Male butterflies use an anti-aphrodisiac pheromone to tailor ejaculates
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Summary

1. When females mate with multiple partners, the risk of sperm competition depends on female mating history. To maximize fitness, males should adjust their mating investment according to this risk. In polyandrous butterflies males transfer a large, nutritious ejaculate at mating. Larger ejaculates delay female remating and confer an advantage in sperm competition. However, large ejaculates are costly, potentially selecting for male adjustment of ejaculate size to the risk of sperm competition.

2. Here, we test if male ejaculate size in the butterfly *Pieris napi* (Lepidoptera) varies with female mating history and thus sperm competition, and whether males assess sperm competition intensity using the male-transferred anti-aphrodisiac Methyl salicylate (MeS) as a cue.

3. First we examined the olfactory ability to detect MeS and if physiological responses differed between the sexes. Both sexes responded to MeS in a dose-dependent manner. However, males were more sensitive to MeS and responded to a ten times lower concentration than females.

4. Ejaculates transferred by males mating with previously mated females were on average 26% larger than ejaculates transferred by males mating with virgin females, which conforms to sperm competition theory and indicates that male *P. napi* tailored their reproductive investment in response to sperm competition. Furthermore, we show that MeS was used by males to assess sperm competition; ejaculates transferred by males mating with virgin females with artificially added MeS were also 26% larger than ejaculates transferred to control virgin females.
5. In conclusion, the study shows how the male-transferred anti-aphrodisiac pheromone not only functions as a male deterrent, but also carries information on female mating history and thus allows males to assess sperm competition intensity.

**Key words**

_Pieris napi_, Anti-aphrodisiac, Pheromone, Olfaction, Mating investment, Nuptial gift, Sexual conflict, Spermatophore size
Introduction

Sperm competition has been recognized as a particularly powerful force that can lead to adaptations in male behavior, morphology and physiology that contribute to competitive fertilization success (Parker 1970, Birkhead & Møller 1998, Simmons 2001). Game theory has been used to develop predictions how male ejaculation strategies should be influenced by variation in sperm competition intensity and sperm competition risk, *i.e.* the probability that females will mate with more than one male - so that sperm will compete for fertilizations (Parker 1990a, b, 1997, 1998, Ball & Parker 1996, 1997, Parker *et al.* 1997, Parker & Ball 2005). Sperm competition games assume that ejaculates are costly - meaning that expenditure on any fertilization must be traded against that of gaining future fertilizations; and also assume that fertilization success is proportional to the amount of sperm transferred to females at mating (Parker 1998). The models predict that increasing risk and intensity of sperm competition from zero to one previous mating should favor the evolution of increased expenditure on ejaculates and there is now evidence suggesting such an effect (Gage & Baker 1991; Gage 1991, 1994; Harcourt, Purvis & Lilies 1995; Hosken 1997, Stockley *et al.* 1997; Byrne, Roberts & Simmons 2002; Pitcher, Dunn & Whittingham 2005, Simmons, Emlen & Tomkins 2007, Larsdotter-Mellström & Wiklund 2009). Although this phenomenon is widespread in insects and other taxa, the mechanisms that allow males to assess sperm competition risk has only recently been investigated. Several studies have shown that intraspecific chemical signals, pheromones, play a vital role for assessing sperm competition risk and intensity, *e.g.* in the beetle *Tenebrio molitor* (Carazo, Font & Alftman 2007), the fruit fly *Drosophila melanogaster* (Friberg 2006), the cricket *Teleogryllus oceanicus* (Thomas & Simmons 2009), the meadow vole *Microtus pennsylvanicus* (delBarco-

Butterfly males produce a complex ejaculate during copulation that can contain both eupyrene (fertilizing) sperm, apyrene (non-fertilizing) sperm (e.g. Silberglied, Shepherd & Dickinson 1984), volatile anti-aphrodisiacs (Andersson, Borg-Karlsson & Wiklund 2000) and accessory gland nutrients that function as paternal investment and/or male mating effort (Oberhauser 1989). For example, the amount of nitrogen transferred in these "nuptial gifts" is equivalent to that in 70 eggs and male-transferred materials increase both female longevity (Karlsson 1998) and fecundity (Wiklund & Kaitala 1995) in the polyandrous butterfly *P. napi*. That male ejaculates are costly is shown by the fact that copulation durations were longer and ejaculates smaller (Svärd & Wiklund 1986, Oberhauser 1988) and contain lower amounts of nutrients (Wedell & Karlsson 2003) in matings involving recently mated males. In polyandrous butterflies larger males enjoy higher paternity (Bissoondath & Wiklund 1997, Wedell & Cook 1998, Solensky & Oberhauser 2009), as they can transfer larger ejaculates that delay female remating for a longer time compared to small ejaculates (Oberhauser 1989, 1992, Wiklund *et al.* 1993, Kaitala & Wiklund 1994, Wiklund & Kaitala 1995, Bissoondath & Wiklund 1997). Larger ejaculates can also contain more sperm (Svärd & Wiklund 1989; but see Cook & Wedell 1996) favouring larger spermatophores under sperm competition. These results all lend support to the hypothesis that sperm competition has played a major role in the evolution of ejaculate size (Bissoondath & Wiklund 1997) and the prerequisites for the game theory (Parker 1998) are thus met, making a polyandrous butterfly a good model for
studying the relationship between sperm competition intensity and mating investment.

In the butterfly *P. napi* (Lepidoptera: Pieridae) females control mating (Bergström & Wiklund 2005). Hence, as males cannot enforce copulations on females, one possibility for a male to enhance his chances of paternity under competition is to manipulate the female into delaying her next mating. Another, non-exclusive, option is to increase the amount of sperm transferred to a female at mating to increase fertilization success under sperm competition. Both of these options can be mediated by a large ejaculate. Yet another advantage of transferring a larger ejaculate, and consequently more anti-aphrodisiacs, under high population densities is that females living under high densities spend the male-transferred anti-aphrodisiacs faster (Andersson, Borg-Karlsson & Wiklund 2004), making it favourable for males to compensate for this. In contrast, under low or no sperm competition males should benefit from saving resources which can be allocated to the next mating; so tailoring mating effort to sperm competition will be selected for. It has previously been shown that males of both *P. napi* and its sister species *P. rapae* can adjust mating investment based on sperm competition risk (Larsdotter-Mellström & Wiklund 2009). Wedell & Cook (1999a,b) have shown that mated *Pieris rapae* males provide a larger spermatophore and more sperm to previously mated females, when compared to virgin females. This indicates that males can tailor their ejaculates in relation to female mating history and sperm competition intensity. If males can distinguish between mated and virgin females they should increase their ejaculate expenditure when mating with non-virgin females (Parker *et al.* 1997). We thus hypothesize that
male *P. napi* mating with previously mated females will invest more than males mating with virgin females, as sperm competition intensity is higher.

So, what cues could males use to determine female mating history? In several insects species olfactory cues have been shown to affect male investment in the ejaculate (Friberg 2006, Carazo, Font & Alfthan 2007, Larsdotter-Mellström & Wiklund 2009, Thomas & Simmons 2009). Male *P. napi* emit a sex pheromone, citral, which makes females acquiesce and accept to mate with a courting male (Andersson, Borg-Karlsson & Wiklund 2007). Male *P. napi* use the concentration of citral in the air to assess the density of males close-by and thus sperm competition risk. Hence, it is conceivable that they also use information from the male-transferred anti-aphrodisiac pheromone to tailor spermatophores to sperm competition intensity and female mating history.

Anti-aphrodisiac pheromones have been identified and described in, among others, *P. napi*, and the closely related *Pieris brassicae* and *Pieris rapae* (Andersson, Borg-Karlsson & Wiklund 2003). An anti-aphrodisiac can initially be beneficial for both males and females (Andersson, Borg-Karlsson & Wiklund 2000). Males benefit from deterring other males from mating with the female, and the female will benefit from less mating harassment that might interfere with egg laying or feeding. However, the cooperation will with time turn into conflict, as the male would benefit from the female only mating with him whereas the female will benefit from the nutrients from new spermatophores (Wiklund & Forsberg 1986, Parker 1998, Andersson, Borg-Karlsson & Wiklund 2000). The anti-aphrodisiac in *P. napi* is the volatile, phenolic ester methyl salicylate (MeS). MeS is a multifunctional compound which has been found to be
active in a number of biological systems e.g. bees and aphids (Williams & Whitten 1983, Pettersson et al. 1994). In *P. napi* MeS is transferred by males at mating and is subsequently emitted by the female as she is courted by additional males, deterring them from attempting to mate with her (Andersson, Borg-Karlsson & Wiklund 2000). According to the same study, males will initially abstain from courting a female even if she is a virgin, if MeS has been artificially added to her abdomen. Males also behave differently when courting virgin and recently mated females; virgin females are courted for eight times as long (Andersson, Borg-Karlsson & Wiklund 2000).

If males can use information from the anti-aphrodisiac, males mating with virgin females with artificially added MeS will invest more than males mating with virgin control females, as this indicates that the female is previously mated. This further means that male *P. napi* should have an olfactory ability to assess differences in concentrations of female emitted MeS.

In this study we investigate the reproductive allocation of male *P. napi* under different female mating history and thus presence and absence of male-transferred anti-aphrodisiac to test if males (i) tailor their ejaculate expenditure to female mating history and thus sperm competition and (ii) use the male-transferred pheromone MeS as a cue when assessing sperm competition, predicting that males mating to either mated females or virgin females with added MeS will transfer larger ejaculates. Furthermore, we investigate the physiological olfactory sensitivity to MeS and whether ability differs between the sexes. Due to the need in males to accurately evaluate the mating status of the females, we hypothesize that males should have a higher sensitivity to MeS than females.
Material and methods

Study species

The green-veined white butterfly, *P. napi* is a polyandrous temperate butterfly species that is generally bivoltine and the second annual generation enters diapause in the pupal stage (Tolman & Lewington 1997). Larvae feed on a variety of crucifers, from the Brassicaceae family. Females control mating (Bergström & Wiklund 2005) and female fitness increases with number of matings (Wiklund *et al.* 1993, Karlsson 1998). In the wild females mate between 1 and 5 times during their lifetime, with an average of 2.67 times (Bergström, Wiklund & Kaitala 2002, but see Larsdotter Mellström & Wiklund 2010). Females usually remate after an average of five days (Kaitala & Wiklund 1994). The male ejaculate, on average, corresponds to around 10-15 % of male body mass (*e.g.* Svärd & Wiklund 1989).

The butterflies used in the study were F1 and F2 directly developing offspring of >25 wild-caught *P. napi* from southern Sweden. In the laboratory, eggs were laid and larvae reared on the natural host plant *Armoracia rusticana* (Brassicaceae). Larvae were reared in a climate cabinet (Termaks KB 8000L) under conditions securing direct development (23:1h light:dark at 23 °C) and were allowed to feed *ad libitum* throughout larval development until pupation. On the day of eclosion, after releasing the meconium, butterflies were weighed to the nearest mg on a Sauter AR 1014 electrobalance, individually marked and transferred to a cold room (8°C) for a maximum of 7 days, until sufficient numbers of adult butterflies had emerged for an experiment to start.
Functional imaging of olfactory responses to MeS

To investigate the sensitivity to MeS in the butterflies we performed functional Ca\(^{2+}\) imaging of the primary olfactory centre in the brain, the antennal lobes. The antennal lobes consist of a number of spherical neuropils called glomeruli. Each glomerulus receives converging olfactory receptor neurons (ORN) housing a specific receptor. Optophysiological measurements of odour-evoked activity in the AL will show activity in all accessible glomeruli (and thus ORNs) responding to a specific volatile, in this case MeS.

First of all we conducted immunohistochemical stainings of the brain to investigate the glomerular structure of the antennal lobes (Carlsson et al. 2011, 2013). In brief: brains of male and female butterflies were dissected in 0.1 M sodium phosphate buffer (PB) and fixed overnight at 4°C in 4% paraformaldehyde in PB. After careful rinsing in PB the brains were preincubated overnight in 5% normal goat serum in phosphate-buffered saline with 0.25% Triton-X (PBS-Tx). Brain tissue was incubated for 72 hours in mouse monoclonal anti-synapsin (anti- SYNORF1, 1:20; Developmental Studies Hybridoma Bank, Iowa City, IA). For detection of antiserum we used an Alexa 546-tagged secondary antibody (1:1000; Invitrogen). The brains were mounted in 80% glycerol and subsequently scanned with a Zeiss LSM 780 META (Zeiss, Jena, Germany) confocal laser scanning microscope and images were obtained with a 10x air objective.

Animal preparation for imaging was similar to previous studies in other butterfly species (Carlsson et al 2011). Briefly, animals were placed in a 1000 µl pipette tip with the tip cut open to fit the head. The protruding head at the narrow end was fixed
in this position with dental wax. Mouthparts were removed to reduce movements
during the experiments. A window was cut in the head capsule between the
compound eyes and the tissue covering the brain was removed to uncover the
antennal lobes. The membrane-permeant fluorescent calcium dye (Calcium Green-1
AM, Molecular Probes) was dissolved in physiological saline (Christensen &
Hildebrand 1987) with 20% Pluronic F-127 (Molecular Probes) to a final
concentration of 30 µM. A drop of this dye solution was bath applied to the exposed
brain and the preparation was incubated for about 60 min at 4°C. The brain was
subsequently rinsed several times with physiological saline to remove excessive dye.
Bath application with Calcium Green-1 AM potentially stains different types of cells in
the antennal lobe. However, odour-evoked responses are supposed to originate
mainly from input neurons (Galizia et al 1998, Sachse & Galizia 2003).

The imaging set-up consisted of an air-cooled 12-bit slow-scan CCD camera
(Olympus U-CMAD3) mounted to an upright microscope (Olympus BX51WI)
equipped with a water immersion objective (Olympus, 20x/0.95). Calcium green-1
AM was excited at 475 nm (500 nm SP; xenon arc lamp, Polychrome V, Till
Photonics) and fluorescence was detected at 490/515 nm (DCLP/LP). The set-up
was controlled by the software Tillvision 4.0 (Till Photonics). Four-fold symmetrical
binning resulted in image sizes of 344×260 pixels with one pixel corresponding to an
area of 1.25 µm×1.25 µm.

Ten µl of MeS, (Sigma Aldrich CAS-#: 119-36-8) diluted in hexane or 10 µl of hexane
(control) were applied onto a rectangular piece of filter paper (20x5 mm). MeS was
diluted in decadic steps from 1:10-1:100 000. Filter papers were inserted into glass
Pasteur pipettes and were renewed every day. A humidified and charcoal-filtered continuous air stream (1 l/min) was ventilating the antenna ipsilateral to the recorded antennal lobe through a glass tube (5 mm inside diameter). The glass tube ended ~10 mm from the distal part of the antenna. An empty Pasteur pipette was inserted through a small hole in the glass tube, blowing an air stream of 0.1 l/min. Another air stream (0.1 l/min) was blown through the odour-laden pipette by a computer-triggered puffer device (Syntech, Hilversum, The Netherlands) during 2 s (starting at frame 12) into the continuous stream of air. During stimulation, the air stream was switched from the empty pipette to the odour-laden one in order to minimize the influence of added air volume. One odorant stimulation experiment lasted 12.5 s and was recorded with a sampling rate of 4 Hz corresponding to 50 frames. The time course was as follows: 3 s clean airstream (frame 1–12), 2 s stimulus airstream (frame 13–20), and 7.5 s clean airstream (frame 21–50). We used at least 60 sec interstimulus periods to reduce adaptation.

With the software Till-Vision we constructed false-colour coded images of relative changes of fluorescence intensity during the peak time of activity. By visual inspection of activity maps we drew a circular region of interest (ROI, 20 pixels diameter corresponding to 25 µm, which is roughly 50% of the diameter of an average glomerulus) round the centre of highest activity. An additional ROI was drawn in an area with minimal activity, which served as a control. In an earlier study in moths it was demonstrated that activity foci correspond to individual glomeruli (Carlsson & Hansson 2003). The mean pixel value within a ROI was calculated for each time-point (50 frames) in a sequence and exported to Microsoft Excel. In Excel we first made a temporal median filtering of data over three consecutive frames.
Secondly we calculated the relative fluorescence (dF/F) where F was defined as the
mean value of frames 3-10. To correct for bleaching we subtracted the values of the
control ROI from the values of activity ROI for each recording. A response was finally
defined as the mean of frames 16-21 (peak of activity). All responses in an animal
was normalised to the strongest response in that animal. Responses to MeS were
then compared to responses to hexane.

Mating allocation experiment
Male and female butterflies were randomly assigned to the treatments and
treatments were randomly assigned to the mating cages in the laboratory room. The
flight cages (0.7 x 0.7 x 0.5m) have solid plastic walls and one side covered by a
mesh net. They were located in a room with large windows and 400W HQIL lamps
over the cages to simulate daylight. In the cages Kalanchoe sp. plants with drops of
20% sucrose solution on the flowers for feeding, were present. An A. rusticana leaf
was also present to allow females from the mated treatment to lay eggs. The bottom
of the cages was covered with paper soaked with water to maintain high humidity.
The lamps were turned on between 0900 and 1700 hours and each experiment
allowed to run for a maximum of 5 days.

The 3 treatments (virgin females with MeS artificially added to their abdomen, mated
females and virgin females) were designed to test the impact of female mating
history and the presence of MeS, on male spermatophore investment. Mated females
were acquired by releasing 20 males and 20 females into a cage, three days before
an experiment started. When a copulation occurred, the mating butterflies were
isolated from the rest in a jar covered by a net and replaced. When the butterflies
separated, the females were stored in an 8°C refrigerated room for a maximum of five days and minimum of 30 minutes, until they were needed for the experiment. Males were discarded from the experiment.

At the start of an experiment 2 µl of the chosen treatment solution was applied, 5-15 minutes before the experiment began, on the abdomen of the females with a micropipette (Andersson, Borg-Karlsson & Wiklund 2003). The MeS solution was acquired by diluting 99% pure MeS 1:100 in hexane (Andersson, Borg-Karlsson & Wiklund 2000). 2 µl of the solution will then yield 20 ng of MeS on the female, which was found to be the biologically relevant amount in Andersson, Borg-Karlsson & Wiklund (2000). Control virgin females were applied with 2 µl of hexane, as were mated females. Ten females were then released into a cage where 10 virgin males had been allowed 24 hours of prior acclimation, to be fully sexually mature.

Each treatment was replicated in three or more cages. Male to female ratio in the mating cages was kept constant at 10M:10F. The cages were inspected continuously for mating pairs and on discovery pairs were isolated in a plastic jar covered by a net, in the cage, until separation. When a mating couple was removed, two new butterflies with the same treatment were added.

When copulation occurred, the time was recorded, the mated butterflies were weighed, frozen within 15 minutes (-18°C) after separating and the females dissected to extract and weigh the ejaculate in their bursa; ejaculates (wet weight) were weighed to the nearest µg on a Cahn 28 Automatic electrobalance. Both spermatophores of previously mated females were weighed in order to separate the
spermatophores from the two copulations and get the weight of the spermatophore from the experiment copulation.

Statistics

All statistical tests were performed in R (Version 2.10.1, 2009) or Prism 6 (GraphPad Software Inc.).

Results

Physiological responses to MeS

Immunohistochemical staining of the brains show that both sexes of *P. napi* have about 65 glomeruli in the antennal lobe (63-67, n=4 of each sex), which is comparable to the closely related species *P. brassicaceae* (Rospars 1983). About 30 of the glomeruli are clearly tractable for imaging recordings (Fig 1A). We successfully recorded MeS evoked activity from 7 males and 10 females. A response to MeS was seen as activity in a single focal region (Figs 1B-C) corresponding to an individual glomerulus (Carlsson & Hansson 2003).

Both sexes responded to MeS in a dose-dependent manner (Fig 1D). When we analysed the sensitivity as the lowest dose that elicited a response that differed significantly from that of the solvent it became evident that males were more sensitive than females (ANOVA followed by Tukeys post hoc test). At the lowest dose tested (1:100 000), only males responded.

Mating allocation experiment
A total of 46 males were mated in the three treatments (virgin n=23, virgin+MeS n=16 and mated n=7). The time from the experiment started until mating occurred differed (Kruskal-Wallis $X^2 = 12.33$, df = 2, $P = 0.002$) between treatments (days±SD; virgin 1.2±1.1, virgin+MeS 1.7±1.3, mated 3.3±1.0). Matings with mated females took longer than both virgin (pairwise Wilcoxon tests, Bonferroni correction; $P=0.002$) and virgin+MeS females ($P=0.039$). There was no difference between virgin and virgin+MeS females ($P=0.596$).

For data on spermatophore investment we used a linear model (Spermatophore weight ~ male weight x treatment). As we predicted, there was a significant difference (Ancova: $F_{2,40} = 6.26$, $P=0.004$) in ejaculate weight between treatments. The effect of male weight and the interaction male weight x treatment were non-significant (Ancova: male weight $F_{1,40} = 3.66$, $P=0.063$, male weight x treatment $F_{2,40} = 0.18$, $P=0.84$). However, as male weight came very close to significantly affecting the result and as male weight in previous studies (e.g. Svärd &Wiklund 1989; Wiklund & Kaitala 1994; Bissoondath & Wiklund 1996; Wedell & Cook 1998) has been shown to affect spermatophore size, we chose to also use a relative investment term (spermatophore weight/male weight) to account for any differences in male weight between treatments. The allometric relationship between male weight and spermatophore size is discussed in e.g. Wiklund & Kaitala (1994) and Bissoondath & Wiklund (1996). Relative investment also differed between treatments (Anova; $F_{2,43}=6.41$, $P=0.004$). Mating investment transferred to mated females (average±95%CI; 16.6±2.2%) and virgin females with MeS (16.6±2.3%) were significantly larger (Tukey, 95% confidence level) than investment in the control, virgin female, treatment (13.2±4.0%) (Fig 2). Thus, the relative investment increases
about 26% both when males mate with previously mated females and with virgin females with MeS compared to virgin control females.

**Discussion**

In this study we show that male *P. napi* have an ability to accurately determine the level of intra-sexual competition and adjust reproductive expenditure accordingly. We also show for the first time that the male-transferred anti-aphrodisiac MeS is used as a cue to allocate ejaculate investment according to female mating history. Furthermore, using Ca\(^{2+}\) imaging we conclude that both sexes can detect MeS and changes in concentrations, and that males are more sensitive than females.

Both male and female butterflies responded physiologically to MeS, in that a short (2s) exposure to the volatile elicited a change in [Ca\(^{2+}\)] in specific glomeruli in the AL. The higher sensitivity in males may reflect their need for an accurate evaluation of the female MeS emission. Interestingly, in a previous study it was shown that females are more sensitive than males to the male-emitted aphrodisiac pheromone citral (Andersson et al 2007). Thus, the actions of these two pheromones are mirrored in the respective receiver’s olfactory system. Sexual dimorphism in pheromone systems is very common in insects. However, as opposed to e.g. moths, where usually only males possess an enlarged subpopulation of glomeruli, the macroglomerular complex, receiving input from pheromone sensing neurons, no difference in AL size and glomerular number between the sexes in *P. napi* was observed. Lack of sexual dimorphism in the AL has also been observed in other species of butterflies (Rospars 1983, Carlsson et al 2013). The differences in sensitivity can have several explanations; the most plausible is that the number of
receptor neurons housing a receptor sensitive to MeS is higher in males than females. The reason for female detection of MeS may be a need for auto-detection (Ref) or that MeS has another informative value for females. MeS is in fact also a common volatile compound emitted from several plant species (ref). For example, MeS is emitted from several host plants of the moth *Manduca sexta*, and females respond physiologically and behaviourally to the compound (Fraser et al 2003).

A previous study (Forsberg & Wiklund 1989) has demonstrated that female *P. napi* are most unattractive to males directly after mating and that courtship duration increases with time elapsed since mating. The amount of MeS applied in this study (20 ng per female) was chosen to be ecologically relevant (Andersson, Borg-Karlsson & Wiklund 2000). What we could not control was rate of evaporation. As indicated by the fact that virgin+MeS females mate sooner than mated females, artificially added MeS will most likely evaporate faster than male-transferred MeS (which is enclosed in the spermatophore and is secreted by the female after mating). It has previously been shown that females living under high densities spend the male-transferred anti-aphrodisiacs faster (Andersson, Borg-Karlsson & Wiklund 2004), so the rate of anti-aphrodisiac loss is not constant in the wild either. However, as the male investment is similar between mated females and virgin+MeS females we contend that males do use MeS as a cue for ejaculate investment and that the time before mating seems less important.

Our result that males delivered larger ejaculates in the presence of MeS, shows for the first time that the anti-aphrodisiac is used by males to assess sperm competition intensity and female mating history. The results are also interesting from the
perspective of how the anti-aphrodisiac has assumed dual functions – (1) deterring other males from mating with the female and (2) allowing males to assess sperm competition. This is mirroring the effect of male sex pheromone (citral) in this species whereby males judge sperm competition risk by “eavesdropping” on the sex pheromone release from other males nearby (Larsdotter-Mellström & Wiklund 2009).

The fact that males use the presence of an anti-aphrodisiac pheromone to tailor ejaculates provide yet another mechanism that males could use to judge female mating history, besides previously suggested ones - e.g. physical contact with previous spermatophores at mating (Wedell & Cook 1999a).

Thus, two different olfactory signals, MeS and citral (Larsdotter-Mellström & Wiklund 2009) affect the ejaculate size in P. napi. MeS signals female mating history and citral signals male density and thus competition. In both situations males would benefit from accurate detection of these signals and subsequent tailoring of their ejaculate.

Future studies should adress this very interesting connection between the olfactory and reproductive systems. Information from OSNs activated by MeS or citral is processed in specific glomeruli just as information from food odours (Carlsson et al in prep). However, are projection neurons from glomeruli in the AL activated by MeS or citral projecting to other regions than projection neurons mediating information on e.g. food odours or oviposition cues? If so, how are these regions connected to the male reproductive system?
The results of this study, that males mating under sperm competition transfer larger ejaculates whereas males under no intra-sexual competition save their resources for future fertilizations, conform well to sperm competition game theory (Parker 1990a, b, 1998, Parker et al. 1997, Parker & Ball 2005) and establish the *P. napi* model system as one very well suited to addressing these questions. It has been suggested by Wedell & Cook (1999a) that even though the sperm competition models were developed for a situation where males compete with ejaculates of two or more males (Ball & Parker 1996, 1997, Parker *et al.* 1997) the sperm competition situation might be more dependent on the number of sperm transferred than the number of matings. In view of our results that male *P. napi* can adjust ejaculate size to female mating history, using MeS, it would of course be interesting to see whether they also strategically change the composition of the ejaculate and transfer proportionately higher numbers of eupyrene sperm when mating competition is high, as has been suggested in *P. rapae* (Wedell & Cook 1999a). This question warrants further experiments.

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**References**


Figure legends

**Figure 1.** Methyl salicylate-activated responses in the AL of male and female *P. napi.*

A The brain of a male *P. napi* with the ALs highlighted in red (projection of a stack of confocal images). Scale bar = 500 µm. **B-C** False-colour coded images of odour-evoked activity to MeS (1:1000) and control (hexane solvent) superimposed on wide-field images of the lobes (B male, C female). Each response is scaled to the upper 50% of its intensity range. Focus of activity (corresponding to an individual glomerulus) is outlined. D, dorsal; M, medial. AN, antennal nerve; AL, antennal lobe. Scale bar = 100 µm. **D** Dose-response curves showing normalised responses to five doses of MeS and to the control. A box plot shows the median normalised response to an odorant (horizontal line), the 25th and 75th percentile (lower and upper margin of the box) together with the minimum and maximum values (whiskers). Red coloured bars indicate significant difference from the control (ANOVA followed by Tukeys post hoc test).

**Figure 2.** Relative mating investment (mean ± 95% CI) when transferred to virgin females, virgin females with added MeS and previously mated females. Ejaculates transferred to mated females and virgin females with MeS were significantly larger than ejaculates in the control virgin female treatment. a,b denote significant post hoc contrasts (Tukey, 95% confidence level)
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Males (n=7)

Control 1:100 000 1:10 000 1:1000 1:100 1:10

Normalised response

Concentration

Females (n=10)

Control 1:100 000 1:10 000 1:1000 1:100 1:10

Normalised response

Concentration