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Multiparasitism Resolves the Apparent Paradox of High Male Pheromone Investment Despite Frequent Within-Host Mating in a Parasitoid

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ABSTRACT

The production of sex pheromones can impose significant costs on the signalling sex if the precursor substances are limited. Therefore, maintaining a costly pheromone system requires an increase of mating opportunities associated with its use. For a long time, it was assumed that mating in the parasitoid *Nasonia girault*i takes place prior to emergence within the host. Mated females, however, no longer respond to the male sex attractant raising the question of why males afford to produce the costly chemical signal. Here we show that males invest about a quarter of their teneral lipid reserves in pheromone production and release more than 60% of the stored pheromone after successful mating likely to attract more virgin females that are about to emerge. Most *N. girault*i females emerging from hosts exclusively parasitized by this species, however, were mated and therefore unresponsive to the male pheromone. In contrast, when hosts were parasitized also by the sympatric congeneric species *N. vitripennis*, most *N. girault*i females emerged unmated and were attracted by the pheromone of conspecific males. Multiparasitism is widespread among the two *Nasonia* species studied here, thus explaining the investment of fitness-relevant amounts of resources in a functional pheromone system in *N. girault*i males.

1 | Introduction

Sexual reproduction requires potential partners to find and recognize each other which is achieved in many insects through the use of sex pheromones (Blomquist and Vogt 2020; Wyatt 2014). Pheromone communication is associated with costs for both the sender and the receiver of the chemical signal. The sender must bear the production costs of the pheromone (Foster and Johnson 2011; Harari et al. 2011; Steiger and Stökl 2014) and runs the risk of being detected by natural enemies eavesdropping on the chemical signal (White et al. 2022). The receiver bears the physiological travel costs and may also be captured by natural enemies when responding to the calling sex (Acharya 1995; Svensson et al. 2004). Therefore, maintaining a chemical communication system only makes sense if it increases the chances of successful mating and should be selected against if it imposes significant costs but offers no benefits. The relative costs of sender and receiver are supposed to determine which sex produces the signal (Johansson and Jones 2007). When pheromone production is inexpensive and the cost of searching is high, females are more likely to emit the chemical signal, whereas costly pheromones and low travel costs tend to favor male-produced pheromones. This general principle rests on the idea that females invest more in offspring production than males do and should thus invest less in mate attraction (Johansson and Jones 2007).

In the parasitoid wasp genus *Nasonia* (Hymenoptera, Pteromalidae), it is the males that produce abdominal sex pheromones to attract virgin females (Mair and Ruther 2019; van den Assem et al. 1980). The genus includes four species, *N. vitripennis*, *N. girault*i, *N. longicornis* and *N. oneida*, all of which parasitise pupae of numerous cyclorraphous fly species occurring in

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bird nests (Darling and Werren 1990; Raychoudhury et al. 2010). The genus *Nasonia* is a prime example of the existence of reproductive interference (RI) (Gröning and Hochkirch 2008; Shuker and Burfield-Steel 2017) with males typically displaying courtship behavior also toward heterospecific females (Giesbers et al. 2013). Depending on the species constellation, heterospecific mismating occurs with varying frequency, but in almost all constellations, this results in females producing either suboptimal all-male broods or no offspring at all due to *Wolbachia*-mediated cytoplasmic incompatibility (Bordenstein et al. 2001; Breeuwer and Werren 1990). Hence, the male *Nasonia* pheromones are required to transfer information in a species-specific manner to decrease the risk of RI. Males of all species produce (4*R*,5*S*)-5-hydroxy-4-decanolide (RS-HDL) as a major pheromone component which is synergized by the minor component 4-methylquinazoline (MQ) (Kurtanovic et al. 2022; Niehuis et al. 2013; Ruther et al. 2014, 2008). Only males of the cosmopolitan *N. vitripennis* have additionally significant amounts of (4*R*,5*R*)-5-hydroxy-4-decanolide (RR-HDL) in their pheromone blend which plays a role in prezygotic isolation of sympatric *Nasonia* species (Kurtanovic et al. 2022; Niehuis et al. 2013; Ruther et al. 2014). In *N. vitripennis*, the pheromone is synthesized from linoleic acid (Blaul and Ruther 2011) in the males' rectal vesicle (Abdel-Latief et al. 2008). Key steps in the biosynthesis are epoxidation of the double bond at position 12, epoxide hydrolysis and chain shortening of the resulting 12,13-dihydroxylated oleic acid by β-oxidation (Ruther et al. 2020). The precursor linoleic acid originates either from the teneral lipids obtained from the host during development or can be synthesized from oleic acid by the involvement of the Δ12-desaturase Nvit_D12a (Semmelmann et al. 2019), an enzyme that also occurs in *N. giraulti* (Broschwitz et al. 2021). The pheromone is deposited to substrates by dabbing and streaking movements of the abdominal tip, a behaviour referred to as abdomen dipping (Barrass 1969; Steiner and Ruther 2009; van den Assem et al. 1980). Pheromone production in *Nasonia* species is age-dependent (Kurtanovic et al. 2022; Ruther et al. 2014, 2007), but reported pheromone quantities are typically highly variable, because quantification often only considered the extractable glandular amounts not taking into account that males might have released significant amounts of the pheromone already before sampling. This complicates the assessment of total metabolic costs imposed on *Nasonia* males when investing in sex pheromone production.

The response of *Nasonia* females depends on their mating status: Only virgin females are attracted, while their preference ceases immediately after mating (Kurtanovic et al. 2022; Ruther et al. 2014, 2007, 2010). In *N. vitripennis*, this olfactory switch is mediated by oral fatty acid ethyl esters applied by the males to the females' antennae during courtship (Ruther and Hammerl 2014) and is modulated by dopamine release in the females' brain (Lenschow et al. 2018). *N. vitripennis* is sympatric with *N. giraulti* in eastern North America often found within the same blowfly pupa (Darling and Werren 1990; Grillenberger et al. 2009). *N. vitripennis* and *N. giraulti* differ in an important aspect of their biology; that is, the site of mating. While *N. vitripennis* mates outside the host but still at the natal patch, *N. giraulti* was long thought to mate almost exclusively in the host prior to emergence (within host mating, WHM) (Drapeau and Werren 1999; Leonard and Boake 2006). Given the high WHM rate and the mating-induced behavioural switch in the pheromone response in *Nasonia* species, the question arises why *N. giraulti* males produce a costly sex pheromone. A possible answer to this question has been given by a study reinvestigating the WHM rate of *N. giraulti* under multiparasitism (Trienens et al. 2021). The authors demonstrated that the WHM rate of *N. giraulti* females decreased significantly, if the natal host was multiparasitized by *N. vitripennis* (Trienens et al. 2021).

In the present study, we investigate the metabolic costs associated with pheromone production by *N. giraulti* males by considering both the released and glandular pheromone amounts as well as the availability of fatty acid precursors and study the behavioural context of pheromone release. We furthermore test the hypothesis that males bear these costs to increase their mating chances with unmated females emerging from multiparasitized hosts.

2 | Materials and Methods

2.1 | Insects

The *N. giraulti* strain used in this study was kindly provided by Evelyn Verhulst (University of Wageningen). It was originally thought to be *N. oneida* but sequencing of the nuclear genes phosphoglucose isomerase and ATP synthase coupling factor F using the primer pairs PGIF1, PGIR1 and ATPf-a, ATPF-b (Raychoudhury et al. 2010) revealed the species to be *N. giraulti*. For the multiparasitism experiment, females of the *N. vitripennis* strain Phero01 (originally collected from bird nests in Northern Germany) were used. Parasitoid wasps of both species were reared on freeze-killed puparia of the green bottle fly *Lucilia caesar* as described previously (Steiner et al. 2006) at 25°C and approximately 60% relative humidity and a daily light: dark cycle of 16:8 h. To obtain a sufficient number of male *N. giraulti* for the preparation of pheromone extracts and the pheromone quantification experiment, all-male broods were produced by providing unmated females with hosts (haplodiploidy).

2.2 | Pheromone Amounts Produced and Released by Males of Different Ages

To determine the age dependent total pheromone production in *N. giraulti* males, deposited pheromone amounts and those that remained in the pheromone gland were quantified separately in individual 0, 1, 2, and 3-days old males ($n = 10$ for each age). To evaluate the age of the males as precisely as possible, male pupae were isolated from hosts 2 day before the expected eclosion and isolated into 1.5-mL Eppendorf tubes (perforated 3× with a needle to ensure air supply). Males found newly eclosed in the morning were defined as being 0-day old. These males were then placed singly in 89 × 5 mm inner diameter thermal desorption (TD) glass tubes (Supelco, Bellefonte, PA, USA) and kept there for 1, 2, or 3 days. TD tubes were filled at one end with 50 mg of Tenax TA porous polymer adsorbent (Supelco), which was fixed by fine-mesh metal screens (Supelco), leaving a free space of approximately 55 × 5 mm on the opposite side of the tube for the male wasp. Both ends of the tube were capped with metal caps to prevent the wasps from escaping. After the respective exposure

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times, males were removed from the tubes and frozen (−20°C) for pheromone gland analyses. Before TD analysis of the tubes, 1 μL of an internal standard solution containing 200 ng/μL methyl decanoate (Sigma-Aldrich, Deisenhofen, Germany) in methanol was applied to the Tenax layer of the TD tubes by using a GC syringe (Hamilton). Then the tube was purged for 5 min (adsorbent upwind) with charcoal-cleaned nitrogen at a flow rate of 60 mL min^{-1}. By this means, the solvent was removed, and both the volatilized proportion of the pheromone marking and the residues remaining on the glass surface were subjected to quantitative analysis by GC/MS. For quantification of glandular pheromone amounts, males were thawed, and abdomens were separated from the bodies using fine forceps (Dumont jeweller forceps no. 5, Merck, Darmstadt, Germany). Abdomens were transferred to 1.5-mL glass vials with 190-μL conical glass inserts (CZT, Kriftel, Germany) and extracted without any further dissection for 30 min in 30 μL dichloromethane (DCM) containing 10 ng μL^{-1} methyl decanoate as internal standard. After removal of the abdomens, extracts were directly used for GC/MS analysis. Abdomens of 0-day old males were directly extracted without first keeping them in TD tubes.

2.3 | Context of Pheromone Release

To investigate the context of pheromone release and the possible correlation of abdomen dipping and pheromone release, naïve, 2-day-old males ($n = 10$ per treatment) were placed singly in TD tubes and pheromone deposition was quantified for 10 min after exposure to one of the following stimuli: (a) none (control for the determination of spontaneous pheromone release), (b) contact to a female and (c) copulation with a female. For (b) and (c), a female was placed in the tube and removed again either after the male had contacted the female shortly with his antennae (courtship and copulation prevented) or after successful copulation with the female had been observed. Directly afterwards, the frequency of abdomen dipping shown by each male was recorded for 10 min under a stereomicroscope. After this observation period, males were removed and freeze-killed. Pheromone amounts released by males in the TD tubes and the amounts remaining in the pheromone glands were quantified by GC/MS as described before.

2.4 | Analysis of Total Fatty Acids in Males

To estimate the energetic investment of *N. giraulti* males in the production of the fatty acid-derived sex pheromone, total fatty acids of 0 and 3-days old males were quantified after converting extractable raw lipids into fatty acid methyl esters (FAME). To this end, 0 and 3-days old males ($n = 10$ each) were killed by freezing and homogenised after thawing with a pestle in 1.5 mL Eppendorf vials after adding 100 μL dichloromethane containing 1 μg 2-methyloctadecanoic acid as an internal standard. The homogenates were kept at room temperature for 30 min. Subsequently, the extracts were transferred to 1.5 mL glass vials. The insect residues were washed with an additional 100 μL dichloromethane (without internal standard) and unified lipid extracts were dried under a stream of nitrogen. The residues were re-suspended in 200 μL of methanol and 20 μL of acetyl chloride (10%, dissolved in methanol) and transesterified for 1.5 h at 75°C–80°C in a 20-well thermostatically controlled heating block (VWR, Darmstadt, Germany). Thereafter, 200 μL of a sodium hydrogen carbonate solution (5%) were added and FAME were extracted with 200 μL hexane (10 s vortexing). The hexane phase was concentrated to ca. 20 μL and used for GC/MS analysis.

2.5 | Preparation and Purification of Pheromone Extracts

Abdomens of 2–3-day old freeze-killed males were separated from the bodies after thawing as described above and extracted without any further preparation for 30 min in batches of 30–60 abdomens with 30 μL DCM per abdomen. Extracts were transferred to 1.5-mL glass vials, and the remaining abdomens were washed again with 300 μL DCM. Combined pheromone extracts were carefully (avoidance of splashing) concentrated to 100–200 μL under a stream of nitrogen. Subsequently, pheromone extracts were purified by solid phase extraction using 100 mg Chromabond silica gel cartridges (Macherey-Nagel, Düren, Germany). Columns were conditioned with 4 mL each of DCM, methanol, and DCM again. Raw extracts were applied to the column and rinsed twice with 400 μL DCM. Pheromone components were eluted with 500 μL methanol. The cleaned pheromone extract was dried under a stream of nitrogen, and residues were re-dissolved in 100 μL of DCM. The concentration of RS-HDL in the pheromone extract was determined by GC/MS (see below) and adjusted to 175 ng μL^{-1}.

2.6 | GC/MS Analysis

TD analyses were performed with an automated TD-20 thermal desorber (Shimadzu GmbH, Duisburg, Germany) coupled to a Shimadzu QP2010 Plus GC/MS system. Volatiles were desorbed from the adsorption tubes for 8 min at 250°C with helium at a flow rate of 60 mL min^{-1} and cryo-focused at −15°C on an internal Tenax trap. Subsequently, volatiles were injected into the GC/MS by heating the internal trap to 280°C for 5 min at a split ratio of 1:20. Helium was used as carrier gas at a linear velocity of 37.8 cm s^{-1}. The mass spectrometer was operated in electron ionization (EI) mode at 70 eV with a mass range of m/z 35–600 and a detector voltage of 1.1 kV. The temperature program started at 80°C, increased at 5°C min^{-1} to 200°C and then at 25°C min^{-1} to 300°C (held for 15 min). Pheromone extracts were analysed on the same instrument using liquid injection (1 μL) in splitless mode with an AOC 20i autosampler. Assuming equal response factors of RS- and RR-HDL, calibration curves were generated for both TD and liquid injection by analysing known amounts (TD: 50–500 ng, split 1:20, liquid injection: 2.5–75 ng) of synthetic RR-HDL together with known amounts of the internal standard methyl undecanoate (TD: 200 ng, liquid injection: 10 ng). Apart from the major component RS-HDL, trace amounts of 4-methylquinazoline and RR-HDL were detected in the chromatograms (< 0.5% of the total pheromone amount) but were not considered for evaluation of the male investment in pheromone production.

FAME analysis was accomplished with the same instrumentation but using a different temperature program (start at 150°C,

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increased at 3°C min^{-1} to 300°C, held 15 min). FAME were identified by comparison of retention times and mass spectra with those of authentic reference chemicals (reference mixture of 37 FAME, Sigma-Aldrich). Total amounts of FAME in *N. giraulti* samples were determined by integrating the peak areas of the identified FAME in the total ion current chromatograms and relating them to the peak area of the esterified internal standard 2-methyloctadecanoic acid methylester.

2.7 | Behavioural Bioassay

The pheromone response of *N. giraulti* females was investigated using the two-choice olfactometer designed for the previous pheromone studies with *Nasonia* wasps (Kurtanovic et al. 2022; Ruther et al. 2014). The olfactometer consisted of a glass plate with a circular walking arena (9 cm diameter) divided into four equally sized sectors. A spherical cavity in the centre of the arena served as release point for the test insects. A spherical cavity (1 cm diameter, 4 mm depth) was located in each of the four sectors at the same position, in a distance of 3 cm to the central cavity. Two opposite cavities were used to present test and control stimuli. The arena was enclosed by a 2 mm high plastic rim and covered by a transparent lid made of acrylic glass, allowing observation of the wasp from above. At the start of the experiment, an open, grey plastic cylinder was placed on the lid to minimize the effects of external visual stimuli. A desk light (60 W incandescent lamp) was centred approximately 50 cm above the arena for uniform illumination. The bioassays were conducted at room temperature. Before each experiment, discs of filter paper were impregnated with 2 μL of the pheromone extract (representing 350 ng RS-HDL) or DCM (control). After evaporation of the solvent (30 s), discs were put into test and control cavity of the olfactometer and the residence time of females (single parasitism: $n = 25$, multiparasitism: $n = 39$) in the cavities was observed for 5 min using The Observer XT observational software (Noldus Information Technology, Wageningen, The Netherlands). After each test, the arena was rotated clockwise by 90° to avoid positional bias. After every four tests, the arena was carefully cleaned with ethanol and deionized water and dried at 60°C for at least 30 min. Preliminary tests have demonstrated that this cleaning frequency is sufficient to avoid memory effects. The observer was blind to the treatments and females were tested only once.

2.8 | Pheromone Response of *N. giraulti* Females

To investigate the pheromone response under near natural conditions, females were tested after having been collected in the moment when voluntarily leaving the natal host. In Experiment 1, these hosts had been parasitized by *N. giraulti* females only (single parasitism) and in Experiment 2 by both *N. giraulti* and *N. vitripennis* (multi parasitism). For Experiment 1, parasitized hosts containing wasps about to emerge (14–15 days after parasitisation) were transferred to Petri dishes and inspected carefully for the presence of exit holes. Emerging male wasps were immediately removed to prevent mating with the females outside the host. 1–2 females per host were collected when leaving the host and isolated in Eppendorf tubes for being tested in the pheromone bioassay ($n = 25$). For Experiment 2, multiparasitized hosts were produced under controlled conditions. Unparasitized hosts were exposed for 3 h to 2–3-days old, unmated *N. vitripennis* females for oviposition and subsequently offered for 24 h to 2–3-days old, mated *N. giraulti* females. Virgin *N. vitripennis* females are constrained to produce only male offspring (haplodiploidy) and were chosen to make sure that any female offspring emerging from the multiparasitized hosts is *N. giraulti*. Subsequently, multiparasitized hosts were kept for 13–14 days under rearing conditions. After this period, they were inspected for emerging *N. giraulti* females which were collected immediately after emergence ($n = 39$) to be tested in the pheromone bioassay as described for Experiment 1. Only those females were selected for the bioassay that emerged from successfully multiparasitized hosts, meaning that both *N. vitripennis* and *N. giraulti* males emerged from the same host as the female. To assess their mating status, all females tested in Experiments 1 and 2 were provided after the bioassay with five unparasitized hosts for oviposition. Offspring emerging from the parasitized hosts 2 weeks later were sexed and those mothers that produced all-male broods were considered having been virgin. Females that produced no offspring, only diapausing larvae, or too few offspring (< 4) to reliably assess offspring sex ratio were excluded from the analysis of the WHM rate.

2.9 | Statistical Analysis

All statistical analyses were performed with PAST 5.0.2.statistical software (Hammer et al. 2001). Shapiro–Wilk test was used to test for normality. As the data were not consistently normally distributed, non-parametric tests were used for all statistical analyses. A significance level of $\alpha = 0.05$ was considered for all tests. Released and glandular pheromone amounts as well as the frequency of abdomen dipping shown by *N. giraulti* males were compared with the Kruskal–Wallis *H*-test. In the case of a significant difference, pairwise comparisons between treatments were performed using Mann–Whitney *U*-tests with Bonferroni-Holm correction for multiple testing (Holm 1979). Spearman's rank correlation was used to assess the relationship between abdomen dipping frequency and amount of released pheromone. A linear regression model was fitted additionally. The residence times in test and control cavities of the olfactometer were compared with Wilcoxon matched pairs test.

3 | Results

3.1 | Pheromone Amounts Produced and Released by Males of Different Ages

The analytical approach of this study allowed for the first time the simultaneous quantification of released and glandular amounts of the major sex pheromone compound RS-HDL in individual *N. giraulti* males over the first 3 days of their life thus allowing conclusions about their physiological investment in pheromone production. Males eclose with no pheromone in their abdomens and total pheromone production (released

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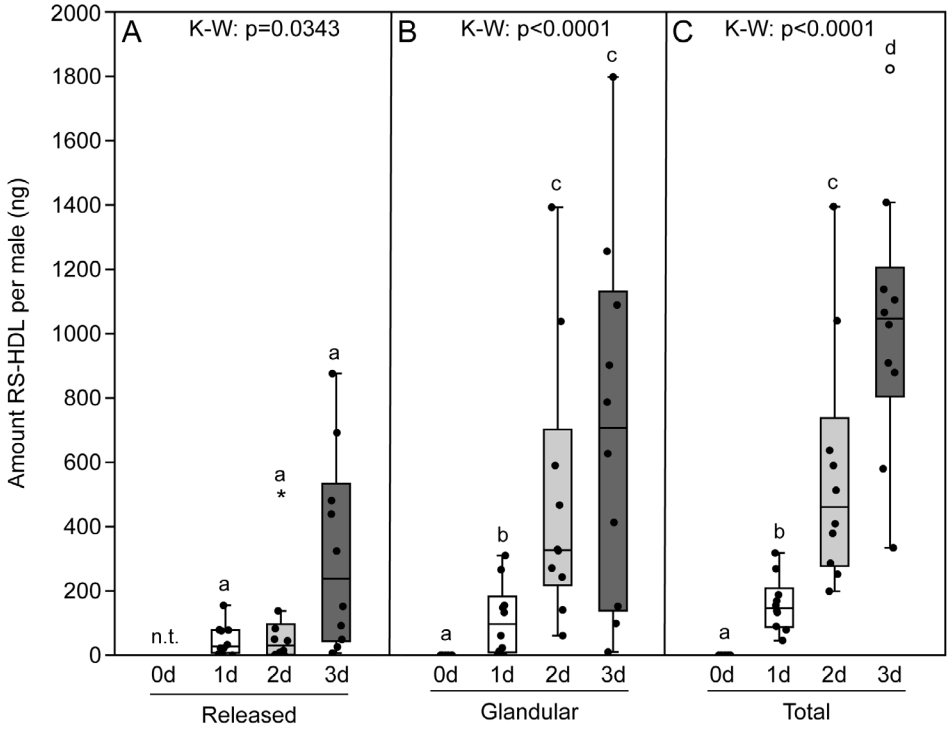

FIGURE 1 | Age dependency of pheromone production and release in *Nasonia girault*i males. Boxplots show amounts of the major pheromone component RS-HDL released spontaneously by *N. girault*i males at different ages (A), pheromone amounts that remained in the pheromone gland at the same time points (B) and total (released + glandular) amounts (C, $n = 10$ per age group). Newly eclosed males (0 day) were immediately extracted and thus, released pheromone amounts were not tested (n.t.). Box-and-whisker plots show 25% and 75% quartiles (upper and lower end of the boxes), median (horizontal line in between), 1.5× the interquartile range (whiskers), and outliers (°, means > 1.5× interquartile range; *, means > 3× interquartile range). Different lowercase letters indicate significant differences between the age groups of released, glandular and total pheromone amounts, respectively ($p < 0.05$, statistical analysis by Kruskal–Wallis test (K–W), followed by pairwise Mann–Whitney *U*-tests with Bonferroni–Holm correction).

+ glandular) increases within the first 3 days (mean ± SEM 1 day: 158 ± 27 ng, 2 day: 570 ± 120 ng, 3 day: 1027 ± 130 ng; Kruskal–Wallis: H = 6.738, df = 2, $p < 0.0001$, Bonferroni-Holm-corrected Mann–Whitney: all comparisons $p < 0.05$; Figure 1A–C, all data presented in the results can be found in the file data.xlsx in the Supporting Information). The pheromone amounts released spontaneously during the first 3 days differed significantly (1 day: 48 ± 15 ng, 2 day: 84 ± 50, 3 day: 313 ± 96 ng; Kruskal–Wallis: H = 6.738, df = 3, $p = 0.0343$). The amounts released spontaneously were equal during Day 1 and 2 (Bonferroni-Holm-corrected Mann–Whitney: $p = 0.9096$) and increased on Day 3, although after Bonferroni-Holm correction these differences were no longer significant (1 day vs. 3 day: $p = 0.0633$, 2 day vs. 3 day: $p = 0.0751$). Also, the amounts of glandular pheromone differed significantly between males of different ages (0 day: 0 ng, 1 day: 111 ± 35, 2 day: 486 ± 133, 3 day: 713 ± 181 ng; Kruskal–Wallis: H = 25.95, df = 3, $p < 0.0001$). Males eclosed with no pheromone, increased glandular pheromone amounts during Days 1 and 2 (Bonferroni–Holm corrected Mann–Whitney: all comparisons $p < 0.05$) while glandular pheromone amounts remained constant on Day 3 (2 day vs. 3 day: $p = 0.4274$). The percentage of available pheromone released by *N. girault*i males spontaneously during the first 3 days of their life was highly variable (0.7%–97.0%, mean ± SEM: 40% ± 12%). Considering the stoichiometry of the biosynthesis (1 mol pheromone from 1 mol linoleic acid) (Ruther et al. 2020) and the molecular masses of RS-HDL (186 Da) and linoleic acid (280 Da), males needed approximately 1.5 ± 0.2 μg of the pheromone precursor for pheromone production during the first 3 days of their life.

3.2 | Context of Pheromone Release

The behavioural context had a significant impact on the pheromone release of 2-day old *N. girault*i males within a 10 min observation period (spontaneously: 0.4 ± 0.1 ng, contact: 4.1 ± 3.9 ng, mating: 242 ± 44 ng; Kruskal–Wallis: H = 20.02, df = 2, $p < 0.0001$, Figure 2A). Recently mated males released significantly more pheromone than control males and those that had merely contacted a female (Bonferroni–Holm corrected Mann–Whitney: both comparisons $p = 0.0004$) while the two latter treatments did not differ ($p = 0.2185$). Abdomen dipping behavior was influenced in a similar manner by the behavioural context (Kruskal–Wallis: H = 19.48, df = 2, $p < 0.0001$, Figure 2C). While control males and those with mere contact to a female hardly showed abdomen dipping (Bonferroni–Holm corrected Mann–Whitney: $p = 0.5171$), this behaviour was shown significantly more often by recently mated males (control vs. mated and contact vs. mated: $p = 0.0003$). Frequency of abdomen dipping and released pheromone amounts were significantly correlated with each other (Spearman's rank correlation: $r_s = 0.92$, $p < 0.0001$, Figure 2D). Analysis of the glandular pheromone amounts of

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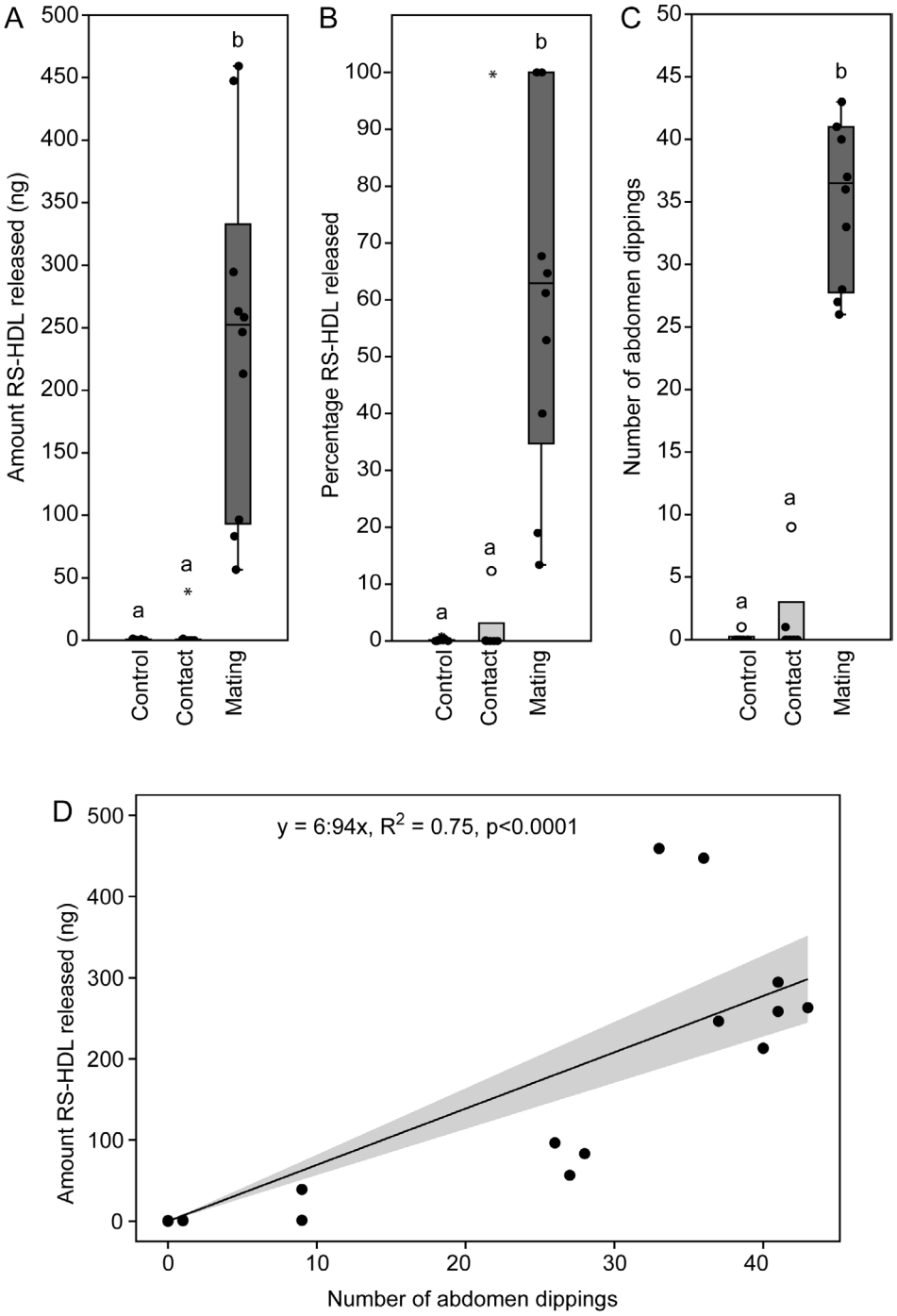

FIGURE 2 | Context of pheromone release and abdomen dipping in *N. giraulti* males. Boxplots show (A) the amounts (B) the percentage of total available amounts of the pheromone component RS-HDL released by 2-day old *N. giraulti* males during a 10-min observation period and (C) the number of abdomen dippings performed by these males during this time. Males were subjected to one of the following stimuli prior to the observation: None (control), contact with a female or mating with a female. Box-and-whisker plots show 25% and 75% quartiles (upper and lower end of the boxes), median (horizontal line in between), 1.5× the interquartile range (whiskers), and outliers (°, means > 1.5× interquartile range; *, means > 3× interquartile range). Different lowercase letters indicate significant differences between the treatments ($p < 0.003$, statistical analysis by Kruskal–Wallis test, followed by pairwise Mann–Whitney *U*-tests with Bonferroni-Holm correction). (D) Correlation between released pheromone amounts and number of abdomen dippings (analysed by a Spearman's rank correlation). Note that several data points are overlapping at 0/0. Grey area indicates 95% confidence interval.

recently mated males revealed that the released pheromone amounts corresponded to 61.9% ± 10.1% of the total amounts available in the pheromone gland at the time of the experiment (Figure 2B).

3.3 | Analysis of Total Fatty Acids in Males

Newly eclosed males possessed approximately 6.2 ± 0.69 μg total fatty acids with six compounds accounting for more than 95%:

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TABLE 1 | Fatty acid amounts in ng (mean ± SE) of newly eclosed (0 day) and 3-day old *N. giraulti* males determined as fatty acid methyl esters after transesterification of raw lipid extracts.

Name	Trivial name	0 day $n = 13$	3 day $n = 10$
Dodecanoic acid	Lauric acid	9 ± 2	5 ± 1
Tetradecanoic acid	Myristic acid	26 ± 3	12 ± 2
Hexadecadienoic acid[a]	—	9 ± 1	2 ± 1
Hexadecenoic acid[a]	—	144 ± 21	42 ± 11
(9*Z*)-hexadec-9-enoic acid	Palmitoleic acid	396 ± 57	162 ± 55
Hexadecanoic acid	Palmitic acid	1125 ± 131	422 ± 129
Heptadecanoic acid	Margaric acid	11 ± 2	9 ± 3
(9*Z*,12*Z*)-octadeca-9,12-dienoic acid	Linoleic acid	1412 ± 171	474 ± 124
(9*Z*)-octadec-9-enoic acid	Oleic acid	1999 ± 213	910 ± 233
(9*Z*,12*Z*,15*Z*)-octadeca-9,12,15-trienoic acid	α-linolenic acid	526 ± 59	190 ± 41
Octadecenoic acid[a]	—	109 ± 13	35 ± 9
Octadecanoic acid	Stearic acid	405 ± 39	205 ± 43
Eicosanoic acid	Arachidic acid	8 ± 1	3 ± 1
	Total	6180 ± 690	2470 ± 640

[a]Position and configuration of the double bond(s) not determined.

palmitoleic acid, palmitic acid, linoleic acid, oleic acid, α-linolenic acid and stearic acid (Table 1). The sex pheromone precursor linoleic acid accounted for 22.4% ± 0.5% of the fatty acids, the second largest amount after oleic acid (32.6% ± 0.7%). After 3 days, total fatty acids decreased significantly to approximately 2.5 ± 0.64 (Mann–Whitney *U* test: $p = 0.0032$). During the first 3 days of their life, males consumed approximately 3.7 μg (60%) of the total fatty acids, including 0.94 μg (66%) of the teneral linoleic acid reserves.

3.4 | Pheromone Response and Mating Status of *N. giraulti* Females

The pheromone response of *N. giraulti* females depended on the parasitisation status of the natal host. Females collected at the time of emergence from natal hosts that had only been parasitised by *N. giraulti* females did not respond to the sex pheromone of conspecific males (Figure 3A, single). The lack of pheromone response could be explained by their mating status, as 96% of them produced female offspring and thus emerged mated from their natal host (Figure 3B, single). In contrast, those females collected at the time of emergence from natal hosts that had been parasitised by both *N. giraulti* and *N. vitripennis* females significantly preferred the male sex pheromone (Figure 3A, multi). Consistently, 93% of these females produced all-male offspring and emerged thus unmated from their natal hosts (Figure 3B, multi).

4 | Discussion

The present study demonstrates that *N. giraulti* males invest significant amounts of their teneral lipid resources in the production of the major component of their abdominal sex attractant and economize the use of the costly pheromone by releasing it when mating chances are high. It furthermore resolves the apparent paradox that males of a species thought to mate preferably within the host invest in the production of a costly sex attractant to which mated females no longer respond. Under multiparasitism with the microsympatric species *N. vitripennis*, most *N. giraulti* females emerge as virgins and do respond to the male sex pheromone.

During the first 3 days of their life, *N. giraulti* males invested roughly a quarter of their teneral lipid reserves in the production of RS-HDL. The calculated amount of linoleic acid required for pheromone production even exceeded the available teneral reserves of this fatty acid, demonstrating that males must have converted significant amounts of teneral oleic acid to linoleic acid by using their Δ12-desaturase (Broschwitz et al. 2021; Semmelmann et al. 2019). The gene encoding this enzyme shows by far the most male-biased expression pattern of all genes in both *N. vitripennis* and *N. giraulti* (Wang et al. 2015), underscoring the importance of linoleic acid for males of both species. In *N. vitripennis*, males developing in hosts that had been experimentally enriched in linoleic acid had three times as many spermatozoa in their seminal vesicle and produced significantly more of their sex pheromone than controls supplemented with other fatty acids (Blaul and Ruther 2011; Brandstetter and Ruther 2016), suggesting a trade-off between pheromone production and fertility also in *N. giraulti* males. These may use their linoleic acid either for pheromone or sperm production, so high pheromone production is likely to come at the cost of reduced sperm production.

Spontaneous pheromone release occurred in *N. giraulti* males not before the age of 3 days when pheromone production maximizes, and the pheromone gland is well filled. The pheromone release, however, was strongly context dependent. This context dependency differed slightly from the congeneric species studied

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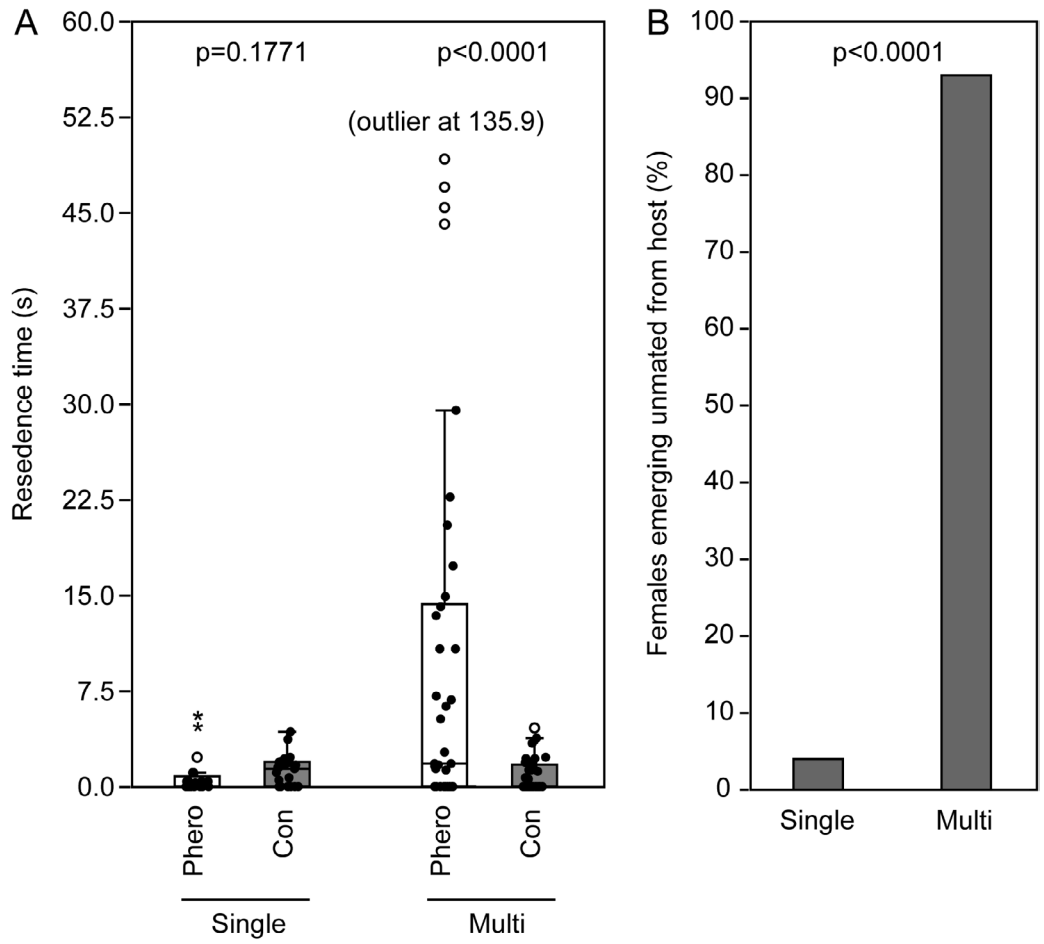

FIGURE 3 | Effect of the parasitisation status of the natal host on the response of *N. giraulti* females to the male sex pheromone and their mating status. (A) Boxplots show the time that *N. giraulti* females spent in cavities of a two-choice olfactometer containing filter papers either treated with a male pheromone extract (Phero) or the pure solvent (Con) during a 5-min observation period. Box-and-whisker plots show 25% and 75% quartiles (upper and lower end of the boxes), median (horizontal line in between), 1.5× the interquartile range (whiskers), and outliers (°, means > 1.5× interquartile range; *, means > 3× interquartile range). Tested females were collected in the moment emerging from the host that had been parasitized either by *N. giraulti* females alone (single, $n = 25$) or by both *N. giraulti* and *N. vitripennis* females (multi, $n = 39$). (B) Percentage of females tested in the pheromone experiment that produced all-male offspring and thus had emerged unmated. Statistical analysis by (A) Wilcoxon matched pairs test and (B) 2×2 Chi^2-test.

so far. While in both *N. vitripennis* and *N. longicornis* increased pheromone release was shown after both mere contact with a female and successful mating (Kurtanovic et al. 2022; Steiner and Ruther 2009), an increase of abdomen dipping behaviour and pheromone release required prior mating success in *N. giraulti* males. When this happened, males released more than 60% of the available pheromone in one go and several males emptied their pheromone gland completely. This suggests that the males go all in when having mated, because the time window for successful mating is small once the first female has left the host, and other females follow in rapid succession. In agreement with this idea, much faster succession of emergences has been observed in *N. giraulti* in mesocosm experiments comparing the emergence patterns of *N. vitripennis* and *N. giraulti* (Mair and Ruther 2018).

The mechanism underlying the increased emergence of virgin *N. giraulti* females from multiparasitized hosts has been investigated by (Trienens et al. 2021). The authors demonstrated that the high incidence of WHM in *N. giraulti* is controlled by the behaviour of conspecific males which, unlike *N. vitripennis* males, refrain from gnawing exit holes into the host thereby decelerating the females' emergence. Under multiparasitism, however, many *N. giraulti* females emerged prior to mating through the exit holes gnawn by the microsympatric *N. vitripennis* males. Males of *Nasonia* species are protandric and complete their development approximately 1 day earlier than females (Moynihan and Shuker 2011). Additionally, *N. vitripennis* develops approximately 1 day faster than *N. giraulti* under comparable rearing conditions (Clark et al. 2010). This increases the chance that exit holes gnawn by *N. vitripennis* males are present when *N. giraulti* females complete their development in multiparasitised hosts.

Previous studies suggest that multiparasitism is a realistic scenario in natural populations of the two sympatric *Nasonia* species. Early reports emphasized that *N. vitripennis* and *N. giraulti* are routinely collected from the same bird nests and frequently occur in the very same blowfly hosts (Darling and Werren 1990). One of the rare studies dealing with the biology of *Nasonia* species under field conditions confirmed that multiparasitism between *N. giraulti* and *N. vitripennis* is not the exception but appears to be the rule in eastern

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North America. The authors sampled parasitized fly pupae from 64 bird nests and found both species in 29% of the nests. Strikingly, *N. giraulti* was never found without *N. vitripennis* (Grillenberger et al. 2009). Hence, the emergence of virgin *N. giraulti* females is likely much more frequent under field conditions than previously thought based on lab experiments with pure breeding lines (Drapeau and Werren 1999; Leonard and Boake 2006).

The present study sheds light on the reason why males use fitness-relevant amounts of fatty acids to maintain their pheromone system functional but raises new questions concerning the benefits of the responding females. Unlike *N. longicornis* females which are sympatric with *N. vitripennis* in western North America and discriminate against the *N. vitripennis* specific RR-HDL (Kurtanovic et al. 2022), *N. giraulti* females are unable to do so and respond to both the conspecific two-component pheromone (RS-HDL + MQ) and the more complex pheromone blend of the sympatric *N. vitripennis* males (RS-HDL + RR-HDL + MQ) (Niehuis et al. 2013). Thus, virgin females run the risk of encountering these heterospecific males, which court females of *N. giraulti* and *N. vitripennis* with the same vigor (Mair et al. 2017). Mismating, however, leads to all-male progeny due to the *Wolbachia*-mediated cytoplasmic incompatibility (Breeuwer and Werren 1990) and thus imposes fitness costs on the respective *N. giraulti* females. Hence, *N. giraulti* females depend on mechanisms other than the male abdominal sex attractant to increase the chance of mating with suitable conspecific males. One possibility is rejection of males during courtship, but (Giesbers et al. 2013) reported a copulation rate of approximately 80% between *N. giraulti* females and *N. vitripennis* males. A recent investigation by (Mair et al. 2017), however, provided evidence that the ability of females to discriminate against heterospecific males during courtship is age-dependent in *N. giraulti* females. While 85% of 2-day old *N. giraulti* females consented to mating with *N. vitripennis* males, newly eclosed females rejected 65% of them (Mair et al. 2017). In conclusion, mating of *N. giraulti* females with a suitable conspecific male is ensured by the high WHM rate under single parasitism conditions, while under multiparasitism the male sex pheromone plays an important role. Due to the lack of species specificity in their pheromone response, however, there is a residual risk of costly mismating with sympatric *N. vitripennis* males, which the females compensate for through—albeit incomplete—mate discrimination during courtship.

Author Contributions

Martina Wendler: conceptualization, methodology, investigation, formal analysis, writing – review and editing. Verena Engl: investigation, formal analysis, writing – review and editing. Joachim Ruther: conceptualization, methodology, formal analysis, supervision, writing – original draft.

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Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

All data presented in this paper are available online in the Supporting Information.

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