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Chemical Ecology of the Parasitoid Wasp Genus *Nasonia* (Hymenoptera, Pteromalidae)

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The use of chemical cues and signals is essential for communication in insects. Wasps of the genus *Nasonia* (Hymenoptera, Pteromalidae) are gregarious parasitoids that lay their eggs into puparia of cyclorrhaphous flies. During their life cycle, various kinds of semiochemicals are used: (1) a male abdominal sex pheromone that attracts females and induces site fidelity in males, (2) a female-derived contact sex pheromone eliciting courtship behavior in males, (3) an oral male aphrodisiac eliciting receptivity signaling in females and causing a switch in the females' olfactory preferences, (4) chemicals derived from host habitat and host puparia used in olfactory host finding by female wasps, and (5) chemicals used by females to assess the quality and parasitization status of potential hosts. We review the literature on the chemical ecology of *Nasonia* spp. following the wasps' life cycle from emergence to oviposition. We depict biosynthetic pathways where available, discuss ecological implications, highlight differences among *Nasonia* species, summarize insights into their olfactory perception and associative learning abilities, and point out gaps in our understanding of the chemical ecology of these parasitoids to be addressed in future studies.

Keywords: biosynthesis, chemical communication, jewel wasp, life cycle, olfactory perception, parasitic wasp, pheromones, semiochemicals

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INTRODUCTION

Chemicals are highly important to insect life enabling them to locate food and prey, to find, recognize and evaluate potential mating partners, to mediate the complex interactions in societies as well as to avoid natural enemies and suboptimal living conditions (Cardé and Baker, 1984; Symonds and Elgar, 2008; Wyatt, 2014). Even though other sensory stimuli such as visual and tactile cues are also employed by insects, chemicals often predominate in communication processes in several stages of the insects' life cycles. Since the identification of bombykol, the sex pheromone used by females of the silkworm moth *Bombyx mori* for long-range attraction of males (Butenandt et al., 1959), and the following establishment of the field of chemical ecology in the early 1960s, particular focus has been laid, among other topics, on the identification of chemical cues and signals used by insects to find mating partners and locate adequate foraging, and oviposition sites (Greenfield, 1981; Cardé and Baker, 1984; Renwick, 1989; Landolt, 1997; Pichersky and Gershenzon, 2002; Wyatt, 2014). Setting out for a specific destination (e.g., a mating site or oviposition site) in an often complex environment usually necessitates different stages of searching behavior. This includes the location of an adequate habitat using long-range volatiles and narrowing down the search to ever smaller spatial scales until finally making use of non-volatile

substances to recognize mating partners or assess the quality of a food item or host by direct contact (Vinson, 1976). On the long range, the process of mate finding often involves the release of highly volatile sex pheromones by one sex and the attraction to these pheromones by individuals of the other sex (Greenfield, 1981). On the short range, males usually recognize females based on chemicals distributed over the females' cuticle (Lockey, 1988; Blomquist and Bagnères, 2010) and courtship frequently involves the transfer of a species-specific sex pheromone which allows females to assess not only the species of the courting male but also its quality (Birch and Hefetz, 1987; Scott et al., 1988).

In parasitic wasps, hymenopteran insects developing in or on different life stages of other arthropods, chemical cues and signals are used in a plethora of different situations. For mate finding, some species rely on volatile aggregation pheromones released by the aggregating wasps (e.g., in *Barchymeria intermedia* and *B. lasus*, Mohamed and Coppel, 1987). In other species, host-associated volatile cues are attractive from a distance for both sexes thus enabling encounters of potential mates at these host patches (e.g., alarm pheromones produced by host-associated mites, Ruther and Steidle, 2000; or herbivore-induced plant volatiles, Xu et al., 2016; Xu and Turlings, 2018). Mostly, however, individuals of the opposite sex are attracted directly via volatile sex pheromones released by either females (Quicke, 1997; Ruther, 2013) or less frequently males (e.g., *Melittobia digitata*, Cônsoli et al., 2002; or *Nasonia vitripennis*, Ruther et al., 2007). At mating sites, intraspecific competition for mates sometimes involves the application of territorial markings by males (e.g., *Urolepis rufipes*, Cooper and King, 2015; *N. vitripennis*, Mair and Ruther, 2018). During encounters, female-derived cuticular lipids elicit courtship behavior in males (e.g., *Roptrocerus xylophagorum*, Sullivan, 2002) and in some species, courting males transfer an aphrodisiac from their antennal (e.g., *Leptopilina spp.*, Weiss et al., 2015) or oral (e.g., *N. vitripennis*, Ruther et al., 2010) glands to the female's antennae. After mating, females typically use host-associated cues such as host pheromones or herbivore-induced plant volatiles to locate adequate hosts (Vinson, 1976; Steidle and van Loon, 2002; Fatouros et al., 2008; Dicke, 2009; Turlings and Erb, 2018). At oviposition sites, females of some species have been found to make use of marking pheromones to avoid competition with other females (e.g., in *Epidinocarcis lopezi*, van Dijken et al., 1992; Nufio and Papaj, 2001), and in other species, females avoid aggression by releasing appeasement pheromones after having lost disputes with "owners" of already paralyzed hosts (e.g., *Goniozus legneri*, Goubault et al., 2006).

For decades, *Nasonia vitripennis* has served as a model for the study of parasitic wasp behavior (Whiting, 1967; van den Assem, 1986) and has since developed into a model in various other fields of biological research (Gadau et al., 2008; Werren and Loehlin, 2009; Werren et al., 2010; Schurmann et al., 2012; Ruther, 2013; Groothuis and Smid, 2017; Tappert et al., 2017). The rather recent identification of the three other *Nasonia* species (Darling and Werren, 1990; Raychoudhury et al., 2010a) now opens up the opportunity to switch from merely mechanistic studies of chemical ecology that identify the chemical signals and cues used in different contexts of intraspecific interactions and interactions between wasps and hosts, to comparative and evolutionary studies that investigate chemical signal evolution and its consequences on interspecific interactions and sympatry. *Nasonia* wasps are easy to breed in the lab, easy to handle and due to their phylogenetic species structure and distribution pattern (differently related species occurring in sympatry and allopatry; **Figure 1**) form an exceptional model system for the study of the evolution of species-specific chemical communication and other reproductive isolation mechanisms. In particular, the combination of closely related species occurring in allopatry and less closely related species occurring in sympatry allows the differentiation between species characteristics resulting from phylogenetic relatedness and those that have been shaped by natural selection on unfit hybrids. Furthermore, the availability of the whole genome sequences (Werren et al., 2010) and the growing molecular toolbox for the *Nasonia* species (Lynch and Desplan, 2006; Lynch, 2015; Li et al., 2017) open up valuable opportunities to investigate biosynthetic pathways in detail and get new insights into insect chemical perception in general, both of which are fundamental for the understanding of the evolutionary processes shaping chemical communication in nature.

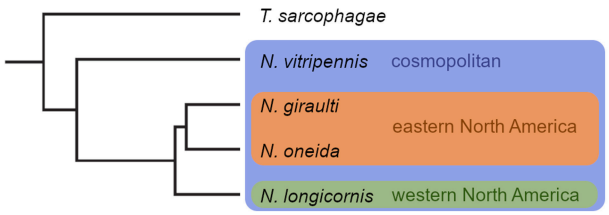

FIGURE 1 | Phylogenetic relationships and distribution of the four *Nasonia* species. *N. vitripennis* is cosmopolitan and occurs in sympatry with all other *Nasonia* species. *N. longicornis* is allopatric to *N. girault* and *N. oneida*. Phylogenetic tree originally published in Niehuis et al. (2013).

THE GENUS *NASONIA*

Wasps of the genus *Nasonia* (Hymenoptera, Pteromalidae) are parasitoids of pupae of various cyclorrhaphous flies (Diptera) found in nests of hole-breeding birds and on rotting carcasses (Darling and Werren, 1990; Raychoudhury et al., 2010a). The wasps are ectoparasitic and lay their eggs onto the surface of the fly pupa inside the fly puparium. Larvae feed on the host from the outside of the host body, pupate inside the host puparium and emerge from the puparium after eclosion. *Nasonia* wasps are gregarious, i.e., females lay more than one egg per host, and frequently several females lay their eggs into the same fly puparium (Grillenberger et al., 2008). Courtship and copulation happen at the natal host patch after emergence and females disperse after mating (King et al., 2000; Grillenberger et al., 2008; Ruther et al., 2014).

The genus *Nasonia* consists of four species. *Nasonia vitripennis* (Walker, 1836) (*Nv*), the species most intensely studied, is cosmopolitan and occurs sympatrically with all other *Nasonia* species (Raychoudhury et al., 2010b; **Figure 1**). *Nasonia*

longicornis (Darling and Werren, 1990) (*Nl*) inhabits western North America, whereas *N. giraulti* (Darling and Werren, 1990) (*Ng*) is restricted to eastern North America, and *N. oneida* (Raychoudhury et al., 2010a) (*No*) is merely known from two locations in New York State, co-occurring with *Ng* and *Nv*. Sympatric *Nasonia* species are frequently found on the same host patch and may even develop in microsympatry, i.e., within the same host individual (Darling and Werren, 1990; Grillenberger et al., 2009b; Raychoudhury et al., 2010a,b). In the natural environment, it is therefore likely that adult females and males of two sympatric species encounter and interfere on shared host patches. All *Nasonia* species, except for the species pair *Ng/No*, are reproductively isolated by *Wolbachia*-induced cytoplasmic incompatibility resulting in paternal chromosome loss after fertilization with heterospecific sperm (Breeuwer and Werren, 1990; Bordenstein et al., 2001). Females having mated with heterospecific males are not able to produce hybrid offspring. Instead, because *Nasonia*, like all hymenopterans, are haplodiploid, eggs fertilized by heterospecific sperm either die or develop into male offspring similar to unfertilized eggs (Breeuwer and Werren, 1990; Tram et al., 2006). As females of *Nasonia* usually mate only once during their lifetime, interspecific copulations are particularly costly for them (Liou and Price, 1994). Mechanisms involving adaptations in chemical communication have therefore evolved between *Nasonia* species to avoid and counteract the risks and costs of copulating with the wrong partner (e.g., Giesbers et al., 2013; Ruther et al., 2014).

The chemical communication system, particularly that of *Nv,* is one of the best understood in insects. Studies on semiochemicals used by *Nv* have revealed pheromones and allelochemicals which are highly important for the wasps in almost all stages of their life: males use sex pheromones to scent mark territories and arrest females after emergence from the host (e.g., Ruther et al., 2007). They use female-derived contact sex pheromones to recognize potential mates (e.g., Steiner et al., 2006). During courtship, the male mounts the female and performs specific courtship movements coupled with the transfer of an aphrodisiac from the male's cephalic glands to the female's antennae to induce female receptivity (e.g., van den Assem et al., 1980b). After mating, females use host habitat cues to locate new host patches (e.g., Frederickx et al., 2013) and are able to assess host quality and the status of pre-parasitization by other females through chemical inspection with their ovipositors (e.g., King and Rafai, 1970; Blaul and Ruther, 2011; for a summary of the semiochemicals used in *Nasonia* see **Table 1** and **Figure 2**).

In this article, we review literature on the chemical ecology of all four *Nasonia* species following the wasps' life cycle from emergence to oviposition (**Figure 2**). Focus is laid on chemical stimuli used by the wasps at different stages of their lives (**Table 1**) including, where available, information on biosynthetic pathways and ecological implications. Recent studies have revealed that the different *Nasonia* species differ in far more aspects of chemical communication and behavioral strategies than previously thought (Leonard and Boake, 2006; Niehuis et al., 2013; Ruther et al., 2014; Giesbers et al., 2016; Mair et al., 2017; Mair and Ruther, 2018). After discussing information available for *Nv,* differences to the other three *Nasonia* species are thus highlighted where they are known. In addition, we give a short overview about what is known about the wasps' olfactory associative learning abilities and olfactory perception, including antennal morphology, sensory sensillae, and the genetic basis of chemosensory receptors and odorant binding proteins.

Territoriality and Mate Acquisition—The Male Abdominal Sex Pheromone

Behavioral Strategies of *N. vitripennis* Males

Males of *Nv* emerge protandrously, i.e., they mostly emerge from the host prior to females by chewing an emergence hole into the fly puparium [Giesbers et al., 2016; Mair and Ruther, 2018; **Figure 2**(1)]. The first male often emerges several hours earlier than the following males and builds up a territory on the host which is defended aggressively against all other males emerging later or intruding from nearby hosts (van den Assem et al., 1980a; van den Assem, 1986; Leonard and Boake, 2006; Mair and Ruther, 2018). Subordinate males stay close to the territory and interfere with the territorial male regularly by challenging its position on the host. Although the territorial male is often the largest male in a group, experience (i.e., having experienced being in the territorial position and winning aggressive interactions) is more important than body size for the outcome of such male-male contests (Mair and Ruther, 2018). The territorial structure of a group can persist over longer time periods, but territoriality becomes increasingly unstable with increasing group size, finally resulting in scramble competition for females emerging from the host later (van den Assem et al., 1980a). Although this needs further investigations, group dynamics likely become more complex when several hosts occur in close proximity on a host patch giving territorial males the opportunity to enlarge their territory to include adjacent hosts and allowing subordinate males to potentially become territorial on hosts from which wasps have not yet emerged (van den Assem et al., 1980a; but see also Mair and Ruther, 2018). Emerging females are mounted and courted by the first male they encounter. By dominating the position on the host from which females are about to emerge, a territorial male should thus get prioritized access to copulations with females. However, observations under semi-natural conditions have been unsuccessful in demonstrating a fitness benefit for territorial males over subordinate males in the lab (Mair and Ruther, 2018). In contrast to territorial males, subordinate males gain copulation opportunities by following an alternative reproductive strategy (Mair and Ruther, 2018). They frequently mount the female together with the territorial male, position themselves on the female's abdomen, sneak in when the female signals receptivity and copulate with the female instead of the courting territorial male. In addition, when two or more females emerge in a quick succession, it may happen that the territorial male is still occupied with courting the first female when the second female emerges, giving nearby subordinate males the opportunity to court and mate with the female themselves (Mair and Ruther, 2018).

TABLE 1 | Semiochemicals used by the four *Nasonia* species, *N. vitripennis* (*Nv*), *N. girault*i (*Ng*), *N. longicornis* (*Nl*), and *N. oneida* (*No*).

				Nv	*Ng*	*Nl*	*No*	Semiochemical class	Source	Function	References
Territoriality and courtship	(1)	Male abdominal sex pheromone	RS-HDL[a]	+	+	+	+	Sex pheromone Marking pheromone	Rectal vesicle	Female attractant	Ruther et al., 2007, 2009, 2014; Abdel-Latief et al., 2008; Niehuis et al., 2013; Diao et al., 2016
			RR-HDL[b]	+	–[c]	–[c]	–[c]	Sex pheromone Marking pheromone	Rectal vesicle	Female attractant Synergist of RS-HDL	
			MQ[d]	+	+	+	+	Sex pheromone Marking pheromone	Rectal vesicle	Male arrestment Synergist of RS-HDL	Ruther et al., 2008, 2011; Niehuis et al., 2013
	(2)	CHCs[e] CLs[f]		+	+	+	+	Sex pheromone Contact pheromone	Distributed over the cuticle	Species and sex recognition	Carlson et al., 1999; Steiner et al., 2006; Niehuis et al., 2010; Raychoudhury et al., 2010a; Buellesbach et al., 2013, 2018; Mair et al., 2017; Bien et al., 2019
	(3)	Male oral sex pheromone		+	+	+	+	Sex pheromone	Mandibular gland?	Triggers receptivity Pheromone switch Receptivity switch Switch to host-seeking behavior	van den Assem and Visser, 1976; van den Assem et al., 1980b, 1981; Steiner and Ruther, 2009a; Ruther et al., 2010; Ruther and Hammerl, 2014; Boulton and Shuker, 2015
Host finding	(4)	DMDS[g]		+	?	?	?		Host habitat (decaying meat)	Finding host habitat	Frederickx et al., 2013
		Birds' nest odors		+	?	?	?		Host habitat (birds' nests)	Finding host habitat	Peters, 2011
		Host odors		+	?	?	?	Kairomones	Host pupa	Finding host	Steiner and Ruther, 2009b; Peters, 2011
Host quality assessment	(5)	Host odors?		?	?	?	?	Kairomones	Host pupa	Discrimination of host species?	Rivers and Denlinger, 1995b
		Linoleic acid		+	?	?	?	Kairomone	Host pupa	Indicates high quality of a host	Blaul and Ruther, 2011
		Eggs/venom/venom-induced changes in haemolymph		+	?	?	?	Pheromone/ allelochemically induced kairomone	Wasp eggs/venom gland/host pupa	Assessment of pre-parasitization	King and Rafai, 1970; King and Skinner, 1991; Shuker and West, 2004

+, substance(s) present in or used by the respective species; –, substance(s) absent; bold, species in which the use and function of the respective substance(s) has been shown in experimental bioassays; ?, no data available; numbers correspond to section numbers in the text and stages in the wasps life cycle (see ***Figure 2****).*

[a] *(4R,5S)-5-hydroxy-4-decanolide.*

[b] *(4R,5R)-5-hydroxy-4-decanolide.*

[c] *RR-HDL found in traces.*

[d] *4-methylquinazoline.*

[e] *cuticular hydrocarbons.*

[f] *cuticular lipids (CHCs + polar lipids).*

[g] *dimethyldisulfide.*

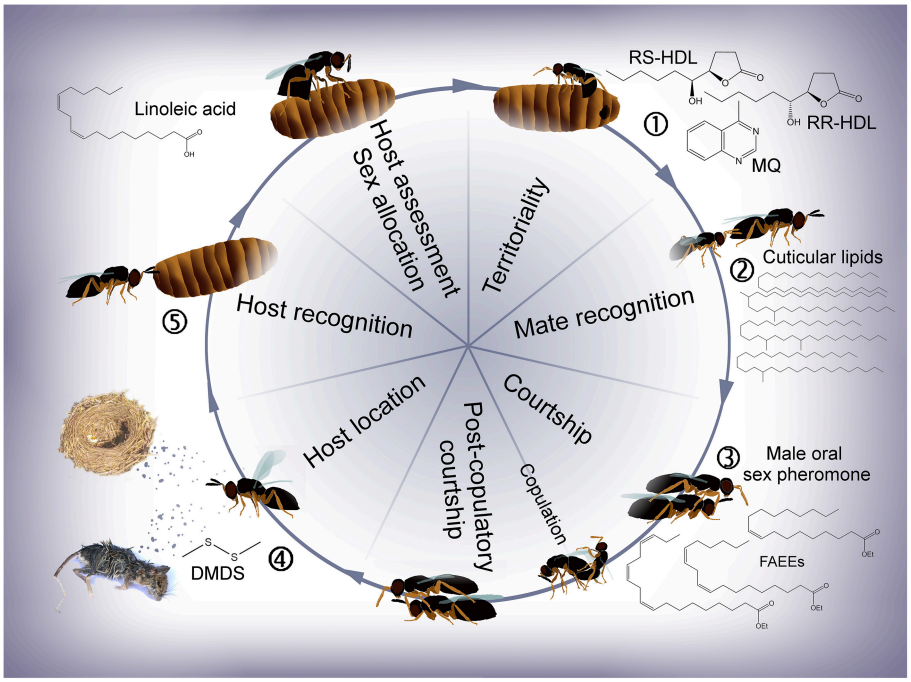

FIGURE 2 | Life cycle of *Nasonia* with emphasis on the different stages at which semiochemicals are involved. (1) Males apply an abdominal sex pheromone to the natal host and its surroundings to attract and arrest emerging females. (2) Males recognize females based on the females' cuticular hydrocarbons (CHCs, *N. vitripennis*) or cuticular lipids (CLs, = cuticular hydrocarbons + polar lipids, *N. giraulti*). (3) During courtship, a male oral sex pheromone is transferred to the female's antennae, females discriminate between conspecific and heterospecific mating partners and post-copulatory courtship induces a switch in the females' receptivity. (4) Females find new hosts based on olfactory cues. (5) Chemical stimuli are most likely involved during host recognition, host assessment and sex allocation (according to local mate competition theory). RR-HDL, (4*R*,5*R*)-5-hydroxy-4-decanolide; RS-HDL, (4*R*,5*S*)-5-hydroxy-4-decanolide; MQ, 4-methylquinazoline; FAEEs, fatty acid ethyl esters; DMDS, dimethyldisulphide.

Territorial Markings as Honest Signals of Male Quality

Territoriality in *Nv* is accompanied by the application of pheromonal scent marks deposited by the territorial male on the surface of the host and areas nearby by performing dapping and streaking movements with its abdomen over the substrate, a behavior termed "abdomen dipping" (Barrass, 1969; Steiner and Ruther, 2009a; Ruther et al., 2011; Mair and Ruther, 2018). Marking behavior is intensified after contact to females and exhibited particularly often after successful copulation (van den Assem, 1986; Steiner and Ruther, 2009a). Territorial males show marking behavior more often than subordinate males (Mair and Ruther, 2018). Nevertheless, subordinate males exhibit marking behavior outside the central territorial area, likely at spots where they have previously encountered and eventually copulated with a female (Steiner and Ruther, 2009a; Mair and Ruther, 2018). The pheromonal markings are highly attractive for both females (virgin) and males, but males do not distinguish between their own markings and those laid by their competitors (van den Assem, 1986; Ruther et al., 2011). By eliciting site fidelity in both sexes, the pheromone prevents virgin females from moving away from the host after emergence and enables males to locate spots where females have been encountered before, either by themselves or by other males. Once deposited, the pheromone markings are attractive for ca. 2–3 h exceeding by far the usual time gap of <50 min between two consecutive emergences (Steiner and Ruther, 2009a; Mair and Ruther, 2018). A chemical basis for the attractiveness of the markings has been suggested already in 1980 (van den Assem et al., 1980b). The components of the abdominal marking pheromone have been identified as the two stereoisomers (4*R*,5*S*)- and (4*R*,5*R*)-5-hydroxy-4-decanolide (RS-HDL and RR-HDL, respectively) and the minor pheromonal component 4-methylquinazoline [MQ; Ruther et al., 2007, 2008, 2011; Steiner and Ruther, 2009b; **Table 1**(1)]. All three components are synthesized in the male rectal vesicle and are absent in females (Abdel-Latief et al., 2008). Males are attracted only by MQ whereas RS-HDL and RR-HDL do not elicit any specific behavioral responses (Ruther et al., 2011). In contrast, *Nv* females are attracted by RS-HDL alone which is synergized by both RR-HDL and MQ (Ruther et al., 2007, 2008; Steiner and Ruther, 2009a; Niehuis et al., 2013). In bioassays, the strongest attraction of females has been observed when all three components were presented together (Niehuis et al., 2013). In addition, the pheromone response of females is dose dependent with females preferring higher deposited amounts over lower ones (Ruther et al., 2009; Blaul and Ruther, 2011). After copulation, however, females are no longer attracted to the abdominal sex pheromone, become restless instead and

switch to host-seeking behavior (Ruther et al., 2007, 2010, 2014; Steiner and Ruther, 2009b).

RS- and RR-HDL occur typically in the rectal vesicle at a 2:1 ratio and sex pheromone titers as well as the amount of pheromone actually deposited by males are associated with male body size and with male mating history (Ruther et al., 2009; Blaul and Ruther, 2012). Larger males produce and deposit more pheromone (up to 1 μg total HDL) than smaller males (<0.5 μg total HDL; Blaul and Ruther, 2012), and male pheromone titers decrease with repeated marking activity following after each copulation (more than 60% reduction of HDL in males having mated once; Ruther et al., 2009; Blaul and Ruther, 2012). Markings deposited by multiply mated males are thus less attractive to females than markings deposited by virgin males. The fact that significant sperm depletion occurs already after seven consecutive matings indicates that pheromone quantity may be used by females during mate choice as an honest signal of male fertility (Ruther et al., 2009). Even if having moved away from the territory when mounted on the female's back during courtship and copulation, territorial males usually return to their territory before intensifying marking activities (Mair and Ruther, 2018). Instead of applying new pheromonal spots elsewhere, they thus strengthen the signal of their earlier pheromonal deposits by adding further amounts of the pheromone. Females attracted to stronger pheromonal signals are therefore also attracted to reproductively and territorially successful males.

Further evidence that the amount of pheromone is indeed an honest indicator of male quality comes from studies on the biosynthetic pathway of the major pheromone components RS-HDL and RR-HDL (**Figure 3**). A chemical sexual signal can become honest, if its production is costly for the producer (Zahavi, 1975; Johansson and Jones, 2007). Often pheromone production involves biosynthetic pathways connected to other important metabolic functions effecting, for example, the individual's immunological defense against pathogens (Rantala et al., 2003) or the production of gametes (Thomas and Simmons, 2009). Linoleic acid ((9*Z*,12*Z*)-octadeca-9,12-dienoic acid, LA), a polyunsaturated fatty acid (PUFA), is needed for the production of sperm in animals (Wathes et al., 2007). Stable isotope labeling experiments revealed that LA is also a precursor for the biosynthesis of HDL in *Nasonia* males (Blaul and Ruther, 2011). This indicates a trade-off between abdominal sex pheromone production and the production of sperm. Consistently, males emerging from hosts artificially enriched in LA produce both larger amounts of sperm and larger amounts of HDL (Blaul and Ruther, 2011).

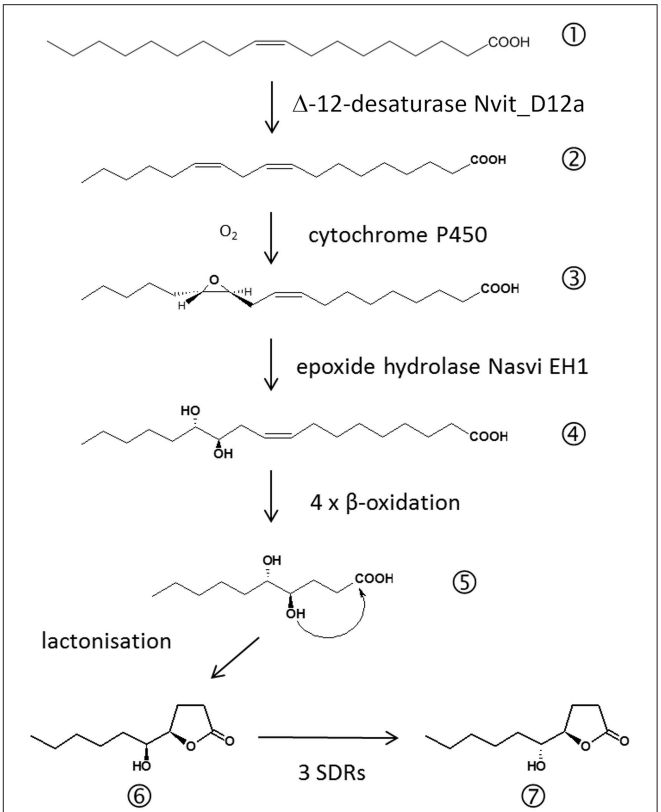

FIGURE 3 | Biosynthetic pathway of the major *Nasonia* male abdominal sex pheromone components. (1) oleic acid, (2) linoleic acid, (3) 12,13-epoxy-(9*Z*)-octadecenoic acid, (4) 12,13-dihydroxy-(9*Z*)-octadecenoic acid, (5) 4,5-dihydroxydecanoic acid, (6) (4*R*,5S)-5-hydroxy-4-decanolide (RS-HDL), (7) (4*R*,5*R*)-5-hydroxy-4-decanolide (RR-HDL).

Biosynthesis of the Male Marking Pheromone

Recent studies on the pheromone biosynthesis revealed that *Nv* has a Δ12-desaturase enabling them to synthesize LA from oleic acid (OA; Blaul et al., 2014; Semmelmann et al., 2019b; see **Figure 3**). Wang et al. (2015) found a predicted desaturase gene Nasvi2EG017727 to be 800-fold higher expressed in *Nv* males while a highly similar paralogue was expressed unbiasedly in both males and females. Functional characterization of the two gene products Nvit_D12a and Nvit_D12b subsequently revealed that both have in fact Δ12-desaturase activity. However, only Nvit_D12a was found to be expressed in the male pheromone gland suggesting that it acquired a specialized function in *Nv* for the production of LA as a pheromone precursor (Semmelmann et al., 2019b). The fact that *Nv* males are capable of synthesizing LA by themselves challenged the postulated importance of LA as a limited resource. However, a recent study (Brandstetter and Ruther, 2016) revealed that males albeit synthesizing LA still benefit from the dietary uptake of LA during larval development. Males reared on LA enriched hosts were able to produce significantly higher amounts of HDL than males reared on hosts enriched in OA indicating that the conversion of OA into LA is a costly process (Brandstetter and Ruther, 2016). The next step of the HDL biosynthesis is an epoxidation of LA to 12,13-epoxy-(9*Z*)-octadecenoic acid. This conversion is typically catalyzed by cytochrome P450 enzymes (CYP450; Oliw, 1994). CYP450 genes are highly abundant in the *Nv* genome (Oakeshott et al., 2010), but the question which of the 92 candidate genes is involved in the pheromone biosynthesis needs further investigation. The next step of the HDL biosynthesis is the hydrolysis of 12,13-epoxy-(9*Z*)-octadecenoic acid to 12,13-dihydroxy-(9*Z*)-octadecenoic acid by the epoxide

hydrolase Nasvi-EH1 (Abdel-Latief et al., 2008). Gene expression experiments using *in situ* RT-PCR suggested that this step occurs in the rectal papillae, twins of secretory organs adjacent to the rectal vesicle (Davies and King, 1975; Abdel-Latief et al., 2008). Four steps of chain shortening by β-oxidation and lactonization of the resulting 4,5-dihydroxydecanoic acid then eventually leads to RS-HDL. RR-HDL is produced in *Nv* males by epimerization of RS-HDL using short-chain dehydrogenases/reductases (SDRs) encoded by the three genes NV10127, NV10128, and NV10129 positioned on chromosome 1 (Niehuis et al., 2013; Ruther et al., 2016). The sequences of the *Nasonia* SDR genes are highly similar to those of enzymes catalyzing the deactivation of prostaglandins which serve various hormonal functions in insects (Stanley, 2006). This suggests that the SDRs epimerizing RS- to RR-HDL in *Nv* have evolved secondarily from these enzymes by gene duplication and neofunctionalization (Niehuis et al., 2013; Ruther et al., 2016). The three homologous SDRs differ significantly in their ability to epimerize RS- to RR-HDL. The investigation of the underlying sequence-activity relationships by site-directed mutagenesis experiments revealed that six amino acids at the C-terminus were crucial for the efficient epimerization of RS- to RR-HDL (Semmelmann et al., 2019a).

Strategies at the Natal Host Patch in the Other *Nasonia* Species

The *Nasonia* species differ profoundly in the behavior they exhibit at the natal host patch: while virtually all females of *Nv* emerge from the host puparium as virgins, almost all females of *Ng* mate inside the host prior to emergence (Drapeau and Werren, 1999; Leonard and Boake, 2006; Giesbers et al., 2013; Ruther et al., 2014), and within-host-mating (WHM) rates in *Nl* and *No* lay between those of *Nv* and *Ng* (Leonard and Boake, 2006; Giesbers et al., 2013). Giesbers et al. (2016) suggested that the high WHM rate in *Ng* results from the fact that *Ng* males refrain from chewing an exit hole into the puparium and thus impede the emergence of virgin females. Consistently, in *Ng*, females emerge prior to males (Mair and Ruther, 2018). Instead of building up territories, *Ng* males readily disperse from the host after emergence and engage less often in aggressive interactions than males of *Nv* (Leonard and Boake, 2006; Mair and Ruther, 2018). Nevertheless, *Ng* males produce similar amounts of HDL as males of *Nv* (Ruther et al., 2014) and use the abdominal sex pheromone to mark the substrate in the surroundings of the natal host patch (Mair and Ruther, 2018). Mair and Ruther (2018) suggested that *Ng* marking, although useless when all females are already mated, is of adaptive importance in microsympatry with *Nv*. When wasps of *Nv* and *Ng* develop within the same host individual, an increased number of *Ng* females emerges as virgins because *Nv* males chew an exit hole into the puparium through which virgin *Ng* females can escape (Giesbers et al., 2016). In these situations, *Ng* males marking the substrate with abdominal sex pheromone may be able to attract and copulate with these unmated females (Mair and Ruther, 2018). Future observations of wasps emerging naturally from hosts multiparasitized by both *Nv* and *Ng* could give valuable insights into the dynamics occurring between these two species at the natal host patch in microsympatry.

A further difference between the *Nasonia* species concerns the composition of the marking pheromone. The male abdominal sex pheromone of *Nl, No,* and *Ng* as well as the one of the closely related species *Trichomalopsis sarcophagae* consists of RS-HDL and MQ, lacking significant amounts of the third component RR-HDL (Niehuis et al., 2013). This suggests that RS-HDL/MQ is the ancestral pheromone composition and RR-HDL has evolved in *Nv* as an adaptation to avoid interspecific mating caused by signal interference in areas of sympatry. Consistent with this hypothesis, RR-HDL has no effect on the pheromone response of *Ng* females while it synergizes the response to RS-HDL in *Nv* females (Niehuis et al., 2013). Strikingly, genes encoding the SDRs that catalyze the epimerization of RS- to RR-HDL are also present in the genome of *Ng* and *in vitro* assays showed that the enzymes are also capable of catalyzing the epimerization albeit with a decreased efficiency. However, proteomic analyses of the pheromone glands showed a much higher expression in *Nv* suggesting that differential SDR gene expression underlies the pheromone difference between *Nv* and the other *Nasonia* species (Ruther et al., 2016). Why *Nv* is the only *Nasonia* species that evolved an additional pheromone component remains unclear so far, but likely involves the differences in the mating behavior, in particular the complete lack of WHM and thus strong dependency on the correct identification of conspecific male sex pheromone markings to find mates in this species.

The behavior of males of *No* at the natal host patch is largely unknown. Males of *No* produce less HDL than males of *Ng* and three quantitative trait loci (QTL; on chromosomes 1, 4, and 5, respectively) have been identified that are significantly correlated with male pheromone quantity in these two species (Diao et al., 2016). However, the functional characterization of genes at these loci as well as the ecological implications of this difference in pheromone quantity necessitates further investigation. It would be interesting to study whether HDL production and the release of the abdominal sex pheromone are further correlated to different behavioral strategies at the natal host patch, e.g., rendering pheromone deposition by *No* males less important than in the other *Nasonia* species.

Nl is characterized by relatively low WHM rates of about 10% (Drapeau and Werren, 1999; Leonard and Boake, 2006; Giesbers et al., 2013) and engage in aggressive interactions on the host after emergence (Leonard and Boake, 2006) indicating that *Nl* males may exhibit territorial behavior similar to that of *Nv* males. The amount of HDL produced by males of *Nl*, however, has not been investigated so far. A detailed comparative study of male and female behavior of all *Nasonia* species at the natal host patch coupled with behavioral bioassays using multiparasitized hosts may prove valuable for the understanding of how differences in the pheromone communication at the natal host patch have evolved and how the different *Nasonia* species are able to coexist in areas of microsympatry. In addition, little is known about the behavioral variability within *Nasonia* species and how this variability influences interspecific dynamics in the field.

Male Mate Recognition—Female Derived Contact Sex Pheromones

When encountering another individual, males have to decide whether or not the individual is a possible mating partner to appropriately adjust subsequent decisions [**Figure 2**(2)]. In *Nv*, females are typically followed and courted by the males, whereas male competitors are either attacked or dominated and chased off (van den Assem et al., 1980a). In addition, as a measure of prezygotic reproductive isolation, males should avoid courting heterospecific females or at least prefer conspecific over heterospecific ones if costs imposed by courting and copulating with the wrong mating partner are considerably high. In insects, male courtship is often induced by chemical stimuli distributed over the females' cuticle (Lockey, 1988; Howard and Blomquist, 2005; Blomquist and Bagnères, 2010; Wyatt, 2014). These chemicals can be washed off using non-polar solvents such as hexane or pentane, which indicates that the chemicals eliciting courtship behavior are cuticular lipids [CLs; **Table 1**(2)]. One major class of CLs are the cuticular hydrocarbons (CHCs) which primarily function as a protection shield against desiccation (Lockey, 1988; Gibbs, 1998) and play important roles in recognition processes in a wide variety of insect taxa (Singer, 1998; Blomquist and Bagnères, 2010). CHCs are the most abundant CLs found on the insects' cuticular surface and are easily detectable and identifiable by gas chromatography and mass spectrometry (GC/MS). Other CLs are the more polar lipids such as aldehydes, alcohols, ketones, wax esters, or non-volatile fatty acid derivatives (NFADs) some of which are not detected by standard GC/MS methods without prior derivatization (Buckner, 1993; Kühbandner and Ruther, 2015). As a result, they are often neglected in studies investigating mate recognition in insects and the term CHCs is often erroneously used interchangeably with CLs. To unambiguously show the behavioral effect of CHCs in recognition processes it is, however, necessary to separate the CHCs from the more polar CLs prior to their use in behavioral bioassays. This can be achieved easily by fractionating complete CL extracts on silica gel columns. In addition, recent studies have shown that more polar lipids (Yasui et al., 2003; Eliyahu et al., 2008; Kühbandner et al., 2012; Salerno et al., 2012; Stökl et al., 2014; Keppner et al., 2017) and even peptides (Turillazzi et al., 2006) can elicit behavioral responses in insects and are likely used as contact sex pheromones far more often than previously thought.

Male Mate Recognition in *N. vitripennis*

In *Nv*, males recognize females based on the females' CHCs alone and do not rely on additional, more polar compounds (Steiner et al., 2006). The CHC profile in *Nasonia* detectable by classical GC/MS techniques consists of hydrocarbons ranging from C25 to C37 including n-alkanes, mono-, di-, tri-, and tetramethylalkanes as well as few alkenes (Carlson et al., 1999; Steiner et al., 2006; Niehuis et al., 2010; Buellesbach et al., 2013, 2018; Mair et al., 2017). Both quantitative and qualitative differences in the composition of CHCs exist between females and males of *Nv* (Carlson et al., 1999; Steiner et al., 2006; Buellesbach et al., 2013, 2018). Compared to males, *Nv* females possess higher relative amounts of hydrocarbons with chain lengths shorter than C30 as well as higher relative amounts of methyl-branched alkanes with central branching positions (e.g., 9-, 11-, 13-, 15-methylalkanes or 9,x-, 11,x-, 13,x-, 15,x-dimethylalkanes) whereas males possess higher relative amounts of methyl-branched alkanes with marginal branching positions (e.g., 3-, 5-, 7-methylalkanes or 3,x-, 5,x-, 7,x-dimethylalkanes) as well as higher relative amounts of alkenes (Steiner et al., 2006; Buellesbach et al., 2013, 2018). Males of *Nv* use the sex-specific differences in the CHCs to distinguish females from males by means of antennal contact during encounters (Steiner et al., 2006). In bioassays, female CHCs applied to dummies (solvent-washed male corpses) elicited arrestment and courtship behavior including copulation attempts, whereas male CHCs did not (Steiner et al., 2006; Mair et al., 2017).

In addition to sex-specific differences, the composition of CLs differs among all four *Nasonia* species (Carlson et al., 1999; Steiner et al., 2006; Raychoudhury et al., 2010a; Buellesbach et al., 2013; Mair et al., 2017). More specifically, compared to females of *Ng*, females of *Nv* possess higher relative amounts of n-alkanes and monomethylalkanes, whereas females of *Ng* possess higher relative amounts of di-, tri-, and tetramethylalkanes (Mair et al., 2017). In addition, Niehuis et al. (2010) showed that males of *Nv* possess larger relative amounts of the three alkenes 9-C31ene, 9-C33ene, and 7-C33ene than males of *Ng*. However, in bioassays with fractionated female extracts (containing only CHCs), *Nv* males showed courtship and copulation attempts equally often toward both dummies applied with CHCs of *Nv* females and those applied with CHCs of *Ng* females (Mair et al., 2017). Furthermore, in bioassays with living couples, *Nv* males seem to even court females of *T. sarcophagae*, a species closely related to the genus *Nasonia* which possesses a relatively similar CHC composition compared to *Nv* (Niehuis et al., 2013; Buellesbach et al., 2018). Overall, *Nv* males are hardly selective in the choice of their mating partners (Giesbers et al., 2013; Buellesbach et al., 2014, 2018) indicating that mating with the wrong partner does not impose considerable fitness costs on them. Although repeated courtship and mating reduce male longevity (Burton-Chellew et al., 2007b), this has probably only little effect on overall male fitness in nature considering that males with continuous contact to females lived for more than 9 days in bioassays, a time span in which most or all females have typically emerged from the host puparium and mated already (Mair and Ruther, 2018). In addition, males can mate multiple times before suffering from sperm depletion (seven or more matings; Whiting, 1967; Ruther et al., 2009; Chirault et al., 2016). Apart from losing time and energy spent in misdirected courtship, single mistakes in mate choice are thus not severely costly for *Nv* males.

Male Mate Recognition in the Other *Nasonia* Species

Similar to *Nv*, males of the other three *Nasonia* species also engage in courtship and copulation with heterospecific females (Giesbers et al., 2013; Buellesbach et al., 2014; Mair et al., 2017). Solely in *Ng* it has been shown that males show significant discrimination against heterospecific mating partners in living couples: They refrain from starting courtship more often when confronted with females of *No* than with conspecific females (Buellesbach et al., 2014) and start courtship faster when

confronted with conspecific as compared to *Nv* females (Mair et al., 2017). This preference for conspecific females was absent, however, in bioassays with dead females and when males were confronted with female extracts (CLs) or fractionated female extracts (CHCs) applied to dummies (Giesbers et al., 2013; Mair et al., 2017). This indicates that *Ng* males use additional species-specific characteristics such as visual cues, tactile cues or differences in the females' behavior to differentiate between con- and heterospecific mating partners. Furthermore, the class of substances used by males in the recognition of females differs between *Nv* and *Ng*. While males of *Nv* rely solely on the females' CHCs (Steiner et al., 2006; Mair et al., 2017), conspecific courtship in *Ng* males is only induced when confronted with complete CL extracts of conspecific females including the more polar lipids (Mair et al., 2017). Surprisingly, although conspecific female CHCs are not sufficient to induce courtship in *Ng* males, heterospecific female CHCs are (Mair et al., 2017). A shift to other chemical messengers used in mate recognition must therefore have happened in *Ng* (Mair et al., 2017). However, the cause leading to this shift and the reasons why heterospecific female CHCs remain attractive to *Ng* males are still unclear.

In *No*, bioassays with complete CL extracts indicate that males of *No* might be able to discriminate against heterospecific females belonging to any other *Nasonia* species (Buellesbach et al., 2013; Giesbers et al., 2013). In mating trials with living couples, however, males of *No* courted con- and heterospecific females equally often (Buellesbach et al., 2014). Nevertheless, a more detailed comparative study of the different males' discriminative abilities which, instead of merely looking at presence/absence of courtship, includes more subtle behavioral parameters such as temporal behavioral patterns is widely lacking to date. In addition, the chemical basis of mate recognition in *No,* and *Nl* has not been solved in detail yet and the potential effects that male discriminatory behavior has in more natural environments under microsympatry still needs to be elucidated. Based on the evidence gained so far, it is, however, unlikely that males of *Nasonia* contribute much to interspecific prezygotic reproductive isolation among the *Nasonia* species.

A recent study using direct laser desorption/ionization mass spectrometry revealed the additional presence of some very long chain alkanes and alkenes on the cuticles of *Nv*, *Ng,* and *Nl* which were not detected in previous studies (Bien et al., 2019). Whether these chemicals play a role in the species-specific mate recognition, however, needs further investigation.

Courtship and Female Mate Discrimination—The Male Oral Sex Pheromone

In contrast to males, females of all *Nasonia* species discriminate against heterospecific males [Raychoudhury et al., 2010a; Giesbers et al., 2013; Buellesbach et al., 2014; Ruther et al., 2014; Mair et al., 2017, 2018; **Figure 2**(3)]. The means by which they achieve discrimination, however, remain unknown to date and the identification of chemical stimuli involved failed so far. One means by which a female gets the possibility to choose between different mating partners is the male's courtship display. During courtship, the male mounts the female and starts moving its head along the female's antennae, a behavior termed head-nodding. Head-nodding comes in repetitive series consisting of species-specific patterns of long and short intervals between nodding movements, and is accompanied by stroking movements of the male's antennae and legs over the female's head and eyes (Barrass, 1960, 1961; van den Assem et al., 1980b; van den Assem and Werren, 1994; Jachmann and van den Assem, 1996; van den Assem and Beukeboom, 2004). Head-nodding cycles typically consist of one slow upward stroke followed by several faster nods, a temporal pattern which is likewise exhibited in all *Nasonia* species but differs in details such as the number of fast nods and the length and number of head-nodding cycles (van den Assem and Vernel, 1979; van den Assem et al., 1980b, 1981; van den Assem and Werren, 1994). Along with these courtship movements, an oral male sex pheromone is transferred from the male's mouthparts to the female's antennae [van den Assem et al., 1980b, 1981; Ruther et al., 2010; Ruther and Hammerl, 2014; **Table 1**(3)] potentially giving the female another means to discriminate between different mating partners. When accepting the courting male, the female shows receptivity by flattening the antennae, lowering the head and opening the genital orifice, and copulation follows (van den Assem and Vernel, 1979; van den Assem et al., 1980b; van den Assem, 1986). After copulation, the male usually returns to the courtship position and performs several more head-nodding movements before unmounting (van den Assem and Visser, 1976). It is likely that during this post-copulatory courtship the male transfers an additional amount of the oral sex pheromone. As a result of courtship, receptivity and copulation, a switch happens in the females' behavior: mated females are no longer attracted to the male abdominal sex pheromone (pheromone switch; van den Assem, 1986; Ruther et al., 2007, 2014; Steiner and Ruther, 2009a; Ruther and Hammerl, 2014; Lenschow et al., 2018), typically refrain from mating again (receptivity switch; Holmes, 1974; van den Assem and Visser, 1976; Grillenberger et al., 2008) and become restless instead and switch to dispersal and host-seeking behavior (King, 1993; King et al., 2000; Steiner and Ruther, 2009b; Ruther et al., 2014).

Receptivity Necessitates the Transfer of the Oral Male Sex Pheromone

The transfer of the oral male sex pheromone during courtship is a prerequisite for the induction of receptivity in females. During head-nodding the male extrudes its mouthparts and transfers the aphrodisiac to the female's antennae (van den Assem et al., 1980b, 1981; Ruther et al., 2010; Ruther and Hammerl, 2014). Males possess a voluminous mandibular gland which has been suggested to be the source of the aphrodisiac (Miko and Deans, 2014). By experimentally covering the mouthparts with a drop of glue, the transfer of the pheromone can be inhibited resulting in the prevention of female receptivity signaling (van den Assem et al., 1980b; Ruther et al., 2010). The substances that are responsible for inducing receptivity are, however, unknown to date (Ruther and Hammerl, 2014) and the establishment of experimental procedures allowing to test pheromonal extracts, fractions of these extracts and synthetic pheromone components has turned out to be rather difficult (personal observation, both authors). van den Assem et al. (1980b) suggested the pheromone

to be volatile. They puffed the headspace of courting couples into a chamber containing a constrained couple with a sealed male and reported that the female became receptive. Our frequent attempts to repeat this experiment, however, were unsuccessful so far. We therefore suggest that the ominous aphrodisiac of *Nv* males is non-volatile. Females of *Nv*, *Ng,* and *Nl* typically show receptivity during the first upwards stroke in a head-nodding series (van den Assem and Werren, 1994). It is thus likely that the pheromone has to be applied in due time with this movement.

The Pheromone Switch

After mating, *Nv* females are no longer attracted to the male abdominal sex pheromone. This pheromone switch occurs fast (within minutes) and is long-lasting (at least 6 days; van den Assem, 1986; Ruther et al., 2007, 2014; Steiner and Ruther, 2009b; Ruther and Hammerl, 2014). As during copulation a single male usually transfers sufficient sperm to fertilize all eggs the female is likely able to lay (Holmes, 1974; Chirault et al., 2016), not being attracted to male territorial markings is reasonable for mated females and gives them the opportunity to switch to host-seeking behavior instead (Ruther et al., 2007; Grillenberger et al., 2008). In addition, Lenschow et al. (2018) argue that the pheromone switch has evolved as a result of a male strategy to prevent females from encountering and copulating with other males. Responsible for the pheromone switch is the application of the male oral sex pheromone to the females' antennae during courtship (Ruther et al., 2010). Courtship movements, copulation, the transfer of sperm or ejaculate and post-copulatory courtship, on the other hand, are not necessary (Ruther et al., 2010; Ruther and Hammerl, 2014). In bioassays, the pheromone switch can even be triggered by merely bringing the antennae of virgin females into contact with male-derived head extracts (Ruther and Hammerl, 2014). More precisely, the active components of the oral sex pheromone that elicit the pheromone switch have been identified as the three fatty acid esters ethyl oleate, ethyl linoleate and ethyl α-linolenate (Ruther and Hammerl, 2014). The mere antennal contact with these three substances thus resulted in a change in the females' response to the abdominal sex pheromone. A study on the neuromodulatory mechanisms underlying this behavioral plasticity showed that it involves the release of dopamine (DA; Lenschow et al., 2018). In bioassays, feeding the DA receptor antagonist chlorpromazine prevented the pheromone switch while the injection of DA into virgin females rendered them unresponsive to the abdominal sex pheromone. As dopamine is also involved in appetitive olfactory learning (Waddell, 2013; Lenschow et al., 2018), this suggests that DA plays a key role in mediating olfactory plasticity in *Nv*. The pheromone switch in *Nv* females is not only induced by conspecific males but can be similarly induced by heterospecific males and a similar switch has been demonstrated in females of *Ng* (Ruther et al., 2014). It is thus likely that the pheromone switch and the substances eliciting the switch are not species-specific but instead represent an ancestral state in the *Nasonia* genus.

The Receptivity Switch

The receptivity switch is a second switch that happens in females after mating and concerns the females' willingness to re-mate. Females of *Nv* typically mate only once during their lifetime (Holmes, 1974; van den Assem and Visser, 1976; Grillenberger et al., 2008). When being courted by a second male after having already mated previously, females usually refuse to become receptive and re-mating does not occur (Holmes, 1974; van den Assem and Visser, 1976). In contrast to the pheromone switch, however, this receptivity switch is connected to the post-copulatory courtship exhibited by males after copulation (van den Assem and Visser, 1976; Boulton and Shuker, 2015). The actual copulatory act and the transfer of seminal fluids, on the other hand, are not important (van den Assem and Visser, 1976). van den Assem and Visser (1976) observed in behavioral bioassays that females showed a second receptivity signal during post-copulatory courtship. They thus hypothesized that females need to show receptivity twice before the receptivity switch happens. As courtship involves the transfer of a pheromone, it is likely that this is also true for post-copulatory courtship. The male oral sex pheromone consists of various compounds that are not involved in the pheromone switch (Ruther and Hammerl, 2014). It is thus likely that some of these compounds are involved in the receptivity switch, a hypothesis which still necessitates further investigation. Reported re-mating rates differ among *Nasonia* species and between different *Nasonia* strains (Leonard and Boake, 2008; Geuverink et al., 2009). In addition, re-mating appears to increase in strains having been reared in the laboratory over prolonged time (van den Assem and Jachmann, 1999; Burton-Chellew et al., 2007a). Studies investigating the receptivity switch therefore need to take into account the individual history of the investigated strains and should ideally work with field-collected outbred strains.

Olfactory Host Finding—The Role of Host Habitat Odors and Host Kairomones

Olfactory Host Finding in *N. vitripennis*

After mating, females of *Nv* become restless and start searching for new hosts [King, 1993; King et al., 2000; Ruther et al., 2014; **Figure 2**(4)]. Because hosts are usually distributed patchily in the environment occurring in birds' nests and on rotting carcasses, finding adequate hosts is a challenging task for females. Using four polymorphic microsatellites, Grillenberger et al. (2008) found a clear isolation between populations of *Nv* at two sampling sites in the Netherlands and Germany located about 300 km apart. Nevertheless, *Nv* females are able to disperse over long distances as indicated by the lack of genetic population substructure over a range of 100 km in a field study in the US using eight microsatellite markers (Grillenberger et al., 2009a). During long-distance dispersal, females are supposedly drifting as aerial plankton by the use of wind currents to reach new habitats (Grillenberger et al., 2009a). For orientation over intermediate and short distances, on the other hand, active chemotaxis toward host habitat odors has been suggested (Whiting, 1967). Consistently, material taken from birds' nests and carcasses attracts mated *Nv* females [Peters, 2011; Frederickx et al., 2013; **Table 1**(4)]. One compound which has been shown to be particularly attractive to mated females is dimethyldisulphide (DMDS), a major component found in the headspace of decaying meat (Kasper et al., 2012). Mated females tested in olfactometer experiments showed a clear affinity toward DMDS whereas all

other tested compounds of rotten meat extracts did not elicit preferential behavior (Frederickx et al., 2013). Adult *Nv* arrive at carcasses at an early stage of decomposition when adult host flies and larvae are already present, but host pupae have not developed yet (Voss et al., 2009). After arriving at a suitable host habitat, females most likely rely on short-range or contact kairomones originating from host fly puparia to find and identify adequate hosts on a carcass or inside birds' nests, respectively. In bioassays using a still-air olfactometer, females preferred right after mating the odor of hosts over that of conspecific males (Steiner and Ruther, 2009b).

Olfactory Host Finding in the Other *Nasonia* Species

Female dispersal and the chemical basis of host location in the other *Nasonia* species have not been investigated to date. Due to their similar life history, morphology and general chemosensory abilities, it is, however, likely that all *Nasonia* females use similar modes of transport and chemotaxis. Whether they respond to the same chemical compounds during their search for new host patches, however, needs further investigation. As *Ng* is specialized on pupae of *Protocalliphora*, it is likely that females of *Ng* (and likely also females of *No*) are not attracted to odors originating from carcasses but rely solely on cues associated with birds' nests.

Oviposition and Sex Ratio Adjustment—Chemical Assessment of Host Quality

When females encounter a host puparium after arrival at a new host patch, they need to decide firstly whether to lay eggs, secondly how many eggs to lay and thirdly how many eggs shall develop into females or males, respectively [**Figure 2**(5)]. *Nasonia* wasps, like all hymenopterans, are haplodiploid, i.e., females are diploid and emerge from fertilized eggs, whereas males are haploid and develop from unfertilized eggs (Heimpel and de Boer, 2008). During oviposition, mated females are capable of actively adjusting the sex ratio of their offspring in response to varying environmental factors essential for offspring survival and performance such as the number, quality, and parasitization status of the hosts (Verhulst et al., 2010).

Host Quality and Offspring Fitness

A high nutritional value of host pupae positively influences offspring condition which is reflected in a shorter development time and increased body size of both female and male adults (Rivers and Denlinger, 1995a; Hoedjes et al., 2014). Females emerging from hosts of lower quality are smaller and produce fewer eggs. Similar to most parasitoids, females of *Nasonia* are supposed to be unable to biosynthesize fatty acids *de novo* (Visser et al., 2010). Despite compensation for this inability through host feeding in the course of oviposition, lipids acquired prior to emergence are thus essential and limiting for adult females (Rivero and West, 2002, 2005; Sykes et al., 2008). In addition, males having developed in hosts rich in linoleic acid produce higher amounts of HDL and sperm, and are thus most likely able to attract and inseminate more females (Blaul and Ruther, 2011). The amount of host nutrients available per individual, however, decreases with increasing parasitoid clutch size or when a host is parasitized by more than one female. In addition, due to local mate competition (LMC) between male offspring at the natal host patch after emergence, females typically produce increased numbers of males under superparasitism to increase the mating success of their male offspring in the competition for mates (Werren, 1980, 1983; Shuker et al., 2006; Burton-Chellew et al., 2008). Therefore, it is crucial for the females' fitness to optimize clutch size based on the amount of nutrients available (i.e., host quality) and offspring sex ratio based on the status of pre-parasitization of the encountered host.

Host Quality Assessment

When encountering a host, the female inspects the fly puparium from outside with her antennae and the tip of her abdomen before drilling into it with her ovipositor (Edwards, 1954; King and Rafai, 1970). The tip of the ovipositor possesses pores containing chemoreceptors which are likely used to chemically inspect the status of the host [Edwards, 1954; King and Rafai, 1970; **Table 1**(5)]. If the host is deemed suitable for oviposition, venom is injected into the fly pupa, and eggs are laid onto the pupa inside the puparium (Wylie, 1965; Ratcliffe and King, 1967). During the process of drilling, *Nv* females are able to assess the nutritional quality as well as the pre-parasitization status of the host pupa (Wylie, 1965; King and Rafai, 1970; Blaul and Ruther, 2011).

Nv females discriminate between pupae of different fly species and prefer pupae of *Sarcophaga* spp. over those of *Musca domestica* (Rivers and Denlinger, 1995a). In addition, they discriminate between different host sizes and hosts with different nutritional values (Rivers and Denlinger, 1995a; Blaul and Ruther, 2011). On dead or small hosts, fewer eggs are laid, and offspring sex ratios are shifted in favor of males (Rivers and Denlinger, 1995a). In addition, females prefer LA-enriched hosts over those poor in LA (Blaul and Ruther, 2011). Given the essential function of LA in the production of sperm and HDL, this is highly adaptive. Additional cues used for the assessment of host quality are, however, to be discovered and offer promising opportunities for further research.

Assessment of Pre-parasitization

In addition to general host quality, *Nv* females are able to discriminate between parasitized and non-parasitized hosts (Wylie, 1965; King and Rafai, 1970; Holmes, 1972; Werren, 1980; King and Skinner, 1991; Shuker and West, 2004) and are even able to distinguish hosts pre-parasitized by conspecific females from those previously parasitized by heterospecific ones (Ivens et al., 2009). In general, pre-parasitized hosts are rejected more often (Ivens et al., 2009). If females decide to oviposit nonetheless, fewer offspring and higher relative numbers of males are produced (Wylie, 1965; Holmes, 1972). The means by which ovipositing females assess the parasitization status of hosts has not been identified yet, but chemical messengers are likely involved (Wylie, 1965; King and Rafai, 1970; Holmes, 1972). Holmes (1972) hypothesized that female venom injected into the fly pupa during oviposition might act as a cue and King and Rafai (1970) recognized changes

in the hosts' haemolymph composition after the injection of female venom.

Cues potentially used by females to assess the hosts' pre-parasitization status are the venom itself and venom-induced changes in the host pupae. The venom of *Nv* is acidic and consists of different amines, peptides and non-glycosylated proteins (Rivers et al., 2006). In total, 79 proteins and peptides have been identified that belong to different functional classes (de Graaf et al., 2010; Martinson et al., 2017; effects of individual compounds are discussed in detail in Danneels et al., 2010). The most obvious effect of *Nv* venom on host pupae is the developmental arrestment followed by the death of the pupae after some time depending on both the fly species and pupal age (Rivers and Denlinger, 1994a). After envenomation, a blackening of the injection site can be observed, a result of melanization of the damaged tissue initiated by the host as a defense mechanism (Rivers et al., 1993; Siebert et al., 2015). A cascade of metabolic changes is subsequently initialized in the host (Rivers and Denlinger, 1994b, 1995b; Rivers and Yoder, 1996; Rivers et al., 2002a,b; Martinson et al., 2014, 2016, 2019; Mrinalini et al., 2015) leading, among other effects, to the mobilization of nutrients for the developing wasp larvae and increased fatty acid biosynthesis (Rivers et al., 1993, 2002a; Rivers and Denlinger, 1995b; Mrinalini et al., 2015). The tremendous changes in the composition and relative amount of different substances found in hosts after envenomation have notable potential of being used in host assessment by female wasps. Which substances are of relevance during host assessment and whether the used substances are constituents of the venom or rather emerge by means of physiological changes induced by venom injection remains, however, to be elucidated. Further investigations of the chemoreceptors found on the ovipositor as well as controlled bioassays might be promising.

Host Discrimination in the Other *Nasonia* Species

Females of *Ng* are also able to discriminate between different host species and prefer pupae of *Protocalliphora sialia* over those of *Sarcophaga bullata* (Desjardins et al., 2010) which is in accordance with the occurrence of *Ng* in birds' nests rather than on carcasses. Bioassays on host discrimination in *Nl* and *No* have not been conducted so far, but it is likely that they are similarly able to distinguish different host species and host qualities. Also, taking a deeper look into the venom composition of the other three *Nasonia* species might give insights into mechanisms used by females to further differentiate between con- and heterospecifically pre-parasitized hosts.

ASSOCIATIVE OLFACTORY LEARNING

Associative Olfactory Learning in *N. vitripennis*

The olfactory localization of hosts by *Nasonia* females is not completely inherent but employs a dynamic learning and conditioning scheme that depends on the memory of host-associated chemical cues. Females of *Nv* are able to memorize odors they perceive during host assessment and oviposition and use these cues during the subsequent search for new hosts. The inspection of a single host puparium for 1 h in the presence of the synthetic volatile furfuryl heptanoate (FFH; the conditioned stimulus) for example led to the preference of FFH in subsequent olfactometer bioassays (Schurmann et al., 2009). This conditioning, however, was non-permanent and the preference vanished after 4 days. Nevertheless, Schurmann et al. (2012) were able to increase memory effects to 6 days by elongating the duration of inspection, repeating conditioning procedures, and allowing the females to perform host-feeding or oviposition in the presence of the conditioned stimulus (cinnamon odor). The physiological mechanisms leading to the formation of a long-term memory are dependent on protein synthesis which is indicated by decreased memory retention to a maximum of 3 days after the injection of the transcription inhibitor actinomycin D after training (Schurmann et al., 2012).

The neuromodulatory mechanisms underlying associative learning in insects involve a variety of different chemical messengers which interact at different levels of the olfactory system (Menzel and Muller, 1996). Two neuromodulators found to be of specific importance in learning are the two biogenic amines dopamine (DA) and octopamine (OA; Unoki et al., 2005, 2006). While DA has usually been associated in insects with aversive learning (i.e., learning to avoid a stimulus when it is associated with a negative experience or punishment), OA has predominantly been associated with appetitive learning (i.e., learning to prefer a stimulus when it is associated with a positive experience or reward Schwaerzel et al., 2003; Unoki et al., 2005). However, although OA seems to be sufficient in inducing appetitive learning in some insects (e.g., honeybees and crickets; Hammer and Menzel, 1998; Matsumoto et al., 2015), it has been shown lately that appetitive learning involves both DA and OA in *Drosophila melanogaster* (Burke et al., 2012). Similar to *D. melanogaster*, a recent study indicates that both DA and OA are also important for appetitive learning in *Nv* (Lenschow et al., 2018). In behavioral bioassays using oviposition as a reward, mated females of *Nv* have been readily conditioned to being attracted again to the male abdominal sex pheromone, thus reversing the pheromone switch. However, after having been fed DA or OA receptor antagonists prior to conditioning, appetitive learning was prevented. In addition, the oviposition reward could be mimicked completely by the injection of DA and partially by the injection of OA into the abdomen of mated females prior to exposure to the male sex pheromone. This indicates that, similar to *D. melanogaster*, DA and OA act in a concerted manner on different levels in the olfactory system during appetitive learning in *Nv* (Burke et al., 2012; Lenschow et al., 2018).

Differences in Olfactory Learning Among the *Nasonia* Species

Memory retention after associative olfactory conditioning differs among *Nasonia* species (Hoedjes et al., 2012; Hoedjes and Smid, 2014). In experiments involving only one single conditioning event, memory lasted up to 5 days in *Nv* and *Nl*, whereas in *Ng* the effect already vanished after 2 days (Hoedjes et al., 2012). In addition, in contrast to females of *Nv,* females of *Ng* depended on a second conditioning treatment to form

long-term memory (Hoedjes and Smid, 2014). One possible reason why these differences in memory retention have evolved is the difference in host preference among the *Nasonia* species (Hoedjes et al., 2011, 2012). *Nv* and *Nl* are generalists which accept pupae of a wide variety of different fly species as hosts (Darling and Werren, 1990; Desjardins et al., 2010). Different fly species prefer different habitats such as birds' nests (e.g., bird blowflies, *Protocalliphora* spp.; Bennett and Whitworth, 1992) or carcasses (e.g., several species of Sarcophagidae (flesh flies) and Calliphoridae (blow flies); Denno and Cothran, 1976) for oviposition, and the availability of fly pupae in different habitats in the field likely changes over time (Villet et al., 2017). A dynamic learning scheme which allows females to focus during the search for hosts on odors connected to recent successful oviposition experiences might thus be particularly adaptive in these two species (Schurmann et al., 2009, 2012; Hoedjes et al., 2011, 2012). In contrast, *Ng* is primarily specialized on pupae of *Protocalliphora* spp. which are obligate parasites of birds (Darling and Werren, 1990). Considering the consistency of relying solely on birds' nest odors, depending on stronger innate preferences during host-seeking might therefore be advantageous for them (Hoedjes et al., 2011, 2012).

OLFACTORY PERCEPTION—ANTENNAL MORPHOLOGY, ODORANT-BINDING PROTEINS AND CHEMOSENSORY RECEPTORS

The body parts most obviously used in chemoreception in *Nasonia* are the wasps' antennae, but chemosensory sensillae have also been found on the wasps' tarsal claws, maxillary palps, labial palps, and on the female's ovipositor (Slifer, 1969). Three types of chemosensory sensillae have been identified in *Nv* (Slifer, 1969; Wibel et al., 1984): thick-walled sensillae are bristle-shaped hollow chitinous structures which contain a single pore on the sensillar tip where the dendrites are exposed. Thick-walled sensillae are mainly located on the antennal tip (11th and 12th antennal segments) as well as on tarsal claws, maxillary and labial palps. In contrast, thin-walled sensillae and multiporous plate sensillae are perforated by many small pores. While thin-walled sensillae are bristle-shaped, plate organs are prominent elongated structures connected tightly to the cuticular surface. Both thin-walled sensillae and multiporous plate sensillae are located on segments three to eleven, sparing the antennal tip (Slifer, 1969). The abundance of the different types of sensillae is sex-specific, with females carrying more multiporous plate sensillae, and thick-walled sensillae than males (Slifer, 1969; Wibel et al., 1984).

Insect sensillae are filled with sensillar lymph, an aqueous liquid which contains various proteins such as odorant-binding proteins (OBPs) and chemosensory proteins (CSPs), i.e., small water-soluble proteins which help to transport the often hydrophobic odor molecules through the aqueous liquid (Vieira and Rozas, 2011; Vieira et al., 2012; Pelosi et al., 2014). During olfaction, odor molecules enter the sensillum through the sensillar pores, bind to OBPs or CSPs and diffuse to chemosensory receptors which are located in the dendritic membrane of chemosensory neurons reaching into the sensillar lymph (Vieira and Rozas, 2011). The chemosensory receptors interact with the odor molecules, and the presence of this interaction is subsequently communicated via electrical signals to the brain where the information is processed (antennal lobe, mushroom bodies, lateral horn). Chemosensory receptors are commonly classified into two main groups: odorant receptors (ORs) associated with the perception of food odors and pheromones and gustatory receptors (GRs) which include, among others, highly conserved carbon dioxide receptors (Jones et al., 2007; Kaupp, 2010).

The number of gene loci coding for OBPs, CPSs and chemosensory receptors are often discussed as indicators of the complexity of olfactory behavior in animals (Robertson and Wanner, 2006; Robertson et al., 2010). In the genome of *Nv*, 90 predicted OBPs and 10 predicted CSPs have been identified, and 59 of these OBPs have full expressed sequence tags (EST) support indicating that at least two thirds of the OBP sequences are also expressed (Werren et al., 2010; Vieira et al., 2012; Pelosi et al., 2014). All 90 OBPs of *Nv* have orthologs in *Nl* and *Ng* (Vieira et al., 2012). Compared to the 21 OBPs and 6 CSPs in the honey bee *Apis mellifera* and the 52 OBPs and 4 CSPs in *Drosophila melanogaster* (Sánchez-Gracia et al., 2009; Pelosi et al., 2014), the genus *Nasonia* thus seems to possess an exceptionally large OBP and CSP gene family. In accordance with the high number of OBPs and CSPs, the number of loci coding for ORs in *Nv* is also notably large compared to other insects (225 as compared to 170 in *A. mellifera* and 62 in *D. melanogaster*; Robertson and Wanner, 2006; Robertson et al., 2010). In accordance with the *Nasonia* lifestyle, although possessing 58 predicted GRs, no predicted carbon dioxide receptors have been found in *Nv* (Robertson et al., 2010).

The *Nasonia* OBPs, ORs, and GRs have not yet been functionally characterized. Neither the ORs detecting the different pheromone components nor the olfactory sensillae housing the pheromone sensing olfactory receptor neurons have been identified so far. The neurophysiological mechanisms underlying the stereospecific perception of chiral semiochemicals by insects are poorly understood. A comparative study of the *Nasonia* olfactory system offers therefore great opportunities to achieve major advances, because the species specificity of the chemical information is encoded by the chiral stereoisomers RS- and RR-HDL which are perceived differentially by *Nv* and the other *Nasonia* species, respectively (Niehuis et al., 2013). The availability of whole genome sequences and annotated olfactory gene families for the *Nasonia* species will facilitate the application of molecular tools to unravel the mechanisms underlying the perception of a newly evolved chiral pheromone component. Hence, the *Nasonia* model system has the potential to enable important inferences on the molecular basis of enantioselective pheromone perception in insects.

OUTLOOK

On a mechanistic level, the chemical communication system of *N. vitripennis* is meanwhile one of the best understood in insects. The chemicals that have been identified and whose pheromonal function has been demonstrated experimentally include the three components of the male abdominal sex

pheromone, the female-derived sex pheromone enabling male mate recognition, and the components of the oral male sex pheromone that induce the pheromone switch in females (see **Table 1**). Nevertheless, there are still gaps in our knowledge on the species' chemical ecology, concerning in particular the identification of the constituents of the oral sex pheromone that induce receptivity in females, the substances that induce the post-mating receptivity switch, the chemical cues used by females to find and recognize adequate hosts, and the chemical messengers enabling females to distinguish between parasitized and non-parasitized hosts. More pronounced than in *N. vitripennis* are the gaps in our knowledge of the chemical ecology of the other three *Nasonia* species. The number of studies investigating the different species in a comparative approach, however, has grown in recent years revealing more and more differences in their behavioral strategies, their chemical communication and ecology.

In general, research in the field of chemical ecology has shifted during the last years from mere mechanistic approaches to more complex studies that investigate the evolution of chemical signals and their perception instead of their mere function, and, secondly, from male-female and dual-species interactions to approaches analyzing multi-species interactions (Raguso et al., 2015).

Together with the species' distributional patterns, the *Nasonia* genus forms an exceptional system for the study of the evolution of chemical signals and pre-zygotic reproductive isolation in general, and the evolution of pheromonal signals in particular. For example, the two sister species *Ng* and *No* occur in microsympatry in eastern North America and the two species are separated from the allopatric species *N. longicornis* in western North America (**Figure 1**). In a comparative approach within the *Nasonia* genus, it might thus be possible to discriminate between reproductive species characteristics that likely result from phylogenetic relatedness and species differences that likely evolved through natural selection caused by post-zygotic reproductive isolation. This is particularly interesting, as males of *No* appear to produce significantly smaller amounts of HDL indicating that *No* males do not rely on sex pheromone markings during mate acquisition (Diao et al., 2016). Furthermore, the genes encoding the epimerization of RS- to RR-HDL in *Nv* males are known (Niehuis et al., 2013; Ruther et al., 2016), and switching off RR-HDL production in *Nv* using RNA interference or the CRISPR/Cas 9 system (Li et al., 2017) combined with behavioral observations and fitness measurements in multiparasitized hosts might give valuable insights into the selection pressures that led to the evolution of RR-HDL in *Nv*.

So far, it is not fully understood, how the male abdominal sex pheromone of *Nasonia* has evolved and how the evolution was driven by selection pressures imposed by other co-occurring species. This question could be further addressed by investigating in more detail the differences and similarities in the pheromone communication and behavior of species which occur in (micro)sympatry with *Nasonia* such as *Muscidifurax*, *Spalangia* or *Pachycrepoideus* (Rueda and Axtell, 1985; Rueda et al., 1997). In this context, the genera *Urolepis* and *Trichomalopsis* will be of particular interest, because they are the closest known relatives of *Nasonia* and have been suggested to form a monophyletic taxon with *Nasonia*, the so-called "*Nasonia* group" (Burks, 2009). Males of the only two species of these genera studied so far, *T. sarcophagae,* and *U. rufipes,* produce male abdominal sex pheromones. However, while the pheromone composition of *T. sarcophagae* equals the pheromones of *Ng, Nl,* and *No* (Niehuis et al., 2013), *U. rufipes* males use the monoterpenoid 2,6-dimethyl-7-octen-1,6-diol to attract virgin females (Ruther et al., 2019). The evolutionary pressures underlying this unusual switch between fatty acid and isoprenoid metabolism in the *Nasonia* group deserve further research activities in the future.

The exceptional role of fatty acid-derived compounds in the intraspecific communication of *Nasonia* raises the question whether these species are capable of synthesizing fatty acids *de novo* from nutritional carbohydrates. While the fatty acid biosynthetic machinery is highly conserved in most taxa it has been suggested to be lost in most parasitic wasps including *N. vitripennis*. It has been argued that parasitoid wasps get sufficient lipids from their host resulting in a loss of lipogenesis during evolution due to environmental compensation (Visser et al., 2010, 2012). However, in many species, the lack of lipogenesis has been concluded from experiments involving rather unsubtle techniques such as gravimetry or colorimetry and re-investigation with stable isotope labeling techniques will be necessary to consolidate the lack of lipogenesis in *Nasonia* and other parasitic wasps.

A similarly important but still unanswered question in chemical ecology concerns the molecular mechanisms underlying the stereoselective perception of chiral pheromone components (Lassance and Löfstedt, 2013). The evolution of the additional male sex pheromone component RR-HDL in *Nv* is accompanied by the females' stereoselective perception of RS- and RR-HDL. The comparative characterization of ORs detecting RS-HDL and RR-HDL in *Nasonia* species could thus help getting novel insights into the role of chirality in the evolution of chemical communication in insects.

Finally, during the past decades, *Nasonia* has proven to be an excellent lab animal, while laborious field studies with these tiny insects are rare (Grillenberger et al., 2009a,b). Hence, one of the major challenges of future *Nasonia* research will be the step from well-controlled lab experiments and microcosm approaches to field studies investigating chemical communication and orientation of the *Nasonia* species in the wild. The ever-expanding techniques available for *Nasonia* in chemical ecology and genetics, combined with behavioral ecology and some already existing experience with field studies put this genus in the prime position to solve these questions.

AUTHOR CONTRIBUTIONS

Both authors conceptualized the paper and approved the final version for publication. MM wrote the first draft and revised the manuscript. JR reviewed and edited the manuscript.

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