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Male sex pheromone in the parasitoid wasp *Nasonia longicornis*: Chemical and behavioral analyses

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The use of sex pheromones for the attraction of potential mating partners is widespread in insects. Species-specificity of these chemical signals is essential, particularly in closely related species with overlapping habitats. In parasitoid wasps of the genus *Nasonia*, it is the males that produce sex pheromones in their rectal vesicles. The genus consists of four species: *N. vitripennis* (*Nv*), *N. giraulti* (*Ng*), *N. oneida* (*No*), and *N. longicornis* (*Nl*). The cosmopolitan species *Nv* is sympatric with *Ng* and *No* in eastern North America and with *Nl* in the west. Interspecific courtship is common in *Nasonia* although hybridization is prevented in most combinations by *Wolbachia*-mediated cytoplasmic incompatibility. The pheromone of *Nv* males differs from all *Nasonia* congeners by an additional component that is believed to ensure precopulatory isolation from the sympatric congeners. Detailed investigations on these interactions, however, exist only for the species combination *Nv*/*Ng*. Here, we report the results of chemical and behavioral investigations on the sex pheromone of *Nl* males. The pheromone consists of (4*R*,5*S*)-5-hydroxy-4-decanolide (RS) and 4-methylquinazoline (MQ) as a minor component which are produced only after eclosion. Pheromone titers peaked 2 days after eclosion and remained constant on day three. The pheromone is deposited by abdomen dipping which males increasingly exhibited after mating or brief contact with a female. The presence of hosts containing females about to emerge did not increase marking behavior. Site fidelity of males at their own pheromone markings is mediated by MQ. Both natural and synthetic pheromone attracted virgin but not mated females and both RS and MQ are required for female attraction. Females collected during emergence from the host responded likewise to the pheromone and 84% of them produced mixed-sex offspring showing that most of them emerge unmated. *Nl* females preferred pheromone extract from conspecific males to extract from *Nv* males, and the addition of the *Nv*-specific component (4*R*,5*R*)-5-hydroxy-4-decanolide (RR) to the *Nl* pheromone made them unresponsive. The present study demonstrates that *Nl* uses the male sex pheromone in a similar way as previously shown for *Nv* and *Ng*. Furthermore, it shows that *Nl* females use the *Nv*-specific pheromone component RR to avoid costly sexual interactions with *Nv* males in regions of sympatry.

KEYWORDS

mate finding, parasitic wasp, precopulatory isolation, sex attractant, species-specificity, (4*R*,5*S*)-5-hydroxydecanolide, (4*R*,5*R*)-5-hydroxydecanolide, 4-methylquinazoline

Introduction

In most insects, the mate finding process is mediated by volatile sex pheromones, chemical signals that are released by one sex to attract the other (Wyatt, 2014). To guide the signal recipient effectively and reliably to the sender, sex pheromones need to be species-specific. Being species-specific, sex pheromones provide long-range precopulatory isolation even in sympatric species with overlapping mate recognition signals (Smadja and Butlin, 2009).

An excellent model system to study such processes is the parasitic wasp genus *Nasonia* (Ashmead, 1904). During the past decades, the genus has been frequently used to answer questions concerning all aspects of parasitic wasp biology (Werren and Loehlin, 2009; Werren et al., 2010; Lynch, 2015; Mair and Ruther, 2019; Wang et al., 2020). It consists of four species: *N. vitripennis* (*Nv*), *N. giraulti* (*Ng*), *N. oneida* (*No*) and *N. longicornis* (*Nl*; Darling and Werren, 1990; Raychoudhury et al., 2010; Werren et al., 2010). All species parasitize the pupae of various cyclorraphous fly species. The typical habitat of *Nasonia* wasps is in the nests of cavity-nesting birds. *Nv* is cosmopolitan and sympatric with *Ng* and *No* in eastern North America and with *Nl* in the west. In areas of sympatry, *Nasonia* species occur often microsympatrically, i.e., heterospecific offspring develop within the same host individuals (Darling and Werren, 1990; Grillenberger et al., 2009; Raychoudhury et al., 2010). Hence, interspecific interactions between males and females of sympatric species are likely to occur. Interspecific mating in *Nasonia* occurs in the lab with varying frequency but does not lead to hybrid offspring in most combinations of species due to *Wolbachia*-mediated cytoplasmic incompatibility (Breeuwer and Werren, 1990; Bordenstein et al., 2001; Giesbers et al., 2013). Hence, fertilized eggs of females that mated with a heterospecific male die or develop into male offspring due to the haplodiploid sex determination in Hymenoptera (Tram et al., 2006). The resulting all-son-offspring impose evolutionary costs on the respective females because the mating system of *Nasonia* is characterized by local mate competition and female-biased offspring sex ratios are common (Werren, 1983; Grillenberger et al., 2009; Ivens et al., 2009). Given that *Nasonia* females typically mate only once in their life, they depend on reliable information to reduce the probability of interspecific mating.

Males of all *Nasonia* species produce sex pheromones in their rectal vesicle (Abdel-Latief et al., 2008). Three pheromone components have been identified in *Nasonia* males: the two diastereomers (4*R*,5*S*)- and (4*R*,5*R*)-5-hydroxy-4-decanolide (RS and RR; Ruther et al., 2007) and 4-methylquinazoline (MQ) as a minor component (Ruther et al., 2008; Figure 1A). RS and MQ are produced by males of all *Nasonia* species as well as by males of the closely related species *Trichomalopsis sarcophagae*, whereas *RR* is specific for *Nv.* This suggests that the ancestral sex pheromone of *Nasonia* males consisted solely of RS and MQ and that the additional occurrence of RR is a derived state that evolved in the *N. vitripennis* lineage (Niehuis et al., 2013). RR has been shown to be synthesized from RS by short-chain dehydrogenases/reductases (SDRs; Niehuis et al., 2013; Ruther et al., 2016). The chemical identities of the pheromones have been established in all *Nasonia* species, but detailed analyses of the biological function are so far available only for the model organism *Nv* and, to a lesser extent, for *Ng* (Ruther et al., 2014; Mair and Ruther, 2019). *Nv* males eclose without pheromone, but pheromone titers increase within the first 2 days after eclosion and remain constant on day three (Ruther et al., 2007). Males deposit pheromone by dragging their abdominal tips over substrates, a behavior referred to as 'abdomen dipping' (Barrass, 1969; van den Assem et al., 1980). *Nv* males economize pheromone deposition by engaging in abdomen dipping more frequently after contact or mating with virgin females (Steiner and Ruther, 2009), i.e., if the chance of mating with (additional) females is high. The mating system of *Nv* is characterized by territorial males that monopolize parasitized hosts containing females about to emerge. Apart from showing aggressiveness towards male competitors (Leonard and Boake, 2006), dominant males often mark monopolized hosts to prevent emerging females from dispersing unmated (Mair and Ruther, 2018). *Nv* males are able to identify intact hosts (without exit holes) containing females about to emerge (Prazapati et al., 2022). It is unknown, however, whether the presence of those hosts also affects male marking.

Pheromone markings, abdomen extracts and synthetic blends of the ternary *Nv* pheromone (RS + MQ + RR) and the binary *Ng* pheromone (RS + MQ) all attract conspecific females (Niehuis et al., 2013). The exclusive occurrence of RR in *Nv* strongly suggests that this compound has a function in precopulatory isolation and reduces the risk of interspecific sexual interactions in sympatric *Nasonia* species both in eastern and western North America. The role of individual pheromone components, however, has so far only been studied in the eastern species pair *Nv*/*Ng*. In the initial study addressing this question (Niehuis et al., 2013), virgin females of both species were attracted to RS when presented alone (Figures 1B,C). The attractiveness of RS was synergized by the minor component MQ in both *Nv* and *Ng*, whereas MQ had no effect when presented alone. Likewise, RR was neither

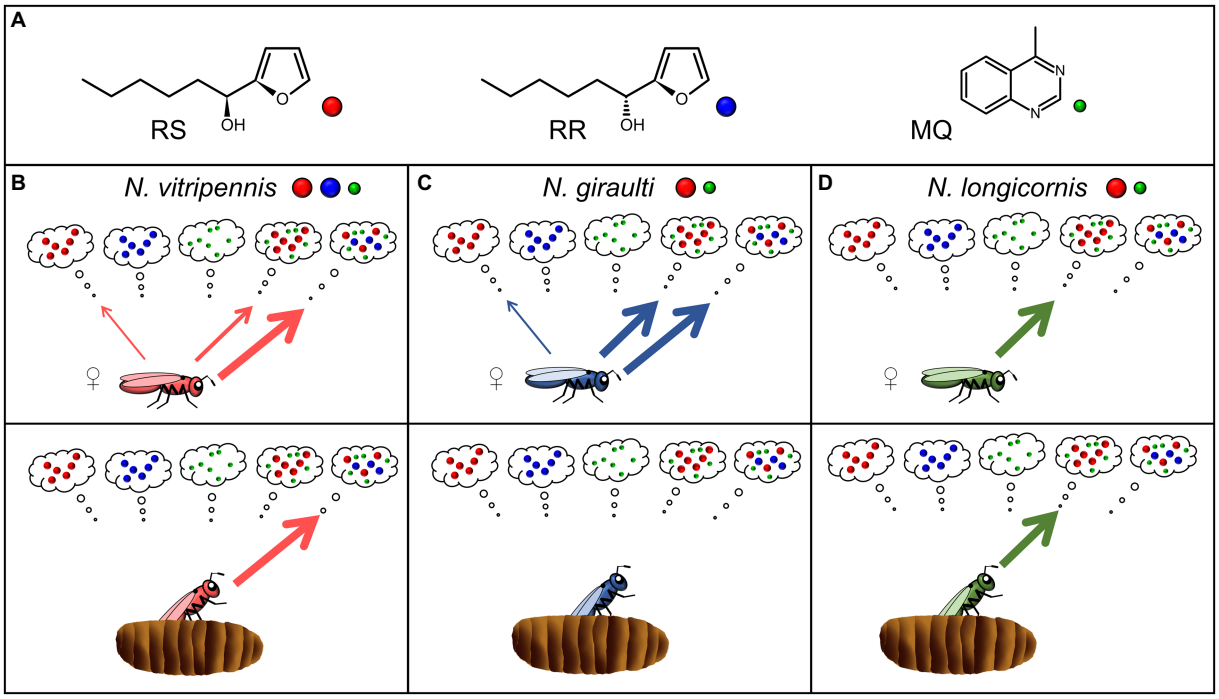

FIGURE 1
(A) Structures of the components of the male abdominal sex pheromones in the genus *Nasonia* (4*R*,5*S*)-5-hydroxy-4-decanolide (RS), (4*R*,5*S*)-5-hydroxy-4-decanolide (RR), and 4-methylquinazoline (MQ). **(B–D)** Scheme summarizing the female responses of the hitherto investigated species *Nasonia vitripennis* (Niehuis et al., 2013; Ruther et al., 2014), *Nasonia girault* (Niehuis et al., 2013; Ruther et al., 2014), and *Nasonia longicornis* (this study) to the male sex pheromone components and combinations thereof. Pheromone composition of the different species is indicated by colored dots near the species names. The upper panels show the response of 2-d-old virgin females excised from the hosts, the lower panels refer to females collected at the moment of emergence from the host (for more details see text).

attractive for *Nv* nor for *Ng* virgin females when presented alone, but the addition of RR to the binary *Ng* blend (*RS* + MQ) synergized the attractiveness of this blend for *Nv* whereas this modification had no effect on *Ng* females. Hence, females of both species responded to the pheromone of heterospecific males, but *Nv* females preferred the ternary conspecific pheromone blend over the binary heterospecific one (Niehuis et al., 2013). The preference for the heterospecific pheromone blend in either species raised the question whether this is associated with evolutionary costs. In the case of *Ng*, this is unlikely, because *Ng* differs from its *Nasonia* congeners by showing a very high within-host-mating (WHM) rate, i.e., mating in *Ng* occurs already inside the host prior to female emergence (Drapeau and Werren, 1999). Given that mating makes *Nv* and *Ng* females unresponsive to the male sex pheromone (Ruther et al., 2010, 2014; Ruther and Hammerl, 2014) it is unlikely that the response to the ternary *Nv* pheromone blend has fitness consequences for them. In fact, females collected at the moment of emergence from the host were not even attracted to the conspecific pheromone (Ruther et al., 2014; Figure 1C). As for *Nv*, mating occurs almost exclusively outside the host within a few minutes after emergence of the females (Mair and Ruther, 2018) and thus, the attraction of females to the binary *Ng* blend might be problematic. However, a second study addressing the *Nv*/*Ng* pheromone interaction in more detail revealed that the attraction of virgin *Nv* females to the binary *Ng* blend is age-dependent (Ruther et al., 2014). Newly eclosed females and those collected at the moment of emergence from the host did not respond to the binary *Ng* blend but strongly preferred the conspecific ternary blend (Figure 1B). Only with increasing age (≥1d), when most females would have been mated and dispersed under natural conditions anyway (Mair and Ruther, 2018), do *Nv* females became less choosy and responsive to the heterospecific binary pheromone blend (Ruther et al., 2014). Nothing is known about the pheromone response and the role of individual pheromone components in *Nl* females. This species shows intermediate WHM rates (Drapeau and Werren, 1999; Trienens et al., 2021) and thus many females emerge as virgins and mate outside the host. Therefore, *Nl* females should discriminate more strongly against the ternary *Nv* pheromone blend than *Ng* females to avoid costly sexual interactions with *Nv* males in regions of sympatry.

The abdominal sex pheromone of *Nv* males is not only attractive for virgin females but also mediates site fidelity of the pheromone-marking males using their own pheromone markings (van den Assem et al., 1980). This behavior could also be interpreted as an economization of pheromone use because

males have a way to stay near and return to their own pheromone markings rather than needing to deposit a new one. The minor component MQ has been shown to mediate this phenomenon in *Nv* (Ruther et al., 2011), whereas nothing is known about the male response to the pheromones in the other *Nasonia* species.

In the present study, we fill a knowledge gap about pheromone communication in the genus *Nasonia* by investigating chemical and behavioral aspects of the male *Nl* sex pheromone. We study the age dependence of pheromone titers in males, examine the context of pheromone deposition and elucidate the role of the different pheromone components in mediating the attractiveness of the pheromone for both females and males. We hypothesize that the sex pheromone of *Nl* males attracts virgin females and mediates site fidelity in males whereas the *Nv*-specific pheromone component RR contributes to precopulatory isolation between the sympatric species *Nl* and *Nv*.

Materials and methods

Insects

The *Nl* strain NLMN8510 used in this study was kindly provided by B. Pannebakker (University of Wageningen). Wasps were reared on freeze-killed pupae of the green bottle fly *Lucilia caesar* at 25°C under a 16:8 light:dark regime. To obtain wasps of known age and mating status, fully melanized pupae were isolated from host puparia and kept singly in 1.5-mL microcentrifuge tubes. Tubes were checked daily in the morning and newly eclosed wasps were defined as being 0 day old. Mated females were obtained by observing couples under a stereomicroscope until copulation was completed. To obtain enough males for the pheromone isolation, all-male broods were produced by using virgin females for parasitization. Because *Nasonia* wasps are haplodiploid, virgin females produce all-son offspring. In all experiments, wasps were used only once.

Pheromone extraction

Sex pheromone extracts of *Nl* and *Nv* males for bioassays were obtained by extracting batches of 30 abdomens from 2-d-old freeze-killed males with 30 µl of dichloromethane (DCM) per abdomen. After 30 min, the abdomens were removed, and raw extracts were cleaned by solid phase extraction as described previously for *Nv* (Ruther et al., 2007). One microliter aliquots of cleaned extracts (representing one male equivalent) were used in the bioassays.

To investigate the age dependence of male pheromone titers, the abdomens of 0-, 1-, 2-, and 3-d-old males ($n = 10$ per age) were dissected and extracted singly with 30 µl DCM containing 10 ng µl^{-1} methyl undecanoate (Sigma-Aldrich, Deisenhofen, Germany) as an internal standard. These extracts were analyzed by coupled gas chromatography/mass spectrometry (GC/MS, see "GC/MS analysis").

Bioassays

The response of *Nl* to pheromone extracts and synthetic pheromone components was observed using a still-air, dual-choice olfactometer described previously (Ruther et al., 2014). It consists of a round glass arena (9 cm diameter) with a hole (3 mm diameter) in the center from which the wasps were released. Four symmetrically arranged spherical cavities (1 cm diameter, 4 mm depth, 3 cm distance from the central hole) in the arena served for sample storage and a plastic rim (2 mm height) was used as spacer for a glass plate that covered the arena from above. Natural and synthetic pheromone samples (1 µl) or equal amounts of the pure solvent (control) were applied to discs of filter paper (5 mm diameter). After 1 min of solvent evaporation, paper discs were placed into two opposite cavities of the olfactometer, whereas the two remaining cavities were left empty. A wasp was released into the central hole, and the arena was covered with the glass plate. Finally, an open gray plastic cylinder (11 cm diameter, 6 cm height) was put on the upper glass plate to exclude visual stimuli. The experimental set-up was illuminated from above with a desk light (60 W incandescent lamp, distance 50 cm, centered by eye). The residence times spent by the responding wasps ($n = 25$ for each test if not stated otherwise) in the pheromone-baited and the unbaited control cavity was recorded for 5 min (live observation) using The Observer XT 15.0 observational software (Noldus, Wageningen, The Netherlands). After each observation, the arena was rotated by 180°C to avoid positional bias. After every five observations, the arenas were cleaned with ethanol and deionized water and dried at 60°C. Doses and relative proportions of synthetic pheromones used in the experiments were matched to the amounts found in natural pheromone extracts and pheromone markings deposited by live *Nl* males (see results of this paper) and *Nv* (Blaul and Ruther, 2011), respectively.

Responses of females to natural and synthetic pheromones

In the first series of experiments, we tested responses of *Nl* females (1–2 day old) to the conspecific pheromone and synthetic analogues thereof (Table 1). Virgin females were exposed in the olfactometer to (a) natural pheromone extract of conspecific males (one male equivalent), (b) the synthetic pheromone components RS (200 ng), (c) MQ (0.5 ng) and (d) a combination of RS (200 ng) + MQ (0.5 ng). To investigate whether the *Nv*-specific pheromone component RR alters the behavior of virgin *Nl* females, we also tested their responses to (e) natural pheromone extract from *Nv* against extract from *Ng* males, (f) the ternary heterospecific combination RS (200 ng) + MQ (0.5 ng) + RR (100 ng) against the solvent control, and (g) the ternary blend directly against the binary conspecific combination RS (200 ng) + MQ (0.5 ng). To investigate the impact of the mating status on the pheromone responses of *Nl* females, we tested the responses of (h) mated females to the natural

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TABLE 1 Overview of the behavioral bioassays testing the responses of *N. longicornis* to natural extracts and synthetic pheromone components (for more details see text).

Tested wasps	Treatment 1	Treatment 2	Research question
Virgin females	Extract *Nl*[a] males	DCM[b]	Influence of individual pheromone components on female attraction
	RS[c]+MQ[d]	DCM	
	RS	DCM	
	MQ	DCM	
Virgin females	Extract *Nl* males	Extract *Nv*[e] males	Role of RR in precopulatory isolation between *Nl* and *Nv*
	RS+RR[f]+MQ	DCM	
	RS + RR + MQ	RS + MQ	
Mated females	Extract *Nl* males	DCM	Influence of mating status on female attraction
Females emerging from the host	RS + MQ	DCM	Relevance of within-host-mating for female attraction
Virgin males	Extract *Nl* males	DCM	Influence of individual pheromone components on pheromone-mediated site fidelity in males
	RS + MQ	DCM	
	RS	DCM	
	MQ	DCM	

[a]*Nasonia longicornis*.
[b]Dichloromethane.
[c](4*R*,5*S*)-5-hydroxy-4-decanolide.
[d]4-methylquinazoline.
[e]*Nasonia vitripennis*.
[f](4*R*,5*R*)-5-hydroxy-4-decanolide.

pheromone extract (one male equivalent). Finally, we tested the responses of (i) females collected at the moment of emergence from the host to a combination of RS (200 ng) and MQ (0.5 ng; $n = 39$). The last experiment of this series was performed to investigate whether emerging females are responsive to the male pheromone despite having been exposed to males inside the host thus having had the opportunity of WHM. We additionally controlled the WHM rate of the tested females by offering them hosts for 2 days after the experiment and checking whether they produced daughters. All-son offspring would indicate virginity of the respective female because of the haplodiploid sex determination system in *Nasonia*.

Responses of males to natural and synthetic pheromones

In the second series of experiments, we tested the responses of naïve *Nl* males to their own pheromone and synthetic constituents thereof. Unmated males were exposed in the olfactometer to (a) natural pheromone extract (one male equivalent), (b) RS (200 ng), (c) MQ (0.5 ng) and (d) a combination of RS (200 ng) + MQ (0.5 ng).

Male abdomen dipping behavior

To study the behavioral context of abdomen dipping, individual males were placed into a glass observation chamber (3 cm diameter, 12 mm high) and the frequency of abdomen dipping was recorded for 10 min under a stereomicroscope. Two-day-old males were observed (a) without any additional cue being present, (b) immediately after having had contact with a female but without having copulated, (c) immediately after having mated with a female. Additionally, we observed males (d) in the presence of a parasitized host containing females about to emerge to investigate whether the upcoming emergence of females has a preemptive effect on pheromone deposition behavior. Under our rearing conditions, *Nl* develops within 14 days. Thus, the hosts offered in the last experiment had been parasitized 14 days prior to the experiment but did not yet show any visible exit holes.

Quantification of deposited pheromone

RS and MQ deposited by abdomen dipping of individual males were quantified by GC/MS using thermal desorption (TD) sampling as described previously for *Nv* (Blaul and Ruther, 2011). To this end, empty 89 mm x 5 mm inner diameter TD glass tubes that had been filled at one end with 50 mg of Tenax TA (Supelco, Bellefonte, PA) were used. The adsorbent layer was fixed using fine mesh metal screens (Supelco). One µL of an internal standard solution containing 100 ng µL^{-1} methyl undecanoate (Sigma-Aldrich, Deisenhofen, Germany) in methanol was applied to the adsorbent, and the tube was purged for 5 min with a nitrogen flow of 60 ml min^{-1} to remove the solvent. Subsequently, 2-day-old males were released into the open end of the adsorption tubes and kept there for 10 min. Analogous to the behavioral observations in "Male abdomen dipping behavior," the 2-day-old males were either (a) naïve (unmated), (b) had contact with a female but were

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prevented from mating or (c) were sampled immediately after having mated with a female ($n = 20$ per treatment). After removing the male, TD tubes were purged for 10 min with charcoal filtered air at a flow rate of 60 ml min^{-1} (adsorbent upstream) to trap the volatilized proportions of RS and MQ. By this means, losses of RS and MQ during sampling were kept to a minimum and both the pheromone adsorbed to the glass surface and the volatilized proportion could be sampled. TD was accomplished using an automated Shimadzu TD20 sampler coupled to a QP2010 Plus GC/MS system (Shimadzu GmbH, Duisburg, Germany) as described previously (Blaul and Ruther, 2011).

GC/MS analysis

Aliquots (1 µl) of pheromone extracts were analyzed on a Shimadzu QP2010 Plus GC/MS system fitted with a non-polar BPX5 capillary column (60 m × 0.25 mm inner diameter, 0.25 µm film thickness, SGE Analytical Science Europe, Milton Keynes, UK). Samples were injected at 280°C in splitless mode using an AOC 20i auto sampler. Helium was used as carrier gas at a linear velocity of 37.8 cm s^{-1}. The initial oven temperature of 80°C was increased at 5°C min^{-1} to 280°C and held at this temperature for 15 min. The mass spectrometer was operated in electron ionization (EI) mode at 70 eV, and the mass range was m/z 35–600. RS and MQ were identified by comparison of mass spectra and retention times with those of authentic reference chemicals that were synthesized as described previously (Ruther et al., 2008, 2016). For pheromone quantification, peak areas of RS and MQ were related to the peak area of the internal standard.

Statistical analysis

Data did not meet the assumptions of parametric statistical analysis. Therefore, non-parametric tests were applied using Past 4.05 statistical software (Hammer et al., 2001). Pheromone titers of males of different age, frequencies of abdomen dipping by differently treated males and deposited amounts of RS and MQ were compared by a Kruskal-Wallis *H*-test followed by multiple pairwise Mann–Whitney *U*-tests with Bonferroni correction. Residence times in the test and control cavities of the olfactometer were analyzed by a Wilcoxon matched pairs test.

Results

Male pheromone titers were almost zero in newly eclosed males, increased significantly within the first 2 days after eclosion and remained constant on day three (median RS/MQ 0d: 0/0 ng; 1d: 14/0.1 ng; 2d: 302/0.4 ng; 3d: 318/0.4 ng; Figures 2A,B). In the behavioral bioassays, virgin *Nl* females were attracted to filter paper disks treated with purified abdomen extract or the binary conspecific blend consisting of synthetic

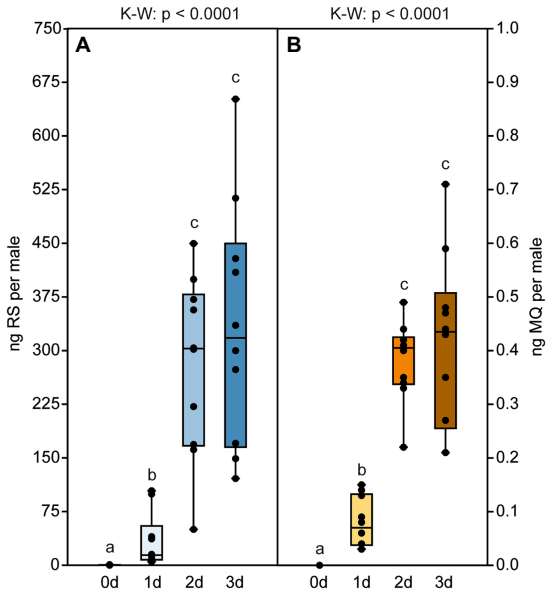

FIGURE 2
Pheromone titers of *N. longicornis* males are age dependent. Amounts of **(A)** (4*R*,5*S*)-5-hydroxy-4-decanolide (RS) and **(B)** 4-methylquinazoline (MQ) in abdomen extracts from *N. longicornis* males of various age. Box and whisker plots show median (horizontal line), 25%–75% quartiles (box), maximum/minimum range (whiskers) and outliers (°>1.5 × above box height; *>3 × above box height). Different lowercase letters indicate significant differences between the ages at $p<0.001$ [$n=10$, data analysis by a Kruskal–Wallis *H*-test (K–W) followed by multiple Bonferroni-corrected Mann–Whitney *U*-tests].

RS + MQ (Figures 3A,D). However, they neither preferred RS nor MQ over the solvent controls when these chemicals were offered alone (Figures 3B,C). Virgin females preferred purified extract of conspecific males over extract of *Nv* males. They did not prefer the ternary heterospecific pheromone blend consisting of synthetic RS + MQ + RR when tested against the solvent control. When given the choice between the ternary hetero- and the binary conspecific pheromone blends, *Nl* females preferred the latter (Figures 3E–G). Mated *Nl* females were no longer attracted to the male sex pheromone (Figure 4A). However, females collected at the moment of emergence from the host were attracted to the conspecific binary pheromone blend indicating that most of them had emerged unmated (Figure 4B). This was confirmed by the analysis of the offspring's sex, because following olfactometer bioassays most (84.4%) offspring-producing females had only sons, indicative of their virgin mating status.

Nl males were attracted to their own pheromone in the olfactometer tests. They preferred both the natural pheromone and the binary blend consisting of synthetic RS + MQ over the solvent control. When given the choice between RS or MQ alone and the solvent control, males preferred MQ but not RS indicating that MQ underlies the preference of males for their own pheromone (Figures 5A–D).

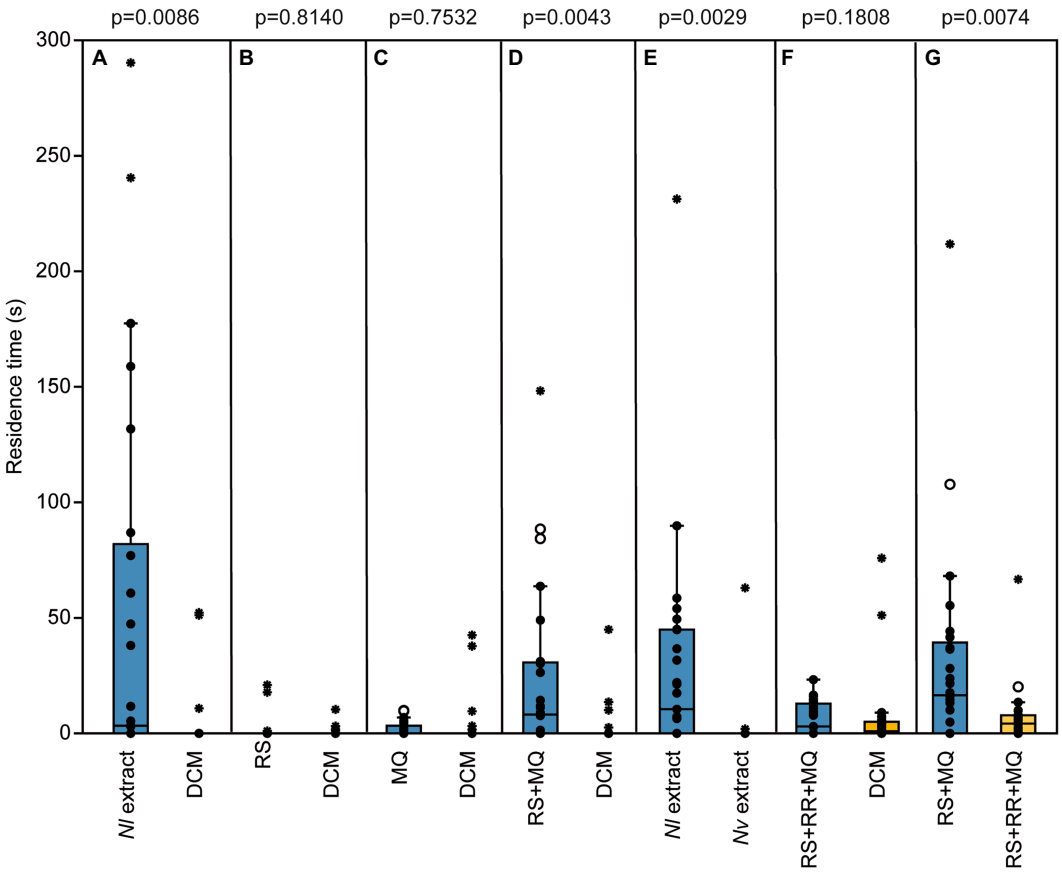

FIGURE 3
The male sex pheromone attracts virgin *Nasonia longicornis* (*Nl*) females species-specifically. Residence time of virgin *Nl* females in the two odor fields of a static two-choice olfactometer when given the choice between dichloromethane (DCM, control) and **(A)** one male equivalent of a natural *Nl* pheromone extract, **(B)** 200 ng of synthetic (4*R*,5*S*)-5-hydroxy-4-decanolide (RS), **(C)** 0.5 ng of synthetic 4-methylquinazoline (MQ), **(D)** the synthetic pheromone blend of *Nl* males (200ng RS+0.5ng MQ), and **(F)** the synthetic pheromone blend of *N. vitripennis* (*Nv*) males (200 ng RS+0.5 ng MQ+100 ng (4*R*,5*R*)-5-hydroxy-4-decanolide (RR)). Furthermore, virgin females were given direct choice between **(E)** natural pheromone extracts from *Nl* and *Nv* males and **(G)** the synthetic pheromone blends of *Nl* and *Nv* males [treatments **(D)** and **(E)**]. Box and whisker plots show median (horizontal line), 25%–75% quartiles (box), maximum/minimum range (whiskers) and outliers (°>1.5 × above box height; *>3 × above box height; *n*=25, data analysis by a Wilcoxon matched-pairs test).

Naïve, 2-d-old males exhibited the typical abdomen dipping behavior that has been shown to serve pheromone deposition in *Nv* males (Steiner and Ruther, 2009). Both contact with a female and successful mating equally increased abdomen dipping frequency in *Nl* males (Figure 6A). In contrast, the presence of a host containing females about to emerge did not increase male abdomen dipping frequency (Figure 7A) and males showed this marking behavior almost exclusively in the vicinity of a host but hardly on a host itself (Figure 7B). Quantification of deposited pheromone after abdomen dipping revealed that 2-day-old, mated males deposited significantly more RS than naïve ones of the same age, while males with only female contact released intermediate amounts (median RS unmated: 1.5 ng; contact: 12 ng; mated: 176 ng; Figure 6B). As for the minor component MQ, deposited amounts showed a similar pattern (Figure 6C), however, statistical analysis did not reveal any significant differences between treatments (median MQ unmated: 0 ng; contact: 0.01 ng; mated: 0.2 ng).

Discussion

The present study demonstrates that *Nl* uses the male abdominal sex pheromone in a similar way as previously described in the congeneric species *Nv* and *Ng*. Furthermore, it shows that the *Nv*-specific pheromone component RR mediates precopulatory isolation not only in the eastern North American species pair *Nv*/*Ng* but also in the western pair *Nv*/*Nl*. Like in *Nv* and *Ng* (Ruther et al., 2007, 2010, 2014), exclusively virgin *Nl* females were attracted to the conspecific male pheromone. The vast majority of *Nl* females emerged from the host as virgins showing that mating occurs predominantly outside the host and that the male pheromone is relevant for finding an adequate

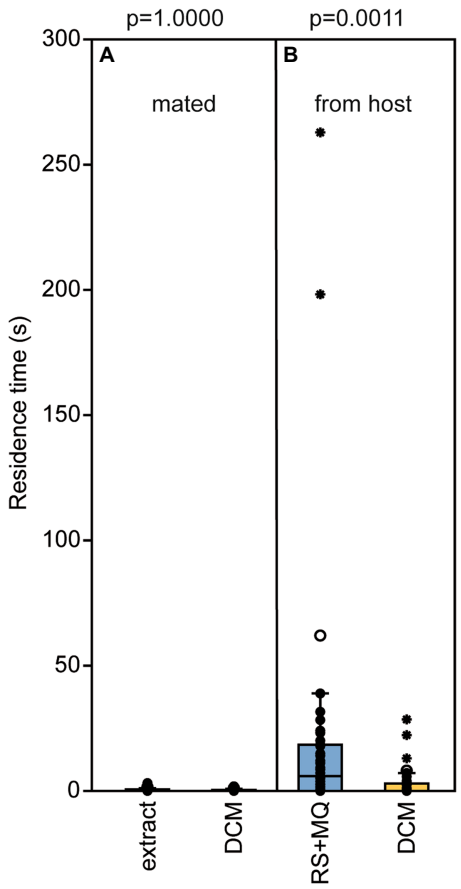

FIGURE 4
Newly emerged, but not mated, *Nasonia longicornis* females are attracted by the male pheromone. Residence time of *N. longicornis* females in the two odor fields of a static two-choice olfactometer when given the choice between dichloromethane (DCM, control) and **(A)** one male equivalent of a natural *N. longicornis* pheromone extract (mated females) or **(B)** a combination of synthetic (4*R*,5*S*)-5-hydroxy-4-decanolide (RS)+0.5 ng 4-methylquinazoline (MQ; females were collected during the process of emergening from the host). Box and whisker plots show median (horizontal line), 25%–75% quartiles (box), maximum/minimum range (whiskers) and outliers (°>1.5 × above box height; *>3 × above box height; n=25, data analysis by a Wilcoxon matched-pairs test).

mating partner. In contrast to *Nv* and *Ng* (Niehuis et al., 2013), RS is not attractive for *Nl* females when presented alone. Rather, the presence of synergistic MQ is needed to attract *Nl* females. The addition of the *Nv*-specific pheromone component RR to the conspecific binary blend RS + MQ made *Nl* females unresponsive, and they preferred both natural and synthetic conspecific binary blends over the respective ternary heterospecific blends when exposed to them simultaneously. This demonstrates that *Nl* females discriminate more strongly against the *Nv*-specific pheromone component RR than previously reported for *Ng* (Niehuis et al., 2013). This might be explained by the lower WHM rate in *Nl* (Drapeau and Werren, 1999; Trienens et al., 2021) that makes encounters between virgin *Nl* females and *Nv* males in shared habitats more likely than in the eastern species pair *Nv/Ng*. The high WHM rate in *Ng* has been shown to be due to the males refraining from gnawing exit holes through which virgin females might escape. A recent study, however, has demonstrated that *Ng* shows much lower WHM rates when developing in microsympatry with *Nv*, because virgin *Ng* females emerge through exit holes gnawed by *Nv* males (Trienens et al., 2021). This suggests that, under conditions of microsympatry, *Ng* females are prone to get involved in costly sexual interactions with *Nv* males if they do not discriminate against the ternary pheromone blend produced by these sympatric heterospecific males. Therefore, it remains to be investigated whether *Ng* females having developed in microsympatry with *Nv* discriminate more strongly against the *Nv*-specific pheromone component RR than those having developed in the absence of *Nv*. The strong discrimination of *Nl* females against the *Nv*-specific pheromone component RR comes along with a low discriminatory ability shown by these females during courtship. In a previous study, *Nl* females accepted more than 80% *Nv* males as mates while vice versa *Nv* females accepted less than 30% of *Nl* males (Giesbers et al., 2013). This suggests that *Nl* females rely mainly on precopulatory isolation based on the male abdominal sex pheromone to avoid costly interspecific mating with *Nv* males, while *Nv* females use both the species-specific composition of the male pheromones (Niehuis et al., 2013; Ruther et al., 2014) as well as courtship-related cues (Giesbers et al., 2013) to decrease the risk of mismating with sympatric congeneric males.

Nl males economize both pheromone production and deposition. Like in *Nv* and *Ng* (Ruther et al., 2007, 2014), *Nl* males eclose without any pheromone in the rectal vesicle, and pheromone production starts only after eclosion. In *Nv*, newly eclosed males typically remain inside the host for approximately 1.5 days before emerging from the host and most males eclose and emerge before the females (protandry; Moynihan and Shuker, 2011). Assuming a similar scenario in *Nl*, emerging males should have produced sufficient pheromone to attract and arrest virgin females. *Nl* males show abdomen dipping increasingly if mating chances are high, i.e., after contact or successful mating with a female. Once an exit hole has been gnawed by protandrous *Nasonia* males, females may emerge from the host in rapid succession (Mair and Ruther, 2018). Hence, the presence of one female increases the probability of meeting others and pheromone deposition is likely to enable additional mating opportunities. This is supported by our TD analyses of deposited pheromone amounts: Mated *Nl* males deposited *ca.* two-thirds of their available pheromone which is probably a significant investment. The presence of hosts (without showing exit holes) containing females about to emerge is obviously not sufficient to trigger this investment. Pheromone markings remain attractive for virgin *Nasonia* females for a

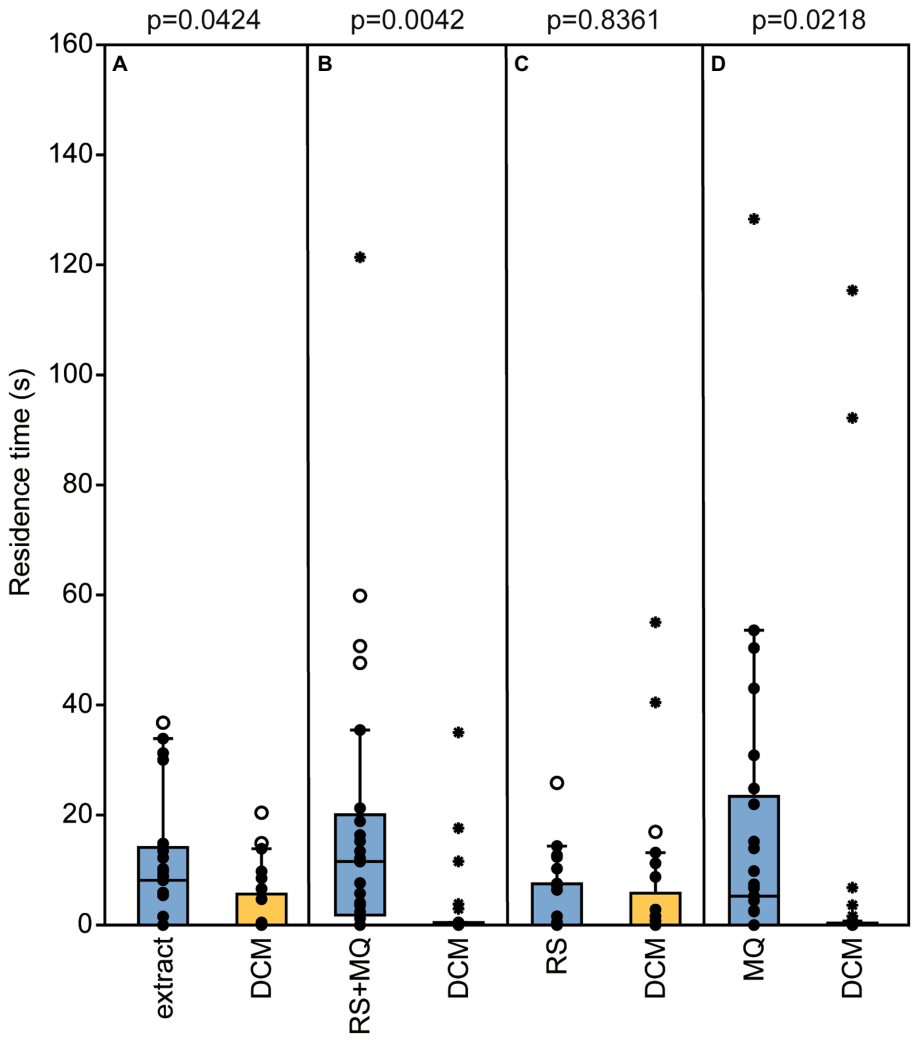

FIGURE 5
Nasonia longicornis males are arrested by their own pheromone. Residence time of unmated *N. longicornis* males in the two odor fields of a static two-choice olfactometer when given the choice between dichloromethane (DCM, control) and **(A)** one male equivalent of a natural *N. longicornis* pheromone extract, **(B)** a combination of 200 ng of synthetic (4*R*,5*S*)-5-hydroxy-4-decanolide (RS)+0.5 ng 4-methylquinazoline (MQ), **(C)** 200 ng RS, and **(D)** 0.5 ng MQ. Box and whisker plots show median (horizontal line), 25%–75% quartiles (box), maximum/minimum range (whiskers) and outliers (°>1.5 × above box height; *>3 × above box height; *n*=25, data analysis by a Wilcoxon matched-pairs test).

maximum of 2 h (Steiner and Ruther, 2009). Hence, males likely refrain from depositing higher pheromone amounts in the presence of female containing hosts, because they are unable to reliably estimate the time of female emergence when exit holes are missing. Dominant *Nv* males pheromone-marked monopolized hosts to prevent females from dispersing unmated. In these experiments, however, exit holes were already present (Mair and Ruther, 2018). Site fidelity at the sites of pheromone deposition is another strategy of economic pheromone use in *Nasonia*. As previously shown for *Nv* (Ruther et al., 2011), *Nl* males use the trace component MQ but not the major component RS to recognize the pheromone markings at which virgin females are likely to appear. In this context, 0.5 ng MQ was sufficient to mediate site fidelity in *Nl* males and to enhance responses of virgin *Nl* females. This falls into place with much lower MQ titers determined in *Nl* males (*ca.* 0.5 ng per male) when compared to values reported previously for *Nv* (*ca.* 3 ng per male; Ruther et al., 2008).

After this study, *No* remains the only *Nasonia* species to be investigated in detail with respect to responses of females and males to male sex pheromone. Given that *No* is sympatric with both *Nv* and *Ng* in eastern North America, *No* females likely encounter both the binary pheromone blend of *Ng* males and the ternary pheromone blend of *Nv* males, with the former blend being identical to that of conspecific *No* males. However, *No* has been reported to possess much lower titers of the binary pheromone blend (RS + MQ) than *Ng* (Diao et al., 2016) suggesting that *No* no longer uses the male abdominal sex

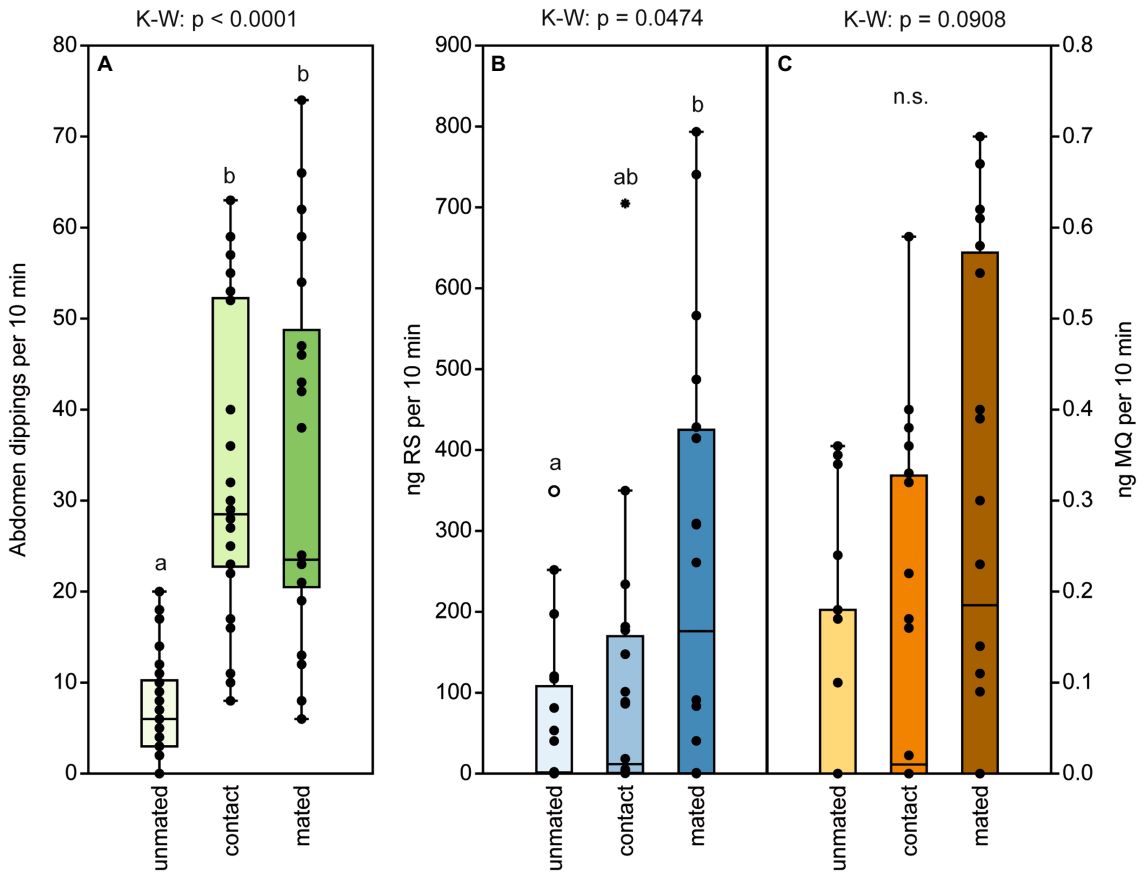

FIGURE 6
Male abdomen dipping serves to deposit pheromone and increases in frequency after mating and contact with a female. **(A)** Frequency of abdomen dipping shown by males within a 10-min observation time. Males were either unmated (naïve), had contact with a female, or were allowed to mate with a female. **(B)** Amounts of (4*R*,5*S*)-5-hydroxy-4-decanolide (RS) and **(C)** 4-methylquinazoline (MQ) deposited by males of the three treatments within 10 min in a thermal desorption tube. Box and whisker plots show median (horizontal line), 25%–75% quartiles (box), maximum/minimum range (whiskers) and outliers (°>1.5 × above box height; *>3 × above box height). Different lowercase letters indicate significant differences between the treatments at $p<0.05$ [n.s. = non significant, $n=10$, data analysis by a Kruskal–Wallis *H*-test (K–W) followed by multiple Bonferroni-corrected Mann–Whitney *U*-tests].

pheromone in the context of mate finding. Further studies are needed to fully understand the role of male sex pheromones in precopulatory isolation of the three sympatric *Nasonia* species in eastern North America.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Author contributions

JR designed the study, supervised AK, and wrote the first draft of the manuscript. AK performed the experiments and analyzed the data. JH synthesized and provided a sample of (4*R*,5*S*)-5-hydroxy-4-decanolide. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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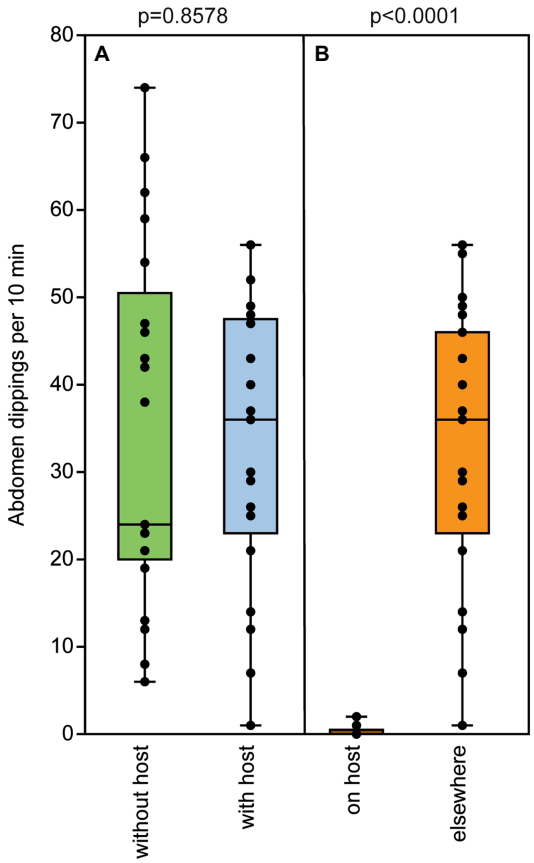

FIGURE 7
The presence of a parasitized host does not influence male marking behavior. **(A)** Frequency of abdomen dipping shown by mated males within a 10-min observation time in the absence or presence of a host containing females about to emerge. **(B)** Location of abdomen dipping by mated males when a host was present. Box and whisker plots show median (horizontal line), 25%–75% quartiles (box), maximum/minimum range [whiskers; *n*=25, data analysis by a **(A)** Mann–Whitney *U*-test and **(B)** Wilcoxon matched-pairs test].

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