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Sexual dimorphism and sex pheromone detection in *Aphidoletes aphidimyza*

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Abstract

Sexual dimorphism, particularly at the level of sensory and locomotor organs, is usually attributed to sexual selection. Antennae are notably developed in males of species that need to detect a sex pheromone at low concentration or at long distance. In addition to their role in intrasexual selection, antennae can be seen as important ornaments in intersexual selection. Antennae of *Aphidoletes aphidimyza* are clearly sexually dimorphic (males have longer antennae than females, with highly developed sensilla) while females emit a sex pheromone for mating. Males with longer and more symmetrical antennae than others could be more successful in reaching the source of sex pheromone, especially if they can fly properly. A morphometric study was first conducted, to apprehend the variability of antennae, wings and tibias in lab conditions. The length of the antennae of male *A. aphidimyza* is impressive and the right antenna is longer than the left antenna. Secondly, choice experiments were conducted in a Y-shaped olfactometer with males of *A. aphidimyza* facing the sex pheromone. The relationship between choice patterns and morphology of males was then studied, but no link was found between the morphology of males and their behaviour while exposed to the sex pheromone, although males were indeed attracted by the olfactometer arm containing the sex pheromone.

Keywords

Antennae asymmetry; *Aphidoletes aphidimyza*; insect; males; morphometry; olfactometry; sexual dimorphism

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Introduction

Sexual dimorphism – differences between the two sexes of the same species (Punzalan & Hosken, 2010) – is ubiquitous in animals and particularly in insects. Its evolution is usually attributed to sexual selection (intrasexual as well as intersexual selection) but may also result from sex-specific niche divergence (Shine, 1989; Moore, 1990). Sexual size dimorphism is really widespread (Stillwell et al., 2010) but other characters may differ between sexes, like horns in the beetle genus *Onthophagus* (Coleoptera: Scarabaeidae), which are mainly present in males (Emlen et al., 2005).

Intrasexual selection promotes traits helpful to locate and reach a mating partner. For instance, it may result in males having locomotor or sensory organs that are more developed than those of females (Darwin, 1871; Cézilly & Allainé, 2016; Johnson et al., 2017, Elgar et al., 2018). The case of antennae is thus rather particular since they have a role in the perception of pheromones (Elgar et al., 2019). In some species of moths, males have well-developed antennae, enabling them to detect female sex pheromone at a very low concentration or over very long distances (Shiel et al., 2015; Elgar et al., 2018). To achieve this, feathery antennae are useful for sampling a large amount of air (Chapman, 1998) and long antennae offer much space for sensilla (Schneider, 1964; Chapman, 1998) and extend the sweep breadth (Johnson et al., 2017). However, in a wide phylogenetic analysis, Symonds et al. (2011) showed that no clear relationship appeared between antennal shape and pheromone titer after removing the effect of body size. Antennae can also act as important ornaments in intersexual selection. Indeed, males bearing longer and more symmetric antennae can be favoured for mating because their antennae indicate their genetic quality (Møller & Zamora-Munoz, 1997; Koshio et al., 2007). However, few studies have investigated the natural variations in antennal morphology or size and their effect on the perception of sex pheromones and the related behaviour (Elgar et al., 2018, 2019).

Like most Aphidoletini, the cecidomyiid *Aphidoletes aphidimyza* (Rondani, 1847) preys on aphids at the larval stage (Harris, 1966). The antennae of this species present a pronounced sexual dimorphism: males exhibit very long, curved and feathery antennae (fig. 1) while females have short straight antennae (Markkula & Tiittanen, 1985; Zhang & Yang, 2008; Tabadkani et al., 2012a, b, 2013). The antennae of males are composed of 12 flagellomeres, each bearing three typical circumfila (Harris, 1966; Gagné, 1994; Fedotova, 2014), which are a type of sensilla only present in Cecidomyiidae (Zhang & Yang, 2008). For mating, *A. aphidimyza* females generally hang by the front legs in a spider web and emit a sex pheromone – (2*R*, 7*S*)-diacetoxytridecane (Choi et al., 2004a) – to attract a male. Once a male lands on the web, it moves to the female frontally while wing-fanning. The mating takes place during the scotophase in a "face to face" position in the spider web. During mating, the male first extends its wings and then folds them against its body, like the female (Harris, 1973; van Schelt & Mulder, 2000; van Lenteren et al., 2002; van Lenteren & Schettino, 2003).

Figure 1. Male of *Aphidoletes aphidimyza*. Photo © Christophe Salin.

In *A. aphidimyza*, antennae and wings of males are thus important for reproduction. As the very striking sexual dimorphism at this level suggests, long antennae of males could be a specialization to ensure a better detection of the female sex pheromone. Antennae could also be an ornament that constitutes a handicap during flight, due to their long size relative to the body size. Wings are used by males to fly to the female for mating in the spider web and their movements during mating are standardised. Males with longer and more symmetrical antennae should reach the source of sex pheromone more easily, and wide wings could help them to cope with their long antennae (Emlen, 2001; McCullough & Emlen, 2013; Painting & Holwell, 2013).

The aim of this research was thus to better characterize the variability in the morphology of males of *A. aphidimyza* and to analyse the male behaviour in the presence of female sex pheromone considering their morphology. First, to evaluate the phenotypic variability of males of *A. aphidimyza* under lab conditions, the length of antennae, wing size and length of hind tibias were analysed. Secondly, to determine the consequences of antennal size for the perception of female pheromones, choice experiments were conducted with males in a Y-shaped olfactometer.

Materials and methods

Aphidoletes aphidimyza individuals were reared following the study of Kohandani et al. (2017), which was inspired by Le Goff et al. (2016). The aphid preys, *Myzus persicae* (Sulzer, 1776), were reared on sweet pepper plants, *Capsicum annuum* L. Once infested, plants were given to *A. aphidimyza* for laying eggs. Rearing took place in a room at 23°C, with a photoperiod of 16 hours and a relative humidity of 60-70%. Plants with eggs were then placed in another cage and larvae ready to pupate were recovered from the sand under the plants. Pupae were separated from the sand and placed at 25°C until the emergence of adults.

Morphometry

To analyse the size range of *A. aphidimyza* males in lab conditions, 49 individuals aged less than 24 hours were sampled and stored in a freezer at −20°C. These males were placed on a microscopic slide in a Hanks' balanced salts solution (98 g/l) so that the antennae, tibias and wings were well flattened and pictures were taken. The following parameters were measured:

- Length of both antennae (which is the flagellum total length, without scape and pedicel, but considering the antennal curvature).
- Left hind tibia length, as a proxy of body size (see Jann et al., 2000; Blanckenhorn et al., 2003).
- Right wing area.

The antennal symmetry was estimated by subtracting the length of the left antenna from the length of the right antenna.

As actual wing loading (body weight on wing area, Danforth, 1989) could not be calculated because it was not possible to weigh individuals without breaking the fragile antennae, the wing loading was evaluated by dividing the cube of the tibial length by the wing surface area, in mm^2.

Olfactometry

To relate the morphology of *A. aphidimyza* males to their movement towards the sex pheromone, olfactometry choice experiments were carried out. Because *A. aphidimyza* is nocturnal, pupae were placed under a reverse photoperiod (dark from 9 a.m. to 5 p.m.) after they were sieved from the sand (approximately one week before emergence of the adults – Le Goff et al., 2016) so that experiments could run in the daytime. Pupae were isolated in microtubes of 1.5 ml with a piece of cotton swab soaked in a 1:3 solution of sugar and water to allow adult feeding. For each replicate, one fed virgin male less than 72 hours old was used. Experiments were performed in a Y-shaped glass olfactometer of 25 mm ID with a central branch of 20 cm and side arms of 18 cm at 60° (fig. 2), following Choi et al. (2004a). All

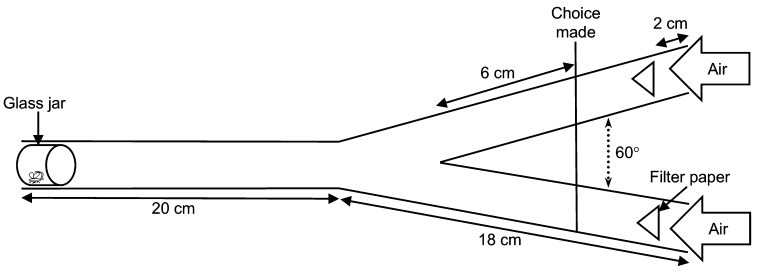

Figure 2. Configuration of the Y olfactometer, with the glass jar containing the insect on the left, and the filter papers folded in a cone shape on the right.

tests were carried out at 23.5°C, between 12 and 5 p.m., in the dark under a red light (40 W red bulb, not to disturb the males' nocturnal behaviour, as insects are not sensitive to this wavelength – Schaefer & Wilkinson, 2004). A flow of ultrapure synthetic air was blown through a charcoal filter at 1.6 mL/s to the base of each Y olfactometer arm and then toward the central branch where the insect was placed. One ng of $(2R, 7S)$-diacetoxytridecane (synthetic female sex pheromone, prepared according to a procedure of Choi et al., 2004a) dissolved in 100 μL of pentane was used in the base of one arm and 100 μL of pentane alone was used in the second arm as control. The solutions were micropipetted on a filter paper folded into a cone shape under an extractor hood and left 5 minutes to allow the evaporation of pentane prior to use. The filter papers were then placed at 2 cm from the orifice of the arms. A line was traced under the olfactometer 6 cm after the beginning of the arms. The olfactometer and the arms where sex pheromone and pentane alone were placed were rotated, so that the pheromone was placed in the left arm the same number of times as in the right arm.

To start a replicate, a male of *A. aphidimyza* trapped in a glass jar was introduced into the central branch of the olfactometer to allow a choice between the arm containing the female sex pheromone and the arm without pheromone. If the tested male did not leave the glass jar after 5 minutes or did not make a choice within 10 minutes after exiting the jar, it was considered "not responding". All the other insects were considered "responding". The time spent to exit the jar as well as the time needed to cross the line traced under the olfactometer (choice made, end of test) were recorded. The mode of movement the male used to approach the odour source (flight or walk) was also recorded. All the males ($n = 78$) were stored in a freezer at -20°C after the experiment in order to conserve them for further measurements. After each replicate, whether the male was responding or not, the apparatus was washed and a new source of pheromone, a new control and a new insect were used for the next test.

The same parameters as described in the subsection "Morphometry" (see above) were measured and calculated for all the males. However, some changes were made: (1) only the first six flagellomers of the antennae were measured (corresponding to half the antenna, due to frequent breakages of the last flagellomers when males were retrieved from the device) and (2) the right hind tibia was measured instead of the left hind tibia.

Statistical analysis

Statistical analyses were performed using GraphPad Prism version 5.0 software for Mac. All tests were performed with the significance level (P) set at $\alpha = 0.05$ and under a two-tailed hypothesis.

Morphometry. Margin errors indicated are standard errors (SEs). A D'Agostino & Pearson omnibus normality test was applied and the data were normally distributed, except for the difference between the lengths of the left and right antennae (which gives an estimation of the antennal symmetry).

To check differences in length between the two antennae of the same individual, a Wilcoxon matched-pairs test was performed, as these differences did not follow a Gaussian distribution.

A Wilcoxon signed-rank test was used to determine whether differences in antennal length are distributed symmetrically around zero.

Olfactometry. A binomial test was used to determine if the proportion of males attracted by the olfactometer arm with the pheromone differed from random (sign test, 50/50 repartition). The same test was applied to establish whether males going to the selected olfactometer arm did it by flying as much as by walking (sign test, 50/50 repartition).

A D'Agostino & Pearson omnibus normality test was used and not all the data were normally distributed. Accordingly, a Mann-Whitney test was applied to compare the morphology of responding and not-responding males.

Spearman correlation coefficients were calculated to determine if morphology influences the time spent to exit the jar and to make a choice.

Results

Morphometry

Antennae. The length of male right antennae ranged from 2.42 to 3.07 mm with a mean of 2.80 ± 0.02 mm ($N = 49$). The length of left antenna ranged from 2.36 mm to 3.05 mm with a mean of 2.77 ± 0.02 mm ($N = 49$). Surprisingly, a significant difference was observed between the lengths of left and right antennae of individual males (Wilcoxon matched-pairs test: $N = 49$, $P = 0.018$) (fig. 3). The mean

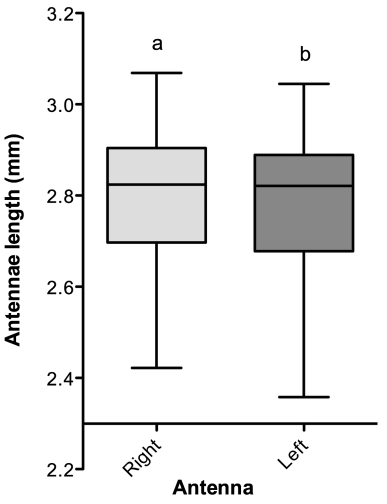

Figure 3. Comparison of antennal lengths of a single male (Boxplot, $N = 49$, different letters represent significantly different means according to a Wilcoxon matched-pairs test; the error drawn on the graph corresponds to the SE).

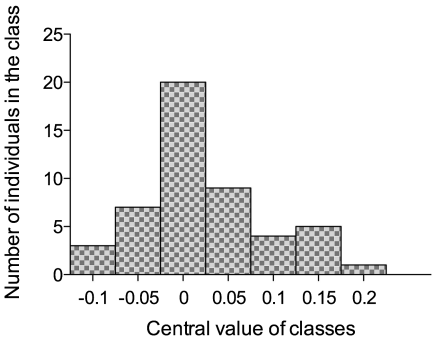

Figure 4. Frequency distribution of the differences between the lengths of right and left antennae ($N = 49$).

difference between both antennae of the same individual (estimation of the antennal symmetry) is 0.03 ± 0.01 mm, with a minimum of −0.12 mm, a maximum of 0.29 mm and a coefficient of variation of 265.26%. The difference between the lengths of right and left antenna does not distribute symmetrically compared to zero (Wilcoxon signed rank test: $N = 49$, $P = 0.019$) (fig. 4). The D'Agostino & Pearson omnibus normality test indicates that this distribution is not consistent with a normal distribution either ($K^2 = 11.51$, $N = 49$, $P = 0.003$).

Tibia and wing size and relationships with antennae. The length of the left hind tibia ranged from 0.78 mm to 1.05 mm with a mean of 0.92 ± 0.01 mm ($N = 49$). The area of the right wing ranged from 0.97 mm^2 to 1.52 mm^2 with a mean of 1.29 ± 0.02 mm^2 ($N = 49$). The estimation of the wing loading ranged from 0.48 mm to 0.75 mm with a mean of 0.62 ± 0.01 mm ($N = 49$).

Olfactometry

Out of 78 males, 27 individuals (34.6%) entered the olfactometer within 5 minutes and 21 (26.9%) made a choice within 10 minutes after having entered. Of these, 17 (81.0%) males were attracted by the arm with the pheromone, which is significantly different from chance (sign test: $N = 21$, $P = 0.007$).

Most of males that selected an arm of the olfactometer went flying (17 individuals) rather than walking (four individuals) and this is not random behaviour (sign test: $N = 21$, $P = 0.007$).

No difference was observed between the morphology of responding ($N_1 = 17$) and not responding males ($N_2 = 40$) (Mann-Whitney test applied to the length of half of the right antennae [$U = 331.0$, $P = 0.882$], differences between half of the right and half of the left antenna [symmetry, $U = 325.5$, $P = 0.807$], body sizes [estimated by the length of the right hind tibia, $U = 289.0$, $P = 0.378$], and area of the right wings [$U = 313.0$, $P = 0.644$]).

No difference was apparent between males attracted by the arm with the sex pheromone and the ones attracted by the control arm, because we were able to

measure the morphological parameters for only two of the males attracted by the control.

Males took on average 90 ± 25 s to exit the jar and an additional 86 ± 31 s to make a choice ($N = 17$). There was no linear relationship between the time spent to exit the jar or to make a choice and the morphology of responding males.

Discussion

As expected, the antennae of *Aphidoletes aphidimyza* males are remarkably long (a mean length for the right antennae of 2.80 mm and for the left ones of 2.77 mm) compared to the size of the species (body presenting the same size or smaller than the antennae) or to the female antennal size (1.05 mm according to Zhang & Yang, 2008). In comparison, the antennal length of a wild individual trapped in Russia was 2.85 mm (at a body size of about 2 mm long – Markkula & Tiittanen, 1985; Jandricic, 2013; Fedotova, 2014), which is similar to the results obtained here. The symmetry of antennae (length of the left antenna minus length of the right one) presents a huge coefficient of variation (265.26%). The mean length of the left hind tibia is 0.92 mm (also consistent with the 0.93 mm of Fedotova, 2014) and the mean of estimated wing loading is 0.62 mm. To calculate a realistic wing loading, it would be interesting to find a way to weigh males without breaking their fragile parts (antennae, tibias and wings) (Danforth, 1989).

The absolute difference between the lengths of both antennae is not striking (fig. 3) but significant. However, it is difficult to decide between directional asymmetry (concerning traits that present a mean of the right side minus the mean of the left side differing from zero) and antisymmetry (traits for which the frequency distribution of the right side minus the left side is not normal) (Van Valen, 1962; Palmer & Strobeck, 1986, 1992; Demers, 1999). These types of asymmetry differ from fluctuating asymmetry because they do not only result from random perturbations in the environment during development but have in part a genetic basis and thus are not good indicators of developmental stability. The frequency distribution of the differences between the lengths of the left and right antennae seems to present a skewed distribution (fig. 4), that could be generated by the presence of both directional asymmetry and antisymmetry (Palmer & Strobeck, 1992). Evidence of left-right asymmetries in invertebrates is reported more and more, suggesting a lateralization process even for simple brains. These population-level asymmetries may be the result of selective social pressures due to the coordination of asymmetric organisms, which is more likely to occur in social species (Frasnelli et al., 2012). However, recent studies showed that it is not always the case (Freelance et al., in press). It cannot be excluded that population-level asymmetries of solitary species may be connected with mating aspects and the recognition ability of conspecifics and thus may result in interactions these solitary species have with congeners. In our case, this astonishing asymmetry of antennae should still be considered with caution (Demers, 1999) because the difference found between the lengths of both

antennae (0.03 mm) is only 0.01 mm above the measurement error (0.02 mm) of the length of the whole antennae. However, small differences were also found in the number of putative olfactory sensillae of *Apis mellifera ligustica* Spinola, 1806, which were correlated with the preferential use of one antenna (Frasnelli et al., 2010).

For males with long and symmetric antennae, it could be relatively easy to reach the origin of sex pheromone emission (Shiel et al., 2015). However, the link between antennae, tibia and wing size and the perception of the pheromone in olfactometry is not clear. Roughly a third of the males exited the jar, almost four out of five of them made a choice and they chose the arm with the sex pheromone in 81% of the cases. Despite all this, the morphology of males cannot be related to their ability to respond to sex pheromone, nor to the time spent in making a choice. Perhaps other morphological parameters could explain the choice behaviour toward the sex pheromone. For example, examining the composition in sensillae could be interesting, but these are difficult to assess because of their very small size. The number of sensillae can be linked to the area of the antennae (Schneider, 1964; Chapman, 1998), but the type of sensilla could be more important in the perception of sex pheromones (Zhang & Yang, 2008) than the antennal length itself. Moreover, antennae could be an ornament that is important in the selection of a male by females, which has not been assessed yet. Maybe the results could also be improved by carrying out the experiments at the beginning of the dark period, as the adults are known to be the most active at the beginning of the night (Jeoung et al., 2003). Besides, analyses for olfactometry were conducted on the six first flagellomeres out of 12 only, because of the difficulties experienced with collecting males from the device after the experiments. This potentially leads to underestimating the variability between males that could arise from a difference at each flagellomere or from an inequality of the length of the end of the antenna that was lost, as the terminal flagellomere is slightly different from the others (Gagné, 1994). Results could maybe also be improved by conducting experiments with females of *A. aphidimyza* instead of synthetic pheromone or by leaving the males more time to choose one arm of the olfactometer.

In accordance with what could be expected from the habits of the species in relation to mating, males flew to the selected arm much more often than they walked, even if the space to fly was relatively limited, which is quite different from natural circumstances. Indeed, other experiments with *A. aphidimyza* used larger devices (Choi et al., 2004b; Watanabe et al., 2016) than the olfactometer used in this study. An experiment with more space to fly or with a faster air flow could indicate whether the impressive antennae are really a handicap while moving (especially for small-bodied insects; Symonds et al., 2011) or if they can offer advantages, like improving the lift or helping to stabilize the insect during flight (Symonds et al., 2011).

Our results do not reveal a link between the morphology of males and their behavioural response to sex pheromone. The evident sexual dimorphism of *A.*

aphidimyza requires further testing to understand how and why this particular morphology developed. More generally, research on the way selection acted on insect antennae (structure and size) still needs more investigations (Elgar et al., 2018, 2019). Mating experiments where females of *A. aphidimyza* have the choice between different male phenotypes (intersexual selection) or where males are in competition for the same female (intrasexual selection) are thus needed. Indeed, we highlighted a phenotypic variability of males under constant lab conditions and this variability will need to be investigated further. Exacerbating phenotypic differences between males, for example by giving different diets at the larval stage or by rearing the insects for experiment at different but not stressful temperatures could improve results.

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