig. 6.4; Wilcoxon signed-ranks test: searching and following: $Z = -1.651$, $N = 48$, $P = 0.099$; mounting: $Z = -1.749$, $N = 48$, $P = 0.080$; mating: $Z = -0.369$, $N = 48$, $P = 0.712$; remounting: $Z = -0.488$, $N = 48$, $P = 0.625$).

There was a tendency for small males to need less time to detect the female. The small male was significantly more often the first to mount the female (small male: 32; large male: 16; binomial test: $P = 0.029$; Fig. 6.5). The mounting duration was about twice as long and the remounting time was shorter than in experiment 1.

![Figure 6.5: Number of large and small males first mounting the female in the competition experiment. Asterisk indicates a significant difference at $P < 0.05$ (*) (two-tailed binominal test).](image)

### 6.5 DISCUSSION

The results of the present study add another puzzle piece to our understanding of pheromone communication in the model organism *N. vitripennis* by showing that male pheromone titres correlate with body size. Large males have much more pheromone at their disposal than small males and therefore also release higher amounts after the first three matings, as indicated by our calculated quantification of pheromone deposits. After the first mating this difference was most pronounced and females clearly preferred pheromone doses
calculated for large males over those of small males in our open arena bioassay. However, females of *N. vitripennis* are able to discriminate even more subtle differences of pheromone doses (Ruther et al., 2009), suggesting a general advantage for large males. Pheromone deposits (of medium-sized males) remained attractive for at least 2 h (Steiner & Ruther, 2009b), and it can be assumed that this time is even longer for large males. Considering furthermore that *N. vitripennis* females typically mate only once (Holmes, 1974) with the first male they encounter (van den Assem, 1986; Moynihan & Shuker, 2011), large males should gain clear fitness benefits from being more attractive from a distance for virgin females.

The outlined scenario raises the question of why there appears to be no selection on females to produce larger sons, because male *Nasonia* wasps are typically smaller than the females (Rivers & Denlinger, 1995). A possible explanation is the missing size effect in male-male competition (see below), which may select against male size. Furthermore, females also benefit from producing large daughters (Rivero & West, 2002), and thus selection on male and female size might neutralize each other. Finally, *Nasonia* females are host-limited (i.e. they have only one host at a time to lay eggs). Hence, unidirectional selection on male size would probably cause fitness costs because of competition between sons and daughters for limited nutritional resources. Male size, however, might be selected for indirectly by the host-finding ability of the mother, as females have been shown to prefer high-quality hosts for oviposition (Blaul & Ruther, 2011) and host quality in turn does influence offspring size of either sex (Rivers & Denlinger, 1995). A considerable proportion of females mate immediately after emerging from the host, with a dominant male monopolizing the exit hole (van den Assem et al., 1980a). Early emerging males have better mating chances in this situation, suggesting that development time rather than male size is under sexual selection (Moynihan & Shuker, 2011).

For those males competing aggressively for females directly at the exit hole, the abdominal sex pheromone presumably does not play an important role, but subordinate males might lure virgin females away from the control of the dominant male by a strong pheromone signal. Hence, the *Nasonia* system matches nicely the sexual advertisement scramble competition model by Parker (1983). This model predicts that males with an above average advertisement level (here: deposition of higher pheromone dose) will have better mating chances by attracting more females. However, detailed observations of the wasps at a larger scale under field or semifield conditions are necessary to establish this scenario finally.

Our behavioural observations suggest that small males are not disadvantaged when competing directly with a large male for a virgin female. This confirms previous studies (Burton-Chellew et al., 2007; Moynihan & Shuker, 2011) in which the outcome of male-male
competition has also been measured. In these studies, however, the authors neither observed the behavioural details nor enforced direct interaction between the competitors, as has been done in the present study. In our experiments small males won almost 60% of the contests and mounted the female first in two-thirds of all observations (Fig. 6.5). Considering furthermore that small males needed significantly less time to recognize a female in the no competition experiment (Fig. 6.3), our results suggest that small males have greater courtship agility. In none of our behavioural parameters were small males inferior to large ones. This was also true of their ability to elicit female receptivity, suggesting that the oral aphrodisiac pheromone (van den Assem et al., 1980b; Ruther et al., 2010) is not used by the females to discriminate males according to size at this stage of the courtship sequence.

Mating competition between males is a well-documented topic in many species of insect (Wong & Candolin, 2005), and the impact of male size has been frequently studied in this context (Blanckenhorn, 2005). Even though large males have been found to have greater mating chances in a number of insects (Partridge & Farquhar, 1983; Savalli & Fox, 1999; Lomborg & Toft, 2009; Jorge & Lomonoaco, 2011), a higher mating success of smaller males is not unusual in nature (Steele & Partridge, 1988; Moya-Larano et al., 2007; Blanckenhorn et al., 2008). Males of *Drosophila subobscura*, for instance, regurgitate food during courtship and larger males have greater courtship success in mating contests because they offer bigger food drops to females (Steele & Partridge, 1988). However, small males are superior in tracking the female during the courtship dance and thus are more successful during courtship. Small males of the seed beetle *Stator limbatus* have an advantage in mating competition, because they show higher courtship agility and reach females before larger males (Moya-Larano et al., 2007). Similar results were obtained by McLachlan & Allen (1987) when studying the mating behaviour in six species of Diptera that mate in flight. Small males had a higher mating success because of their aerobatic ability. However, although small males of *Scathophaga stercoraria*, *D. melanogaster* and *D. pseudoobscura* were faster than their larger conspecifics in attaining females, they lost mating contests in a competitive situation owing to the more aggressive courtship behaviour of larger males (Partridge & Farquhar, 1983; Partridge et al., 1987; Blanckenhorn et al., 2008).

The higher pheromone titres of large males in the present study can be best explained by a higher availability of nutritional resources for these males, because body size has been shown to correlate with host quality (= host species) and host size in *N. vitripennis* (Rivers & Denlinger, 1995). A correlation between the quality and/or quantity of larval diet and male sex pheromone titres has also been found in some other insects: for instance, in the red flour beetle, *Tribolium castaneum* (Ming & Lewis, 2010), and the arctiid moth *Utetheisa ornatrix*.
As we used only one host species for rearing our wasps, it is probably the quantity of the nutrients rather than the quality that caused the size differences of males in our study. Rivers & Denlinger (1995) found a negative correlation between the relative clutch size (number of wasps per milligram of host) and host size in *N. vitripennis*, resulting in a higher nutrient availability per wasp with increasing host size and consequently in larger wasps emerging from larger hosts.

The biosynthesis of the male sex attractant HDL starts from LA (Blaul & Ruther, 2011). It is therefore likely that fatty acids are the nutritional resource responsible for the observed pheromone differences in this study. The availability of LA and other polyunsaturated fatty acids is also crucial for sperm production (Wathes et al., 2007). Consequently, *N. vitripennis* males from hosts experimentally enriched in LA have been shown to have both higher pheromone titres and sperm numbers (Blaul & Ruther, 2011). A similar link between pheromone titre and sperm number exists for the parameter body size, because large males not only have more pheromone (present study) but also higher sperm numbers (Clark et al., 2010), as is the case in many other insects (e.g. Savalli & Fox, 1998; Ponlawat & Harrington, 2007; Teuschl et al., 2010). As *N. vitripennis* is a prospermatogenic species (Boivin et al., 2005), sperm limitation might become a problem for multiply mated small males earlier than for large males. A significant sperm limitation in medium-sized *N. vitripennis* males was detectable already after seven successive matings (Ruther et al., 2009).

We conclude that body size is, as well as age, mating history (Ruther et al., 2009) and the availability of LA in the larval diet (Blaul & Ruther, 2011), a factor influencing pheromone communication in *N. vitripennis*. Females not being inseminated immediately at the exit hole might increase their chance of mating with a male of sufficient fertility by orienting along concentration gradients of the male sex attractant. Hence, the females’ preference for the strongest pheromone signal at the natal site helps them to avoid later constraints of sperm limitation as they probably will not get any other mating opportunities once they have left the natal site. The preference of females for a stronger pheromone signal may be considered as an example for passive attraction sensu Parker (1983), as they increase the probability of mating with a higher quality male indirectly rather than by discriminating actively between attributes of individual males. Small males are likely to be less preferred by females using the chemical signal for mate finding but may compensate for this disadvantage at least partly by greater agility in mating contests.
6.6 Acknowledgments
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Personal Contribution:
I made all experiments and created all figures of this paper. I wrote the text of the paper as a first version.
Behavioural switch in the sex pheromone response of *Nasonia vitripennis* females is linked to receptivity signalling

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7.1 ABSTRACT

The benefits for insects using chemical information may depend on their physiological state and the behavioural context in which the chemical stimuli are perceived. Hence, insect behaviour is often characterized by olfactory plasticity. Mechanisms triggering changes in the postmating behaviour of insect females are typically mediated by constituents of the male ejaculate. Here we show that the behavioural switch displayed by mated females of the jewel wasp *Nasonia vitripennis* in response to a male abdominal sex pheromone is independent of the transfer of a male ejaculate. Rather, our results suggest a pheromone interaction in that prior exposure of a female to one pheromone and her subsequent receptivity signal modulated her response to a second one. We tested the pheromone response of females that had been experimentally prevented from receiving sperm or from experiencing certain elements of the male courtship behaviour. We show that the behavioural switch in *N. vitripennis* females is linked to the receptivity signal shown by females in response to a male-derived oral aphrodisiac during precopulatory courtship.
7.2 INTRODUCTION

The chemical sense is of crucial importance for the orientation of insects in complex environments. Many pivotal competences of insects such as the ability to locate mates, food sources, and oviposition sites as well as to avoid natural enemies and suboptimal living conditions are mediated by chemical messengers (Wyatt, 2003; Schoonhoven et al., 2005). In many cases, however, benefits resulting from the exploitation of these so-called semiochemicals depend on the physiological state of the responder, the behavioural context in which they are perceived, and a number of environmental parameters (Anton et al., 2007). In fact, responding to chemical cues and signals at the wrong time might be costly rather than beneficial. In the context of pheromone-mediated sexual communication, for instance, the ability of the responding gender to detect a sex attractant should coincide with its own fertility and receptivity of potential mating partners (Anton et al., 2007). On the other hand, it might be detrimental for individuals that mate only once (typically the females) to orient towards a sex attractant once mating has occurred since this might cause fitness costs as a result of the waste of energy or sexual harassment (Gay et al., 2009). Therefore, sex pheromone-guided behaviour of insects has to be plastic, that is, adapted to conditions under which the benefits of a behavioural response are likely to outweigh possible costs. Most insect species studied so far with respect to olfactory plasticity in sex pheromone communication belong to the Lepidoptera and Diptera (reviewed by Anton et al., 2007). Apart from environmental parameters such as light (e.g., Kanno, 1981), diel periodicity (e.g., Cardé et al., 1974) and temperature (e.g., Cardé & Roelofs, 1973; Linn et al., 1988), a number of intrinsic factors such as age (Turgeon et al., 1983), hormone titres (Gadenne et al., 1993; Anton & Gadenne, 1999) and mating status (e.g., Jang, 1995; Gadenne et al., 2001) modulate sex pheromone responses in these taxa. Males of the black cutworm moth *Agrotis ipsilon*, for instance, do not respond to the female sex pheromone until sexual maturity is reached (Gadenne et al., 1993). Then, they can mate only once per night and therefore do not respond to the female sex pheromone once a spermatophore has been transferred. After having replenished the sperm and accessory gland fluid supplies, males become responsive again (Gadenne et al., 2001). Female Mediterranean fruit flies, *Ceratitis capitata* show a behavioural switch after mating with a shift of their olfactory preference from the male sex attractant to host odours (Jang, 1995). This effect is mediated by proteinaceous molecules from the male accessory gland being transferred to the female together with sperm during copulation (Jang, 2002). In *Drosophila*, sex peptides from male accessory gland secretions have been shown to trigger remating refractoriness and affect female reproductive activity in many other ways (Gillot, 2003; Chapman et al., 2003; Yapici et al., 2008). The sex peptide/receptor system is thought to be highly conserved in insects (Werren, et al., 2010).
but appears to be missing in *Apis mellifera* and *Nasonia vitripennis*, the only two hymenopteran insects with sequenced genomes so far (Werren et al., 2010). This raises the question how mating-dependent behavioural changes which have been reported at least for *N. vitripennis* are mediated in these insects.

*Nasonia vitripennis* is a gregarious pupal parasitoid of numerous fly species (Whiting, 1967). The stereotypic courtship behaviour of *N. vitripennis* males has been studied in detail (Barras, 1960; van den Assem & Vernel, 1979; van den Assem et al., 1980b; van den Assem & Werren, 1994; Steiner et al., 2006). When encountering a female, the male immediately mounts her and shows vellicating wing movements together with a typical head nodding behaviour. Head nodding is typically accompanied by chewing movements of the mouthparts which results in the release of a still unknown aphrodisiac pheromone from an oral gland eliciting female receptivity. Involvement of a volatile aphrodisiac was concluded from the observation that courting males whose mouthparts had been sealed by a drop of quick-drying superglue were unable to elicit receptivity in females. Exposure of these handicapped pairs to headspace volatiles from unconstrained pairs, however, restored receptivity in these females (van den Assem et al., 1980b). Within the first 30s of courtship mostly even faster (van den Assem & Vernel, 1979), the female signals receptivity by lowering head and antennae and synchronously opening her genital orifice. After copulation, the male remounts the female and displays postcopulatory courtship often resulting in another receptivity signal by the female. Postcopulatory courtship is crucial for making the female unreceptive for other males (van den Assem & Visser, 1976) and in fact, the majority of females mate only once under natural conditions (Grillenberger et al., 2007). Apart from remating refractoriness, *N. vitripennis* females show also a mating-dependent behavioural switch concerning their sex pheromone response. Only virgin females are attracted to the male sex pheromone consisting of a mixture of (4\(R\),5\(R\))- and (4\(R\),5\(S\))-5-hydroxy-4-decanolides (HDL) and 4-methylquinazoline (MeQ) (Ruther et al., 2007, 2008, 2009). The attractive chemicals are biosynthesized in the male rectal vesicle and released via the anal orifice (Abdel-latief et al., 2008; Steiner & Ruther, 2009b). Within minutes after mating, however, females are no longer attracted to the male pheromone and prefer host odours instead (Ruther et al., 2007; Steiner & Ruther, 2009a).

The present study was performed to investigate the mechanism involved in the behavioural switch of *N. vitripennis* females. We asked in particular whether the reception of a male ejaculate is essential for the modification of the female behaviour or whether the exposure of females to particular elements of the male courtship behaviour is sufficient. We also studied whether the behavioural switch is innate. To achieve this goal, we performed a series of
olfactometer bioassays to test the pheromone response of females that had been experimentally prevented from receiving a male ejaculate or from experiencing particular details of the male courtship sequence.

7.3 MATERIALS & METHODS

Insects

*Nasonia vitripennis* wasps were reared in Petri dishes on freeze-killed puparia of the green bottle fly, *Lucilia caesar* (Insektenzucht, Schürpflingen, Germany) at 25°C and 60% relative humidity with a daily light:dark cycle of 16:8 h. Unmated parasitoids for the experiments were obtained by excising pupae from host puparia 1-2 d prior to eclosion and keeping them singly in 1.5 ml microcentrifuge tubes until emergence. Females used in the olfactometer bioassays were 1-3 d old and unmated. Males used for pheromone extraction were unmated and 2 days old. Manipulations of parasitoids were done with a fine paint-brush.

Conditioning of females at different levels of courtship experience

To study the olfactory responses of females subject to defined elements of the male courtship sequence, we allowed individual pairs to court in a round observation chamber (0.5 cm height, 1 cm diameter) but prevented females experimentally from receiving a male ejaculate or experiencing particular details of the male courtship behaviour, respectively (see below). Additionally we tested unmated females as positive controls (referred to as *virgin*, *N* = 20, Table 7.1).
Table 7.1. Behavioural elements of the *Nasonia vitripennis* courtship sequence for females of the different experience levels before their response to the male sex pheromone was tested in the olfactometer bioassay.

<table>
<thead>
<tr>
<th>experience level of females</th>
<th>precopulatory courtship</th>
<th>copulation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>male mounting</td>
<td>male head nodding</td>
</tr>
<tr>
<td>virgin</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>no postcop</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>no cop</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>no HDL/4-MQ&lt;sup&gt;1&lt;/sup&gt;</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>no aphro&lt;sup&gt;2&lt;/sup&gt;</td>
<td>+</td>
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</tr>
</tbody>
</table>

For an explanation of experience levels, see text and Fig. 1.

<sup>1</sup> Perception of the male sex pheromone during courtship was prevented by using newly emerged males and sealing their abdominal tip.

<sup>2</sup> Release of the aphrodisiac was prevented by sealing the mouthparts of the courting male.

**Experimental exclusion of postcopulatory courtship**

Virgin pairs were allowed to court and mate but postcopulatory courtship was prevented by removing the male before he could remount the female (referred to as *no postcop*, *N* = 20).

**Experimental exclusion of the copulation**

Virgin pairs were allowed to court. However, after male head nodding and the female receptivity signal, pairs were separated with a fine paint-brush (referred to as *no cop*, *N* = 20).

**Experimental exclusion of the male sex attractant**

*Nasonia vitripennis* females are able to learn chemical stimuli associatively (Schurmann et al., 2009). They have been shown to prefer odours to which they were exposed to during a single oviposition event and even learn odours that do not occur in the natural habitat. We therefore performed an experiment to address the question whether the opportunity to perceive the male sex attractant (HDL/4-MeQ) during courtship is essential for the behavioural switch of females. For this purpose, we exposed virgin females to the courtship
of newly eclosed males (age < 30 min) which have been shown to possess no sex attractant yet (Ruther et al., 2007) but do possess the aphrodisiac and thus, are able to elicit female receptivity (Ruther et al., 2009). The absence of the sex attractant in these males was additionally verified by coupled gas chromatography-mass spectrometry (GC-MS) using the methodology and instrumentation described elsewhere (Ruther et al., 2007). To be sure that females had no chance to experience the sex attractant during courtship, the males’ abdominal tip including the pheromone releasing anus was sealed with a drop of quick drying superglue (Pattex, Henkel, Düsseldorf, Germany). For this purpose, males were cooled down on ice for 15 min and the glue was applied to the abdominal tip under a stereo microscope using a fine dissection needle. After the glue hardened (5 min) males were kept for 10 min at room temperature and subsequently they were allowed to court a virgin female. All females in this experiment signalled receptivity by lowering their head and simultaneously opened their genital orifice but copulation did not occur because of the males’ handicap. These females were thus assumed to have experienced full precopulatory courtship in the absence of the male sex attractant (referred to as no HDL/4MeQ, N = 20).

**Experimental exclusion of the male aphrodisiac**

Males in this experiment were prevented from releasing the aphrodisiac from their oral gland and thus, to elicit female receptivity. For this purpose their mouthparts were sealed with a drop of quick-drying superglue using a fine needle as described above for the abdominal tip. Agglutination of eyes and antennae was avoided because this would have hampered the males’ ability to detect females by their sex specific cuticular hydrocarbons (Steiner et al., 2006). After the glue hardened (5 min) males were kept for 10 min at room temperature and subsequently allowed to court a virgin female. Sealed males were able to detect and mount the female, showed the normal head nodding behaviour but were never able to elicit female receptivity. Females were thus assumed to have experienced full male precopulatory courtship apart from perceiving the male aphrodisiac (referred to as no aphro, N = 21 for both virgin females and sealed males). To make sure that the missing receptivity signal in this experiment (see results) was not due to the glue treatment per se, we treated the heads of another group of males with glue but left the mouthparts open. For this purpose, a droplet of glue was applied topically to the head of immobilized males (N = 17). Again agglutination of male antennae was avoided. These males were exposed to virgin females in an observation chamber and courtship and mating were observed under a stereo microscope.
Impact of glue treatment on female pheromone response

To demonstrate that the exposure of females to glue treated males does not influence their pheromone response per se, we performed the following control experiment. We applied drops of the glue to the heads of immobilized virgin females \((N = 21)\) avoiding agglutination of the antennae. After the glue hardened, we tested the response of these females to the male sex attractant.

Preparation of pheromone extracts

Two hundred males were killed by freezing and extracted for 30 min with 1.2 ml of dichloromethane. After removal of the supernatant, the wasps were washed again with 200 \(\mu l\) of dichloromethane. The combined extracts were concentrated to 800 \(\mu l\) under nitrogen and batches of 200 \(\mu l\) were applied to silica gel cartridges (100 mg, International Sorbent Technology, Glamorgan, UK) and rinsed twice with 1 ml of dichloromethane each. The bioactive pheromone components (HDL and 4-MeQ) were eluted with 1 ml of methanol. Methanol fractions were combined and the solvent was carefully removed under nitrogen. The residue was resolved in dichloromethane and adjusted to a concentration representing 100 ng/\(\mu l\) of total HDL. The concentration was estimated by GC-MS as described elsewhere (Ruther et al., 2007). The concentration of 4-MeQ was not estimated but previous analyses revealed a HDL/4-MeQ ratio of approximately 100:1 (Ruther at al., 2008).

Female olfactory responses

Apart from the glue treatment control experiment, bioassays were performed in a linear still-air olfactometer, consisting of a rectangular clear acrylic tube (14 cm x 1 cm x 1cm) which was open at the bottom and divided into two 4-cm test zones at either end and a 6-cm neutral zone in the middle (for more details see Steiner & Ruther, 2009a). We applied 1 \(\mu l\) of the pheromone extract and pure dichloromethane (control), respectively, to filter paper disks (5 mm diameter) and the solvent was evaporated for 2 min. Test and control disks were placed at both ends of the olfactometer and volatiles were allowed to diffuse into the olfactometer tube for 5 min to enable the formation of an odour gradient. Subsequently, \(N.\ vitripennis\) females of the different courtship experience levels were released individually into the centre of the olfactometer tube through a cylindrical entry (0.5 cm diameter) and the time parasitoids spent in test and control zone was recorded for 5 min using the computer
software The Observer 3.0 (Noldus, Wageningen, The Netherlands). The olfactometer was evenly illuminated from above with a microscope light (Type KL 1500, Schott, Mainz, Germany) and rotated by 180° after every observation to compensate for any unforeseen asymmetry of the set-up. The olfactometer was regularly cleaned with ethanol and odour sources were renewed after every tested female. Females of all experience levels were tested 10 min after being separated from the male. Females of the levels no postcop and no cop (Table 7.1) were tested additionally 24 h after the treatment to investigate whether the observed behavioural switch is persistent. The impact of the glue on the female pheromone response was tested in a four cavity olfactometer where test and control paper disks were presented in spherical cavities (1 cm diameter, 4 mm deep) of a round walking arena made from acrylic glass (for the experimental set-up see Steiner & Ruther, 2009b). All other experimental details were as described above.

**Statistical analysis**

Residence times of *N. vitripennis* females in the test and control zones of the olfactometers were compared by a non-parametric Wilcoxon signed-ranks test using Statistica 4.5 scientific software (StatSoft, Hamburg, Germany).

### 7.4 RESULTS

Females without prior courtship experience ("virgin") were strongly attracted to the male pheromone extract (Wilcoxon signed-ranks test: $T = 22$, $N = 20$, $P = 0.0019$, Figure 7.1a) confirming previous results (Ruther et al., 2007; Steiner & Ruther, 2009a) and demonstrating the functionality of the experimental setup. Females that were allowed to mate but did not experience any postcopulatory courtship ("no postcop") were not attracted to the male sex pheromone 10 min (Wilcoxon signed-ranks test: $T = 75$, $N = 20$, $P = 0.263$) or 24 h (Wilcoxon signed-ranks test: $T = 74$, $N = 20$, $P = 0.247$, Figure 7.1b) after being separated from the male. Females that were exposed to precopulatory courtship until the receptivity signal but were not allowed to mate ("no cop"), avoided the male sex pheromone 10 min after
Chapter 7 – Receptivity

separation from the male (Wilcoxon signed-ranks test: \( T = 41, N = 20, P = 0.017 \)) and responded indifferently after 24 h (Wilcoxon signed-ranks test: \( T = 81, N = 20, P = 0.370 \), Figure 7.1c). The opportunity to perceive HDL/4-MeQ during precopulatory courtship and receptivity signalling was not a prerequisite for the behavioural switch to occur since females signalling receptivity towards newly emerged males in the absence of HDL/4-MeQ (“no HDL/4-MeQ”) were still not attracted to the male sex pheromone in the subsequent olfactometer bioassay (Wilcoxon signed-ranks test: \( T = 64, N = 20, P = 0.126 \), Figure 7.1d).

Figure 7.1a-e. Residence times of *Nasonia vitripennis* females in the test and control field of a linear still-air olfactometer. The test field (P) was treated with a purified extract of the male sex pheromone, the control field (C) was treated with the pure solvent. Females were either virgin or subjected to...
different levels of courtship prior to the test (see text and Table 7.1). Residence times of (a) virgin females, (b) females that mated but did not experience postcopulatory courtship (“no postcop”), (c) females that were courted but did not mate (“no cop”), (d) females that experienced full precopulatory courtship in the absence of either the male sex attractant (“no HDL/4-MeQ) and (e) females that were courted but did not mate (“no aphr”). Females were tested either 10 min or 24 h after the courtship treatments. Box and whisker plots indicate the median (horizontal line) with 25th and 75th percentiles (boxes) and maximal/minimal values (whiskers). Asterisks indicate significant differences between pheromone and the control at $P < 0.05$ (*) or $P < 0.01$ (**) (Wilcoxon signed-ranks test, n.s. = not significant).

However, those females that were exposed to precopulatory courtship but did not signal receptivity because of the missing aphrodisiac of their sealed partner (“no aphro”) were attracted to the male sex pheromone (Wilcoxon signed-ranks test: $T = 57, N = 21, P = 0.042$, Figure 7.1e). Sealing the males’ mouthparts did not generally hamper their ability to detect and court a female. Like the unconstrained males, all of them mounted the females and showed the typical head nodding behaviour. The missing receptivity signal in this experiment was not due to the glue treatment per se because all males ($N = 17$) whose heads had been topically treated with glue but had intact antennae and open mouthparts were able to elicit receptivity in females and mated successfully. Also, the glue per se did not influence the pheromone response of virgin females. Females topically applied with a drop of glue nevertheless were strongly attracted to the male sex attractant. Residence times (mean ± SEM) in test and control fields of the olfactometer were $51 \pm 11s$ and $6 \pm 3s$, respectively (Wilcoxon signed-ranks test: $T = 16, N = 21, P < 0.001$).

7.5 DISCUSSION

The results of the present study clearly demonstrate that postcopulatory courtship which has been shown to induce remating refractoriness in *N. vitripennis* females is not essential to switch off their response to the male sex attractant. Not even the reception of a male ejaculate is necessary because also females that were separated from the male before
genital contact was established also showed the behavioural switch. Thus, a mechanism other than the involvement of male accessory gland secretions like in tephretid fruit flies (Jang et al., 1995, 2002) is likely to be present in *N. vitripennis*. This is in agreement with the recent finding that neither sex peptide genes nor the respective receptors are present in the *N. vitripennis* genome (Werren et al., 2010). Rather, our results show that the receptivity signal displayed by the female in response to the male aphrodisiac is sufficient to switch off her response to the male sex attractant. Females subjected to courtship by males with sealed mouthparts which could not release the aphrodisiac during head nodding, did not signal receptivity and thus were strongly attracted to the male sex attractant. Hence, the glue treatment of males did not hamper the female pheromone response per se. In fact, even those females that had been treated themselves with a drop of glue were still attracted to the pheromone. Furthermore, glue-treated males with open mouthparts were able to elicit receptivity. Therefore, it is the exact position of the glue seal (mouthparts) rather than its presence per se that prevents males from eliciting the receptivity signal in females.

Although evidence is increasing that learning can modify sexual behaviour of insects (Dukas, 2006) and *N. vitripennis* has been shown to learn very well (Schurmann et al., 2009), the behavioural switch demonstrated here is innate and not learned because also naïve females that were experimentally prevented from perceiving the male sex attractant before or during courtship showed the behavioural switch after receptivity signalling.

To our knowledge, the mechanism demonstrated here is novel in that prior exposure of the females to one male pheromone (the oral aphrodisiac) and her subsequent receptivity signal modulate her response to another one (the abdominal sex attractant). However, since male aphrodisiac release and female receptivity signal are linked so closely to each other, it cannot be concluded unambiguously whether one of these elements alone would be sufficient to cause this behavioural switch. It remains to be shown that this mechanism is more common in parasitic wasps as suggested by previous work on the genus *Mellitobia* (Hymenoptera: Eulophidae) (Gonzalez et al., 1985).

From an evolutionary perspective, the triggering of a behavioural switch in females might complement the earlier demonstrated induction of remating refractoriness during postcopulatory courtship (van den Assem & Visser, 1976) as a male strategy to monopolize females because it further decreases the probability of females mating again. It might equally, however, be the result of selection acting on the females to optimize their semiochemical use in different phases of life because the fading response to the sex attractant after fertilization coincides with an increasing attraction toward host odours (Steiner & Ruther, 2009a). This shift of olfactory preferences should contribute to the maximization of
reproductive success because it increases the chance of finding oviposition sites and decreases the risk of costly sexual harassment by other males (Gay et al., 2009). The fact that the behavioural switch occurs before the transfer of sperm, bears a certain risk of females dispersing unfertilized from the natal patch if pairs are interrupted between receptivity signalling and copulation. This risk, however, is small because these two behavioural elements are closely connected to each other and interrupted couples are likely to restart courtship.

Compared to many other studies dealing with the plasticity of pheromone-guided insect behaviour (e.g., Gadenne et al., 1993; Jang, 1995; Anton & Gadenne, 1999; Dickens, 2007), the behavioural switch demonstrated here sets in quickly, that is, within a few minutes after receptivity signalling. Furthermore it appears to be rather persistent because both mated females and those that had been separated from the male before copulation remained unresponsive 24 h after the treatment. In fact, an earlier study suggested that the effect persists for the rest of the females’ life because even those mated females that had been given the opportunity to oviposit for 6 days prior to the bioassay did not respond to the pheromone (Ruther et al., 2007).

In a number of insects slow modifications of olfactory-guided behaviour are controlled by juvenile hormone (JH) (reviewed by Anton et al., 2007). In the moth *A. ipsilon* for instance, JH induces a slow increase of the male olfactory response to the female sex pheromone correlating with progressive age and sexual maturation (Gadenne et al., 1993; Anton & Gadenne, 1999). After mating, however, the pheromone response is inhibited in a quick and transient manner until males have replenished their sperm and accessory gland supplies 24 h later. The peripheral olfactory system appeared not to be affected by mating in these studies as indicated by electroantennographic measurements. Rather, a reversible effect on the projection neuron sensitivity in the antennal lobe was found (Gadenne et al., 2001). Biogenic amines such as octopamine and serotonine are candidates to be involved in the rapid modifications of neuron sensitivity (Anton et al., 2007). These molecules may interfere in various ways with the olfactory competences of insects and have been shown to influence insect sex pheromone responses both at the behavioural as well as at the central and peripheral neuronal level (Blenau & Baumann, 2001; Roeder, 2005; Jarriault et al., 2009). Studies are needed to investigate the genetic and neurosensory mechanisms triggering the quick and persistent behavioural switch of *N. vitripennis* females after receptivity signalling and to identify the hormones and neuromodulators involved.
7.6 Acknowledgments
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Personal Contribution:
I made control experiments to demonstrate that the exposure of females to glue treated males does not influence their pheromone response per se.
CHAPTER 8

General Discussion

This doctoral thesis presents interrelated results that provide new insights into the chemical communication of the model organism *Nasonia vitripennis*. By $^{13}$C-labelling and GC-MS analysis, *Nasonia* males have been shown to incorporate linoleic acid (LA) taken up by the host during feeding directly into the sex pheromone components $(4R,5R)$- and $(4R,5S)$ HDL. Thus LA functions as a precursor of the biosynthetic pathway of this pheromone (chapter 2). Furthermore, I have demonstrated that *N. vitripennis*, as only one of relatively few animals, is able to convert oleic acid (OA) into LA and use both fatty acids for pheromone synthesis (chapter 4). For the *de novo* synthesis of LA from OA, *N. vitripennis* must have a $\Delta_{12}$-desaturase. Demonstrating the role of LA and OA as precursors and the ability of *N. vitripennis* to convert LA from OA, I have been able to extend the knowledge about the biosynthetic pathway of HDL (Figure 8.2). I have worked with molecular methods on the identification of the $\Delta_{12}$-desaturase (chapter 5). Two putative $\Delta_{12}$-desaturases have been tested for their functionality by expressing them in yeast cells. For at least one of the genes data suggest its involvement in fatty acid biosynthesis, although no $\Delta_{12}$-desaturase activity has been found. Behavioural assays have indicated that females prefer hosts artificially enriched in safflower oil that is rich in OA and LA to hosts artificially enriched in coconut oil that is poor in these unsaturated fatty acids for ovipositing. Females’ host choice leads to a high HDL amount and in turn to a high mating success of their male offspring. Dissection and microscopy analysis has revealed that males reared on OA and LA-rich hosts have a higher sperm amount than males reared on OA and LA- poor hosts (chapter 2). By rearing hosts of *N. vitripennis* on various beef tissues, simulating its natural habitat on a carcass, I have imitated the host choice situation in the natural environment of *Nasonia*. Hosts vary in their fatty acid composition depending on the fatty acid composition of their food (chapter 3). Male body size is a further attribute that influences the HDL deposits of *Nasonia* males (chapter 6). Larger males are able to release higher amounts of their sex pheromones and therefore attract more virgin females than smaller males but, in the direct competition, smaller males have the same success because of their higher agility. In general, male mating success was only achieved when females showed receptivity mediated by a second pheromone, the so-called “aphrodisiac” (chapter 7). This doctoral thesis includes a wide spectrum of methods such as behavioural analysis, $^{13}$C-labelling, GC-MS analysis and molecular methods. This wide method spectrum ensures a view of the topic from various perspectives. The
interconnection of the results of this doctoral thesis is extended to a hypothetical scenario that might happen in the field and is illustrated in figure 8.1.

Fig 8.1: Hypothetic, schematic illustration concluded from the results of this doctoral thesis. In the field *N. vitripennis* parasitize hosts either on a cadaver or in a nest of cavity breeding birds. In both habitats probably hosts of different fatty acid compositions occur (1). *Nasonia* females favour hosts that are rich in LA and OA for oviposition (2). *Nasonia* offspring is able to convert OA into LA by a $\Delta_{12}$ - desaturase (3). LA has been shown to have a positive effect on females’ egg production and males’
sperm amount (4). OA and LA function as precursors for HDL synthesis and are directly incorporated from the host into HDL (5). A high amount of OA and LA in the hosts probably leads to a large body size of the offspring (6). Males with a large body size have a high HDL titre (7). With a high HDL titre many virgin females are attracted, because females prefer higher HDL deposits to lower ones (8). Females’ preference leads to high mating chances of the males (9). By the aphrodisiac, released by the males during courtship females’ receptivity for mating is elicited (10). The aphrodisiac also leads to a behavioural switch in *Nasonia* females. They are not attracted to HDL anymore (11).

**Central role of unsaturated fatty acids**

In this doctoral thesis, the role of LA in the life of *N. vitripennis* has been shown to be central. First of all, LA is involved in many vital membrane functions of the cells of all animals (Cripps et al., 1986). Furthermore LA can function as precursor for C20 polyunsaturated fatty acids (PUFAs) and these in turn are precursors for eicosanoids synthesis like prostaglandins (Blomquist et al., 1991). Prostaglandins are most abundant in the spermatozoa, at least in vertebrates (Horton, 1969). In insects, prostaglandins are also often involved in reproductive traits and are also thought to play a role in reproduction (Canavoso et al., 2001). They can be transferred from the male to the female during copulation and stimulate egg production of the female (Loher et al., 1981; Stanley-Samuelson, 1994).

A major result of this doctoral thesis was the identification of a further fundamental role of LA in *Nasonia* in addition to this general meaning of LA. In *Nasonia* LA functions as precursor of the male sex pheromone HDL that attracts virgin females and is important for male mating success (chapter 2).

A second major result of this doctoral thesis was the detection that *Nasonia*, as one of only a few insects, is not depended to ingest LA as an essential fatty acid with nutrition but is able to synthesize LA *de novo* from OA (chapter 4) (Cripps et al., 1986). Therefore OA function as further precursor for HDL.

Pheromone synthesis often starts with primary nutrients like fatty acids as precursors. In the orders Lepidoptera and Coleoptera, many pheromones and biosynthetic pathways have been analysed and fatty acids often function as pheromone precursors (for instance: Karlson & Butenandt, 1959; Rule & Roelofs, 1989; Foster & Anderson, 2012; Vanderwel et al., 1990).

The results of this doctoral thesis show that in pheromone synthesis it is possible to avoid the dependence on an essential, limited nutritional resource as precursor by developing an ability to synthesize the limited resource from an abundant resource.
Biosynthesis of HDL

Two new steps in HDL synthesis were investigated in this doctoral thesis. The ability to convert OA into LA by a $\Delta_{12}$-desaturase is the first step in HDL biosynthesis. For the de novo synthesis of LA from OA a $\Delta_{12}$-desaturase is necessary (Figure 8.2,1). This enzyme has previously been known only from plants, fungi and algae (Blomquist et al., 1991; Sperling et al., 2003; de Renobales et al., 1987), until it was characterized first in a cockroach, a cricket and a termite (Blomquist et al., 1982). Maybe further animals possess a $\Delta_{12}$-desaturase activity, in particular when LA has a special function for the animals, for instance in the mating system.

The gene coding for the enzyme $\Delta_{12}$-desaturase in Nasonia could not be identified during this doctoral thesis, although two candidate genes with the highest homology to other insect $\Delta_{12}$-desaturases were successfully expressed in yeast cells. The yeast assay worked well as far we could determine by the $\Delta_{12}$-desaturase activity of the positive control. Further candidate genes, identified by Niehuis et al. (2011), remain to be analysed.

As second new step in HDL synthesis LA is presumably processed by a monoxygenase to threo-12,13-epoxy-octadec-9Z-enoic acid (Niehuis et al., 2012, (Figure 8.2,2)).

It is possible that further fatty acids, in addition to LA and OA, play a role in the biosynthetic pathway of HDL. Despite experiments and efforts (chapter 5) subsequently no more fatty acids as precursors and no more enzymes could be investigated. Maybe the two newly investigated biosynthetic steps have completed the biosynthetic pathway of HDL.

In the final steps of HDL synthesis, as also mentioned in the general introduction, an epoxide hydrolase catalyses the hydrolysis into the erythro diol (Abdel-Latief et al., 2008 (Figure 8.2,3)). The first diastereomer (4R,5S)-HDL is synthesized by chain reduction via $\beta$-oxidations (Figure 8.2,4) and following lactonization (Figure 8.2,5). Under involvement of one of three putative alcohol dehydrogenases, the hydroxyl group at carbon atom five of (4R,5S)-HDL is oxidized to a ketone that is reduced under inversion of stereochemistry to (4R,5R)-HDL, the second diastereomer of the male sex pheromone (Niehuis et al., 2012 (Figure 8.2,6)).
HDL as a quality indicator

Quality indicators correlate "honestly" with positive attributes concerning mate quality. "Honestly" means that signals are insusceptible to cheating or costly in production or maintenance (Johansson & Jones, 2007). Signals depending on a limited resource are known as quality indicators. For instance the pink coloration of house finches or greater flamingos but also of sticklebacks, obtained from carotenoids from the diet, reflect a good nutritional status of the sender and is supposed to be a criterion in mate choice (Hill et al., 2002; Amat et al., 2011; Milinsky & Bakker, 1990). LA, a precursor of HDL, is a limited resource that most animals have to take up with their nutrition. However, it is not a limited resource for Nasonia, because of its ability to biosynthesize LA de novo (Chapter 4). OA is the most common fatty acid in many organisms and functions as further precursor of HDL.
In all the experiments in this doctoral thesis in which females’ mate choice were tested, females used HDL as a quality indicator and preferred higher HDL deposits to lower ones. The HDL amounts of males decrease after the first mating by more than half of the amount of unmated males and the sperm amount also decreases (chapter 6). After further copulations, the HDL amount decreases further (Ruther et al., 2009; Blaul & Ruther, 2011). Simultaneously, sperm depletion occurs, as in many Hymenopteran parasitoid species after multiple mating (Bressac et al., 2008, 2009; Boivin, 2013). By counting the sperm of *N. vitripennis* males, I have directly shown that the HDL amount correlates with the sperm amount of the males. Therefore, HDL still functions as a quality indicator.

Probably it is still an advantage for *Nasonia* males to have direct access to LA from the host and no need for a costly LA synthesis. The Δ12-desaturase activity can be dependent on the temperature, on the amount of substrate and the age of the organism and can be different in each individual case (Batcabe et al., 2000). Batcabe et al. (2000) have shown differing Δ12-desaturase activity depending on the age of the house cricket *Acheta domesticus*. So far, the Δ12-desaturase activity in *Nasonia* was demonstrated in 2-days-old males. In the experiments, 2-days-old *Nasonia* males were used, because, at the age of 2 days, males have their full pheromone titre (Ruther et al., 2009) and all experiments are thus comparable. However, it would be interesting to analyse the LA and the HDL amount again few days earlier or later and at different points of the male mating history in order to be able to describe the Δ12-desaturase activity of *Nasonia* males.

A possibility is that the ability of LA de novo synthesis is used to "top up" the LA amount after HDL synthesis in order to have LA for cell functions. A further option is that *N. vitripennis* males are able to biosynthesize LA de novo after a series of copulations and to produce new HDL to attract females that have dispersed unmated. The question, in this case, is whether new sperm is also produced or whether LA is invested in HDL to obtain mating chances, despite a decreased sperm amount. Two contrary kinds of males exist in insects: (1) synspermatogenic males have no spermatozoa at emergence and produce sperm during their whole life and (2) prospermatogenic males have produced their whole sperm amount at emergence and do not produce any further sperm as adults. In *N. vitripennis*, most sperm production is complete at emergence (Clark et al., 2010). However sperm-depleted *N. vitripennis* males are able to elicit receptivity of females in the same way as males rich in sperm (van den Assem, 1986). Therefore, a Δ12-desaturase activity after copulations and sperm depletion would only be used to attract females with newly synthesized HDL, but not to produce sperm.
In all experiments, copulations occurred in direct succession with only a 10-min break in between for marking and the release of pheromone. In the field, all individuals of *Nasonia* from the same host emerge at about the same time and the mating system is characterized by local mate competition (LMC). Therefore copulations also occur in direct succession. Thus, in natural mating situations, HDL should correlate with the sperm amount and act as an "honest" signal and quality indicator. However, how realistic is it that gregarious parasitoids with a mating system, characterized by LMC can choose a mating partner by means of their pheromone deposits in the field? Eberhard (1996) has described female choice as a small but important part of sexual selection. In the field, dominant males wait at the emergence site of their sisters to copulate and, thus, at least some females will have no opportunity to select their mating partner (van den Assem et al., 1980a). Nevertheless, whereas males copulate with females directly at the emergence site, further females might disperse inseminated and have the opportunity to select a mating partner based on his pheromone amount.

**Host choice of *Nasonia***

All experiments in this doctoral thesis were made with the same host species, namely *Lucilia caesar*. The age of the host was also the same in all experiments. In the field, *N. vitripennis* parasitizes several flies (Whiting, 1967). This variation in the life of *N. vitripennis* was deliberately removed in the laboratory experiments to be able to compare all results with each other. The quality of the host, by contrast, was made variable by diets of different fatty acid composition presented to the fly larvae. The fatty acid composition of the food of the fly larvae was reflected in the hosts (chapter 2, 3). In phytophagous insects, females are known to select, for oviposition, a plant that offers good conditions for their larvae whose only nutrition is the plant on which females lay their eggs. This choice is predicted by the preference performance hypothesis (Jaenike, 1978; Clark et al., 2011). In parasitoids, hosts have a similar role to that of plants for herbivorous insects. Hosts are the only nutrition for the larvae and determine the further growing conditions of the offspring. Therefore, the preference performance hypothesis is transferable from herbivorous insects to parasitoids. The nutritional and also the physiological status of the host influence the oviposition behaviour of parasitoid females and the sex ratio, progeny allocation and development of the offspring. Vinson (1976) has demonstrated that parasitoid females select hosts especially with respect to the odours and chemicals of the host. Females recognize the chemical and physical attributes of the hosts by the insertion of the ovipositor into the host tissues (Rivers & Denlinger, 1995).
Females are known to be able to decide about the mating chances of the offspring by their mate choice. The "sexy son" theory predicts that, by choosing an attractive male as a mating partner, females produce male offspring that are also attractive and that have high mating success (Weatherhead & Robertson, 1979; Qvarnström & Price, 2001). However, in haplodiploid species, the choice of the mating partner does not play a role at least for the next generation, because male offspring emerge from unfertilized eggs. In haplodiploid species the sex ratio depends on the females` sperm management (Bressac et al., 2008).

One of the main new results of this doctoral thesis is that in haplodiploid species females although have a possibility to produce “sexy sons” and to care about mating success of the offspring. Females of haplodiploid species are not only able to choose a host with the best nutritional value for the offspring. When primary nutrients function as sex pheromone precursors, females are able to decide about a high sex pheromone titre and subsequently about high mating chances of the offspring by choosing a host incorporating a high amount of these pheromone precursors (chapter 2).

In chapter 2 it was investigated that Nasonia males from a “LA rich host” (enriched in safflower oil) produce high amounts of HDL that leads to a high mating success and is correlated with a high sperm amount as has been directly shown by sperm counts. The main ingredients of the safflower oil were OA and LA. With current knowledge that Nasonia is not only able to use LA but also OA for HDL synthesis (chapter 4) the designation of “LA rich hosts” in chapter 2 must be renewed now to “hosts rich in LA and OA”.

Host choice situation in the field

Nasonia vitripennis parasitize flies occurring in nests of cavity breeding birds and on carcasses (Peters & Abraham, 2010; Voss et al., 2009). It has been shown that mated N. vitripennis females were attracted to the same chemical substances that also attract their hosts, substances that occur in decaying cadavers (unpublished data).

To find hosts in the field, parasitoids can use pheromones of their hosts as kairomones or odours of the host food (Vet & Dicke, 1992). On that way, parasitoids probably find not a single host but several hosts. However, is it realistic, that hosts in the field have different fatty acid compositions? I had, at least, been able to demonstrate that hosts, reared on tissues with different fatty acid compositions, also exhibit different fatty acid compositions (chapter 2, 3). The tissues were set up to simulate different pieces of a carcass. Therefore, on a carcass female parasitoids probably find hosts of different nutritional value. The fatty acid
composition of the tissues of a cadaver is dependent on the diet of the animal during its lifetime (Wood et al., 2004). However, we do not know the precise nutritional preferences of the fly larvae; their nutrition depends on the oviposition site of their mother and of the size of the carcass.

In nests of cavity breeding birds, hosts can feed on different types of nourishment such as blood from the nestlings or on faeces or dead nestlings (Peters & Abraham, 2010) and therefore they will probably also differ in their fatty acid composition.

For a complete understanding of the natural host choice situation, host pupae from different habitats should be collected and their fatty acid composition should be analysed. Laboratory conditions can differ dramatically from natural conditions. Under field conditions, parasitic wasps will probably be confronted with lower rates of hosts and therefore the oviposition behaviour might change in comparison with that in the laboratory, maybe not in host choice, but at least in the number of eggs laid in a host (Bezemer & Mills, 2002).

**Connecting body size with the amount of fatty acids**

The LA amount and the body size of *N. vitripennis* males were analysed separately (chapter 2, 6). However, the results indicated that these two characteristics were connected. Male body size was shown to have no direct influence in mating success in direct mate competition in *Nasonia* (chapter 6). However, larger male body size leads to higher HDL titres than smaller male body size (chapter 6), in the same way that more OA and LA in the nutrition of the host also leads to higher HDL titres of *Nasonia* males (chapter 2).

The reason for higher pheromone titres of larger males needs to be investigated. A large male emerges from a large host or from a host into which only a few eggs had been laid. Both variants lead to a higher availability of nutritional resources (Rivers & Denlinger, 1995). Supposedly, large parasitoid males had better nutrition during their development than small males (Chown & Gaston, 2010). Small and large males emerge from one and the same host at the same time and, therefore, a large male grows faster by taking in more resources within the developmental time than small males. Larger body size gives the opportunity to accumulate more fatty acids as pheromone precursors, which in turn leads to more pheromone.

Several studies have demonstrated a correlation between male body size and sperm amount and fitness in insects (for instance: Blanckenhorn, 2005; Gage, 1994; Bissoondath & Wiklund, 1997; Schlüns et al., 2003). Because of the correlation between the HDL titre and
the sperm amount, large *Nasonia* males in turn also have more sperm, as was shown at least for *Nasonia* hybrids (Clark et al., 2010).

Large males, reared on an untreated host, have been shown to have about the same amount of HDL (figure 6.1) as a male of normal size reared on a host enriched in OA and LA (figure 2.3). Therefore, *Nasonia* females have a second possibility to produce “sexy sons”, besides laying eggs in hosts rich in OA and LA (chapter 2). Laying fewer eggs in a host leads to a better nutrition of the offspring, to a larger body size and also to “sexy sons” with a high HDL amount. However, simultaneously it leads to fewer daughters and subsequently to a smaller number of descendants and to disadvantages for the mother. Furthermore a female cannot precisely determine the number of eggs that she lays in a host, because more females can lay eggs in one and the same host, although *N. vitripennis* females sometimes reject a parasitized host or lay only a few eggs in it (for instance Wylie, 1970; Werren, 1983; King & Skinner, 1991; Ivens et al., 2009). It is more advantageous for the female and, in turn, for the male offspring if she lays her eggs in LA-rich hosts.

**Aphrodisiac**

One function of the male aphrodisiac is to elicit a receptivity signal of the female in courtship. The results of chapter 7 show, that simultaneously females’ behaviour is switched, females are not attracted to HDL anymore but increasingly to host odour. No mating or transmission of sperm is sufficient for the behavioural switch of the females a few minutes after mating. The behavioural switch complements the second function of the aphrodisiac, the monopolization of females. After copulation, males once again release a chemical substance that prevents female from further matings. It is probably the same substance that has previously evoked the receptivity of the females (van den Assem & Visser, 1976).

High mating rates generally lead to high reproductive success. The reproductive success of females is positively correlated with the number of eggs. Multiply mated females have advantages such as a greater amount of sperm available in the reproductive tract and for the stimulation of egg production. However, females also have disadvantages as multiple mating leads to a shorter lifespan and egg production rate, because of, for instance, the loss of time and energy during mating and the risk of physical injuries (Andersson, 1994; Eberhard, 1996; Arnqvist & Nilsson, 2000; Strassmann, 2001). Males increase their reproductive success when they monopolize females to ensure that only their own sperm is transferred to a female. Monopolizing females is not only common in insects but also in several other animal species. For instance male orb-web spider *Argiope bruennichi* often breaks off a pedipalp
inside a female, fleeing from the aggressive female after copulation, with the effort that females’ genital system is blocked for sperm of further males (Nessler et al., 2007). Well known monopolizing examples exist also in mammals, for instance infanticide in lions (Loveridge, et al., 2007) and postcopulatory genital lock in Canidae (Hart, 1972). An abundant phenomenon in insects is that males release chemicals that deter females from re-mating or that repell other males (Andersson et al., 2000; Eberhard, 1996). In Nasonia a new method was revealed to decrease the risk of multiple mating in females, an inactivation of females’ attraction to a male sex pheromone with another.

Males of Bombus terrestris release a sticky substance that they transfer from their accessory glands that highly efficiently prevent females from re-mating; LA was analysed as the active component incorporated into the “mating plug” (Baer et al., 2000, 2001). In N. vitripennis the chemistry of the aphrodisiac is still unknown, maybe LA might do not only act as a precursor of HDL, but also has an important role with regard to the aphrodisiac, possibly as a precursor or perhaps modified into the aphrodisiac itself. It is known from some male insects that they transmit substances ingested by nutrition with the sperm to females as a gift. The males signal their transferable amount of gift with a courtship pheromone that females use for mate choice (for instance: Eisner et al., 1996; Dussourd et al., 1991; Kelly et al., 2012).

In Nasonia no hint exists that the aphrodisiac functions as a quality indicator for the females. Selection of the male mating partner happens at an earlier stage by choosing the male with the highest HDL deposit representing highest nutritional condition and highest sperm amount. During courtship, Nasonia females seem not to choose males according to the aphrodisiac (chapter 2, 6 & 7). However, males that do not release the aphrodisiac are unable to elicit female receptivity and no copulation occurs (van den Assem et al., 1981, Ruther et al., 2010).

For the females the aphrodisiac might function as a control that a male of the same species is courting. However, the risk remains that N. vitripennis females have chosen a male of a sister species (Niehuis et al., 2012).

For the males the aphrodisiac can be a new investigated method to monopolize females. The ability to switch off females’ attraction to one pheromone with another pheromone decreases the risk of multiple mating of the females.

Conclusion

Unsaturated fatty acids serve not only as nourishments; experiments with Nasonia revealed that unsaturated fatty acids can also function as sex pheromone precursors and an ingestion
of a high amount of them leads to a high sex pheromone titre and subsequently to high mating chances. In parasitoids, hosts are the only source of nutrition during development and it has been shown that the fatty acid composition of the hosts depends on host diet. Therefore, host diet can influence the mating chances of parasitoids.

*Nasonia vitripennis* was identified as one of only few animals and as the first insect of the order Hymenoptera that has to possess a ∆12-desaturase to convert OA into LA. Two putative *Nasonia* genes, with highest sequence homology to desaturase genes of other insects were expressed successfully in yeast cells. However none of the two genes could be determined as a ∆12-desaturase gene. The identification of ∆12-desaturases needs further investigation. Maybe still more animals possess a ∆12-desaturase.

Furthermore the results of this doctoral thesis demonstrate two different, exciting methods of compensation:

First, in haplodiploid species females cannot influence the reproductive success of their sons by choosing an attractive mating partner. Nevertheless, at least *Nasonia* females are able to care about a high reproductive success of their sons by choosing a host incorporating a high amount of sex pheromone precursors. Females` host choice leads to sons with a high pheromone titre and high attractiveness.

Second, smaller *Nasonia* males have less pheromone to attract females than larger males. However in direct competition they compensate this disadvantage with greater agility in courtship and therefore they win the same mating success than larger males.

Finally it was shown, that it is possible to inactivate an attractive effect of a pheromone by the release of another pheromone. *Nasonia* males release a pheromone during courtship that elicit females` receptivity and simultaneously females` attraction to HDL is switched off and thereby the risk of multiple mating in females decrease.
REFERENCES


References


Foster, S. P. & Anderson, K. G. 2012 Synthetic rates of key stored fatty acids in the biosynthesis of sex pheromone in the moth Heliothis virescens. Insect Biochemistry and Molecular Biology, 42, 865-872.


References


References


References


Tres, A., Bou, R., Codony, R. & Guardiola, F. 2009 Dietary n-6 or n-3-rich vegetable fats and α-tocopheryl acetate: effects on fatty acid composition and stability of rabbit plasma, liver and meat. Animal, 3, 1408–1419.


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