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MATING BEHAVIOUR AND THERMAL PLASTICITY OF ADULT MORPHOLOGY IN A SEXUALLY DIMORPHIC FLY

Thèse présentée par Aurore Nicolas en vue de l'obtention du grade de docteur en sciences agronomiques et ingénierie biologique

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"Until one is committed, there is hesitancy, the chance to draw back, always ineffectiveness. Concerning all acts of initiative (and creation), there is one elementary truth the ignorance of which kills countless ideas and splendid plans: that the moment one definitely commits oneself, then providence moves too. A whole stream of events issues from the decision, raising in one's favor all manner of unforeseen incidents, meetings and material assistance, which no man could have dreamt would have come his way. I learned a deep respect for one of Goethe's couplets:

Whatever you can do or dream you can, begin it. Boldness has genius, power and magic in it!"

(Murray, 1951)

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ABSTRACT

The sexual dimorphism, shaped by natural and sexual selections, always fascinated. Antennal sexual dimorphism is common in insects, including Diptera. Males antennae are often more complex than those of females and it is generally due to sexual selection, which results from two processes: the intrasexual selection and the intersexual selection. Sexual selection can, for example, favour an enlargement of antennae to improve pheromones perception (mechanism of scrambles) or to indicate male quality, in case antennae are costly to produce or maintain (handicap principle).

In *Aphidoletes aphidimyza*, males have long curved plumose antennae while females have short straight antennae. The driving factors of antennal sexual dimorphism in this species are unknown. Considering the peculiar reproductive behaviour of *A. aphidimyza*, elaborate antennae of males could potentially have been produced as well by intrasexual selection as by intersexual selection. The hypothesis is thus that sexual dimorphism in *A. aphidimyza* mainly results from sexual selection. The prediction is that longer and more symmetric antennae should improve access of males to a mate or to a better-quality mate. The size of wings, also important for the reproduction and to evaluate the flying capacity of males, whose antennae can possibly impede mobility, should vary as antennae size. Moreover, larger structures should be observed in larger males (isometry).

A methodical rearing method was implemented in the laboratory to obtain enough standardised individuals for the needs of the subsequent experiments and is presented before actually checking the hypotheses. A high relative humidity during the pupal stage and a separation of pupae from their substrate just after their formation optimise the productivity of the rearing method.

First, based on these individuals reared in the laboratory, the morphology of males of *A. aphidimyza* and the influence it could have on the reproductive behaviour were studied. There are positive linear relations between antennae, wing and tibia sizes (proxy of the body size) in males of *A. aphidimyza*. The importance of phenotypical traits which are needed by males to reach a source of sex pheromone was analysed through olfactometry experiments (intrasexual selection). In order to evaluate the roles of intrasexual and intersexual selections in the appearance and the maintenance of sexual dimorphism in *A. aphidimyza,* direct mating experiments were performed. Males

with long antennae relatively to their body size (high antenna to tibia ratio) have a higher probability to gain access to a mate than other males. Fisher's runaway process and the good genes model (two mechanisms of intersexual selection influencing the fitness of the offspring) could explain the preference of females for small-bodied males with long antennae and thus the reinforcement of the "small body with long antennae" trait in males.

Secondly, since size usually matters in mate choice, and since developmental temperature can influence adult size, the effects of developmental temperature on the morphology and on the reproductive behaviour of males of *A. aphidimyza* were investigated. According to the experiments, larger males (body size evaluated by tibia size) have longer antennae and wider wings, whatever the developmental temperature. Males that developed at lower temperature are larger, with larger antennae and wings, than males that developed at higher temperature, as predicted by the temperature-size rule. The antennae size presents a negative allometry while the wing size presents a positive allometry, according to the body size. Olfactometry experiments and mating experiments were then again performed using these males presenting clear morphological differences. The supposition that males of *A. aphidimyza* with longer antennae and especially with a higher antenna/tibia ratio should be favoured in the context of reproduction, particularly if they can fly properly, was not confirmed. Moreover, long antennae have apparently no impact on the flying capacity of males.

Longer and more symmetric antennae do not improve the access of males to a mate, but, in some conditions, a high antenna to tibia ratio does. Consequently, the prediction that sexual selection, through intrasexual and intersexual selections, shaped the antennal sexual dimorphism in *A. aphidimyza* is not completely verified. However, the higher antenna to tibia ratio in mated males gives a partial answer to this questioning about the factors that may cause and maintain sexual dimorphism.

CHAPTER I. GENERAL INTRODUCTION

1. Presentation of the study

Differences of morphology, physiology or behaviour between males and females of a same animal species – called **sexual dimorphism** (Ricklefs & Miller, 2005; Freeman & Herron, 2007; Smith & Smith, 2009) – have always fascinated (Punzalan & Hosken, 2010). Darwin (1871a) already introduced the concept of **sexual selection**, which can improve the reproductive success in a different way for each sex. The sexual selection results from two processes: **intrasexual selection** (competition for the opportunity to mate between individuals of the same sex, generally males) and **intersexual selection** (selection of a mate by individuals of one sex, generally females, among individuals of the other sex based on their different attractivenesses) (Darwin, 1871a; Freeman & Herron, 2007; Smith & Smith, 2009). According to Wallace (1889), dimorphic traits can also appear as a result of the **natural selection**, because of the conspicuousness and the hazard this trait represents for one sex. However, it is only in the works of Fisher (1930, 1958) that the first mathematical models were proposed to better understand the relative roles played by natural and sexual selections in both sexes. Since then, an abundant literature has been produced on the sexual dimorphism under natural or sexual selection, particularly in insects. Indeed, many of them present exaggerated sexually dimorphic traits (Lavine *et al.*, 2015), such as mandibles (Hosoya & Araya, 2005), eyestalks (Cotton *et al.*, 2004), antlers (Dodson, 1997) or antennae (Streinzer *et al.*, 2013).

Antennal sexual dimorphism, which is common in insects (Chapman, 1998), can thus have been shaped by natural selection and by sexual selection. It is present in Diptera (Sivinski, 1997), although very few studies report differences of antennae size between sexes in this order (Bonduriansky & Rowe, 2005), except in gall midges (Gagné, 1994). Males antennae are frequently more complex than those of females and this often follows from sexual selection (Chapman, 1998; Johnson *et al.*, 2017). Sexual dimorphism at the level of antennae could have evolved through both intrasexual selection and intersexual selection (Moore, 1990; Berns, 2013). Indeed, sexual selection could, for example, favour enlarged sensory organs that enhance stimuli perception – a prediction of Darwin (1871a), which, surprisingly, has rarely been investigated directly – or that indicate male quality, if antennae are costly to produce or have

costly consequences (**handicap**) (Zahavi, 1975; Smith & Harper, 2003; Shiel *et al*., 2015; Johnson *et al.*, 2017).

As it happens, the cecidomyid *Aphidoletes aphidimyza* shows a strong sexual dimorphism (Markkula & Tiittanen, 1985): males have long curved and hairy antennae (FIGURE 1) whilst females have short straight antennae (FIGURE 2). This small Diptera is currently used in biological control against aphids (Harris, 1973; Markkula & Tiittanen, 1976; Markkula *et al.*, 1979; Morse, 1981; Gagné, 1994; Křivan & Havelka, 2000; van Schelt & Mulder, 2000; Yano, 2006; Cock *et al*., 2010; van Lenteren, 2011; Fedotova, 2014; Hance *et al.*, 2017). The mating behaviour – involving the use of a sex pheromone by females, hung in a spider web to attract males – and the life cycle of this little insect are complex. The driving factors of sexual dimorphism and the reasons why it is maintained have, to our knowledge, never been studied in this species. It would be interesting to know more about these, to pay attention to produce well-shaped individuals, with the appropriate behaviour, for biological control. Therefore, *A. aphidimyza* is a really exciting and interesting model of study, in spite of its complicated mass rearing (van Lenteren *et al*., 2002; van Lenteren & Schettino, 2003).



FIGURE 1. Head and antennae of a male of *A. aphidimyza*.



FIGURE 2. Head and antennae of a female of *A. aphidimyza*.

As antennae of males of *A. aphidimyza* are probably important for the perception of the sex pheromone (Chapman, 1998), the pronounced antennal sexual dimorphism has probably evolved in the context of the reproduction (Hillbur *et al.*, 2001; Hall *et al.*, 2012). Moreover, females of *A. aphidimyza* should be choosy about the selection of a mating partner because they mate only once and, as they are not parthenogenetic, they cannot lay eggs without having received sperm (van Lenteren *et al.*, 2002; van Lenteren & Schettino, 2003). Antennae could thus be important for intrasexual selection, but also for intersexual selection if females select males based on their antennae. Therefore, the hypothesis is that sexual – intrasexual and/or intersexual – selection largely shaped the impressive antennae of males of *A. aphidimyza*. The prediction is that longer and more symmetric antennae facilitate access of males to a mating partner or to a better-quality mate (Møller & Zamora-Munoz, 1997; Jiménez-Pérez & Wang, 2004; Koshio *et al.*, 2007; Shuker, 2010; Shuker, 2014; Johnson *et al.*, 2017).

Besides, wings are also important for the reproduction of *A. aphidimyza*. Indeed, males have to fly to reach a mating partner (van Lenteren *et al.*, 2002; van Lenteren & Schettino, 2003) and they may need wide wings to cope with their long antennae, which could be a handicap for their flight (Symonds *et al.*, 2011). In order to assess the flying capacity of males with their cumbersome antennae and to interpret correctly the possible differences in mobility, the size of wings will also be evaluated. The body size – estimated by the length of a tibia (Jann *et al.*, 2000; Blanckenhorn *et al.*, 2003) – could also help to make the link between antennae and wings sizes and to weight the results obtained regarding mobility. As antennae and wings of males both seem important in the context of reproduction, their size should vary the same way, with the body size (isometry). Larger structures should thus be found in larger males, these three phenotypic traits being correlated (Koshio *et al.*, 2007).

Before actually checking these hypotheses and their predictions, the next part of the CHAPTER I, on sexual selection, gives an overview of the basics and presents in detail the mechanisms that are involved in the sexual selection and in the appearance of sexual dimorphism.

The CHAPTER II presents the species that is used as model – *Aphidoletes aphidimyza* – and especially its morphology and reproduction. The influences of humidity and of the pupae age at the sieving time on the productivity of the rearing method of *A. aphidimyza* are assessed in the ARTICLE 1 (Le Goff *et al.*, 2016). The life cycle and the rearing technique that was developed in the laboratory and used in the subsequent experiments are explained in that article. Afterwards, the link is made between the theory of natural and sexual selections and the sexual dimorphism of the model species, *A. aphidimyza*. The end of this chapter presents the phenotypic traits that will be measured for the needs of experiments in subsequent chapters.

The CHAPTER III deals with the reproductive behaviour of males of *A. aphidimyza*, according to their morphology, and includes two papers. The first one (ARTICLE 2: Nicolas *et al.*, in prep.b) studies the morphology of males and its consequences on the detection of, and the access to, a source of sex pheromone (intrasexual selection). In the second paper (ARTICLE 3: Nicolas & Hance, in prep.a) the sexual selection is investigated by means of direct mating experiments. This chapter therefore focuses on the ultimate causation (function – Tinbergen, 1963; Freeman & Herron, 2007) of the impressive antennae of males of *A. aphidimyza*.

As the developmental temperature has an influence on the phenotype (developmental plasticity) and could exacerbate morphological differences between males, the CHAPTER VI analyses the impact of temperature on morphology and fitness. The ARTICLE 4 (Nicolas & Hance, in prep.b) details the differences in the development and the morphology of *A. aphidimyza* individuals according to their rearing temperature (proximate causation, mechanism producing long antennae in males – Tinbergen, 1963; Freeman & Herron, 2007). Using these morphological differences, the ARTICLE 5 (Nicolas *et al.*, in prep.a) investigates the implications of the developmental temperature on the reproductive behaviour of males (ultimate causation, function of long antennae).

Following a scientific approach, this thesis gradually analyses the elements that could have played a preponderant role in the appearance and maintenance of sexual dimorphism in *A. aphidimyza* and decodes the influence of developmental temperature on the morphology and the reproduction of *A. aphidimyza*.

2. The theory of sexual selection

2.1. Introduction

2.1.1. The origin

The original concept of natural selection appears for the first time in 1859, in the book *"On the Origin of Species by Means of Natural Selection, Or the Preservation of Favoured Races in the Struggle for Life"* of Charles Darwin. It is now commonly accepted that individuals that have a characteristic that gives them a fitness advantage over the others in a given environment should most likely transmit this feature to the next generation. So, natural selection conserve favourable variations and supress detrimental ones. In a colourful way, this principle was called "the struggle for life". It is also Darwin who highlighted sexual selection, that was already mentioned in his book of 1859, and was analysed in detail in another work ("*The Descent of Man, and Selection in Relation to Sex*"), in two volumes (Darwin, 1871a, 1871b). Individuals that possess the most advantageous features, from the point of view of the competition for reproduction, will leave more progeny. That was logically called "the struggle for reproduction". To summarize, the net selection can be seen as the result of two phenomena: on the one hand, the struggle for existence (natural selection), in which the individuals with useful traits survive and, on the other hand, the struggle for procreation (sexual selection), in which the individuals that are most effective to reproduce will transmit their genetic background, and thus some of their characteristics, to their offspring (Hosken & House, 2011).

2.1.2. General concepts

The **natural selection** is a process acting when individuals composing a population (Endler, 1986; Smith & Smith, 2009):

- Are variable (trait variation).
- Vary in fitness in relation to their variable traits (fitness differences).
- Can transmit these variations, at least in part, to their offspring (inheritance).

To summarize, natural selection occurs when individuals composing a population present variable traits which are inheritable and if some of these traits are advantageous for surviving or reproducing in a given environment. Natural selection is thus a statistical difference of fitness between biological entities and is not an external agent or force (Futuyma, 2005; Freeman & Herron, 2007; Shuker, 2010).

The term **fitness** (also called **reproductive success**, **adaptive value** or **selective value**) is a central notion in natural selection. It is the capacity of an organism to survive and to breed in a given environment (Futuyma, 2005; Freeman & Herron, 2007). Fitness is often evaluated by the number of fertile descendants left to the next generation, because if an organism reproduces, that presupposes it survived (Futuyma, 2005; Smith & Smith, 2009). Fitness is a relative concept because it refers to the ability of an organism to survive and produce a progeny, compared to other individuals of the same population (Freeman & Herron, 2007).

If differences are observed between groups of organisms for survival and reproduction, it is because the individuals composing them differ for one or more traits that affect these components of fitness (Futuyma, 2005). This leads to the idea of **adaptation**, which is a trait enhancing the survival or reproduction of an organism in a given environment, in comparison with organisms without this trait (Freeman & Herron, 2007; Smith & Smith, 2009). An adaptation is therefore shaped by natural selection over the time (Smith & Smith, 2009).

Although natural selection acts on the survival and the reproduction at the individual level, changes are visible at the population level. A selected trait influencing fitness has to be transmissible to the next generation and will more frequently be transmitted if it promotes the survival and the reproduction of its bearer, in comparison with individuals without this trait. Consequently, the average characteristics of the population will change slightly at each generation, in case of directional selection. This change in the population corresponds to the **evolution** by natural selection, organisms with the most beneficial variation of the trait for survival and reproduction being naturally selected (Endler, 1986; Futuyma, 2005; Freeman & Herron, 2007; Shuker, 2010).

It is obvious that an organism will not be able to excel in all fields. Indeed, a trait which is beneficial to the fitness from one point of view (= **benefit** = increase of fitness) could be a handicap (= **cost** = decrease of fitness) from another point of view. Populations of organisms will thus not be able to respond optimally to all selective pressures. Consequently, there will always be compromises, called **trade-offs,** which will tend to optimise the cost/benefit ratio of the targeted characteristics for the fitness. In particular, an organism must always face its physiological needs in addition to responding to external factors (Futuyma, 2005; Freeman & Herron, 2007; Smith & Smith, 2009).

2.2. Sexual selection

The **sexual selection** is described as the struggle for the progeny, complementary to the struggle for the existence (Buican, 1997). Sexual selection results from the competition to obtain a mating partner and its gametes, the quantity as well as the quality of mates and gametes being important (Shuker, 2010; Shuker, 2014). Some individuals with a particular transmissible phenotype will gain easier access to reproduction than others with a different phenotype, leaving more descendants presenting this phenotype (Darwin, 1871a; Andersson, 1994; Futuyma, 2005; Freeman & Herron, 2007). Sexual selection mainly concerns the evolution of traits an organism does not clearly need to survive and to produce offspring according to their gender (gonads and related plumbing). Sexual selection thus explains the appearance of some traits, present in only one sex, that cannot have evolved by natural selection. For example, a red deer could have an offspring even without having antlers. These antlers are a disadvantage in accordance with the natural selection but could give the male an advantage over others in mating with a female (Darwin, 1871a; Reynolds & Harvey, 1994; Smith & Smith, 2009).

Size and symmetry of traits are often positively involved in sexual selection, because they are positively related to the mating success (Thornhill & Møller, 1998; Blanckenhorn *et al.*, 2003; Polak, 2008), as it is the case for the antennae of the male moth *Elcysma westwoodii* (Koshio *et al.*, 2007). Random deviations from the perfect symmetry corresponds to the **fluctuating asymmetry (**Palmer & Strobeck, 1992; Demers, 1999; Graham *et al.*, 2010; Debat, 2016). This fluctuating asymmetry is assumed to reflect the ability of an individual to pursue a stable development of its phenotype in a given environment, known as the **developmental stability** (Van Valen, 1962; Møller, 1993; Møller & Swaddle, 1997; Thornhill & Møller, 1998; Polak, 2008; Graham *et al.*, 2010). The fluctuating asymmetry of traits under sexual selection should thus inform on developmental stability (related to the quality of an individual) although this relation remains controversial, some factors potentially biasing the estimation of the developmental stability (Lens *et al.*, 2002; Blanckenhorn *et al.*, 2003; Koshio *et al.*, 2007).

The reproductive success of females is usually limited by the resources or the time available rather than by the number of partners, contrary to males. Therefore, males are often subjected to a strong sexual selection to gain access to mates, as females are a limiting resource for them. On one hand, males will thus generally compete to mate, as the number and the quality of the mates is important. On the other hand, females will be choosy in their selection of a

mating partner (Bateman, 1948; Trivers, 1972; Thornhill & Alcock, 1983; Futuyma, 2005; Freeman & Herron, 2007; Koshio *et al.*, 2007; Smith & Smith, 2009; Shuker, 2014). This difference between the two genders can result from various factors:

1. The **parental investment** (investment of one parent to take care of one offspring in order to enhance the offspring fitness, with the consequence of decreasing the capacity of the parent to invest in another offspring) is more important for females than for males in many species. From the beginning, the size of female gametes is bigger than that of male (**anisogamy**), which leads to an energy investment in the offspring more important for the female than for the male (Bateman, 1948; Trivers, 1972; Kvarnemo & Ahnesjo, 1996; Futuyma, 2005; Freeman & Herron, 2007; Shuker, 2014; Fromhage & Jennions, 2016). Even if male gametes are generally produced in greater number than female gametes, an ejaculate still seems to represent a smaller total gametic investment per copulation than eggs released by a female (Kokko & Jennions, 2008). So, females are usually more limited by the resources than by the number of mates.
2. The **potential reproductive rate** (maximum number of independent descendants that parents can generate per unit of time) is often higher for males than for females. Males can have more mating partners and each new partner increases their number of descendants. In consequence, even if males take care of their progeny, they are predisposed to compete more for mates than females (Clutton-Brock & Vincent, 1991).
3. Males usually mate more than females – this is sometimes pushed to its apogee when females mate just once and afterwards are unavailable for the other males – which biases the **operational sex ratio** (ratio of the number of males available for mating to the number of females available for mating, at a given time) toward males. Females are thus a limiting resource for males (Clutton-Brock & Vincent, 1991; Kvarnemo & Ahnesjo, 1996; Futuyma, 2005; Kokko *et al.*, 2006).

This competition for reproduction, characterising sexual selection, can materialise in two non-exclusive ways (Andersson & Simmons, 2006; Freeman & Herron, 2007; Hunt *et al.*, 2009; Hosken & House, 2011; Cézilly & Allainé, 2016):

- **Intrasexual selection**, which is the selection between individuals of the same sex. Members of the opposite sex will reproduce with the winners of this competition to mate.

- **Intersexual selection**, which is the selection involving individuals of opposite sexes. Members of one sex will choose the most attractive individuals of the other sex to reproduce.

(Ricklefs & Miller, 2005; Freeman & Herron, 2007; Smith & Smith, 2009).

As said before, it is usually males that compete to mate with females, and females that choose. This is thus the chosen situation for the explanations below, but, in most cases, it can be transposed to the opposite situation.

Moreover, it is important to highlight that the mechanisms presented hereunder are not to be opposed, as they can contribute to shape a same trait (Kokko *et al.*, 2003). The natural and the sexual selections can even act together to shape a same trait, as this trait has to trade-off with all the other traits of the individual which is a finite resource pool (Shuker, 2014).

2.2.1. Intrasexual selection

The intrasexual selection between different phenotypes can occur before copulation (**competition for mating opportunities**), between copulation and fecundation (**sperm competition**) or after fecundation (**infanticide** – killing the progeny of other males –, not presented here). The moment of the competition will determine which kind of trait will be selected (Thornhill & Alcock, 1983; Andersson, 1994; Freeman & Herron, 2007; Cézilly & Allainé, 2016).

2.2.1.1. Competition for mating opportunities

a) Interference competition

The **interference competition** – reduction of the access to a resource by the presence of a competitor (Smith & Smith, 2009) – promotes some characteristics of individuals according to the environment and can influence static or dynamic traits (Cézilly & Allainé, 2016):

- **Static traits**, without variation over short time frames, for example the size of the body or of a weapon. These traits are important in the typical case of male-male fight for the access to females (Freeman & Herron, 2007).
- **Dynamic traits**, with variations over short time frames, for example colours. The great advantage of this kind of trait is that it does not

express itself permanently, which reduces the cost. These characteristics are generally used as ritualized signals to assess the rival during an aggressive courtship (Hurd, 1997) but not directly in the fight itself.

b) Exploitative competition

The **exploitative competition** – reduction of the availability of a resource for the competitors (Smith & Smith, 2009) – can be subdivided in:

- The **mechanism of scrambles** that influences traits which are useful to locate and to reach a mating partner. In this way, competition by exploitation can apply a selective pressure on sensory organs or on traits influencing the mobility. All these traits are generally also used for other purposes, like finding a habitat or avoiding predators (Andersson, 1994; Cézilly & Allainé, 2016).
- The **mechanism of endurance rivalry** that influences traits which are useful to remain reproductively active longer and thus to prevent females from mating with other males (Andersson, 1994).

2.2.1.2. Sperm competition

This kind of competition takes place between sperms of various males for the fecundation of eggs (Parker, 1970; Parker, 1982; Freeman & Herron, 2007). Sperm competition is also characterised by two kinds of interactions (Andersson, 1994; Cézilly & Allainé, 2016): interference competition and exploitative competition.

a) Interference competition

Interference competition corresponds to physical interactions between males or their ejaculate into a female. Males can avoid the copulation of the female with another male by (Cézilly & Allainé, 2016):

- Guarding their mates (as in some birds – Møller & Birkhead, 1991 – or in the desert locust, *Schistocerca gregaria* – Ferenz & Seidelmann, 2003).
- Transmitting substances that inhibit the sexual receptivity of females (as observed in many insects, especially Diptera – Leopold, 1976) or that reduce female attractiveness (for example in Heliconius butterflies – Gilbert, 1976).

- Closing the genital opening of the female by a copulatory plug (like in rodents – Ramm *et al.*, 2005).
- Making injuries to the genital tract of the female (as in many insect species, including Diptera – Johnstone & Keller, 2000).
- Moving (as noticed in the ground beetle *Carabus insulicola* – Takami, 2007) or removing (for instance in the damselfly *Calopteryx maculata* – Waage, 1979) the sperm of other males.
- Inducing the rejection of the rival sperm by the female (as for the damselfly *Ceriagrion tenellum* – Andrés & Cordero Rivera, 2000).
- Mating while they are sperm-depleted, to limit the storage of the sperm of other males (as observed in the parasitoid wasp *Trichogramma evanescens* – Damiens & Boivin, 2006).
- Being well hooked to the female to control the duration of the copulation (as for the snake *Thamnophis sirtalis* – Friesen *et al.*, 2013).

In theory, the spermatozoa of different males can directly interact to decrease the probability of the rival to inseminate the eggs but this remains to prove experimentally (Cézilly & Allainé, 2016).

b) Exploitative competition

Exploitative competition concerns the situation where the fecundation of eggs by the sperm of a male will reduce the value of the clutch for the others. Males can increase their probability of paternity thanks to (Cézilly & Allainé, 2016):

- An increase of their ejaculate size (Martin *et al.*, 1974; Freeman & Herron, 2007).
- The production of more but smaller sperm (Parker, 1982; Parker, 1998).
- Frequent copulations (Møller & Birkhead, 1991; Gage & Morrow, 2003).
- A cooperation between the spermatozoa of a same ejaculate (Pizzari & Foster, 2008). For example, the aggregation of spermatozoa can enhance their motility (Moore *et al.*, 2002), or some spermatozoa can protect the others against spermicidal agents (Holman & Snook, 2006).

2.2.2. Intersexual selection

Regarding intersexual selection, the first possibility is that the preference of the female for a mating partner appears before copulation. In some cases, the preference of the female for a trait of the mating partner is subject to a selection pressure that influences directly her fitness (**direct selection**). In other cases, the selection pressure on the preference of the female for a trait of the mating partner will be indirect, influencing the fitness of her offspring, the male contribution being then at the level of the genes (**indirect selection**). The second possibility concerns cases where the female will show a preference for a mating partner at the time of the fecundation, promoting the sperm of some males to fertilize her eggs (**cryptic choice of females**, not developed here) (Kokko *et al*., 2003; Futuyma, 2005; Hosken & House, 2011; Cézilly & Allainé, 2016).

The word **preference** refers only to the fact that the mating choice of the female is not random, without notion of elaborate reasoning (Thornhill & Alcock, 1983; Cézilly & Allainé, 2016).

2.2.2.1. Direct selection

a) Gain of direct benefits

This corresponds to the preference of the female for males that bring benefits by improving her survival or her fecundity (quality and number of offspring). It could be, for example, some food, parental cares, a protection against predators or avoiding potential costs that represent the acquisition of pathogens. A female can also select a male based on traits which reflect his capacity to offer these direct benefits (Futuyma, 2005; Andersson & Simmons, 2006; Freeman & Herron, 2007; Smith & Smith, 2009; Cézilly & Allainé, 2016; Dhole & Servedio, 2016).

b) Sensory bias

The selection pressure can be exerted on the sensory system of females through a pre-existing preference for some phenotypic traits of males, that will exploit this bias. In this case, the evolution of the preference of the female shaped by the natural selection precedes the evolution of the trait in the mating partner, favoured by the sexual selection. So, it is no co-evolution between the preference of the female and the trait of the male, and there is no genetic correlation between the preference and the trait. This sensory bias in the

preference of the female derived from another context than that of the reproduction, like foraging (Ryan, 1998; Fuller *et al.*, 2005; Futuyma, 2005; Ricklefs & Miller, 2005; Andersson & Simmons, 2006; Freeman & Herron, 2007; Cézilly & Allainé, 2016; Dhole & Servedio, 2016).

c) Sexual conflict

Sexual conflict appears in an interaction between males and females when the evolutionary optimum cannot be reached simultaneously by the two sexes (when the fitness of one partner increases, the fitness of the other partner decreases). The **chase-away model** explains how sexual conflict can lead to a sexually antagonistic coevolution. A chase-away sexual selection generates a **resistance** (increasing selectivity) of the female as a consequence of the amplification of the trait of the male (Holland & Rice, 1998; Pizzari & Snook, 2003; Cézilly & Allainé, 2016). A good example of sexual conflict is given by substances transmitted by males during copulation to prevent future mating of females and thus decrease the risk of sperm competition (Gilbert, 1976; Leopold, 1976; Cézilly & Allainé, 2016).

2.2.2.2. Indirect selection

a) Fisher's runaway process

In the **Fisher's runaway process** (or **runaway sexual selection** or **sexy-sons hypothesis**), a female arbitrarily prefers a trait present in a male. If the preference for the trait and the trait itself are inheritable, the daughters produced will prefer the males with the trait, and the sons will present this attractive trait. The mating success of their offspring will thus be improved (Fisher, 1915; Fisher, 1930; Smith, 1991; Ryan, 1998; Futuyma, 2005; Freeman & Herron, 2007; Cézilly & Allainé, 2016). The evolution of the preference is linked to the evolution of the male trait which is under sexual selection. It is due to the genetic correlation between the preference and the trait, alleles being associated in a same individual more often than that could be expected by chance. A self-reinforcing coevolution of the preference and the trait is thus observed (Ryan, 1998; Kokko, 2001; Andersson & Simmons, 2006; Freeman & Herron, 2007; Hosken & House, 2011; Cézilly & Allainé, 2016). This runaway process will stop when the cost of the trait for the survival of the individual is so high that it will no longer be compensated by the increase of fitness resulting from the reproduction (Thornhill & Alcock, 1983; Ridley, 1993).

b) Good genes model

In the **good genes model** (or **indicator mechanism**), females preferring males with a well-developed indicator trait will be promoted by the natural selection because the development of the trait of the male is linked to his genetic quality, and the survival or the reproduction of their offspring should thus be improved. An indicator trait developed through a mate preference is an **honest signal** (it indicates the relevant quality of the male) if it presents:

- A cost of signalling, referring to the **Zahavi's handicap principle**. This concerns traits that are costly to produce or that have costly consequences on the fitness, and which are thus considered as a **handicap**. This kind of trait is typically too costly for low quality males to develop and thus proves the genetic quality of males producing it.
- A high potential cost of cheating. Sometimes, a trait is not really costly to produce in itself. In this case, the reliability of the signal is ensured by a high potential cost of cheating. This contributes to avoiding as much as possible the appearance of **cheaters** (individuals of low quality pretending to be of high quality).

The reliable trait can then be used by the female as a cue to select a mate with a superior genotype, and the preference and the trait coevolve (Zahavi, 1975; Thornhill & Alcock, 1983; Grafen, 1990; Smith, 1991; Ryan, 1998; Kokko, 2001; Smith & Harper, 2003; Futuyma, 2005; Andersson & Simmons, 2006; Smith & Smith, 2009; Számadó, 2011; McCullough & Emlen, 2013; Cézilly & Allainé, 2016).

The good genes hypothesis has a weak point called **lek paradox**. A **lek** is a grouping of individuals of the same sex, usually males, that will parade on a territory without resources, defending a small area. The males displaying the most convincing courtship or ornaments will be chosen for mating by females prospecting in the lek mating area. Females mate generally with the same very attractive males (polygyny) and males will not provide parental care (Höglund & Alatalo, 1995; Smith & Smith, 2009; Cézilly & Allainé, 2016). In this context, the lek paradox is the fact that, if females systematically choose males of a better genetic quality from generation to generation, better alleles should be fixed in the population. In this situation, the differences still visible between males result from environmental effects, and these phenotypic differences are not inheritable. The advantage won by females choosing better males should thus disappear and so the preference of females for some males, too (Borgia, 1979; Taylor & Williams, 1982; Cézilly & Allainé, 2016).

An influential solution to solve this paradox, called the **Hamilton-Zuk hypothesis**, postulates that the resistance of individuals to diseases is linked to the expression of some phenotypic traits, that are thus honest signals of the quality of the individuals. The permanent coevolution between individuals and their pathogens maintains enough genetic variation and the preference of females for the traits of interest will result in the transmission of genes of resistance to diseases to the offspring. The antagonism between the development of phenotypic traits and the functioning of the immune system of an individual could exist either because the same limited resources are used for the immune system and for the development of the indicator trait, or because, simultaneously, a third factor acts negatively on the immune system and positively on the development of the indicator trait (Hamilton & Zuk, 1982; Ricklefs & Miller, 2005; Smith & Smith, 2009; Cézilly & Allainé, 2016).

c) Genetic compatibility mechanism

This mechanism corresponds to the assumption that the advantages for the offspring come from a good interaction between the genomes of its father and of its mother and this will thus guide the choice of the female. As a consequence, a male that is advantageous for the offspring of a female will not necessarily be good for the offspring of another female, in contrary of what is observed with the good genes model (Colegrave *et al.*, 2002; Cézilly & Allainé, 2016).

2.3. Sexual dimorphism

Males and females differ from each other by **primary sexual characters**, which are organs of reproduction (copulatory organs and gonads), mainly selected by the natural selection. Moreover, the two sexes can also differ by **secondary sexual characters**, which are not directly implicated in the act of reproduction (like the longer tail of males for some birds), mainly selected by sexual selection (Darwin, 1871a; Andersson, 1982; Andersson, 1994; Ricklefs & Miller, 2005). The extravagant secondary sexual characters are generally called **ornaments (**Jennions & Petrie, 1997). These more or less marked morphological, physiological or behavioural differences (other than primary sexual characters) between the two sexes of a same species correspond to the definition of **sexual dimorphism** (Ricklefs & Miller, 2005; Freeman & Herron, 2007; Smith & Smith, 2009; Punzalan & Hosken, 2010). For example, this dissimilarity between males and females is particularly remarkable in Cervidae or in some beetles (Reynolds & Harvey, 1994; Buican, 1997; Post *et al.*, 1999;

Kawano, 2006). When the sexual dimorphism concerns a difference of size between the two sexes, it is called **sexual size dimorphism (SSD)** (Hedrick & Temeles, 1989; Shine, 1989; Kelly *et al*., 2008; Stillwell *et al*., 2010; Berns, 2013; Kishi *et al*., 2015).

2.3.1. Evolution by natural selection

By natural selection, some features appearing at the same time in the two sexes will regress in one sex due to their conspicuousness and the hazard it represents (Wallace, 1889). There is a temporal separation between the appearance of a novel trait and its sex-limitation. The trait appears simultaneously in the two sexes and then will be modified by the natural selection to reach a sex-limited expression (Oliver & Monteiro, 2011). The investment in reproduction, generally greater for females than for males, is often pointed out as a cause of sexual dimorphism. The fecundity selection on females (the fecundity usually increases with the body size) or the care of eggs and juveniles, which often falls to the females, generate different needs according to the gender. These different needs can lead to a different exploitation of the environment by males and females of the same species, called **intraspecific niche divergence** or **intersexual niche partitioning**. Subsequently, evolution of some traits by natural selection to adapt to these ecological niches may occur, leading to the sexual dimorphism (Hedrick & Temeles, 1989; Shine, 1989; Ricklefs & Miller, 2005).

2.3.2. Evolution by sexual selection

By sexual selection, some features appearing in only one of the two sexes will be transmitted to the offspring, because these characteristics make their bearer more effective for reproduction (Darwin, 1871a). There is no temporal separation between the appearance of a novel trait and its sex-limitation, and the sexual selection will maintain and even accentuate this sexual dimorphism (Oliver & Monteiro, 2011). The differences between males and females cannot be explained by natural selection because they may be unfavourable for the survival of their possessor and thus represent a cost from this point of view. On the other hand, the appearance of the trait results in an increased reproductive success of the individuals who present this trait, and thus it constitutes a benefit for the sexual selection (Futuyma, 2005; Freeman & Herron, 2007). Both intrasexual and intersexual selection can vary the reproductive success of one sex and lead to the sexual dimorphism (Hedrick & Temeles, 1989; Moore, 1990; Ricklefs & Miller, 2005; Berns, 2013).

2.3.3. The reality is nuanced

The proposed mechanisms for evolution of sexual dimorphism are not exclusive and a single mechanism cannot explain the complexity of sexual dimorphism (Shine, 1989; Punzalan & Hosken, 2010; Berns, 2013). For example, in the weevil *Rhopalapion longirostre*, natural selection promotes the lengthening of the rostrum of females and sexual selection maintains the small size of the rostrum of males (Wilhelm *et al*., 2015). In *Bicyclus* butterflies, sexually dimorphic wing patterns can also appear by both modes of selection (Oliver & Monteiro, 2011). The evolution of a sexually dimorphic trait can thus arise from natural and sexual selections and can be hampered by a genetic correlation of the trait in males and females (Lande, 1980; Hedrick & Temeles, 1989). Various other constraints may also influence the evolution of sexual dimorphism, like ontogenesis, biomechanics, physiology or performance constraints (Kaliontzopoulou *et al.*, 2008; Stillwell *et al*., 2010; Worthington *et al.*, 2012; Berns, 2013). Moreover, the environmental conditions can also be important, good conditions enabling individuals to fully develop their sexually dimorphic traits (phenotypic plasticity) (Madsen & Shine, 1993; Bonduriansky, 2007; Stillwell *et al*., 2010).

Consequently, there is a fragmented view on the mechanisms, linked to natural and sexual selections, involved in the appearance of sexually dimorphic traits, as there are many non-exclusive possibilities for each situation (Berns, 2013). Moreover, some tracks are still to explore, as the intrasexual selection by the mechanism of scrambles for the evolution of elaborate sensory organs, rarely studied directly, though already proposed Darwin (1871a) (Johnson *et al.*, 2017).

CHAPTER II. BIOLOGICAL MODEL: *APHIDOLETES APHIDIMYZA*

1. Introduction

1.1. Classification

Aphidoletes aphidimyza (Rondani, 1847) is a Diptera of the family Cecidomyiidae, usually called **gall midges** (Kulp *et al.*, 1989; Gagné, 1994; Fedotova, 2014), although *A. aphidimyza* does not produce galls (Boulanger *et al.*, 2018). Palps and antennae are important to recognize genera and species of Cecidomyiids (Harris, 1966). The tribe Aphidoletini to which *A. aphidimyza* belongs is very interesting for biological control because its members are predators, especially on aphids (Harris, 1966; Gagné, 1971; Harris, 1973; Gagné, 1994; Fedotova, 2014).

1.2. Geographical distribution

Aphidoletes aphidimyza can be found in the wild all over the world. It lives especially in the northern hemisphere (Harris, 1973), like in Europe (Harris, 1966), in Japan (Gagné, 1971; Yukawa *et al.*, 1998; Hinomoto *et al.*, 2012; Yamane *et al*., 2012; Higashida *et al.*, 2016; Watanabe *et al.*, 2016; Higashida *et al.*, 2017) or in Iran (Madahi *et al.*, 2013a, 2013b, 2013c; Tabadkani *et al.*, 2012a, 2012c; Tabadkani *et al.*, 2013; Farhoudi *et al.*, 2014; Madahi *et al.*, 2015), where it is common.

1.3. Use in biological control

Aphidoletes aphidimyza is used since the seventies to control aphids in greenhouses (Harris, 1973; Markkula & Tiittanen, 1976; Markkula *et al.*, 1979; Křivan & Havelka, 2000; Yano, 2006; Hance *et al.*, 2017), and since probably 1989 in augmentative biological control (Cock *et al*., 2010; van Lenteren, 2011), in many crops (Markkula & Tiittanen, 1976; Morse, 1981; van Schelt & Mulder, 2000; Boulanger *et al.*, 2018).

The worldwide use of *A. aphidimyza* in biological control (Yukawa *et al.*, 1998) can be explained by the following properties of the species:

1. Even if it feeds on nectar and honeydew at the adult stage, *A. aphidimyza* is a **furtive predator** at the larval stage. The larvae, by their unusual form of foraging behaviour, avoid being detected by their preys. The cohesion of the aphid colonies is maintained, since preys do not show defensive responses to these slowly moving larvae (Lucas & Brodeur, 2001; Sentis, 2010; Sentis *et al.*, 2012; Dumont *et al.*, 2015; Nicolas *et al*., 2015; Kohandani *et al.*, 2017).
2. *Aphidoletes aphidimyza* preys on almost all true aphids, and it is thus a **polyphagous aphid predator** (Uygun, 1971; Harris, 1973; Lefevre & Adams, 1982; Markkula & Tiittanen, 1985; Yukawa *et al.*, 1998; van Lenteren *et al*., 2002; van Lenteren & Schettino, 2003; Higashida *et al.*, 2016; Watanabe *et al.*, 2016; Hance *et al.*, 2017; Higashida *et al.*, 2017).
3. The number of aphids eaten by *A. aphidimyza* is variable and increases with the prey density (Choi *et al.*, 2001; Madahi *et al.*, 2013c; Guo *et al.*, 2014; Madahi *et al.*, 2015; Mottaghinia *et al.*, 2016; Kohandani *et al.*, 2017), with a minimum of 7 *Myzus persicae* required for the development (Uygun, 1971). Moreover, when there are plenty of aphids, larvae kill and eat more preys than it is actually needed for their nutrition (Harris, 1973; Markkula & Tiittanen, 1985). This predator presents a type II functional response (the number of preys consumed by the predator first increases rapidly as the prey density increases; then, this increase slows down as the prey density increases further, to reach a maximum that corresponds to the predator's satiety; in terms of proportion of preys eaten, it decreases with the increase of the prey density – Ricklefs & Miller, 2005; Smith & Smith, 2009) according to Morse & Croft (1987) and Mottaghinia *et al.* (2016) and a type III functional response (the proportion of preys eaten by the predator is low for a low prey density and then increases to a maximum when the prey density is intermediate; next, with the increase of the prey density, the proportion of preys eaten decreases; in terms of numbers of preys eaten as a function of the prey density, this corresponds to a logistic curve that tends towards a maximum value regardless of the future increase in prey density when predator is satiated – Ricklefs & Miller, 2005; Smith & Smith, 2009) according to Madahi *et al.* (2013c).
4. Females of *A. aphidimyza* easily locate aphids by themselves (Markkula & Tiittanen, 1985; Kohandani *et al.*, 2017). A female is able to find a plant infested with aphids among 75 not infested plants

(El-Titi, 1974) and females are able to find aphid colonies at a long distance (about 35 meters, with aphid-infested plants placed every 3 meters along four 45 meter axes radiating from the release point of the predator). In biological control, a diminution of the number of release points is thus possible, without sacrificing efficiency (van Schelt & Mulder, 2000).

5. The number of eggs laid by a female of *A. aphidimyza* increases with the aphid density (El-Titi, 1973; Lucas & Brodeur, 1999; Sentis *et al.*, 2012), and ranges from an average of 70 eggs per female (Uygun, 1971) to about one hundred (Harris, 1973; Madahi *et al.*, 2013a, 2013b) and can even reach roughly 250 eggs per female (Gilkeson, 1987).
6. The pupal stage is convenient for the storage, the shipment and the release of *A. aphidimyza* for use in biological control (Markkula & Tiittanen, 1985; Meadow *et al.*, 1985; van Schelt & Mulder, 2000; Le Goff *et al.*, 2016).
7. Several generations of *A. aphidimyza* can follow one another for a year thanks to the short life cycle – about 3 weeks, between 20 °C and 25 °C (Havelka & Zemek, 1999). In this way, the predator is able to respond to a population increase of its preys (Hance *et al.*, 2017).
8. Populations of *A. aphidimyza* hold from year to year when no incompatible disinfectant is used and when the substrate of culture of plants is conserved (Markkula & Tiittanen, 1985; Alotaibi, 2008).

2. Life cycle and rearing method

2.1. Introduction

The life cycle of *A. aphidimyza* and the rearing method implemented in the laboratory are presented in ARTICLE 1 (Le Goff *et al.*, 2016). A methodical rearing method was necessary to get enough standardised individuals for the needs of the subsequent experiments. With the rearing method developed by Markkula and Tiittanen (1985), it was difficult to recover pupae in the moist sand without damaging them. Therefore, as the individuals must be isolated before to be used in experiments, the rearing method of Markkula and Tiittanen (1985) has been improved by working with dry sand, which can easily be sieved to collect pupae.

The strain of *A. aphidimyza* reared for the needs of this ARTICLE 1, and also for the subsequent articles, is coming from the company Koppert, and it is thus not a wild strain.

2.2. Implementation of the rearing method of *Aphidoletes aphidimyza* according to the biological development

ARTICLE 1♦

Impact of Humidity on the Biological Development of *Aphidoletes aphidimyza* (Diptera: Cecidomyiidae)

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Abstract

Aphidoletes aphidimyza Rondani (Diptera: Cecidomyiidae) is one of the most important predators used in the augmentative biological control of aphids worldwide. However, due to its particular life history, mass rearing *A. aphidimyza* remains difficult. Our results show that a high relative humidity level during pupation optimizes the development of *A. aphidimyza*. By improving humidity levels during pupae storage, we improved the production efficiency and nearly achieved a 100 % adult emergence rate. These results allow us to suggest a new rearing method for the aphid predator *A. aphidimyza*.

Key words

Aphidoletes aphidimyza, humidity, rearing method, life cycle

Introduction

The midge *Aphidoletes aphidimyza* Rondani is a voracious predator of aphids (Diptera: Cecidomyiidae); a single larva can kill between 5 and 80 aphids during its development (Harris, 1973). It has been used since the 1970s with

♦ Aurore Nicolas took part in all the steps of the production of this article 1 (identification of the rearings' problems, conception and implementation of the experiments, obtaining and discussion of the results, redaction and corrections of the article).

various crops in greenhouses and orchards (Adams & Prokopy, 1980; Křivan & Havelka, 2000; Albajes *et al*., 2002; Hance *et al.*, 2017).

The developmental cycle of *A. aphidimyza* is described in FIGURE 1. After fertilization, females require 2 d at 23 °C for egg maturation (Havelka & Zemek, 1999). They then lay eggs in groups near or in an aphid colony (Lucas & Brodeur, 1999). The eggs hatch after 2-4 d, and larvae feed on aphids for 3-7 d (Jeoung *et al*., 2003). In the final larval stage, insects leave the aphid colony to pupate in the soil, where the larvae weave a silk cocoon (Harris, 1973). In *A. aphidimyza*, the pupa is enclosed in a puparium (a hardened exoskeleton of the last larval instar), surrounded by a silk cocoon that traps substratum particles. A few days later, a new adult emerges. The cycle's duration is strongly influenced by temperature and requires ~3 wk between 20 and 25 °C (Havelka & Zemek 1999).

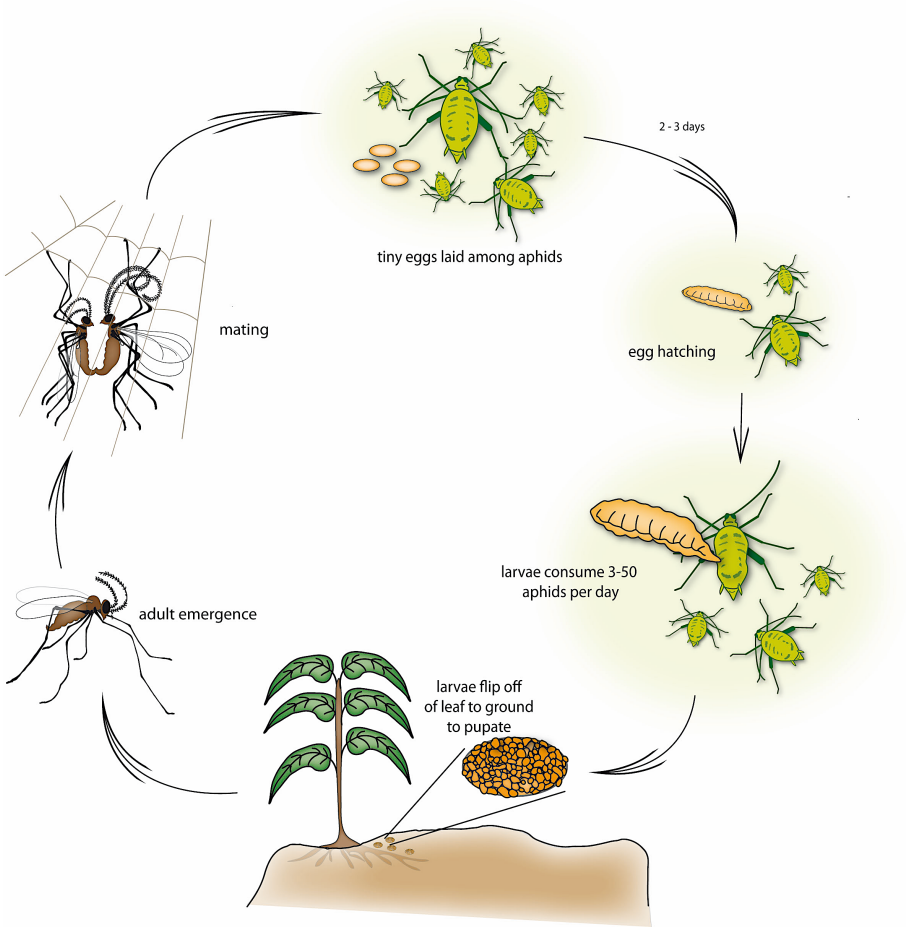


FIGURE 1. Life cycle of *A. aphidimyza* ©Delphine Bourdais.

Biological control by augmentation is a technique widely used for crop protection (Crowder, 2007). One of the major obstacles to its implementation is the difficulty of developing standardized and cost-effective rearing methods for biological control agents. Markkula and Tiittanen (1985) already described a method for mass-rearing *A. aphidimyza*. They noted that soil humidity was important for pupation. They placed the last-instar larvae on moist sand to maintain a high level of humidity and avoid larvae desiccation. However, it is not possible to know the exact number of pupae in the sand with this method because of the difficulty of sifting moist sand without hurting the pupae. Considering these points, our main goal was to improve the method described in Markkula and Tiittanen (1985) by working with dry sand. This would allow us to easily sift the sand and retrieve the pupae. In order to know the impact of our rearing method on adults' emergence, we aimed to evaluate the effect of pupae's age at the moment of sieving and the level of humidity on the productivity of the rearing method.

To evaluate the impact of our rearing method on adult emergence, we worked on the two following critical aspects of rearing *A. aphidimyza*:

1. Pupae retrieval. The substrate in which pupae form must be removed, leaving only pupae. Our hypothesis is that sieving should be performed as late as possible; more mature pupae can endure shocks more easily.
2. Pupae storage conditions. We know that a high level of humidity is required to rear *A. aphidimyza*, especially during pupation (Markkula & Tiittanen, 1985; Gilkeson, 1987). Therefore, our hypothesis is that a high relative humidity level would enhance the adult emergence rate.

Materials and methods

Rearing method

Potted fava bean plants (*Vicia faba L.*, about 45 cm) were infested by the aphid *Myzus persicae* (Sulzer) (Hemiptera: Aphididae) using specimens initially obtained from Viridaxis S.A. (Gosselies, Belgium). *Myzus persicae* is considered to be the most suitable prey for *A. aphidimyza* (Markkula & Tiittanen, 1985). Each aphid-infested plant was placed in a cage (the mating cage, 60 by 70 by 80 cm^3) containing adults of *A. aphidimyza* (the first pupae came from the commercial supplier Koppert B.V., Berkel en Rodenrijs, Netherlands), allowing mated *A. aphidimyza* females to lay eggs on the plant near aphid colonies. After 2 d, the potted bean plant with aphids and *A.*

aphidimyza eggs was removed from the first cage and placed in another cage without adults (the harvest cage, 65 by 63 by 107 cm^3; FIGURE 2). The larvae hatched and fed on aphids *ad libitum*. The fava bean plant was placed so that its leaves were located above six harvest boxes (17.5 by 12.5 by 6.5cm^3; FIGURE 2). These harvest boxes have a fine mesh (784 threads/cm^2) bottom, contained sand, and were placed above another box (17.5 by 12.5 by 12 cm^3) filled with water (FIGURE 3). There was no contact between the sand and the water; however, this arrangement maintained a relatively high level of humidity due to evaporation of the underlying water. A new fava bean plant infested with aphids and *A. aphidimyza* eggs was added to the harvest cage every 2 d, and the plant was removed when no *A. aphidimyza* larvae remained on the plant. A maximum of eight plants were in the harvest cage around the six harvest boxes at any given time (FIGURE 2). Rearing stocks were maintained in a climate room at 22 °C, 41 % relative humidity (RH), and a photoperiod of 16:8 (L:D) h.



FIGURE 2. Harvest cage (65 by 63 by 107 cm^3) with eight Fava bean plants (*Vicia faba* L.) around six harvest boxes.

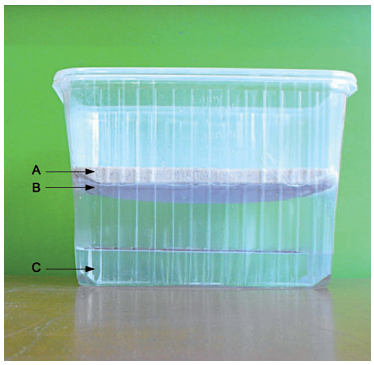


FIGURE 3. Harvest box (17.5 by 12.5 by 12 cm^3). (A) sand with larvae, (B) mesh (784 threads/cm^2), and (C) water.

After sitting for 2 d under the plants, the harvest boxes were placed in a 25 °C climatic chamber at dark until the sand was sieved to recover pupae. Recovered pupae were then placed in an emergence pot (a pot with a mesh bottom above a pot filled with water), which was then placed in a climatic chamber (25 °C, at dark), until emergence. After adults emerged, they were placed in the mating cage with other adults to restart the cycle.

Experiments: impact of sand sieving time and humidity during storage on pupa development and adult emergence

We performed the sand-sieving operation either 2, 4, or 6 d after having placed the harvest boxes in the climatic chamber (25 °C, at dark).

We also varied the relative humidity in harvest boxes by using two different types of lids to close the system: one made of plastic (high relative humidity level [HRH; RH: 99 %]) and one made of mesh (low relative humidity level [LRH; RH: 30 %]). HRH and LRH were measured using a meteorological station (HOBOware® 2.7, Onset Computer Co.).

The two treatments resulted in six combinations (humidity levels x sand sieving time). For each combination, five replications were performed. Each replicate consisted of 100 pupae chosen at random from a same harvest box and placed in an emergence pot in the same conditions as rearing. The number of adults, their sex, and whether they were alive or not were noted every day for 15 d. Four parameters were then calculated to determine the influence of sand sieving time and of humidity:

a. Adult emergence rate (%) (number of emerged adults x 100 / number of pupae);
b. Death rate at harvest time (%) (number of dead adults in the emergence pot x 100 / number of emerging adults);
c. Window of emergence (time between first and last day of emergence);
d. Time for emergence (time between the day we collected the harvest boxes and the day of first emergence).

Statistical Analysis

As our data follow a Poisson distribution, a generalized linear model was performed to test the potential influences of sand sieving time and humidity on adult emergence rate, death rate at harvest time, window of emergence, and time to emergence.

Statistical analyses were performed using R version 2.14.1 Copyright (C) 2011 The R Foundation for Statistical Computing for Mac. All tests were applied under two-tailed hypotheses, and the significance level, P, was set at 0.05.

Results

No significant interaction was observed between the two tested factors on the four measured parameters (adult emergence rate: $\chi^2 = 2.15$, df = 2, P = 0.34; death rate at harvest time: $\chi^2 = 0.05$, df = 2, P = 0.99; window of emergence: $\chi^2 = 0.42$, df = 2, P = 0.81; time to emergence: $\chi^2 = 0.40$, df = 2, P = 0.38).

Our results indicate that HRH induced a higher adult emergence rate ($\chi^2 = 400.85$, df = 1, P < 0.001) but also a slightly higher death rate at harvest time ($\chi^2 = 48.37$, df = 1, P < 0.001) compared with LRH (TABLE 1). The HRH group had a narrower window of emergence ($\chi^2 = 12.45$, df = 1, P < 0.001) compared with the LRH group and a shorter time for emergence ($\chi^2 = 2.68$, df = 1, P < 0.001) than the LRH group (TABLE 1). We also noticed that the silk cocoons of the pupae seemed to be more thoroughly covered by sand in the HRH group than in the LRH group (FIGURE 4).

TABLE 1. Mean (± SE) of adult emergence rate, death rate at harvest time, window of emergence, and time for emergence per level of relative humidity (HRH = high relative humidity, LRH = low relative humidity) and per sand sieving time.

	Relative humidity level		**Sand sieving time**		
	HRH (100%)	**LRH (30%)**	**Day 2**	**Day 4**	**Day 6**
Adult emergence rate (%)	88.22 ± 0.02	53.19 ± 0.07	88.62 ± 0.07	67.64 ± 0.07	61.80 ± 0.10
Death rate at harvest time (%)	3.67 ± 1.00	0.44 ± 0.16	2.31 ± 0.87	2.91 ± 1.42	1.10 ± 0.69
Windows of emergence (days)	4.11 ± 0.43	6.94 ± 0.77	4.92 ± 0.58	5.64 ± 0.87	5.90 ± 1.19
Time for emergence (days)	9.50 ± 0.43	11.31 ± 0.59	9.92 ± 0.24	10.00 ± 0.33	11.30 ± 0.96

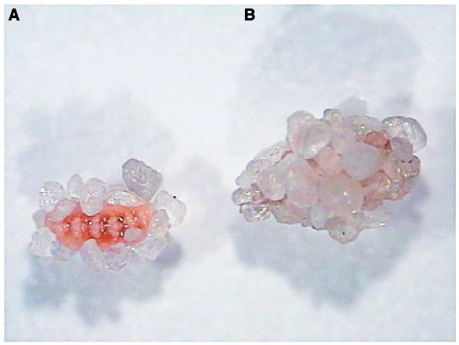


FIGURE 4. Cocoons formed at a low (A) or a high (B) humidity level.

Sand sieving time had a significant impact only on adult emergence rate (χ^2 = 80.98, df = 2, P < 0.001; TABLE 1). For the two relative humidity conditions, adult emergence rate was lower when sand was sifted on the fourth or the sixth day than on the second day (TABLE 1). No influence of sand sieving time was observed for the death rate at harvest time (χ^2 = 7.72, df = 2, P = 0.28), the window of emergence (χ^2 = 0.97, df = 2, P = 0.62), or the time of emergence (χ^2 = 1.08, df = 2, P = 0.07; TABLE 1).

Discussion

Our experiments confirm that a high relative humidity level during pupation facilitates the development of *A. aphidimyza* larvae, resulting in a higher emergence rate, a narrower window of emergence, and faster emergence. It has been shown that a HRH increases the adult emergence rate in *Hermetia illucens* (L.) and other Diptera (Holmes *et al.*, 2012). Wigglesworth (2013) explains that in an LRH condition, water loss through the pupal membranes can be detrimental to the survival of holometabolous insects, resulting in desiccation. Insects respond to desiccation with increases in stress protein 28 and in juvenile hormone as protection. The problem is that these hormones are downregulated between pupation and adult reproduction (Kroeker & Walker, 1991), which could explain our results. Furthermore, it has been shown in several flea species that a low humidity level can reduce the life span of all stages, slow down larval activity, inhibit silk production (causing cocoons to be small and soft), and affect adult emergence rate and size (Krasnov *et al.*, 2001), which may correspond to what we observed in our experiments. The cause of this high sensitivity has been attributed to the inability of flea larvae to close their spiracles, which induces water loss (Krasnov *et al.*, 2001). It is possible that this same mechanism occurs in *A. aphidimyza* and, for instance, an HRH condition may allow larvae to compensate or to decrease for this daily water loss. We also observed, in the LRH condition, that cocoons seemed malformed (FIGURE 4), probably either because larvae must weave their cocoons rapidly to protect against water loss or because they are not in good physical condition to pupate correctly.

Finally, an HRH condition increases the death rate at harvest time compared with an LRH condition, which could be explained by the presence of entomopathogens. Indeed, high relative humidity has long been known to increase the infection rate by entomopathogenic fungus (Luz & Fargues 1999). Testing and estimating the presence of entomopathogenic organisms in our rearing conditions could therefore constitute an interesting future study. Moreover, this higher death rate at harvest time in the HRH condition may also

be explained by the higher pupal density in emergence pots compared with the LRH condition. Indeed, the volume of these pots was quite small (137 cm^3). Therefore, a high density of individuals can lead to collisions between numerous emerging adults before their full sclerotization, leading to injuries that could result in death. To prevent collisions between individuals in an HRH condition, emergence pots should be larger or the pupal density should be decreased. However, this higher death rate is more than offset by a much higher adult emergence rate in the HRH condition.

The sand sieving time influences the adult emergence rate; sieving the sand later reduces the adult emergence rate in both LRH and HRH conditions. This could be explained by the fact that later-stage pupae enter into a critical and sensitive phase of their development that makes them more vulnerable to shocks caused by sand sieving. For example, the insect may be between apolysis (detachment of the pupa cuticle from epidermal cells) and secretion of adult cuticle (Hinton, 1971). Alternatively, it could also be because pupae are clamped in their puparium and are therefore not well protected against collisions. The impact of shocks on the survival of *A. aphidimyza* pupae could be an interesting future study that would help in determining how shipment would affect them. This statement also indicates that when deploying a treatment against aphids with *A. aphidimyza*, pupae shipment should be performed at the beginning of pupation to avoid a decrease of the adult emergence rate in the field to avoid limiting the effectiveness of the aphids' biological management capabilities.

By examining abiotic factors (i.e., humidity) during pupae storage, we improved production efficiency, reaching nearly a 100 % adult emergence rate. The process described here can help researchers improve their own rearing techniques, and this process can also be expanded for mass rearing and commercial production.

Acknowledgments

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3. Reproduction

3.1. Mating

Adults come out of the pupae at the end of the day (Jeoung *et al*., 2003) or at the beginning of the night (van Lenteren *et al*., 2002; van Lenteren & Schettino, 2003) and take off roughly 30 minutes later. The activity period extends from sunset to just before sunrise, or the equivalent under artificial photoperiod (Harris, 1973; van Lenteren *et al*., 2002; van Lenteren & Schettino, 2003), but adults are mostly active at the beginning of the night (Jeoung *et al*., 2003). So, mating usually occurs between the sunset and the sunrise (Harris, 1973). During the photophase, adults of *A. aphidimyza* are generally inactive (Markkula & Tiittanen, 1985; van Lenteren *et al*., 2002; van Lenteren & Schettino, 2003).

For mating, adults of *A. aphidimyza* land in a spider web using tarsi of the first pair of legs. Afterwards, they hold the web only with the front legs or also with one or two leg(s) of the middle pair. Females start to call for a mating partner quickly after having landed in the spider web, generally during the scotophase, but sometimes during the light period. Females call by extruding the end of their abdomen and moving it slowly. They were observed to call for a mate during up to 8 hours, several nights in a row (van Lenteren *et al*., 2002; van Lenteren & Schettino, 2003). The sex pheromone emitted by females during the calls is the (2R,7S)-diacetoxytridecane (Choi *et al.*, 2004a). Attracted males then land in the spider web and move to a female frontally while wing-fanning. To copulate, the male bends its abdomen toward the female to grip its genitalia. The mating preferentially takes place in the spider web (van Schelt & Mulder, 2000), in a face-to-face position, as illustrated in the FIGURE 1 of the ARTICLE 1 (Le Goff *et al.*, 2016). During the mating, the male will first extend its wings and afterwards it will fold them against its body, like the female. The mating lasts between 2 and 3 minutes (van Lenteren *et al*., 2002; van Lenteren & Schettino, 2003).

After mating, the female can stay in the spider web for a few hours, whilst the male flies away. Just after the mating, the female no longer calls and even flies away if a male is approaching. The ending of the call behaviour by mated females and the refusal of mated females to mate again after a first copulation strongly suggest that females are **monoandrous** (a female mates with only one male), while males are **polygynous** (a male mates with several females) (van Lenteren *et al*., 2002; van Lenteren & Schettino, 2003; Tabadkani *et al.*, 2012a). Males are no longer attracted by females for roughly one hour

after the mating. Yet, males can be attracted and try to copulate with other males that have just mated, probably because they are contaminated with the sex pheromone of a female. If there are too many males compared to females, as the males fly more actively than females, the mating can be interrupted prematurely. This can lead to a low reproductive success as unmated females do not lay eggs, which is problematic in mass production (van Lenteren *et al*., 2002; van Lenteren & Schettino, 2003).

The females are supposed to take advantage of the position of the spider webs, which are generally in air currents, to spread their pheromone without making wide movements. After landing in the spider web, the individuals move really slowly so the spider is quickly losing interest in them. The face-to-face mating position could also be an adaptation of *A. aphidimyza* to mate in spider webs quietly. The gall midges are also able to land in and take off from the spider web without being stuck in the web, and even if the spider is seen walking across the web, it does not discover the midges. Consequently, protection against predators gained by *A. aphidimyza* by mating in spider webs might exceed the risk of being eaten by a spider (van Lenteren *et al*., 2002; van Lenteren & Schettino, 2003).

The use of a sex pheromone for the female to attract a male for mating is widespread in gall midges and pheromones are usually produced in very small quantities (Harris & Foster, 1999; van Lenteren *et al*., 2002; Choi *et al.*, 2004a; Hall *et al*., 2012). In order to emit the sex pheromone, females extend their telescopic abdomen, but the abdominal movements of *A. aphidimyza* are rather limited compared to those of other cecidomyiids. The courtship of gall midges is generally very reduced: a male finds a virgin female, tries to copulate and usually succeeds without any movement of the partner. The male mounts on the back of the female or the mating partners copulate in parallel position. Conversely, the mating behaviour of *A. aphidimyza* is obviously more complex (Harris & Foster, 1999; van Lenteren *et al*., 2002; van Lenteren & Schettino, 2003), with copulation that takes place in a spider web (van Schelt & Mulder, 2000), as for some other species of Cecidomyiids (Yukawa *et al.*, 2008; Yukawa *et al.*, 2011).

3.2. Monogeny

The individuals composing the offspring of a single female of *A. aphidimyza* are all of the same sex. This production of unisexual progeny by a female corresponds to the definition of **monogeny** (Sell, 1976; Markkula & Tiittanen, 1985; Stuart & Hatchett, 1991; Tabadkani *et al.*, 2011; Tabadkani *et al.*, 2012a, 2012b, 2012c; Kohandani *et al.*, 2017).

As for other gall midges, the monogeny of *A. aphidimyza* results from the mechanism of sex determination (FIGURE 3), which is chromosome-based, although gametes of males and gametes of females contain the same number of sex chromosomes. During embryogenesis, a postzygotic elimination of chromosomes will lead to a different ratio of sex chromosomes and autosomes in somatic cells of males ($A_1A_2X_1X_2/A_1A_2OO$, where A_1 and A_2 represent autosomes, X_1 and X_2 represent sex chromosomes – haploid in males – and paternally derived chromosomes are after the slash) compared to females ($A_1A_2X_1X_2/A_1A_2X_1X_2$) (Stuart & Hatchett, 1991; Benatti *et al.*, 2010). When a female presents the autosomal dominant genetic factor called "Chromosome maintenance" (Cm), this prevents the elimination of a paternally derived set of sex chromosomes during the embryogenesis. In this situation, all the progeny of the female will have a female-determining karyotype. Females bearing this allele Cm are **thelygenic** (females producing females). The offspring of a female lacking Cm will exclusively present a male-determining karyotype because of the elimination of a paternally derived set of sex chromosomes during the embryogenesis. Females without the Cm allele are thus **arrhenogenic** (females producing males) (Benatti *et al.*, 2010; Tabadkani *et al.*, 2011; Tabadkani *et al.*, 2012a, 2012b, 2012c). So, in *A. aphidimyza*, males have no influence on the sex of their progeny (Sell, 1976).

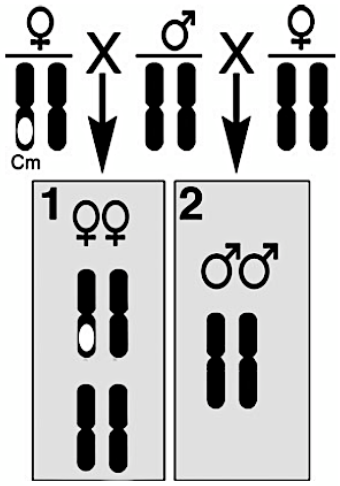


FIGURE 3. Segregation of Cm, represented by the white dot, in monogenous gall midges. The progeny of thelygenic females (Box 1) is composed of the same number of thelygenic females (with Cm) and arrhenogenic females (without Cm), whilst the progeny of arrhenogenic females (Box 2) is composed exclusively of males (Tabadkani *et al.*, 2011, adapted from Benatti *et al.*, 2010).

Monogeny has for consequence to avoid inbreeding, because descendants of a same mother cannot reproduce among themselves as they are of the same gender. At worst, parents will be half-siblings, if they share a same father, preventing complete inbreeding (Tabadkani *et al.*, 2011).

3.3. Sex ratio

The mechanism of sex determination has for consequence that the **sex ratio** (ratio of males to females) is expected to be 1:1 in *A. aphidimyza* (Sell, 1976). Indeed, as the half of the progeny of thelygenic females will present the Cm allele and the other half of females will lack this allele, the ratio of thelygenic to arrhenogenic females in the progeny is 1:1. These ratios will therefore be maintained generation after generation (Sell, 1976; Benatti *et al.*, 2010; Tabadkani *et al.*, 2011; Tabadkani *et al.*, 2012a, 2012b, 2012c).

In the studies addressing the sex ratio of *A. aphidimyza*, the actual values of the sex ratio are pretty close to these theoretical proportions. For example, Tabadkani *et al.* (2012b) report that the sex ratio of individuals from the company Koppert is slightly skewed towards males (53.2 %), while the sex ratio in their experiments ranges from 48.3 % to 58.2 % of males. Other references give other sex ratios: between 28 % and 53 % of males for Havelka &

Zemek (1999), 55 % of males for Heimpel & Lundgren (2000), between 52.8 % and 58.1 % of males for Tabadkani *et al.* (2012a).

Even though the sex ratio at emergence is about 1:1 (Tabadkani *et al*., 2012c), differences often appear between sex ratios estimated at a given times and the theoretical sex ratio, for the following reasons:

1. There is a difference in mortality between male and female gall midges, particularly in harsh conditions (Dorchin & Freidberg, 2004; Tabadkani *et al.*, 2012a, 2012b, 2012c; Tabadkani *et al.*, 2013). For example, the sex ratio of *A. aphidimyza* biases towards females shortly after emergence of pupae (Tabadkani *et al.*, 2012a), but it can also occur before emergence (Tabadkani *et al*., 2012c) and males die quickly after mating (Tabadkani *et al.*, 2012c; Tabadkani *et al.*, 2013). The lifespan is generally longer for females than for males and this can be sufficient to bias the estimations of the sex ratio in natural populations (Tabadkani et al., 2012b; Tabadkani *et al.*, 2013). In *A. aphidimyza*, the longevity of females and males is respectively of 5 days and 3 days for El-Gayar (1976), of 10 days and 7 days for Jeoung *et al*. (2003) and of 6 days and less than 4 days for Tabadkani et al. (2012c).
2. A majority of males of *A. aphidimyza* emerge before the females (van Lenteren *et al*., 2002; van Lenteren & Schettino, 2003). This phenomenon is called **protandry**. The emergence of males can precede that of females by a day (Tabadkani *et al.*, 2012a, 2012c; Tabadkani *et al.*, 2013). The sex ratio is thus skewed towards males at the time of emergence and thereafter progressively towards females (Tabadkani *et al.*, 2012c; Tabadkani *et al.*, 2013). This can lead to erroneous estimations of the real sex ratio when counting individuals of the two genders at a given time, instead of cumulating emergences of the two sexes until all pupae emerged.
3. Sampling errors can occur due to individuals distributing differently in their habitat depending on their gender. When individuals are sampled at a same location, which would gather more individuals of one sex compared to the other sex, the estimation of the sex ratio is wrong. For example, in *A. aphidimyza*, sampling done by taking adults only from plants which are infested with aphids is likely to bias the sex ratio towards females, because these are laying eggs while males are flying (Tabadkani *et al.*, 2013).

4. Sexual dimorphism

4.1. Description

Antennae are known as the main organs of olfaction in insects (Chapman, 1998; Boddum, 2013). Sexual dimorphism in antennae is common and, when males have to locate females by her scent, antennae of males are often more complex than those of females (Chapman, 1998). More complex antennae bear more **sensilla** (sensory hairs) than less complex ones, and feathery antennae are useful to sample a large amount of air. In species using a sex pheromone, the specialisation of antennae to detect mates is thus often well visible (Steinbrecht, 1987; Chapman, 1998). In particular, pheromones produced in tiny quantities seem to have induced enlarged sensilla circumfila in males of many gall midge species, in order for these males to detect them (Hillbur *et al.*, 2001; Hall *et al*., 2012).

The adults of *A. aphidimyza* look like little mosquitoes. They have long and thin legs and they are about 2 mm long (Markkula & Tiittanen, 1985; Jandricic, 2013; Fedotova, 2014). The sexual dimorphism is very marked at the adult stage. With the naked eye, it is possible to see that males have very long curved grey antennae with many bristles, while females have short straight dark antennae (Markkula & Tiittanen, 1985).

Antennae of *A. aphidimyza* are composed of a scape, a pedicel, and then a flagellum made of 12 flagellomers (see FIGURE 1 in CHAPTER I for an illustration of the complete antennae of a male *A. aphidimyza* and FIGURE 4 hereunder for details) (Harris, 1966; Zhang & Yang, 2008; Fedotova, 2014). Six different types of sensilla contribute to the sexual dimorphism. They can be observed on the antennae of *A. aphidimyza*, at the level of the nodes (Zhang & Yang, 2008):

1. **Styloid**: only in males; probably a receptor for sex pheromone or a facilitator to choose a mating partner; present on the second flagellomer.
2. **Cavity**: in greater numbers in males than in females; likely to perceive sounds or vibrations.
3. **Basiconia**: in almost the same number in males and females; present on each flagellomer of males and females.
4. **Trichoidea**: in somewhat greater numbers in males than in females; chemoreceptors; present on each flagellomer of males and females, may be important to detect the female sex pheromone.

5. **Chaetica**: in greater numbers in females than in males; mechanoreceptors; present on each flagellomer of males and females; the most prominent kind of sensilla in both genders.
6. **Circumfila**: less complex in females than in males; probably an olfactory receptor, important in perception of sex pheromone; present on each flagellomer of males and females; this kind of sensilla can only be found in gall midges.

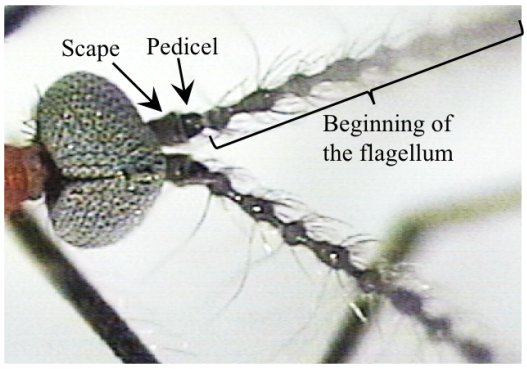


FIGURE 4. Head and base of antennae of a male *A. aphidimyza*.

Flagellomers of males (FIGURE 5 and FIGURE 6) include two nodes each. The distal node, with two series of sensilla circumfila, is greater than the proximal one, with only one series of circumfila. A flagellomer thus bears three separated circumfila, the first and third of each flagellomer presenting loops of irregular lengths, dorsal loops being longer than the flagellomers themselves (Harris, 1966; Gagné, 1994; Zhang & Yang, 2008; Fedotova, 2014). Circumfila, that encircle flagellomers with their loops (Gagné, 1994), could improve the detection of the provenance of the stimuli (Hall *et al*., 2012). Only the distal flagellomer seems to be a little different from others, at the very end, as there is not the long apical neck to connect it with the following flagellomer, and it is the case for most of the males of the Cecidomyiidae (Gagné, 1994).

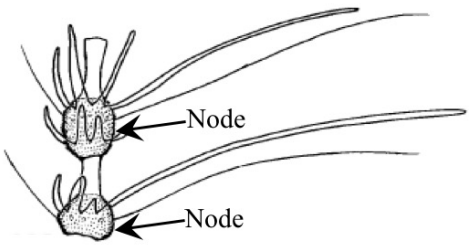


FIGURE 5. Third flagellomer of a male *A. aphidimyza* (Harris, 1966).

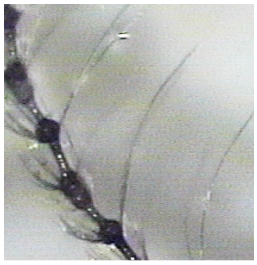


FIGURE 6. Details of flagellomers of a male *A. aphidimyza*.

Flagellomers of females (FIGURE 7) consist of only one relatively long and cylindrical node bearing simple circumfila which form one or two loop(s), with a relatively short internode (Harris, 1966; Zhang & Yang, 2008; Fedotova, 2014). This shape results of the fusion of the two initial nodes of each flagellomer into a single one (Zhang & Yang, 2008).

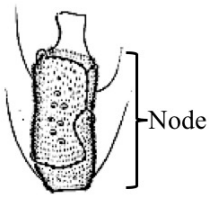


FIGURE 7. Third flagellomer of a female of *A. aphidimyza* (Harris, 1966).

This difference between males and females in the structure of flagellomers induces a difference in the length of the complete antennae (FIGURE 1 and FIGURE 2, in the CHAPTER I). Males have longer antennae – 2 mm for Zhang & Yang (2008) and 2.85 mm for Fedotova (2014) – than females – 1.05 mm according to Zhang & Yang (2008).

Thanks to all these specificities at the level of the antennae, individuals of *A. aphidimyza* can be sexed with the naked eye (Markkula & Tiittanen, 1985; Tabadkani *et al.*, 2012a, 2012b, 2012c; Tabadkani *et al.*, 2013). Gender can be corroborated by the fact that the body of females is generally a little more robust than that of males (Tabadkani *et al.*, 2012c; Tabadkani *et al.*, 2013).

4.2. Theoretical approach

Prior to carrying out the experimental approach (in the next four papers), the theories of natural and sexual selections and sexual dimorphism, already presented in the CHAPTER I, will be applied to *A. aphidimyza*, the model species, and the main hypothesis and prediction of the thesis will be explained. Indeed, the antennal sexual dimorphism observed in *A. aphidimyza* could have been shaped by different non-exclusive factors (Lande, 1980; Hedrick & Temeles, 1989; Shine, 1989; Punzalan & Hosken, 2010; Berns, 2013), as explained in the point 2.3. of the CHAPTER I.

4.2.1. Natural selection

The sexual dimorphism observed in *A. aphidimyza* could result from the natural selection (Wallace, 1889; Hedrick & Temeles, 1989; Shine, 1989; Ricklefs & Miller, 2005; Oliver & Monteiro, 2011). This form of selection generally influences the body size and traits which are linked to a different exploitation of their environment by the two genders (Hedrick & Temeles, 1989; Shine, 1989; Ricklefs & Miller, 2005). Females and males of *A. aphidimyza* use their environment differently for reproduction. Females need to find an aphid colony where to oviposit, contrary to males (El-Titi, 1974; Markkula & Tiittanen, 1985; van Schelt & Mulder, 2000). This could have led to more developed antennae for females, which is the opposite of what is observed in reality. Moreover, females of *A. aphidimyza* do not provide special care to their progeny and both males and females have the same diet (Markkula & Tiittanen, 1985; Watanabe *et al.*, 2014). Consequently, their needs (and their exploitation of the environment) are the same at these levels. Furthermore, the production of very large and complex antennae in males should represent a significant energetical cost and also a cost for the survival of the bearer of such antennae (Emlen, 2001), which is normally not favoured by natural selection (Freeman & Herron, 2007). Contrariwise, natural selection might have strengthened the sexual dimorphism by leading to a simplification of the structure of the antennae of females, compared to males. Nevertheless, it seems quite unlikely that natural selection could have been the preponderant factor in the appearance of these stunning antennae in the males of *A. aphidimyza*.

4.2.2. Sexual selection

For males of *A. aphidimyza*, the costs required to produce and maintain long and feathery antennae could be partially compensated by the benefit of finding a mate or a better-quality mate (Freeman & Herron, 2007; Shuker, 2010; Shuker, 2014) but possibly only the most vigorous males could bear theses costs (Futuyma, 2005; Ricklefs & Miller, 2005). The sexually dimorphic antennae of *A. aphidimyza* could thus result from sexual selection.

In monoandrous species, protandry is particularly common since females copulate just once and males have to compete for virgin females (Forsberg & Wiklund, 1988; Muralimohan & Srinivasa, 2010; Tabadkani, *et al.*, 2012c; Tabadkani *et al.*, 2013). As a matter of fact, females of *A. aphidimyza* are monoandrous, while males are polygynous (van Lenteren *et al.*, 2002; van Lenteren & Schettino, 2003; Tabadkani *et al.*, 2012a), and this mating system

biases strongly the operational sex ratio towards males (Futuyma, 2005; Smith & Smith, 2009). Moreover, the parental investment should be more important in females than in males due to anisogamy (Bateman, 1948; Trivers, 1972; Futuyma, 2005; Freeman & Herron, 2007; Fromhage & Jennions, 2016) and the potential reproductive rate should be lower for females than for males as parental care is not observed in this species (Clutton-Brock & Vincent, 1991). As a consequence, females should be considered as a limiting resource by males and thus males of *A. aphidimyza* should compete to mate, while females should be choosy in their selection of a mate (Futuyma, 2005).

4.2.2.1. Intrasexual selection

When males have to locate a female by her scent – as it is the case in *A. aphidimyza*, since females call a mate with a sex pheromone (Choi *et al.*, 2004a) – antennae of males are often more complex than those of females (Chapman, 1998). Moreover, when males have sensory or locomotor organs more developed than females, it is often due to the competition for mating opportunities, by the mechanism of scrambles, which promotes traits that help to locate and reach a mating partner (Darwin, 1871a; Cézilly & Allainé, 2016). The competition between males for virgin females is corroborated by the fact that being the first to find the female is primordial in monoandrous species (Shiel et al., 2015). Moreover, protandry is observed in *A. aphidimyza* and this is known to increase mating opportunities of early emerged males (Bukowski & Avilés, 2002; Tabadkani *et al.*, 2013). The intrasexual selection is thus likely to be a cause of the sexual dimorphism at the level of antennae in *A. aphidimyza* as the antennae are important for males to perceive the sex pheromone emitted by females and to arrive first to copulate. Long and feathery antennae could thus have been favoured by intrasexual selection (Tabadkani *et al.*, 2012c).

4.2.2.2. Intersexual selection

Antennae of males of *A. aphidimyza* could also correspond to an ornament, like in the cerambycid beetle *Stenurella melanura*, whose males with more symmetric antennae are advantaged by the intersexual selection (Møller & Zamora-Munoz, 1997), or like in the moth *Elcysma westwoodii*, whose males with more symmetric and longer antennae are favoured for mating (Koshio *et al.*, 2007). The fluctuating asymmetry is supposed to inform about the developmental stability, related to the genetic quality, but this relation remains controversial (Lens *et al.*, 2002; Blanckenhorn *et al.*, 2003; Koshio *et al.*, 2007). Nevertheless, females of *A. aphidimyza* could preferentially choose males with

longer and more symmetric antennae, according to the good genes model – elaborate antennae should represent a handicap to fly and should indicate the quality of their bearer – or to the Fisher's runaway process – the attractive and thus advantageous trait being transmitted to the offspring – (see CHAPTER I, point 2.2.2.2.).

4.2.3. Main hypothesis and prediction

The hypothesis is thus that evolution by sexual selection – through intrasexual selection and intersexual selection – largely shaped the impressive antennae of the males of *A. aphidimyza*. The prediction is that longer and more symmetric antennae facilitate access of males to a mate.

To test this prediction, the chosen approach is to refer to the definitions of sexual selection and of evolution by sexual selection. The evolution by sexual selection is based on the same process as by natural selection (Freeman & Herron, 2007), since only the nature of the selected trait changes. The process of sexual selection occurs thus if (Endler, 1986; Freeman & Herron, 2007; Shuker, 2010; Shuker, 2014):

- Some individuals in a population present a variation for a trait.
- The ability to obtain a mating partner (or a better-quality mate) and its gametes varies according to that trait.
- A connection, at least partly independent of the effects of the environment, exists between parents and their progeny for that trait.

The main difference between natural and sexual selections is that sexual selection concerns traits that impact success to mate, rather than traits that impact survival or production of offspring according to the gender (Reynolds & Harvey). The consequence of the sexual selection process will be equivalent to that of the natural selection process: the evolution by sexual selection, which corresponds to slight changes in the average characteristics of the population at each generation. To summarise, if the ability to obtain mates is affected by heritable variation in a trait, the individuals presenting the favourable variation will become more prevalent in the population over the time. Sexual selection comes from competition for mates and can take the form of intrasexual and intersexual selections (Andersson, 1994; Freeman & Herron, 2007; Shuker, 2010; Shuker, 2014).

In practice, the huge antennae of *A. aphidimyza* can still be under sexual selection, or the process of sexual selection could have led to the fixation of this trait. Indeed, if long antennae are very important to obtain a mating partner, males with short antennae could fail to reproduce, and thus not transmit their genes. In this case, the variability observed at the level of antennae for males could just be phenotypic plasticity (Borgia, 1979; Taylor & Williams, 1982; Cézilly & Allainé, 2016) and females could thus choose their mate according to other traits, that are also important for reproduction, like wings or body size.

5. Measured phenotypic traits in males

The morphological parameters of males that will be analysed in the subsequent experiments (next four articles) are presented hereafter.

5.1. Antennae

The length of both antennae (for an illustration of the complete antennae of a male of *A. aphidimyza*, see FIGURE 1 in the point 1. of the CHAPTER I) will be measured, in millimetres. It is the total length of the flagellum that will be assessed, the scape and pedicel will not be considered (structures illustrated in FIGURE 4 in the point 4.1. of the CHAPTER I).

Since antennae are studied for their sexual dimorphism in *A. aphidimyza,* probably shaped by sexual selection, the symmetry in their size will also be evaluated, in addition to their absolute sizes. Indeed, the size and the symmetry of traits under sexual selection (if fluctuating asymmetry reflects the developmental stability, related to the quality of an individual) are often positively related to the mating success (Thornhill & Møller, 1998; Blanckenhorn *et al.*, 2003; Polak, 2008). The symmetry of antennae of an individual will be estimated by subtracting the length of the left antenna from the length of the right antenna, both in millimetres. When the sign of the difference is less relevant than the value of the difference itself, the symmetry of antennae will rather be estimated by the absolute value of the difference between the lengths of right and left antennae.

5.2. Wings

Wings play a role in the mating behaviour of *A. aphidimyza*: standardised movements can be observed during courtship and mating, and individuals have to fly to a spider web to mate (van Lenteren *et al*., 2002; van

Lenteren & Schettino, 2003). As the measurement of wings should give an interesting insight into the mobility of males, the area of the right wing (FIGURE 8) will be measured, in square millimetres. To avoid any ambiguity in the measurements, benchmarks will be taken at the level of the wing veins.

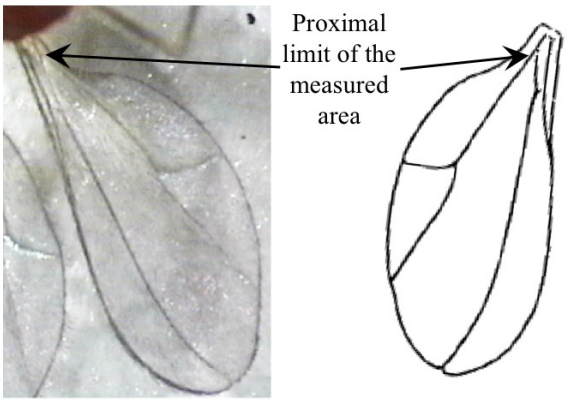


FIGURE 8. Wing of *A. aphidimyza*.
On the left: photograph.
On the right: drawing, from Harris (1966).

5.3. Hind tibias

The length of a hind tibia (FIGURE 9) will be measured as a proxy of the body size (Jann et al., 2000; Blanckenhorn et al., 2003, for another Diptera), in millimetres. The end of the tibia should not to be confused with the first tarsal article, which is very small.

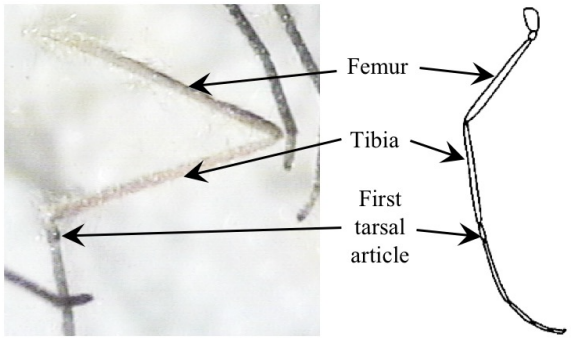


FIGURE 9. Structure of a leg of *A. aphidimyza*.
On the left: photograph.
On the right: drawing, from Harris (1966).

CHAPTER III. MORPHOLOGY AND REPRODUCTIVE BEHAVIOUR OF MALES OF *APHIDOLETES APHIDIMYZA*

1. Introduction

In order to understand, in practice, the role played by sexual selection in the antennal sexual dimorphism in *Aphidoletes aphidimyza,* this chapter will aim at determining whether:

- Antennae of males of *A. aphidimyza* are variable in length or symmetry.
- The ability for males to obtain a mate or a better-quality mating partner varies according to length and symmetry of antennae.
- These variations in antennae length and symmetry are inheritable.

If antennae are indeed a major driver of the access of males to a mate, sexual dimorphism could have been shaped largely by sexual selection.

Through the experiments presented in the ARTICLE 2 (Nicolas *et al.*, in prep.b), the variability in the morphology of males under constant rearing conditions will be assessed and information on sexual selection, especially intrasexual selection, will be brought to light. For that, the morphology of males, in particular length and symmetry of antennae, will be connected to their success to detect and access to sex pheromone in olfactometry choice experiments.

The ARTICLE 3 (Nicolas & Hance, in prep.a) will study the sexual selection through mating experiments, encompassing both intrasexual and intersexual selections. The morphology of the males and their antennae will be linked to the mating status (mated or not mated), to the quality of the obtained mate and to the time to achieve a mating.

2. Morphology of males of *Aphidoletes aphidimyza* and intersexual selection

ARTICLE 2

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, especially in the monoandrous ones. In addition to their importance in intrasexual selection, antennae can be seen as a useful ornament 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 the battle to reach 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 the lab conditions. Length of antennae of males of *A. aphidimyza* is remarkable and the right antenna is longer than the left antenna, even though this asymmetry is not explained by the measured parameters. Moreover, there are positive linear relations between antennae, wing and tibia sizes. Secondly, choice experiments, with males of *A.*

aphidimyza facing the sex pheromone, were conducted in a Y-shaped olfactometer. The relationships between choice patterns and morphology of males were then studied, but no link has been found between the morphology of males and their behaviour while exposed to the sex pheromone, although males were indeed attracted by the arm of the olfactometer that contained the sex pheromone.

Keywords

Antennae, *Aphidoletes aphidimyza*, male, morphometry, olfactometry, sexual dimorphism

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 results 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), mainly present in males (Emlen *et al.*, 2005).

When males have locomotor or sensory organs more developed than females, it is often due to the competition by exploitation. In this way, the intrasexual selection promotes traits that help to locate and reach a mating partner (Darwin, 1871a; Cézilly & Allainé, 2016; Johnson *et al.*, 2017). The case of antennae is thus rather particular since they have a role in the perception of pheromones. Good examples are the well-developed antennae in males of some species of moth (Shiel *et al.*, 2015), enabling them to detect female sex pheromone at very low concentration or over very long distances (Symonds e*t al.*, 2011). To achieve this, feathery antennae are useful to sample a large amount of air (Chapman, 1998) and long antennae offer much room for sensilla (Schneider, 1964; Chapman, 1998) and extend the breadth of sweep (Johnson *et al.*, 2017). Moreover, quickly finding the female is crucial in monoandrous species, to give the male the opportunity to mate (Shiel *et al*., 2015). Antennae can also correspond to a useful ornament in intersexual selection, the male with longer and more symmetric antennae being advantaged for mating because it is an indicator of its genetic quality (Møller & Zamora-Munoz, 1997; Koshio *et al.*, 2007), according to the handicap principle (Zahavi, 1975). Amazingly, the prediction of Darwin on the evolution of sensory organs by sexual selection,

although formulated almost 150 years ago, still lacks corroborating data (Johnson *et al.*, 2017).

Like most Aphidoletini, the cecidomyiid *Aphidoletes aphidimyza* preys on aphids at the larval stage (Harris, 1966). The antennae of this species present a pronounced sexual dimorphism visible with the naked eyes: males exhibit very long, curved and feathery antennae (FIGURE 1) while females have short straight antennae (Markkula & Tiittanen, 1985; Zhang & Yang, 2008; Tabadkani *et al.*, 2012b, 2012c; Tabadkani *et al.*, 2013). The antennae of males are composed of 12 flagellomers, 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, females of *A. aphidimyza* generally suspend by the front legs in a spider web and emit a sex pheromone – (2R,7S)-diacetoxytridecane (Choi *et al*., 2004a) – to attract a male. Once a male lands in the web, it moves to the female frontally while wing-fanning. The mating will take place during the scotophase in a "face to face" position in the spider web. During mating, the male first extends the wings and after folds them against its body, like the female (Harris, 1973; van Schelt & Mulder, 2000; van Lenteren *et al.*, 2002; van Lenteren & Schettino, 2003). After mating, the female stops emitting sex pheromone and refuses subsequent matings, which strongly suggests that this species is monoandrous (van Lenteren *et al.*, 2002; van Lenteren & Schettino, 2003). This might indicate an intrasexual selection by sperm competition (Cézilly & Allainé, 2016) in which males could transmit, during mating, substances that inhibit the sexual receptivity of females to prevent copulation with a rival, as it occurs in other Diptera (Leopold, 1976; Andersson, 1994).



FIGURE 1. Male of *Aphidoletes aphidimyza* ©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 insure a better detection of the female sex pheromone. Antennae could also be an ornament that constitutes a handicap during flight, due to their large size relative to the body size. Wings are used by males to fly to the female for mating in a spider web and their movements during mating are standardised. As both antennae and wings seem important for reproduction of males, their size could vary in the same way, like the body size. Bigger males could thus present larger structures, these three traits being correlated (Koshio *et al.*, 2007). Moreover, males with longer and more symmetrical antennae should reach more easily the source of sex pheromone, and wide wings could help them to cope with their long antennae.

The aim of this research was thus to better characterise the variability in the morphology of males of *A. aphidimyza* and to analyse males behaviour when facing the sex pheromone, in relation to their morphology, which could bring data to support the Darwin's prediction. First, a morphometric study was performed to apprehend the phenotypic variability in *A. aphidimyza* males under the lab conditions. The length of antennae, the area of a wing and the length of a hind tibia, as proxy for the body size (Jann *et al.*, 2000; Blanckenhorn *et al.*, 2003, for another Diptera) were investigated. Secondly, choice experiments with males facing the female pheromone were conducted in a Y-shaped olfactometer. The relationships between choice patterns and morphology of males were then studied.

Materials and methods

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

Morphometry

To analyse the range in size of males of *A. aphidimyza* in the lab conditions, 49 individuals less than 24 hours old were sampled and stored in a freezer at -20 °C. These males were then spread in a Hanks' balanced salts

solution (98 g/l), with the antennae, tibia and wing to measure well flat, and pictures were taken. The following parameters were measured afterwards:

- Length of both antennae (the total length of the flagellum, without scape and pedicel, the curvature of antennae having been well considered).
- Left hind tibia length, as proxy of the body size.
- Right wing area.

For each parameter, the mean of three measures was taken, in order to diminish the variability related to the measurement (as suggested by Palmer & Strobeck, 1986, by Merilä & Björklunds, 1995 and by Blanckenhorn *et al.*, 2003).

The symmetry of antennae was estimated by subtracting the length of left antenna to the length of right antenna.

An indicator of wing loading (body weight on wing area, Danforth, 1989) – which cannot be calculated because the body weight of individuals is unknown – was calculated by taking the ratio of the cube of tibia length divided by area of wing, in mm.

Olfactometry

To relate the behaviour of males of *A. aphidimyza* towards the sex pheromone with the variation in their morphology, olfactometry choice experiments were carried out. Because of the nocturnal habits of *A. aphidimyza*, pupae were placed under a reverse photoperiod (dark from 9 a.m. to 5 p.m.) after sand sieving (approximately one week before adults emergence – Le Goff *et al.*, 2016) to run experiments in the daytime. Pupae were isolated in microtubes of 1.5 ml with a piece of cotton swab soaked in a solution of ¼ sugar and ¾ of water to allow adult feeding. For each replicate, one fed virgin male less than 72 hours old was used. Experiments were realised 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° (FIGURE 2), inspired by Choi *et al.* (2004a). All the 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, to not disturb the nocturne behaviour of individuals 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 arm of the Y olfactometer 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 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 for the evaporation of pentane prior to use. The filter papers were then placed at 2 cm of the orifice of the arms. A line was traced under the olfactometer 6 cm after the beginning of the arms. The position of the olfactometer and the arm where sex pheromone and pentane alone were placed were rotated, so that the pheromone was put the same number of times in the left arm as in the right arm.

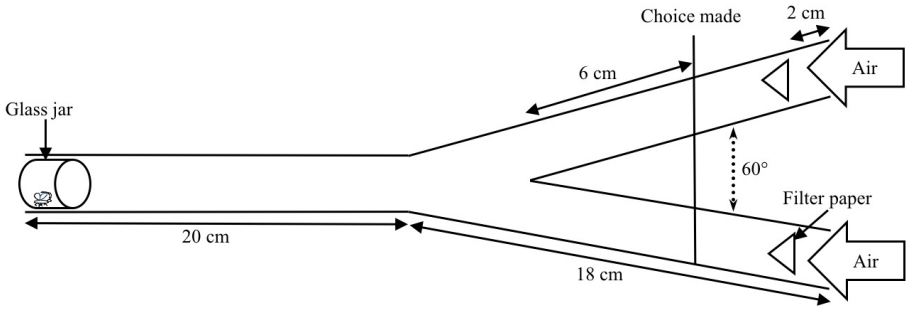


FIGURE 2. Configuration of the Y olfactometer, with the glass jar containing the insect to the left, and the filter papers fold in a cone shape to the right.

To start a replicate, a male of *A. aphidimyza* trapped in a glass jar was introduced in the central branch of the olfactometer in order to choose between the arm containing the female sex pheromone and the arm without. In case the male did not leave the glass jar after 5 minutes or if it did not make a choice within 10 minutes after exiting the jar, the insect was considered "not responding". All the other insects were considered "responding". The time to exit the jar and to cross the line traced under the olfactometer (choice made, end of test) were recorded. The way the male went to the odour source (flight or walk) was also observed. All the males (N = 78) were stored in the freezer after the experiment, 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 "Morphometry" (vide supra) were measured and calculated for all the males, except for antennae for which only the first six flagellomers were measured (half of the antenna because of frequent breakages of last flagellomers when males were retrieved of the device), and for the right hind tibia that was measured rather than the left hind tibia.

Statistical analyses

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

Morphometry

The margin error indicated is standard error (SE).

The standard error on the measurements of antennae was calculated for each individual. Afterwards, the mean of the standard errors for all the individuals was calculated, to obtain the measurement error on antennae, as it is especially important for the asymmetry studies (Palmer & Strobeck, 1986; Merilä & Björklunds, 1995; Lens *et al.*, 2002; Blanckenhorn *et al.*, 2003).

A D'Agostino & Pearson omnibus normality test was applied and the data were normally distributed, except for the difference between the length of the left and the length of the right antenna (estimation of the symmetry of antennae).

To check differences in length between both antennae of a 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 know if differences between antennae length distribute symmetrically compared to zero.

Olfactometry

A binomial test was used to determine if the proportion of males attracted by the arm with the pheromone among responding males differed from a random choice (sign test, 50/50 repartition). The same test was applied to know if the males that went to the selected arm of the olfactometer did it by flying as much as 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 know if morphology influence the time to exit the jar and to make a choice.

Results

Morphometry

Antennae

The lengths of right antennae of males ranged from 2.42 to 3.07 mm with a mean of 2.80 ± 0,02 mm (N = 49). The length of left antennae ranged from 2.36 mm to 3.05 mm with a mean of 2.77 ± 0.02 mm (N = 49). The measurement error for both the right antenna and the left antenna is 0.02 mm. Surprisingly, a significant difference was observed between the lengths of left and right antennae of a same male (Wilcoxon matched-pairs test: N = 49, P = 0.018) (FIGURE 3). The mean difference between both antennae of a same individual (estimation of the symmetry of antennae) is 0.03 ± 0.01 mm, with a minimum of -0.12 mm, a maximum of 0.29 mm and the coefficient of variation of this parameter is 265.26 %. The difference between the lengths of the right antenna and of the left one does not distribute symmetrically compared to zero (Wilcoxon signed rank test: N = 49, P = 0.019) (FIGURE 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).

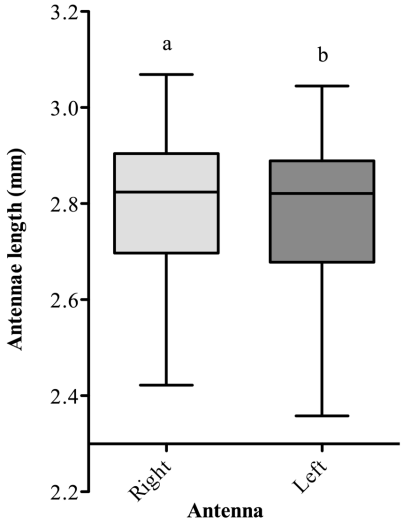


FIGURE 3. Comparison of lengths of antennae of a same male (Boxplot, N = 49, different letters represent significantly different means according to Wilcoxon matched-pairs test; the error drawn on the graph corresponds to the SE).

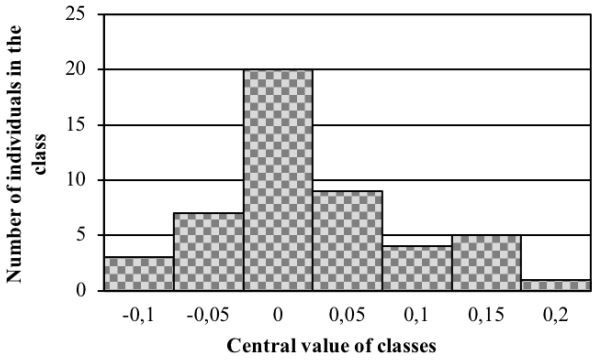


FIGURE 4. Frequency distribution of the differences between the length of right antennae and the length of left antennae (N = 49).

In order to try to determine the relationship between these differences of right and left antennae and the other parameters, the coefficient of determination was calculated for the linear regressions with the differences between both antennae and the size of right antenna ($R^2 = 0.01$), of left antenna ($R^2 = 0.15$), of left hind tibia ($R^2 = 0.00$) and of right wing ($R^2 = 0.01$) and they are all almost null. The variance in the asymmetry of antennae is therefore not explained by the size of antennae nor of the tibia or the wing.

The ratio between lengths of left antenna and of left hind tibia was calculated to weight the variability due to the body size. The comparison of coefficients of variation indicates that there is a little more variability in the lengths of left antenna than in the ratio of the lengths of left antenna to the lengths of left hind tibia (5.65 % against 4.33 % respectively).

Tibia and wing size and relationships with antennae

The length of 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 right wing ranged from 0.97 mm2 to 1.52 mm2 with a mean of 1.29 ± 0.02 mm2 (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).

There are positive linear relations between:

- The length of the right antenna and the body size (estimated by the length of left hind tibia) ($R^2 = 0.67$).
- The length of the left antenna and the body size ($R^2 = 0.57$).
- The length of the right antenna and the area of the right wing ($R^2 = 0.78$).

- The length of the left antenna and the area of the right wing (R^2 = 0.61).
- The body size and the area of the right wing (R^2 = 0.80).

When the influence of body size is removed by dividing the length of antennae and the area of the right wing by the length of the tibia, the linear relation between antennae and wing is no longer present (R^2 drops to zero).

Olfactometry

Twenty-seven males out of the 78 (34.6 %) entered the olfactometer and 21 males out of the 78 (26.9 %) made a choice. 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 (4 individuals) and this is not a random behaviour (sign test: N = 21, P = 0.007).

No difference could be highlighted between the morphology of responding (N_1 = 17) and not responding males (N_2 = 40) (Mann-Whitney test applied on the length of ½ right antennae (U = 331.0, P = 0.882), differences between ½ right and ½ left antenna (symmetry, U = 325.5, P = 0.807), body sizes (estimated by length of right hind tibia, U = 289.0, P = 0.378), area of the right wings (U = 313.0, P = 0.644)).

No difference could be brought out between males attracted by the arm with the sex pheromone and the ones attracted by the control arm, because the morphological parameters for only two of the males attracted by the control are complete which is not enough to make a statistical test.

Males took on average 90 ± 25 s to exit the jar and an additional 86 ± 31 s to make a choice (N = 17). There is no linear relation between the time 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 remarkable, with a mean length for right antennae of 2.80 mm and for left ones of 2.77 mm. In comparison, the length of antennae of a wild individual trapped in Russia was of 2.85 mm (for a body size of about 2 mm long – Markkula & Tiittanen, 1985; Jandricic, 2013; Fedotova, 2014), which is similar to the results obtained here. There is slightly more variability in the length of left antennae (coefficient of variation of 5.65 %) than in the ratio of lengths of left antenna and of left hind tibia (coefficient of variation of 4.33 %), but the difference is weak, so the body size is not the only driver of this variability. The symmetry of antennae (length of the left antenna minus length of the right one) present an enormous coefficient of variation (265.26 %). The mean length of 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 could be interesting to find a way to weigh males without breaking their fragile parts that are also important to measure (antennae, tibias and wings) (Danforth, 1989), or to estimate correctly the weight of males. For example, the relationship between weight and tibia size could be calculated for a sample of individuals reared in the same conditions as the individuals used in the experiments. Afterwards, this relationship could be used for the individuals of the experiments, to convert the tibia size into weight (to be used to calculate a more accurate wing loading).

There are positive linear relations between antennae, wing and tibia sizes. However, when the length of antennae and the area of right wing are reported to the length of tibia, removing the influence of the body size, the linear relation between antennae and wing is no longer present indicating that they are both related to the body size. Generally speaking, great individuals have thus also long antennae and wide wings probably because individuals with long antennae need a big body and wide wings to cope with this exaggerated trait (McCullough & Emlen, 2013; Painting & Holwell, 2013). As larvae of *A. aphidimyza* were fed *ad libitum*, the resources available for the development were not limited. Maybe a rearing with limited resources shall enforce a trade-off between the production of these very long antennae, which represents a significant energy cost, and the development of other body parts (Emlen, 2001).

The absolute difference between the lengths of both antennae is not striking (FIGURE 3) but significant. However, nothing in the measured parameters above gives a hint about what influences the symmetry of antennae. Moreover, it is difficult to conclude between directional asymmetry (mean of

the right side minus the left side differs from zero) and antisymmetry (frequency distribution of the right side minus the left side is not normal) (Van Valen, 1962; Palmer & Strobeck, 1986; Palmer & Strobeck, 1992; Demers, 1999). These types of asymmetry differ from fluctuating asymmetry because they do not only result from random perturbations by environment during the 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 right antenna and of the left one seems to present a skewed distribution (FIGURE 4), that could be generated by the presence of both directional asymmetry and antisymmetry (Palmer & Strobeck, 1992). However, this astonishing asymmetry of antennae should be considered with caution (Demers, 1999) because the difference found between the lengths of both antennae (0.03 mm) differs only by 0.01 from the measurement error (0.02 mm) of the length of the whole antennae.

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 males exited the jar, almost 4/5th of them made a choice and they chose the arm with the sex pheromone in 81 % of cases. Despite all this, the morphology of males cannot be related to the fact they responded or made a choice, nor to the time to make a choice. Perhaps the morphological parameters measured were not the right ones to explain the choice behaviour toward the sex pheromone. For example, control of the composition in sensilla could be interesting, but these are difficult to assess because of their very small size. The number of sensilla can be linked to the area of the antenna (Schneider, 1964; Chapman, 1998), but the type of sensilla could be more important in the perception of sex pheromone (Zhang & Yang, 2008) than the length of antennae itself. Moreover, antennae could be an important ornament 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 only the 6 first flagellomers out of 12, because of the difficulty to collect males of the device after experiments. This potentially leads to under evaluate the variability between males that could arise from a difference at each flagellomer, but also marks itself just at the end of the antenna that has been lost, as the terminal flagellomer is a little different from the others (Gagné, 1994).

In accordance with what could be expected with 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 the reality. 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 if 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 bring out a link between the morphology of males and their behavioural response to the sex pheromone. The evident sexual dimorphism of *A. aphidimyza* requires further testing to understand how and why this particular morphology was shaped. Mating experiments where females have the choice between different male phenotypes (intersexual selection) or where males are in competition for a same female (intrasexual selection) are thus needed. Indeed, we highlighted a phenotypic variability of males under constant lab conditions and this variability cannot be explained. Exacerbating phenotypic differences between males, for example by giving different diets at larval stage or by rearing the insects for experiments at different but not stressful temperatures could improve results.

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3. Mating behaviour and sexual selection in males of *Aphidoletes aphidimyza*

ARTICLE 3

Mating Behaviour of *Aphidoletes aphidimyza* and Importance of a High Antenna to Tibia Ratio in Males for Sexual Selection

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Abstract

Sexual dimorphism is common in insects. Antennae are often sexually dimorphic, especially when females emit a sex pheromone to attract a mate. Moreover, antennae are ornaments in some species. Nevertheless, cumbersome antennae can be a cost for the viability of their bearer. Sexually dimorphic antennae, like those of *Aphidoletes aphidimyza*, thus have probably been favoured by sexual selection, rather than by natural selection. The cost of the ornament for the survival of the male could be compensated by the benefits of getting a mate, or a better-quality mate. The mating of *A. aphidimyza* takes place in the dark and is quite astonishing. The female hangs on a spider web and calls for a mate by emitting a sex pheromone. In *A. aphidimyza*, sexual dimorphism is striking: males have long and feathery antennae while females have short antennae. The antennae of males could derive mainly from sexual selection, as they have an important role in the reproduction. Mating experiments were conducted to determine if longer and more symmetric antennae than those of other males facilitate access to females. For each replicate, two males were released in a cage with two females until a mating occurred. The individuals were then collected and the antennae, the right hind tibia and the right wing of males were measured. The comparison of mated and not mated males reveals that the main morphological difference is the ratio of the length of antenna to the length of tibia. A male with a small tibia, and thus a small body, compared to the length of its antennae has a higher probability to mate than a male with a larger body but proportionally smaller antennae. Moreover, bigger males tend to copulate with bigger females and the time to achieve a mating seems to increase when morphological differences between males are less marked, which possibly indicates an assessment of males by females prior to the selection of a male to

mate. This gives a first insight into the potential causes of the sexually dimorphic antennae of *A. aphidimyza.*

Keywords

Aphidoletes aphidimyza, antennae, male, mating, sexual selection, sexual dimorphism

Introduction

In insects, sexually dimorphic traits are common (Shine, 1989; Stillwell *et al.*, 2010). Males and females of a same species often differ by structures at the level of the head (Emlen, 2001) like mandibles (Kawano, 2006) or antennae (Darwin, 1871a; de Freitas Fernandes *et al*., 2002; Steiner *et al.*, 2010; Wilhelm *et al.*, 2015). As antennae are the main organs of olfaction (Chapman, 1998; Boddum, 2013), their specialization to detect a mate is generally well visible in species using a sex pheromone (Steinbrecht, 1987). When males have to locate females by her scent, antennae of males are often more complex than those of females (Chapman, 1998). Moreover, antennae are considered as an ornament in some species of insects, like in Diptera (Sivinski, 1997). In this case, males with longer (*Cnephasia jactatana*, Tortricidae – Jiménez-Pérez & Wang, 2004) or more symmetric (*Stenurella melanura*, Cerambycidae – Møller & Zamora-Munoz, 1997) antennae (*Elcysma westwoodii*, Zygaenidae – Koshio *et al.*, 2007) are more likely to copulate.

As an ornament, big and plumose antennae of males are supposed to be a cost for the viability of their bearer (Andersson, 1994) and only larger males can bear this cost (Symonds *et al.*, 2011). Extravagant sexually selected traits may be a cost in various ways. For example, they can increase the vulnerability to predation, represent a big energetical investment or disrupt the development of other structures (Emlen, 2001; Cézilly & Allainé, 2016). Big antennae may also impede movement and flight (Symonds *et al*., 2011). All these costs can be compensated by the benefits of getting a mate (Futuyma, 2005) or a mate of better quality (Shuker, 2010; Shuker, 2014). Indeed, large antennae can be useful for a good detection of female pheromones (Johnson *et al.*, 2017) and males with the best developed ornaments could be more likely chosen by females for mating (Jiménez-Pérez & Wang, 2004; Møller & Zamora-Munoz, 1997; Koshio *et al.*, 2007). The symmetry and the size of sexually selected traits (indicating the genetic quality of the male) are indeed often positively related to the mating success (Thornhill & Møller, 1998; Polak, 2008). Bigger antennae should thus be an advantage for sexual selection (Jiménez-Pérez & Wang, 2004;

Koshio *et al.*, 2007). So, sexually dimorphic antennae, like those of *Aphidoletes aphidimyza*, should probably have been shaped by sexual selection rather than by natural selection.

Mating of *A. aphidimyza* takes place during the night (Harris, 1973) and is quite astonishing. Females hang on a spider web and call for a male to copulate by emitting a sex pheromone (van Lenteren *et al.*, 2002; van Lenteren & Schettino, 2003; Choi *et al.*, 2004a). The attracted male then lands in the spider web, moves to the female frontally while wing-fanning and bends its abdomen toward the female to grip its genitalia. During the mating, the male first extends its wings and afterwards folds them against its body, like the female. The mating lasts between 2 and 3 minutes. Just after the mating, the female no longer calls and even flies away if a male is approaching. The ending of the call behaviour by mated females and their refusal to mate again strongly suggest that females are monoandrous (a female copulates with only one male), while males are polygynous (a male copulates with several females) (van Lenteren *et al*., 2002; van Lenteren & Schettino, 2003; Tabadkani *et al.*, 2012a).

In *A. aphidimyza*, sexual dimorphism is striking: males have long, grey, curved and feathery antennae while females have short, dark and straight antennae (Markkula & Tiittanen, 1985). Antennae have a crucial role in the mating of this species. Indeed, males have to locate females by a sexual pheromone, thanks to their antennae (van Lenteren *et al.*, 2002; van Lenteren & Schettino, 2003; Choi *et al.*, 2004a), and larger antennae could be advantageous for them (Johnson *et al.*, 2017). Moreover, as a female is supposed to mate only once (van Lenteren *et al*., 2002; van Lenteren & Schettino, 2003; Tabadkani *et al.*, 2012a), it could be choosy and copulate with a male bearing a more attractive ornament (Jiménez-Pérez & Wang, 2004; Møller & Zamora-Munoz, 1997; Koshio *et al.*, 2007). The hypothesis is thus that impressive antennae of males of *A. aphidimyza* derive mainly from sexual selection, which has never been studied in this species. Longer and more symmetric antennae than those of other males should then facilitate the access to a mate or to a better-quality mating partner.

Wings can also be considered as determinant for the reproductive behaviour of males *A. aphidimyza*. Similar wing movements are usually observed during courtship and mating (see third paragraph for more details) and males have to fly to reach a mating partner (van Lenteren *et al*., 2002; van Lenteren & Schettino, 2003). Moreover, wide wings should be needed to cope with antennae that can hamper the flight due to their size (Symonds *et al*., 2011). Furthermore, the body size is known to influence the mating success of males in many insects and particularly in Diptera (Drosophilidae – Partridge *et al.*, 1987;

Culicidae – Yuval *et al.*, 1993; Scatophagidae – Blanckenhorn *et al.*, 2003; Piophilidae – Bonduriansky & Rowe, 2003). In addition to antennae, the wings and the body will thus also be measured to evaluate their role in the mating success of males.

Since, in insects, female body size is generally related to its fecundity, larger females often being more fecund than smaller ones (Honěk, 1993; Stillwell *et al.*, 2010), a male should prefer mating with a larger female, which represents a reproductive advantage (Saeki *et al.*, 2005). Males with larger antennae could possibly better discriminate between large and small females. It is thus interesting to know whether the size of phenotypic traits of mated males is related to the body size of the female obtained, as body size potentially informs about fecundity, and thus quality, of the mate (Honěk, 1993; Saeki *et al.*, 2005).

Mating experiments were conducted to know if, in a contest situation, the size of the antennae, or of another morphological indicator, may explain the mating success of male *A. aphidimyza*. To test that hypothesis, two males were released in a cage containing two virgin females. Once a mating occurred, the experiment was stopped and the individuals were collected for the measurement of their antennae, tibias and wings. These parameters were compared for successful males and unmated males. The relation between the phenotypic traits of males and the body size of the mate obtained, as well as the relation between the morphological differences between mated and not mated males and the time to achieve a mating, were also analysed. All these results will be discussed hereunder in terms of sexual selection and its implications on antennal sexual dimorphism in *A. aphidimyza*.

Materials and methods

Production of insects

Individuals of *Aphidoletes aphidimyza* Rondani were bred like described in Kohandani *et al.* (2017), inspired by Le Goff *et al.* (2016). The strain of the prey, the aphid *Myzus persicae* Sulzer, was initially provided by Viridaxis S.A. (Gosselies, Belgium). Preys were reared on plants of *Capsicum annuum* L. (sweet pepper). A plant infested with aphids was placed in contact of *A. aphidimyza* to obtain eggs. Plants with eggs of *A. aphidimyza* were then placed in another cage above sand to collect the larvae that were ready to pupate and thus of known age. Afterwards, the sand was sieved to recover pupae. The breeding room was at 23 °C, with a relative humidity of 60 to 70 % and a photoperiod of 16 hours.

Experiments

The aim was to determine the morphological characteristics of successfully mated males in a competition situation (2 females, 2 males).

Individuals used in the experiment were coming from pupae kept between 23 and 24 °C, with a reverse photoperiod (light from 4 p.m. to 8 a.m.), as soon as they were isolated from the sand (roughly one week before adults emergence – Le Goff *et al.*, 2016). The photoperiod was reversed in order to carry out experiments in the daytime, in spite of the nocturnal habits of *A. aphidimyza*. Pupae were separated from each other in microtubes of 1.5 ml before adults emergence. To feed adults after their emergence, a cotton swab tip soaked in a solution of ¾ of the volume of water and ¼ of white sugar was introduced in each microtube.

The experimental arena was constituted of a glass cage (30 by 30 by 40 cm^3) with one lateral wall partially made of plexiglass and just a small part of fine mesh for aeration. To mimic a spider web, a fine mesh was attached to the ceiling of the cage for insects to hang for mating. Temperature ranged between 23 and 24 °C. To observe this species with nocturnal habits, the arena was only lightened with a red light (40 W red bulb) from 8 a.m. to 1 p.m. Insects are actually not sensitive to the red wavelength (Schaefer & Wilkinson, 2004).

For each replicate, 2 fed virgin females, randomly chosen and between 24 and 48 hours old, were released into the glass cage. They were placed in the device 30 minutes before males, for these females to have the time to settle and to begin to emit sex pheromone. With 2 females et 2 males for each replicate, there are potentially 2 individuals presenting different characteristics every time, and a possibility of choice for the two sexes. Thirty minutes after females, 2 fed virgin males, randomly chosen and between 24 and 48 hours old, were added in the cage. Observations were made during 30 minutes. When a mating occurred, the experiment was stopped and the time to obtain the mating was noted. All the individuals were then collected and stored in a freezer at -20 °C, separated between mated and not mated. If no mating occurred in 30 minutes, the experiment was stopped, all the individuals were retrieved and new ones were used for the subsequent experiments. Between two experiments, a little fan was placed in front of the open cage for 15 minutes.

Data collection

Males were placed in a Hanks' balanced salts solution at a concentration of 98 g/l to take pictures of their antennae, wings and tibias (Nicolas *et al.*, in prep.b). Pictures were taken using a binocular microscope at 16x magnification with CamUniversal software version 3.5 for Windows XP. Afterwards, the following phenotypic traits (for an illustration, see CHAPTER II, point 5.) were measured with the ImageJ software for Mac:

- The length of both antennae, without considering the scape and pedicel. It was not possible to measure the full size of antennae of all individuals because of the frequent breakage due to insect activity in the cage or to manipulations for recovering them. In consequence, the length of the first nine flagellomers was used as a proxy of the whole antenna size.
- The right hind tibia length, as a proxy of the body size (like Jann *et al.* (2000) and Blanckenhorn *et al.* (2003) for another Diptera).
- The right wing area, as a proxy of the mobility.

For each parameter, the mean of three measures was taken, in order to diminish the variability related to the measurement (as suggested by Palmer & Strobeck, 1986, by Merilä & Björklunds, 1995 and by Blanckenhorn *et al.*, 2003).

On the basis of measured parameters, others were inferred:

- The symmetry of antennae (|RA-LA|), estimated by the absolute value of the difference between the lengths of right and left antennae, for each individual.
- The ratio of right antenna length to tibia length, to determine whether antenna size is directly related to body size.
- The ratio of wing area to tibia length, to withdraw the body size component from the potential variations in wing area.

Besides, the right hind tibia of females was measured, according to the procedure described for males, to evaluate their body size, as a proxy of their fecundity and their quality.

Males put together in the cage for a same replicate were identified by their mating status (mated or not mated) and compared by pair for the above parameters. It has been determined whether the mated male was smaller or larger than the not mated one for each parameter of each replicate. This was then summed up for all replicates The numbers of mated males that were the smallest or the largest of their replicate for each phenotypic trait were then counted for all replicates (N = 18).

Statistical analyses

Statistical analyses were conducted using GraphPad Prism version 5.0 software for Mac (GraphPad Software, San Diego, California, USA, http://www.graphpad.com). All tests were performed with a significance level (P) set at $\alpha = 0.05$ and under two-tailed hypothesis, unless otherwise stated. The margin error which is mentioned is the standard error (SE).

Morphology of mated vs. not mated males

A binomial test was used to determine whether the proportion of mated males that were the largest male of their replicate, to the total number of replicates, is different from a random repartition (one-tail sign test, 50/50 repartition).

A Student t-test for paired samples was performed to know if the measured parameters are different according to the mating status of the males in one replicate. Only for wing area and ratio of wing area to tibia length, a Wilcoxon matched-pairs test was performed to determine if parameters are different according to the mating status of the males, as the differences between mated and not mated males did not follow a Gaussian distribution.

Influence of phenotypic traits of males on the body size of the mate

The D'Agostino & Pearson omnibus normality test was applied. As not all the data were normally distributed, Spearman correlation coefficients (r_s) were calculated to know if the morphological parameters evaluated in mated males are related to the tibia size of the mated females (proxy of the fecundity and the quality).

Morphology and time to achieve a mating

The D'Agostino & Pearson omnibus normality test was applied. As not all the data were normally distributed, Spearman correlation coefficients (r_s) were calculated to know if the differences of size between the morphological parameters of mated and not mated males are related to the time to obtain a mating.

Results

Morphology of mated vs. not mated males

The number of mated males that had the largest antennae of their replicate cannot be considered as different from the number of mated males that had the smallest antennae, according to the binomial test (TABLE 1). Nor can these numbers thus be considered as different from what would have happened by chance (50/50 repartition). The conclusions are the same for the symmetry of antennae (|RA-LA|), for the length of the right hind tibia, for the area of the right wing and for the ratio of wing area to tibia length. Contrariwise, the number of mated males that had the largest ratio of the right antenna to the right hind tibia can be considered as different from the number of mated males that had the smallest antenna to tibia ratio. There is a higher proportion of mated males that present a higher antenna to tibia ratio.

The mean length of antennae is not different between mated males and not mated males, and the symmetry of antennae neither, according to the paired Student t-tests associated to these means. The conclusions are the same for the means of tibias, wings and their ratios, according to the paired Student t-test and the Wilcoxon matched-pairs tests. Contrariwise, the mean ratio of right antenna to right hind tibia is significantly different between mated and not mated males. The mean ratio is higher for mated males than for not mated males, which means that, on average, mated males have relatively longer antennae, compared to their body size, than not mated males.

TABLE 1. Counts (first line) and results of the associated binomial tests (second line), means (± SE) for mated and not mated males (third and fourth lines) and results of the paired Student t-tests associated to means (fifth line) for the morphological parameters measured and inferred in males (lengths of the right and left antennae, symmetry of antennae, ratio of the right antenna length to the right hind tibia length, length of the right hind tibia, area of the right wing and ratio of the right wing area to the right hind tibia length).

	Right antenna (RA)		**Left antenna (LA)**		**|RA-LA|**		**RA/tibia**	
	Small	**Large**	**Small**	**Large**	**Small**	**Large**	**Small**	**Large**
Number of mated males	11	7	11	7	11	7	5	13
Binomial test (sign test, N = 18)	NS P = 0.240		NS P = 0.240		NS P = 0.240		* P = 0.048	
Mated males	1.90 ± 0.04 mm		1.90 ± 0.04 mm		0.02 ± 0.00 mm		2.34 ± 0.02	
Not mated males	1.92 ± 0.04 mm		1.92 ± 0.03 mm		0.03 ± 0.01 mm		2.28 ± 0.02	
Paired t-test (df = 17)	NS P = 0.645 t = 0.469		NS P = 0.767 t = 0.301		NS P = 0.120 t = 1.637		* P = 0.042 t = 2.205	

	Tibia		**Wing**		**Wing/tibia**	
	Small	**Large**	**Small**	**Large**	**Small**	**Large**
Number of mated males	11	7	12	6	12	6
Binomial test (sign test, N = 18)	NS P = 0.240		NS P = 0.119		NS P = 0.119	
Mated males	0.81 ± 0.02 mm		1.08 ± 0.04 mm^2		1.33 ± 0.03 mm	
Not mated males	0.84 ± 0.02 mm		1.13 ± 0.04 mm^2		1.34 ± 0.03 mm	
Paired t test or Wilcoxon matched-pairs test	NS P = 0.154 t = 1.493 df = 17		NS P = 0.327		NS P = 0.486	

Influence of phenotypic traits of males on the body size of the mate

The Spearman correlation coefficients (r_s), listed below, which characterise the linear relation between morphological parameters of mated males, as presented in TABLE 1, and tibia length of the female mate (proxy of the body size and of the quality) (N = 17) are all relatively low:

- Right antenna length: $r_s = 0.574$ (P = 0.016).
- Left antenna length: $r_s = 0.532$ (P = 0.028).
- Symmetry of antennae: $r_s = -0.387$ (P = 0.124).
- Right antenna length to tibia length ratio: $r_s = 0.167$ (P = 0.523).
- Tibia length: $r_s = 0.445$ (P = 0.073).
- Wing area: $r_s = 0.620$ (P = 0.008).
- Wing area to tibia length ratio: $r_s = 0.605$ (P = 0.010).

It seems that the larger the antennae, wing and wing to tibia ratio of the mated male were, the larger the female mate was. There are thus globally positive linear relations between the measured phenotypic traits of the mated males and the body size (and thus potentially the fecundity and the quality) of their female mate (except for the symmetry of antennae), but the relations are relatively weak, even though they are probably not due to chance.

Morphology and time to achieve a mating

The Spearman correlation coefficients (r_s), listed below, which characterise the linear relation between the difference in size of phenotypic traits between mated and not mated males of a same replicate and the time to achieve the mating (N=18) are all relatively low:

- Right antenna length: $r_s = -0.558$ ($P = 0.016$).
- Left antenna length: $r_s = -0.473$ ($P = 0.048$).
- Symmetry of antennae: $r_s = 0.093$ ($P = 0.713$).
- Right antenna length to tibia length ratio: $r_s = 0.006$ ($P = 0.980$).
- Tibia length: $r_s = -0.519$ ($P = 0.027$).
- Wing area: $r_s = -0.480$ ($P = 0.044$).
- Wing area to tibia length ratio: $r_s = -0.336$ ($P = 0.173$).

It seems that the larger the differences between the antennae, tibia and wing of the mated male and the not mated one of a same replicate were, the shorter the time to achieve a mating was. There are thus globally negative linear relations between the differences of measured phenotypic traits between the mated males and the not mated ones (except for the symmetry of antennae and the antenna length to tibia length ratio) and the time to achieve the mating, but the relations are relatively weak, even though they are probably not due to chance.

Discussion

The most important parameter for males to get to females in the experiments performed was the ratio of the length of the right antenna to the length of the right hind tibia. This ratio was higher for mated males than for not mated males (TABLE 1). Yet, a male having long antennae compared to its body still has a higher probability to mate, than a male with relatively shorter antennae. The other morphological parameters which were measured (length and symmetry of antennae, length of right hind tibia and area of right wing) do not reveal any other difference with regard to the mating status of the male.

It would be interesting to better understand the consequences of the relative size of antennae in terms of mobility, to know if the size of antennae could be considered as a handicap to fly or, on the contrary, if it improves stability or lift (Symonds *et al*., 2011). If it is a handicap, it may be that the antenna to tibia ratio corresponds to an honest signal of the quality of the male according to the Zahavi's handicap principle (intersexual selection). Indeed, if a male can survive with cumbersome antennae compared to the size of its body, it is a hint of its genetic quality. Therefore, males with the longest antennae compared to the size of their body could be preferred by females because transmitting this trait should improve the fitness of their offspring. Males with a higher antenna/tibia ratio could thus obtain an advantage for mating over males with a lower antenna/tibia ratio (Zahavi, 1975; Grafen, 1990; Smith & Smith 2009).

The Fisher's runaway process (intersexual selection) could also be a driving factor of this right antenna to right tibia ratio being higher for mated males than for not mated ones in the mating experiments. In this case, this high ratio would be interesting to transmit to the offspring because females prefer to reproduce with males that have long antennae compared to their body size. Accordingly, the high ratio in males and the preference for this high ratio in females would be transmitted to the next generation, improving the mating success of the offspring (Fisher, 1915; Cézilly & Allainé, 2016).

The mechanism of scrambles applied to intrasexual selection could also explain the sexual dimorphism at the level of the sensory organs that help to find a mating partner as fast as possible (Cézilly & Allainé, 2016). In species where the female emits a scent to mate, males with a better sense of perception would fecundate more females than others (Bateman, 1948). Thanks to their long and complex antennae, males of *Aphidoletes aphidimyza* would thus easily localise a female. Moreover, males with a small tibia, and thus a small body size, could possibly reach a mate faster than larger males. Therefore, since *A. aphidimyza* is a monoandrous species (Shiel *et al*., 2015) and finding the female first is really important, a high antenna to tibia ratio would represent a clear advantage for males.

In order to be transmitted, the advantageous phenotypic traits, like a high ratio of antenna to tibia, must obviously be inheritable. Because all males of the experiments were reared exactly in the same conditions, the part of the variability, observed in traits, that can be attributed to environment must be negligible, compared to the genetic variations. Consequently, there is no reason that differences observed between males were not transmissible.

Mating with a large female can represent a reproductive advantage for a male (Saeki *et al.*, 2005) because the fecundity of females is often related to their body size (Ricklefs & Miller, 2005). Besides, the competition for mates can focus on the quality in addition to the quantity (Shuker, 2014). It follows that the benefit obtained by males of *A. aphidimyza* with longer and more symmetric antennae could have been to copulate with a larger female (size being evaluated by tibia length, as a proxy of the quality) to produce many descendants. However, the relation between the length of antennae of the mated male and the size of the mate is relatively weak, although probably not due to chance. It thus seems that larger antennae do not really give an important advantage to males to copulate with a female of a better quality. The conclusion is the same for the relation between the other phenotypic traits measured in males and the size of the female obtained.

The time to achieve a mating tends to increase when the morphological differences between the mated male and the not mated one of a same replicate decrease, although the relation is weak. This could indicate a choice of the female: if the differences between the two males are less marked, the female could have more difficulties to evaluate the quality of males and to choose among them, and so the time to choose a mate would increase. Surprisingly, the time to achieve a mating is not related to antennae characteristics or to antenna to tibia ratio, even though this ratio seems important for males to obtain a mate.

The prediction that longer and more symmetric antennae than those of other males should facilitate the access to a mating partner or to a better-quality mate in *A. aphidimyza* is not confirmed by the results. Yet, the ratio of the length of the right antenna to the length of the right hind tibia, higher in mated males than in not mated males, gives a partial answer. Long antennae still seem to be important for the reproduction, but in relation to the body size. Moreover, to achieve a mating seems to decrease when differences between males increase, possibly indicating an evaluation of the males by the females before they choose (Fisher's runaway process or Zahavi's handicap principle).

Perhaps the males of the experiments were not different enough of each other to highlight a difference of mating success between them with regard to the absolute size of antennae alone. Maybe a mating preference can only be expressed by a female if there are enough phenotypic differences between males to detect the variability (Petrie, 1983; Jennions & Petrie, 1997).

So, it would be interesting to exacerbate the morphological differences between males used in experiments to see if the conclusions remain the same. Yet, it is very difficult to choose, for each replicate, a small and a large male,

because differences are not really visible to the naked eye. A solution might be to vary the rearing conditions to obtain males with different morphologies. For example, the males of a same replicate could come from rearing at different temperatures. Males from low temperature should be larger than males from high temperature (*Drosophila melanogaster*, Drosophilidae – Zamudio *et al.*, 1995). Another solution could be to feed larvae with different diet quality, as less food induces smaller adults (*Aphidoletes aphidimyza*, Cecidomyiidae – Uygun, 1971) and as males from rich larval diet should be larger than males from poor larval diet (*Telostylinus angusticollis*, Neriidae – Bonduriansky, 2007).

Furthermore, the results obtained on antennae concern the first nine flagellomers, because of the activity of the insects during the experiments and the difficulty to take the males out of the device after the experiments. The differences of size of the antennae between mated and not mated males are thus probably underestimated as a part of the antennae was not measured, which did not prevent to highlight differences of the antenna to tibia ratio according to the status of the male.

At this stage, no firm conclusion can thus be drawn on the causes of the sexually dimorphic antennae of *A. aphidimyza*. The average ratio of the length of the right antenna to the length of the right hind tibia was the main difference between mated and not mated males. The fact that this ratio is higher for mated males than for not mated ones gives a track to follow for future research, but not a definitive answer.

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4. Summary

In the ARTICLE 2 (Nicolas *et al.*, in prep.b), linear positive relations between antennae, tibia and wing were observed. Variations at the level of the antennae, particularly concerning their symmetry, have been highlighted between males of the rearing. Variability is also present in tibias and wings sizes even though individuals were reared under the same constant conditions. The impact of these variations on the intrasexual selection was studied through a choice experiment in olfactometry, with males facing the sex pheromone of the female. The study of the choice patterns in relation to the morphology of males did not highlight a link between the measured phenotypic traits in males and the behaviour of males while exposed to the sex pheromone.

In the ARTICLE 3 (Nicolas & Hance, in prep.a), the effect of the female in the sexual selection was added compared to the experiments of the ARTICLE 2 (Nicolas *et al.*, in prep.b). Mating experiments were performed with two females and two males, in order to evaluate the importance of the antennae for the access to a mate. It appears from these experiments that the most important parameter for males to get to females is the ratio of the length of the right antenna to the length of the right hind tibia. The mean value of this ratio was significantly higher for mated males than for not mated males. In other words, a male having long antennae compared to the size of its body has a higher probability to mate than a male with shorter antennae for the same body size. Furthermore, bigger males seem a little more advantaged than smaller males to obtain a female of good quality, and the fact that a mating took more time to occur when morphological differences between the two males of a same replicate were less marked could indicate a choice of the female for a mate.

The prediction that longer and more symmetric antennae than those of other males should facilitate the access to a mating partner or to a better mate in *A. aphidimyza* is not proven for the moment. Yet, the antenna to tibia ratio, higher in mated males than in not mated males, gives a partial answer. Long antennae still seem to be important for reproduction, but in connection with the body size, while wings do not seem to play an important role in the access to a mate. Females could prefer small-bodied males with long antennae, according to the Fisher's runaway process or to the good genes model, or males with a small body compared to their antennae could easily reach the female, according to the mechanism of scrambles.

At this stage, the facts can be summarized as follows:

- Variability exists in the morphology of males of *A. aphidimyza*, especially in the symmetry of antennae (ARTICLE 2: Nicolas *et al.*, in prep.b).
- The ability to obtain a mate varies according to the antenna to tibia ratio. This ratio is higher for mated males than for not mated males (ARTICLE 3: Nicolas & Hance, in prep.a).
- The heritability of the measured parameters has not been studied in the experiments. Nevertheless, the current results can be analysed. Heritability in its broad sense corresponds to the part of the phenotypic variation (V_P) that is due to the genetic variation (V_G). The phenotypic variation is composed of the genetic variation and the environmental variation (V_E): heritability = $V_G / V_P = V_G / (V_G + V_E)$ (Futuyma, 2005; Freeman & Herron, 2007). Consequently, if the environmental variation is negligible because the individuals are reared in the same conditions, and thus only submitted to very small random effects, the heritability should be high. As a matter of fact, the males of *A. aphidimyza* used in the experiments were reared in the same condition, so the phenotypic variations observed between individuals should be mostly inheritable.

There is thus variability in the morphology of the reared males, the ratio of antenna to tibia seems to be the main driver of the access of males to a mate and this parameter should be heritable. Nothing was brought out in the first experiment, which targeted intrasexual selection, but some conclusions can be drawn after the second experiment, that globalised sexual selection, encompassing intrasexual and intersexual selections. It thus ensues, from the two articles, that intersexual selection is probably preponderant compared to intrasexual selection, for the access to a mate.

Nevertheless, no firm conclusion can currently be drawn regarding the driving factors of the sexually dimorphic antennae of *A. aphidimyza*. However, the fact that the ratio of antenna to tibia is higher for mated males than for not mated males gives a track to follow for future research.

As the males used in the experiments were maybe not different enough from each other to highlight a difference of mating success between them concerning the antennae alone, the rearing should be done at different temperatures. Indeed, it is not possible to sort the males with the naked eye, to select only small ones and large ones. Consequently, to test that hypothesis, the morphological differences between males used in the experiments should be exacerbated by rearing conditions. Males reared at low temperature should be larger than males reared at high temperature, as observed for *Drosophila melanogaster* (Diptera: Drosophilidae – Zamudio *et al.*, 1995) and as predicted by the temperature-size rule (see CHAPTER IV, point 2.3.2.) (Ray, 1960; Atkinson, 1994; Atkinson & Sibly, 1997; Davidowitz & Nijhout, 2004; Kingsolver & Huey, 2008). Moreover, the relations between the size of different parts of the body should be **isometric** (relative proportions of body parts remain constant while the absolute body size varies, according to the developmental temperature – Nedvěd, 2009). In the next chapter, our goal will thus be to produce males of different sizes and to analyse their mating success according to their size.

CHAPTER IV. INFLUENCE OF TEMPERATURE ON MORPHOLOGY AND REPRODUCTIVE BEHAVIOUR OF MALES OF *APHIDOLETES APHIDIMYZA*

1. Introduction

As a reminder, the global goal of this thesis is to know what had a preponderant impact on the appearance of the antennal sexual dimorphism in *Aphidoletes aphidimyza*. In the previous chapter, no effect of antennae alone has been highlighted on the mating success of males, but the antenna to tibia ratio was the main driver of the access of males to females, a high ratio being more favourable for the male to mate. Moreover, the time to achieve a mating is slightly influenced by the fact that morphological differences are pronounced or not between males presented to a female, the time tending to be shorter when differences are more pronounced.

To be able to draw a more solid conclusion on the causes of the sexual dimorphism in *A. aphidimyza* and on the role of the size and symmetry of the antennae of males in their success to mate, the idea is to manipulate males morphology through rearing conditions. Indeed, the targets of selection are the phenotypic traits, whether they were shaped by environment (plasticity) or by the genome. The selection of the phenotypic traits will thus be the same whatever the origin of the variations, and could lead to evolution if the phenotypic traits are inheritable (Smith & Smith, 2009 – more explanations in the point 3. of this chapter). Consequently, new experiments were performed with males reared at different temperatures, in order to maximize the morphological differences between these males. The consequences of the different rearing conditions on the development, the morphology and the reproductive behaviour of males of *A. aphidimyza* will be studied and discussed in relation to the sexual dimorphism and in the perspective of the climate change. As temperature was the main factor that varied between the tested males, the effects temperature can have on ectotherms and particularly on insects will be summarised in the next part.

2. Effects of temperature on body size and fitness of ectotherms

2.1. General concepts

Phenotypic plasticity is the ability to produce different phenotypes from a genotype under the action of environmental conditions (Freeman & Herron, 2007; Smith & Smith, 2009; Dingemanse *et al.*, 2010; Martel *et al.*, 2011). The phenotypic traits concerned by the plasticity can be, for example, of morphological, physiological or behavioural nature (Clobert & Sinervo, 2016).

The **reaction norm** is the range of phenotypic expressions produced by a single genotype under different environmental conditions (Smith & Smith, 2009). As the reaction norm is a graphical representation of the environmental dependence (abscissa) of the phenotype (ordinate) for a genotype (Beldade *et al.*, 2011), the reaction norm is non-horizontal for a genotype presenting phenotypic plasticity (Dingemanse *et al.*, 2010; Foray, 2011). A particular case is the **thermal reaction norm**, which is a function relating the phenotype expressed from a given genotype to the environmental or body temperature (Angilletta *et al.*, 2003).

The **developmental plasticity** is the irreversible phenotypic plasticity occurring during the **growth** – increase in size (Kingsolver & Huey, 2008) or in biomass (size of cells) (van der Have & de Jong, 1996; Berg & Merritt, 2009) – and the **development** – changes in life stage or differentiation (number of cells) (van der Have & de Jong, 1996; Kingsolver & Huey, 2008; Berg & Merritt, 2009) – of individuals. This plasticity is particularly useful for animals with a short lifespan that have to face the seasonal conditions, as the environmental changes are slow compared to their lifespan (Ricklefs & Miller, 2005; Smith & Smith, 2009). Conversely, **behavioural plasticity** is reversible (Clobert & Sinervo, 2016).

2.2. Environment and evolutionary process

The environment plays two roles in the evolutionary process (FIGURE 10): it interacts with the developmental process to produce the phenotype (phenotypic plasticity) and it makes the link between the phenotype of an organism and its fitness (Scheiner, 1993). Phenotypic traits are the targets of the selection and the selective agents are the environmental causes of the differences of fitness between the individuals of a population. Genotype and alleles frequencies will be modified in a population as a result of natural selection, even if it acts on the phenotype (Smith & Smith, 2009).

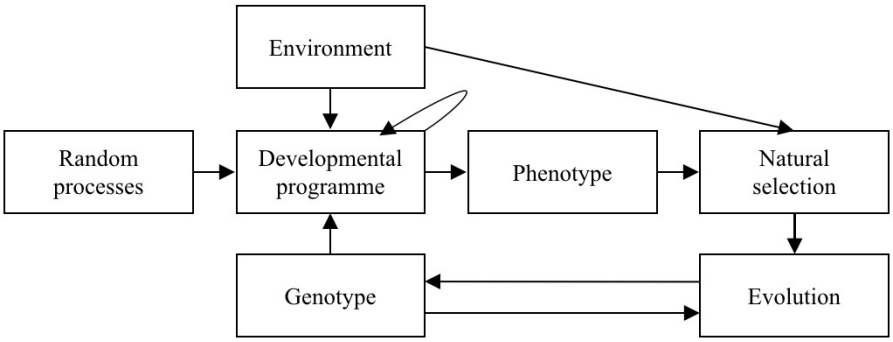


FIGURE 10. Diagram of the links between development, environment, genetics and evolution (adapted from Scheiner, 1993).

2.3. Temperature, body size and fitness

Insects are **poikilotherms,** meaning that their body temperature is variable, and since they are also mostly **ectotherms**, the determination of their body temperature results mainly from the external environment (Atkinson, 1994; Smith & Smith, 2009). The ectotherms, which represent 99,9 % of the Earth species (Atkinson & Sibly, 1997; Daufresne *et al.*, 2009), easily exchange heat with the environment. Environment thus plays an important role in poikilotherms by controlling the rates of their metabolism and activity (Smith & Smith, 2009). Temperature is probably the most restrictive environmental factor in ectotherms (Foray, 2011) and can act, for example, on development time, size or behaviour of insects (Hance *et al.*, 2007).

The relations between temperature, body size and fitness in ectotherms are described by three general rules called “bigger is better”, “hotter is smaller” and “hotter is better” (Kingsolver & Huey, 2008). These three general rules,

explained in the subsequent sections, can be put together into a path diagram (FIGURE 11) which describes the impacts of temperature on critical rate processes throughout the life cycle (Kingsolver & Huey, 2008).

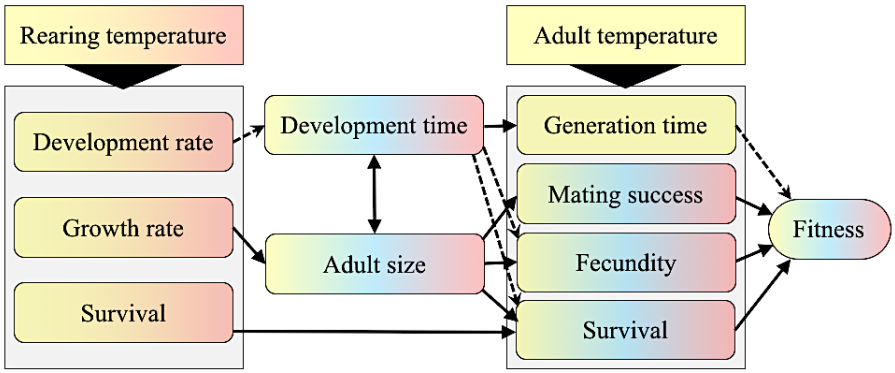


FIGURE 11. Diagram of the combined effects of the three general rules ("bigger is better" in blue, "hotter is smaller" in red and "hotter is better" in yellow) linking body size, temperature and fitness for ectotherms. Single headed arrows are used for causal connections, double headed arrows for correlations, solid lines for positive connections and dashed lines for negative connections. Relations are monotonic but not necessarily linear (adapted from Kingsolver & Huey, 2008).

2.3.1. "Bigger is better"

"Bigger is better" rule states that a larger body size is usually associated with a greater fitness in a population. This rule thus describes the patterns of phenotypic selection among phenotypic variations found in the populations (Colinet *et al.*, 2007; Kingsolver & Huey, 2008).

In the diagram (FIGURE 11, in blue), adult body size contributes positively to mating success, fecundity and survival, that contribute positively to the fitness. However, a higher adult body size requires a longer development time and thus contributes negatively to the fecundity and survival, and also to the fitness. The respect of the rule "bigger is better" thus depends on the relative importance of the positive links between size and fitness and of the negative ones between development time and fitness (Kingsolver & Huey, 2008).

More than 79 % of the studies follow the "bigger is better" rule, mainly with regard to terrestrial insects. Larger individuals present a higher survival (in 76 % of cases), a higher fecundity (in 85 % of cases) and a higher mating success (in 74 % of cases) than smaller individuals (Kingsolver & Pfennig, 2004; Kingsolver & Huey, 2008).

2.3.2. "Hotter is smaller"

"Hotter is smaller" rule states that development at higher temperatures induces smaller adult body sizes (Ray, 1960; Atkinson, 1994; Atkinson & Sibly, 1997; Davidowitz & Nijhout, 2004; Kingsolver & Huey, 2008). In other words, most of ectotherms grow slower and reach a larger body size at maturity in colder conditions (Angilletta & Dunham, 2003; Angilletta *et al.*, 2004; Colinet *et al.*, 2007; Karl & Fischer, 2008). This **temperature-size rule** describes the phenotypic plasticity (a thermal reaction norm) of a genotype. The genotype and the temperature during development (environmental conditions) affect the adult size (phenotypic trait). The relation between the rearing temperature and the size for a genotype (the thermal plasticity of the body size) is thus described by the temperature-size rule, which predicts a negative slope for this thermal reaction norm (Angilletta *et al.*, 2004; Kingsolver & Huey, 2008; Ghosh *et al.*, 2013).

In the diagram (FIGURE 11, in red), a higher rearing temperature increases the rates of development and of growth and the development time decreases, if the thermal sensitivity of development rate is higher than that of growth rate (Davidowitz & Nijhout, 2004; Kingsolver & Huey, 2008). A reduced adult size results and the times to reach adulthood and reproduction decrease (van der Have & de Jong, 1996; Kingsolver & Huey, 2008).

In almost 84 % of the studies on ectotherms analysed by Atkinson (1994), a higher rearing temperature induced a smaller size at a given stage of development. For Diptera, the percentage goes up to 94 % of cases. The temperature-size rule does not apply to **stressful temperatures** – temperatures that generate difficulties to reach maturity or increased daily mortality rate – for growth or differentiation (Atkinson, 1994; Kingsolver & Huey, 2008).

This temperature-size rule for ectotherms is the equivalent of the **Bergmann's rule** for endotherms, stipulating that a higher temperature induces a smaller body size and hence that the body size tends to increase with the latitude. The warm regions are thus supposed to be inhabited by small-bodied species (Bergmann (1847) cited by James (1970), by Atkinson (1994), by Blanckenhorn & Hellriegel (2002), by Daufresne *et al.* (2009) and by Vinarski (2014)). The Bergmann's rule is thus to associate with natural environments and the temperature-size rule is to associate with laboratory experiments (Angilletta & Dunham, 2003). As the Bergmann's rule cannot be assumed to apply to insects (Shelomi, 2012), more data are needed to estimate the adequacy of the Bergmann's rule for these ectotherms (Vinarski, 2014).

2.3.3. "Hotter is better"

"Hotter is better" rule states that higher optimal temperatures are often associated with a greater maximal performance at the optimal temperature. This rule describes the evolution of the differences in reaction norm among genotypes or species (Kingsolver & Huey, 2008).

In the diagram (FIGURE 11, in yellow), a species of genotype having a low rearing temperature has low development and growth rates (Charnov & Gillooly, 2003) and consequently a long development time. A longer development time increases the generation time and decreases the fecundity and survival and thus decreases fitness (rate of population growth). As the effects of rearing and adult temperatures are not distinguishable, the reasoning will be the same for a lower temperature at adulthood, which will decrease the fitness (Kingsolver & Huey, 2008).

The information available about the "hotter is better" rule is relatively limited, but supports the rule, especially for terrestrial insects (Kingsolver & Huey, 2008).

2.3.4. Concluding remarks on the three general rules

It is obvious in the diagram (FIGURE 11) that temperature has an impact on critical rate processes during development and adulthood, and thus on phenotype and resulting fitness. Moreover, development time and adult body size are two key phenotypic traits, often strongly correlated, which are both consequences of temperature-dependant processes and causes of variation in fitness (Kingsolver & Huey, 2008).

As a rule corresponds to an empirical generalization coming from observations in different studies of biological systems, the three rules discussed suffer from exceptions (Angilletta *et al.*, 2004; Kingsolver & Huey, 2008). For example, in some situations, a smaller size can be selected (Zamudio *et al.*, 1995; Moya-Laraño *et al.*, 2007, Ismail *et al*., 2012), because a large size induces a decrease in fitness – due to viability costs (because of long development, fast growth, great detectability, high energy needs, …), decreased mating success (due to reduced agility or high energy needs) or decreased reproductive success (due to late reproduction) (Blanckenhorn, 2000).

Generally speaking, each rule interacts in complex ways with others, sometimes synergistically, sometimes antagonistically. So, each situation requires a careful study of the processes acting (Kingsolver & Huey, 2008; Wu *et al*., 2011).

2.4. Implications of climate change

Further warming and changes in the components of the climate system will occur due to the continued emissions of greenhouse gases. In these conditions, the global surface temperature change is likely to exceed 1.5 °C, relative to the average from year 1850 to 1900, by the end of the 21st century (IPCC, 2013). In the absence of policies to mitigate the climate changes, there is even a high probability to reach between 1.7 and 4.9 °C of warming (Wigley & Raper, 2001). Due to the climate change, the global mean temperatures should thus increase in the coming years but, furthermore, the extreme cold temperature events should become less frequent. Heat waves will very probably occur with increased frequencies and durations (IPCC, 2013; Lhotka *et al.*, 2018).

It is therefore important to understand the way the species can cope with climate change (Abram *et al.*, 2017), which will depend on the kind of change as well as on the living organism itself (Hugues, 2000; Sinclair *et al.*, 2003; Parmesan, 2006; Deutsch *et al.*, 2008; Tewksbury *et al.*, 2008; Williams *et al*., 2008; Beldade *et al.*, 2011; Huey *et al*., 2012; Vasseur *et al.*, 2014). The **vulnerability** – susceptibility to a negative impact – of a species to environmental change is determined by the **exposure** – extrinsic factors – and the **sensitivity** – intrinsic factors – of the organism to environmental change. The **sensitivity** of a species is mediated by the **resilience** – capacity to recover from perturbations – and the **adaptive capacity** – capability to adapt to change by evolutionary modifications and plastic ecological responses – of this species (Smith *et al*., 2000; Turner *et al.*, 2003; Williams *et al*., 2008; Huey *et al*., 2012). Generally speaking, organisms have three ways to cope with changes in environmental conditions (Bale *et al*., 2002; Parmesan, 2006; Beldade *et al.*, 2011; Foray, 2011):

- Move to a more appropriate habitat, which can lead to species distribution changes.
- Adapt to their new environmental conditions through natural selection, with changes at the genetic level across generations.
- Adjust to their new environmental conditions through phenotypic plasticity, without changes at the genetic level.

The effects of temperatures on ectothermic organisms have long been known, going from the physiology to the behaviour (Denlinger & Lee, 1998; Denlinger & Yocum, 1998; Moiroux *et al.*, 2010; Abram *et al.*, 2017). Development and survival of ectotherms are very strongly related to extern temperature. As ectotherms have evolved in a given range of environmental conditions to which they are adapted, occurrences of extreme conditions are a major challenge for these species (Hance *et al.*, 2007). The trend concerning the impact of the climate change on the size of ectotherms is that smaller individuals will result from the increase of temperature caused by the global warming (Gardner *et al.*, 2011; Sheridan & Bickford, 2011), according to the temperature-size rule (Ray, 1960; Atkinson, 1994; Atkinson & Sibly, 1997; Davidowitz & Nijhout, 2004; Kingsolver & Huey, 2008). Indeed, even a small variation of the temperature (of a few degrees – as predicted by Wigley & Raper (2001) for the climate change) can have consequences on the body size of insects (for examples, see Shirai, 1992; Sibly, 1997; Colinet *et al.*, 2007). Decreasing body size has besides been suggested as the third universal response to the global warming, with the changes in phenology and distribution of species (Daufresne *et al.*, 2009; Gardner *et al.*, 2011). This decrease in size will have negative impacts on the fitness in different ways (through mating success, fecundity and survival) according to the "bigger is better" rule (Kingsolver & Pfennig, 2004; Kingsolver & Huey, 2008).

3. Experimental approach

As explained in the previous section (point 2.3.), the temperature must have a significant effect on the phenotype and on the fitness of *A. aphidimyza*, as an ectotherm. When temperature increases, the size of males of *A. aphidimyza* is supposed to decrease (temperature-size rule, see point 2.3.2. hereabove) and so is the fitness, as a consequence ("bigger is better" rule, see point 2.3.1. hereabove). The use of different developmental temperatures can be a way to obtain males with clearly different phenotypes in order to test the influence of these phenotypes on their capacity to reproduce.

In short, the reasoning presented in the CHAPTER II, point 4.2.3. and in the CHAPTER III, point 1. was to determine whether antennae of males of *A. aphidimyza* are variable (trait variation), whether the ability of males to reproduce varies with the characteristics of their antennae (fitness differences) and whether these variations are inheritable (inheritance). In this CHAPTER IV, the focus will be placed on the trait variations and on the fitness differences. In the ARTICLE 4 (Nicolas & Hance, in prep.b), the phenotypic variability will be accentuated by using different rearing temperatures (developmental plasticity),

hence the variations observed will of course not be inheritable. However, the results of the experiments to evaluate the fitness of males according to their phenotype (ARTICLE 5: Nicolas *et al.*, in prep.a) will remain the same whether these phenotypes result mainly from the genome expression alone or with a predominant effect of the environment. Indeed, natural selection (and thus sexual selection) acts on the phenotypic traits, while selective agents cause fitness differences among individuals in a population (Freeman & Herron, 2007; Smith & Smith, 2009). Fitness differences according to variations of phenotypic traits due to phenotypic plasticity or to genetic variability can be considered as equivalent in this context. In the ARTICLE 5 (Nicolas *et al.*, in prep.a), experiments will thus be performed to evaluate the fitness of males by their capability to reach a source of pheromone and to mate, according to their phenotype. These experiments will give indications on the selective pressure acting in the competition to obtain a mating partner, which could actually lead to evolution if phenotypic traits were inheritable (Smith & Smith, 2009; Boivin G., personal communication, March 12, 2015).

The ARTICLE 4 (Nicolas & Hance, in prep.b) will explore the dissimilarities in the development of individuals and in the morphology of males of *A. aphidimyza* produced at different temperatures. The individuals reared at high temperature should develop faster and reach a smaller body size than those reared at low temperature (Angilletta & Dunham, 2003; Angilletta *et al.*, 2004; Karl & Fischer, 2008). Moreover, even though allometry could possibly be induced by different developmental temperatures and affect relative proportions of body parts of males differently, the prediction is that the size of the different parts of the body should change in an isometric way in the experiments (Nedvěd, 2009). Males reared at high temperature should be smaller and should have smaller antennae and wings than males reared at low temperature, and the relative sizes of these structures should remain constant. The consequences of these results in the perspective of changes induced by global warming will be evoked.

The ARTICLE 5 (Nicolas *et al.*, in prep.a) will study the implications of the potential differences highlighted in the previous article on the reproductive behaviour of the males of *A. aphidimyza*. This aims at strengthening the conclusions of the CHAPTER III on the role of sexual selection in sexual dimorphism and on the importance of antennae and tibia sizes in the access of males to a mate, using males reared at different temperatures and thus presenting greater morphological differences. In order to connect mating behaviour of males with their morphology, mating and olfactometry experiments will then be

done again, but this time with these males reared at different temperatures. The implications in the sexual dimorphism will be addressed.

4. Thermal plasticity of development and morphology of *Aphidoletes aphidimyza*

ARTICLE 4

The Size of Males of *Aphidoletes aphidimyza* Reared at Different Temperatures Conforms to the Temperature-Size Rule

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Abstract

According to the temperature-size rule, development time and adult body size of ectotherms decrease when these develop at higher temperatures. The general morphology can also be modified (allometric growth), or sometimes the proportions between the sizes of different body parts are maintained (isometric growth). In the current context of climate change, this raises concerns about the many consequences it can have in terms of fitness. For instance, development time, size and morphology of males of *Aphidoletes aphidimyza* influence their access to a mate. Sexually dimorphic antennae, wings and their relation to the body size are potentially important traits for mating success. However, for *A. aphidimyza*, no data are available on developmental plasticity of these traits induced by temperature. The predictions are that pupae obtained at high temperature should be smaller than those obtained at low temperature and individuals reared at high temperature should develop faster than those reared at low temperature. Moreover, males reared at high temperature should be smaller and have shorter antennae and smaller wings than males reared at low temperature, presenting an isometric growth of these parts in connection with temperature. In order to test these aspects, *A. aphidimyza* individuals were reared under three different temperature conditions. Characteristics of pupae and parameters related to development and emergence as well as morphology of adult males were then determined. It results from these analyses that pupae are globally smaller at higher temperature and that duration of the pupal stage is shorter at high than at low temperature. The highest rate of emergence was observed at temperature close to the optimum (approximately 23 °C).

Furthermore, males reared at higher temperature are globally smaller, with longer antennae and narrower wings compared to their body size, and more symmetric antennae, than those obtained at lower temperature.

Keywords

Allometry, *Aphidoletes aphidimyza*, temperature, development, morphology, male, temperature-size rule

Introduction

The temperature-size rule is well known to describe the phenotypic plasticity of ectotherms in relation to variations of temperature (Angilletta *et al.*, 2004; Colinet *et al.*, 2007; Kingsolver & Huey, 2008; Ghosh *et al.*, 2013) and was deducted from laboratory experiments (Angilletta & Dunham, 2003). According to this rule, also called "hotter is smaller" (Kingsolver & Huey, 2008), development at a higher temperature induces a reduction of development time and of adult body size (Ray, 1960; Atkinson, 1994; Atkinson & Sibly, 1997; Davidowitz & Nijhout, 2004; Kingsolver & Huey, 2008). In the current context of climate change, this rule become even more important. Indeed, the global surface temperature increase of Earth is likely to exceed 1.5 °C by the end of the 21st century, in comparison with the average between 1850 and 1900 (IPCC, 2013). There is even a high probability to reach 4.9 °C of warming in the absence of climate mitigation policies (Wigley & Raper, 2001).

For most ectotherms, the extern temperature has an impact on the development time but also on the general morphology (Angilletta & Dunham, 2003; Colinet *et al.*, 2007; Hance *et al.*, 2007). For example, with changes of developmental temperature, allometric relations were found between body size and antennae of two species of Chironomids, *Echinocladius martini* (Orthocladiinae) and *Australopelopia prionoptera* (Tanypodinae) (McKie & Cranston, 2005). Moreover, it was shown, for *Drosophila simulans* (Drosophilidae), that not only size of wings changes with developmental temperature but also their shape, in a non-isometric way (Debat *et al.*, 2003). Contrariwise, the relations between the sizes of body parts could also remain unchanged despite differences of temperature (isometric growth) (Nedvěd, 2009).

Since *Aphidoletes aphidimyza* is supposed to be a monoandrous species (van Lenteren *et al*., 2002; van Lenteren & Schettino, 2003; Tabadkani *et al.*, 2012a), a faster development of males could increase their mating opportunities, as they would emerge earlier, and protandry would thus be favoured (Bukowski & Avilés, 2002; Rhainds, 2010; Tabadkani *et al.*, 2012c; Tabadkani *et al.*, 2013). Moreover, some morphological traits, like sexually dimorphic antennae (Markkula & Tiittanen, 1985; Zhang & Yang, 2008), body size, and the ratio of both, could play a role in the access of males to a mate (Nicolas & Hance, in prep.a). Indeed, with their antennae, males have to locate females that emit a sexual pheromone (Steinbrecht, 1987; Chapman, 1998; van Lenteren *et al.*, 2002; van Lenteren & Schettino, 2003; Choi *et al.*, 2004a). Since, in this species, females are known to mate only once, they should be choosy. In this context, antennae of males could play the role of an ornament indicating male quality (Sivinski, 1997). Moreover, wings also play a role because males have to fly to reach a mating partner, in spite of their cumbersome antennae. Furthermore, the movements of the wings in relation to reproduction are standardised. Indeed, a male approaches a female while wing-fanning, then first extends its wings before folding them against its body during the mating (van Lenteren *et al*., 2002; van Lenteren & Schettino, 2003).

As a matter of fact, a higher, but not stressful, developmental temperature induces a smaller development time in *A. aphidimyza* (Uygun, 1971; Havelka, 1980; Morse & Croft, 1987; Choi *et al.*, 2001; Jeoung *et al.*, 2003; Higashida *et al.*, 2016; Higashida *et al.*, 2017). Contrariwise, there is no data on the differences of morphology induced by different temperatures, even though the temperature-size rule predicts a smaller size for individuals of *A. aphidimyza* produced at higher temperature. Consequently, with rearing at a higher temperature, pupae should be smaller, duration of the pupal stage should be shorter and male adults should be smaller, with smaller antennae and narrower wings. The changes of size observed in wings and antennae should be isometric compared to variations of the body size, as wings and antennae, which can impede movement due to their size (Symonds *et al*., 2011), are both potentially involved in flight ability. Wings and antennae being probably also involved in mating success of males of *A. aphidimyza* (Nicolas & Hance, in prep.a; Nicolas *et al.*, in prep.b), sexual selection could be influenced by their phenotypic differences produced by temperature.

In order to study the consequences of the temperature on *A. aphidimyza*, rearing experiments have been performed at three different conditions of temperature. Plants with aphids and eggs of *A. aphidimyza* laid in the same rearing conditions have been dispatched in three climatic rooms at different temperatures. When pupae have been recovered in the different rooms, some of them have been isolated and some others have been measured. Parameters related to the development and the emergence, as well as morphology of adult males (body size, antennae and wings), have been studied.

Materials and methods

Rearing

The first pupae were obtained from Koppert B.V. (Rodenrijs, Netherland). Individuals of *Aphidoletes aphidimyza* Rondani were reared like described in Kohandani *et al.* (2017), inspired by Le Goff *et al.* (2016). The strain of the prey, the aphid *Myzus persicae* (Sulzer), was initially provided by Viridaxis S.A. (Gosselies, Belgium). Preys were reared on plants of *Capsicum annuum* L. (sweet pepper). Every two days, three plants infested with aphids were taken from the mass rearing of aphids and placed in the mass rearing of *A. aphidimyza* to obtain eggs. After two days, the three plants with aphids and eggs of *A. aphidimyza* were removed. Each of these plants with eggs was then dispatched in one of the three different climatic rooms, with a relative humidity between 50 and 60 % and white light of nearly 20,000 lumens from 6 p.m. to 8 a.m. (reverse photoperiod). The photoperiod was reversed in order to carry out experiments in the daytime, in spite of the nocturnal habits of *A. aphidimyza*. In each room, plants were bended above sand in a harvest box to collect larvae that are ready to pupate. Every two days, the harvest box was replaced by a new one with clean sand. The previous box was closed with a lid and sieved two days later to recover the pupae, which were then immediately put back in the corresponding climatic room.

The following temperatures of development (constant day and night) were used in the different climatic rooms:

- Low temperature (low t°): 17.6 ± 1.0 °C.
- Intermediate temperature (intermediate t°): 22.6 ± 1.0 °C. It is the usual rearing temperature because it is close to the optimum for *A. aphidimyza* (23 ± 1.5 °C, according to Havelka & Zemek, 1999).
- High temperature (high t°): 27.8 ± 1.0 °C.

Some pupae were isolated in microtubes of 1.5 ml, before emergence of adults. To feed the adults, a cotton swab tip soaked in a solution of ¾ of the volume of water and ¼ of white sugar was put in each microtube. Some of the other pupae were placed in a freezer for subsequent measurements of the characteristics of the cocoons.

Data collection

Characteristics of pupae

A binocular microscope at 16x magnification with CamUniversal software version 3.5 for Windows XP was used to take pictures of pupae coming from the freezer. To compare the characteristics of the pupae produced under the three conditions, the following measurements have been taken, with the ImageJ software for Mac, on the cocoons surrounding the pupae ($N_{low\ t°}$ = 30, $N_{intermediate\ t°}$ = 24, $N_{high\ t°}$ = 24):

- Cocoon length (measurement along the main axis), in mm.
- Cocoon width (measurements along the small axis), in mm.

For each parameter, the mean of three measures was taken, in order to diminish the variability related to the measurement (as suggested by Palmer & Strobeck, 1986, by Merilä & Björklunds, 1995 and by Blanckenhorn *et al.*, 2003).

Development time and emergence

The emergence of adults from isolated pupae was checked every two days and four parameters were selected to compare the progress for the three temperatures of development (Le Goff *et al.*, 2016) ($N_{low\ t°}$ = $N_{intermediate\ t°}$ = $N_{high\ t°}$ = 17):

- Duration of pupal stage, in days (proxy of the development time, number of days between the removal of the harvest box and the emergence of adults).
- Window of emergence, in days (time between first and last day of emergence).
- Emergence rate, in % (number of emerging adults/number of isolated pupae * 100),
- Sex ratio, in % (number of males/number of emerging adults * 100).

Morphology of adult males

After emergence, males were placed in a Hanks' balanced salts solution at a concentration of 98 g/l to take pictures of antennae, wings and tibias. Pictures were taken using a binocular microscope at 16x magnification, with CamUniversal software version 3.5 for Windows XP. Afterwards, the following parameters were measured, with the ImageJ software for Mac, to determine the role of the temperature of development on the morphology of males ($N_{low\ t°} = N_{intermediate\ t°} = N_{high\ t°} = 21$):

- Length of the flagellum of both antennae (12 flagellomers) without scape and pedicel, in mm.
- Length of the right hind tibia, as a proxy of the body size (like Jann *et al.* (2000) and Blanckenhorn *et al.* (2003) for another Diptera), in mm.
- Area of the right wing, in mm^2.

For each parameter, the mean of three measures was taken, in order to diminish the variability related to the measurement (as suggested by Palmer & Strobeck, 1986, by Merilä & Björklunds, 1995 and by Blanckenhorn *et al.*, 2003).

To compensate for body size and to determine possible allometry, the following parameters were calculated:

- Antenna to tibia ratio (RA/T) representing the length of the right antenna divided by the length of the right hind tibia.
- Wing to tibia ratio (W/T) representing the area of the right wing divided by the length of the right hind tibia, in mm.
- Symmetry of antennae (RA-LA) estimated by the difference between the length of the right antenna and the length of the left one, in mm.

Statistical analyses

Statistical analyses were conducted using GraphPad Prism version 5.0 software for Mac (GraphPad Software, San Diego, California, USA, http://www.graphpad.com). All tests were performed with a significance level (P) set at $\alpha = 0.05$ and under two-tailed hypothesis. The margin error which is mentioned is the standard error (SE).

Characteristics of pupae

As the data were normally distributed (D'Agostino & Pearson omnibus normality test) and homoscedasticity for length of cocoon was controlled (Bartlett's test for equal variances), a one-way ANOVA and a Tukey's multiple comparison test were used to compare the data of the different temperatures of development. A Kruskal-Wallis test and a Dunn's multiple comparison test were applied to the data concerning width of cocoon as there was heteroscedasticity (Bartlett's test for equal variances).

Development time and emergence

As the data were normally distributed (D'Agostino & Pearson omnibus normality test) and there was homoscedasticity (Bartlett's test for equal variances) for duration of pupal stage, window of emergence and sex ratio, a one-way ANOVA and a Tukey's multiple comparison test were used, to know if temperature of development had an effect on these parameters. Emergence rates were compared with a Kruskal-Wallis test and a Dunn's multiple comparison test as there was heteroscedasticity (Bartlett's test for equal variances).

Morphology of adult males

As the data were normally distributed (D'Agostino & Pearson omnibus normality test) and there was homoscedasticity (Bartlett's test for equal variances) for length of antennae and area of right wing, a one-way ANOVA and a Tukey's multiple comparison test were used to compare these data at the three temperatures of development. A Kruskal-Wallis test and a Dunn's multiple comparison test were applied to the data concerning length of tibia, RA/T, W/T and RA-LA, to know if temperature of development had an effect on these parameters, as some data were not normally distributed or there was heteroscedasticity.

Results

Characteristics of pupae

Pupae cocoons of individuals that developed at low t° were longer (ANOVA: $F_{2,75} = 13.46$, $P < 0.001$) and larger (Kruskal-Wallis test: K (2) = 25.72, $P < 0{,}001$) than those that developed at intermediate t° and at high t° (TABLE 1). There is no significant difference of size between cocoons produced at intermediate t° and at high t°.

TABLE 1. Length and width of cocoons (mean ± SE) depending of developmental temperature. Different superscript letters represent significantly different means according to the statistical tests.

	Cocoon length (mm)	Cocoon width (mm)
Low t°	2.76 ± 0.04^{a}	1.81 ± 0.03^{a}
Intermediate t°	2.53 ± 0.05^{b}	1.65 ± 0.02^{b}
High t°	2.41 ± 0.06^{b}	1.58 ± 0.10^{b}

Development time and emergence

The duration of pupal stage was significantly different between the three temperatures of development (ANOVA: $F_{2,48} = 587.5$, $P < 0.001$). The pupal stage lasted roughly twice as long at low t° as at high t° (TABLE 2). The emergence rate was not different between low t° and high t°, but was higher at intermediate t° than at low t° and at high t° (Kruskal-Wallis test: $K(2) = 27.82$, $P < 0{,}001$). For the window of emergence (ANOVA: $F_{2,48} = 3.17$, $P = 0.051$) and the sex ratio (ANOVA: $F_{2,48} = 0.66$, $P = 0.523$), the three temperatures of development did not show differences.

TABLE 2. Parameters related to emergence for the three temperatures of development (mean ± SE). Different superscript letters represent significantly different means according to the statistical tests.

	Duration of pupal stage (days)	Window of emergence (days)	Emergence rate (%)	Sex ratio (%)
Low t°	17.89 ± 0.20^{a}	4.18 ± 0.33^{a}	64.25 ± 1.78^{a}	43.06 ± 2.80^{a}
Inter. t°	10.65 ± 0.17^{b}	3.03 ± 0.36^{a}	85.51 ± 1.52^{b}	46.59 ± 3.21^{a}
High t°	9.49 ± 0.19^{c}	3.01 ± 0.39^{a}	65.06 ± 3.66^{a}	41.26 ± 3.94^{a}

Morphology of adult males

Globally, adults produced at low t° were bigger than those produced at intermediate t°, which were themselves bigger than those produced at high t° (FIGURE 1). The sizes of left antenna (ANOVA: $F_{2,60} = 44.32$, $P < 0{,}001$), right antenna (ANOVA: $F_{2,60} = 34.28$, $P < 0{,}001$), right hind tibia (Kruskal-Wallis test: $K(2) = 36.97$, $P < 0{,}001$) and right wing (ANOVA: $F_{2,60} = 48.21$, $P < 0{,}001$) decreased between low t°, intermediate t° and high t°. RA/T was lower at low t° than at intermediate t° and high t° (Kruskal-Wallis test: $K(2) = 45.03$). On the other hand, W/T was higher at low t° than at intermediate t° and high t° (Kruskal-Wallis test: $K(2) = 22.51$, $P < 0{,}001$). The asymmetry of antennae (RA-LA) was more pronounced in adults at low t° than in adults at high t° (Kruskal-Wallis test: $K(2) = 7.40$, $P = 0{,}025$).

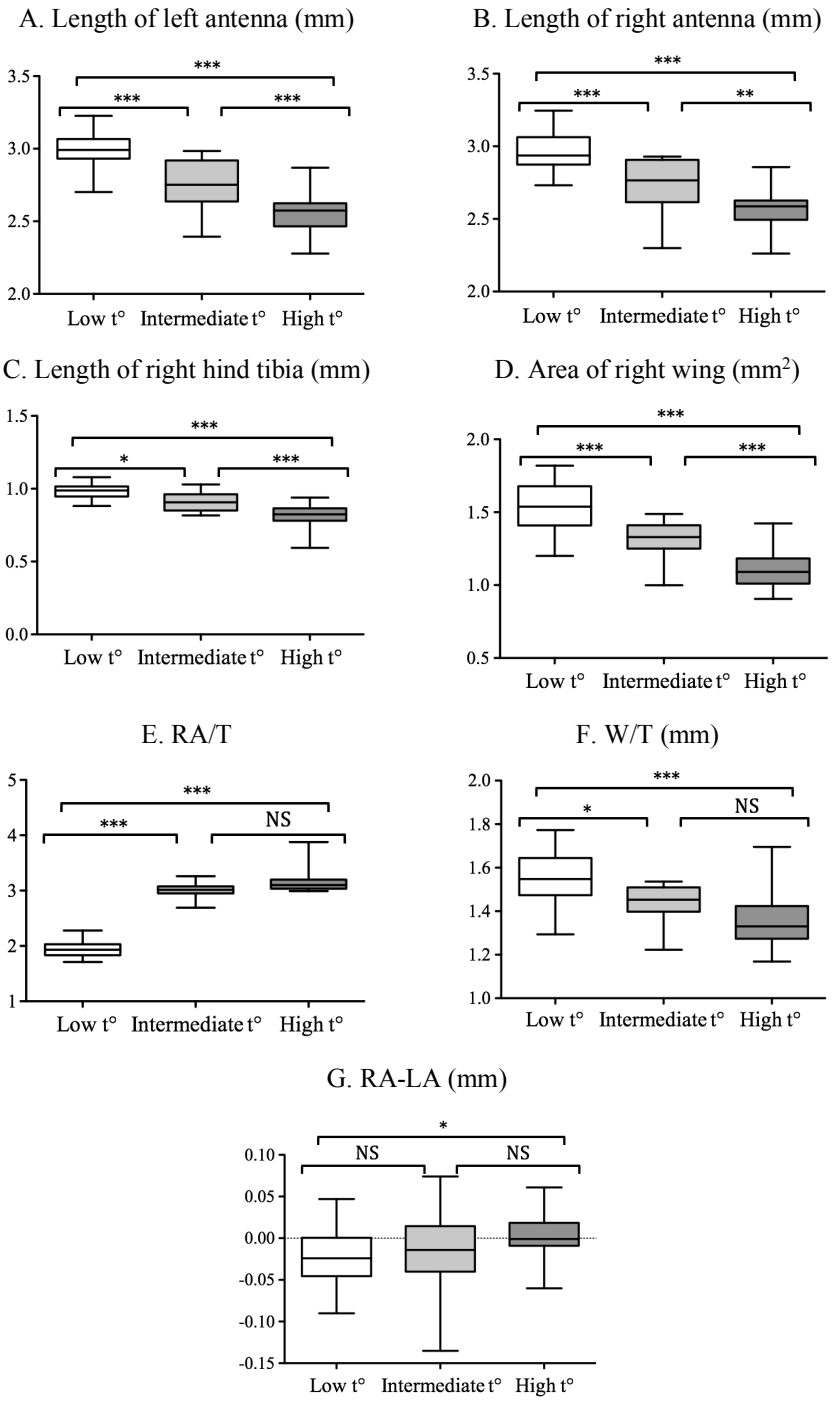


FIGURE 1. Length of left antenna (A), length of right antenna (B), length of right hind tibia (C), area of right wing (D), RA/T (E), W/T (F) and RA-LA (G) depending on temperature of development.

Discussion

Temperature during development clearly impacts the size of males of *Aphidoletes aphidimyza* at adulthood. As the temperature-size rule predicted (Ray, 1960; Atkinson, 1994; Atkinson & Sibly, 1997; Davidowitz & Nijhout, 2004; Kingsolver & Huey, 2008), the males of *A. aphidimyza* reared at a higher temperature were smaller than those reared at a lower temperature, as it appears based on lengths of tibias. The morphological difference is more pronounced between the higher and the intermediate temperatures than between the intermediate and the lower temperatures. The size of the tibia, hence of the body, is thus a plastic phenotypic trait under the action of temperature (Angilletta *et al.*, 2004; Kingsolver & Huey, 2008; Ghosh *et al.*, 2013). *A. aphidimyza* should be added to the 84 % of studies on ectotherms, and to the 94 % of studies on Diptera that follow the temperature-size rule (Atkinson, 1994). The average length of the tibia of males reared at intermediate temperature (0.91 mm at 22.6 °C) is consistent with previous results (Nicolas *et al.*, in prep.b: 0.92 mm at 23 °C) and with observations of Fedotova (2014) (0.93 mm for a wild individual). Even though fecundity selection and sexual selection are known to select larger body sizes, they can be counterbalanced by opposing selective forces favouring small body sizes due to viability costs or lower mating/reproductive success (Zamudio *et al.*, 1995; Blanckenhorn, 2000; Moya-Laraño *et al.*, 2007, Ismail *et al.*, 2012), and, thus, the outcome of the situation would be interesting to study.

As expected, the absolute size of the antennae of males of *A. aphidimyza* decreased when the temperature of development increased. The same trend was observed for the area of the right wing, which also decreased with the augmentation of the rearing temperature. Males developed at a lower temperature, and having thus longer antennae and larger wings, could have a higher mating success (Jiménez-Pérez & Wang, 2004 – Torticidae; Koshio *et al.*, 2007 – Zygaenidae) or could present a better fly ability (Shirai, 1992 – Plutellidae).

However, we also expected a proportional decrease of the sizes of all body parts, thus at a same rate, when the developmental temperature increases (isometric growth). In this case, the antenna to tibia ratio and the wing to tibia ratio should have remained constant under the three developmental conditions (Daly, 1985). Contrariwise, these ratios did not remain constant in the experiments: the antenna to tibia ratio increased (negative allometry) while the wing to tibia ratio decreased (positive allometry) with the increase of temperature. This means that males reared at higher temperature had longer antennae and smaller wings, compared to their body size, than those reared at

lower temperature. The antenna/tibia and the wing/tibia ratios thus do not support the emitted prediction concerning their stability while temperature changes. Instead of an isometric relation between body size, antennae and wings with variations of developmental temperature, allometric growth was observed like in Debat *et al.* (2003) for antennae and like in McKie & Cranston (2005) and Siomava *et al.* (2016) for wings.

It thus seems that a trade-off exists between the antennae and wings sizes and the body size. In a previous experiment, males with a higher antenna to tibia ratio than others had more probabilities to copulate (Nicolas & Hance, in prep.a). In consequence, a trade-off between body reduction and antennae augmentation seems to be a possible hypothesis to explain our observation. Conversely, a decrease of the relative size of wings while the relative size of antennae increases seems not favourable. Indeed, if its antennae become more cumbersome compared to its body size, the male should have more difficulties to fly, and a decrease of the relative size of wings at the same time should really impede the mobility. The mating success potentially gained by the male thanks to its antennae could be counterbalanced by the difficulty to fly to a mating partner because of its wings. It would be interesting to test the males produced at the different temperatures in mating experiments, to determine what will have a preponderant effect on the reproduction.

As predicted by the temperature-size rule (Ray, 1960; Atkinson, 1994; Atkinson & Sibly, 1997; Davidowitz & Nijhout, 2004; Kingsolver & Huey, 2008), the duration of the pupal stage, and thus the development time of *A. aphidimyza*, were reduced when temperature increased. This reduction is consistent with observations of other studies on *A. aphidimyza* (Havelka, 1980; Choi *et al*., 2001; Jeoung *et al.*, 2003; Higashida *et al.*, 2016 and Higashida *et al.*, 2017). The difference of duration of pupal stage was more pronounced between the lowest (17.9 days) and the other temperatures than between the intermediate (10.7 days) and the highest temperature (9.5 days). The pupal stage in the experiments lasted nearly twice more time at the lowest temperature than at the highest. Contrariwise, the window of emergence (between 3 and 4 days) was not different between the three developmental conditions, maybe because the differences between these conditions were underestimated as the emergences were checked every two days. It could be interesting to reduce the time between two observations in order to be able to highlight smaller differences. Nevertheless, these observations on the development time and on the window of emergence could have an impact on the mating success of males. As the females of *A. aphidimyza* are supposed to mate just once (van Lenteren *et al*., 2002; van Lenteren & Schettino, 2003; Tabadkani *et al.*, 2012a), males that develop faster, and thus potentially emerge at the beginning of the window of emergence, could

have more mating opportunities than those emerging a few days later (Bukowski & Avilés, 2002; Rhainds, 2010; Tabadkani *et al.*, 2012c; Tabadkani *et al.*, 2013).

Globally, the differences in size observed in pupae obtained at different temperatures of development give an indication on the differences that can be expected in the same conditions at the adult stage. Even though last stages of development, especially adults, are more appropriate to observe the temperature-size rule (Atkinson, 1994), the results concerning the characteristics of pupae (evaluated by cocoon size) also seem to conform to the rule. As prognosticated, the size of pupae decreased when the rearing temperature increased. This is true as well for length and width of the cocoon. However, the size of the pupae obtained at the higher temperature cannot be considered as different from the size of the pupae obtained at the intermediate temperature. Perhaps the effect of the different developmental temperatures on the size had not yet had the time to manifest for those conditions, what could also explain that differences seemed less marked in pupae than in adults. In order to improve the results, it would be good to find a way to more precisely measure the size of pupae, which are enclosed in a puparium surrounded by a cocoon trapping some substratum particles (Le Goff *et al.*, 2016). Besides, the size of the substrate particles (grains of sand) could lead to undervaluation of differences between pupae, which maybe prevented to highlight differences between intermediate and high temperatures.

The emergence rate observed at the intermediate temperature (85.51 %) is distinctly higher than those observed at the lower (64.25 %) and higher (65.06 %) temperatures, which are almost the same. It is certainly because the intermediate temperature is close to the optimal temperature to rear *A. aphidimyza* (Havelka & Zemek, 1999). The best emergence rate obtained here is higher than those obtained by Markkula *et al.*, 1979 (58 %) and Yukawa *et al.*, 2008 (66.2 %) and close to the one obtained by Le Goff *et al.*, 2016 (88,22 %) in their best rearing conditions. As the sex ratio cannot be considered as different for the three temperatures of development (43.06 %, 46,59 % and 41,26%, respectively at low, intermediate and high temperature), the differences observed in the emergence rates do not imply a differential mortality between males and females. Sex ratios obtained are close to the expected one according to the mechanism of sex determination of *A. aphidimyza* (Sell, 1976: 50 %) and also close to the ones obtained by Havelka & Zemek, 1999 (28 % - 53 %); Heimpel & Lundgren, 2000 (55 %); Tabadkani *et al.*, 2012a (52.8 % - 58.1 %) and Tabadkani *et al.*, 2012b (48.3 % - 58.2 %).

The asymmetry of antennae (RA-LA) was a little more pronounced in males reared at lower temperature (0.02 ± 0.01 mm of difference between the two antennae) than in males reared at higher temperature (0.00 ± 0.01 mm of difference between the two antennae). These values are lower than those previously observed (Nicolas *et al.*, in prep.b: 0.03 mm of difference between the two antennae). This could indicate that the antennae, in addition to being longer at a high temperature, are better conformed. Lower temperatures seem to be more stressful than higher temperatures. Higher temperatures would then definitely be more favourable to antennae than lower temperatures. Males developing antennae a little more symmetric than others because they grew in a warmer part of the environment could have more probabilities to copulate, because their antennae could be preferred by the choosy females, if antennae represent an ornament (Sivinski, 1997), like in Møller & Zamora-Munoz (1997) for a Cerambycidae.

With the climate change, the extreme temperatures events, as heat waves, should become more frequent (Easterling *et al.*, 2000; Karl & Trenberth, 2003; Hance *et al.*, 2007). Consequently, experiments with a short exposure of individuals to extreme temperatures during their development could be interesting to perform, in order to compare the results with the data obtained here. Indeed, the effects of extreme, stressful, temperatures, could be different from the effects of temperatures that are close to optimum, and not stressful.

Globally, the results obtained on *A. aphidimyza* with rearing at different temperatures are in accordance with the temperature-size rule. On the other hand, the antennae and wing sizes relative to body size of males have been affected differently by these different temperatures (allometric growth), even though the absolute size of these structures evolved globally in the same direction as the body size. Antennae presented a negative allometry while wings presented a positive allometry compared to body size, with the changes of developmental temperature. This new information on *A. aphidimyza* gives indications about what could happen in the next decades due to the climate change, not only for this species, but also for other Diptera, Arthropods and even ectotherms, as the scope of the temperature-size rule is very large. More experiments are needed, also with other species, to validate these conclusions and to determine the implications of these differences of development and size of the whole body and its parts, related to temperature, on the fitness of the organisms.

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5. Implications of the morphology on the reproductive behaviour of males of *Aphidoletes aphidimyza*

ARTICLE 5

Reproductive Behaviour of Males of *Aphidoletes aphidimyza* According to their Developmental Temperature

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Abstract

Sexual dimorphism can be shaped by natural and sexual selections. Both intersexual and intrasexual selections can lead to sexual dimorphism. The antennal sexual dimorphism, especially when males have more developed antennae than females, is often produced by sexual selection. Antennae of males can be selected for a better perception of sexual signals emitted by females (intrasexual selection). Oversized antennae of males can also be induced by a female preference to reproduce with males with more developed antennae (intersexual selection). Males of *Aphidoletes aphidimyza* exhibit curved antennae, which are as long as their body, while antennae of females are short and straight. This morphological difference between the two sexes is probably due to sexual selection. Indeed, females of *A. aphidimyza* mate just once. They emit a sex pheromone to attract males to copulate and thus long antennae of

males could give them a better capacity to locate sexually receptive females. Moreover, females could preferentially choose males with well-developed antennae, for example because the handicap of long antennae in the mobility of males prove their genetic quality. Males of *A. aphidimyza* produced at a higher temperature are smaller and present a higher ratio of the length of antenna to the length of tibia, which is an important trait to get a mate. Males with a higher ratio of antenna to tibia could locate and reach a source of sex pheromone more easily and be more readily accepted by a female, especially if they can fly properly. Males coming from low or high rearing temperatures were used to test the impact of their morphology on their mating success. For each replicate, one female was in contact with two males produced at different temperatures, in a cage. When a mating occurred, individuals were retrieved and antennae, tibias and wings were measured and compared between mated and not mated males. The impact of the developmental temperature on the capacity of males to find and reach a source of sex pheromone versus a control was studied using a Y-shaped olfactometer. The males, produced at two different temperatures, were subjected to two different air flow speeds, to carry the scents. The movement patterns of males were then analysed in accordance with their rearing temperature and the air flow speed, to determine their capacity to perceive sex pheromone and to move toward it. The importance of the antennae size compared to the body size in the context of the reproduction is not confirmed by the mating experiments. In olfactometry, even though the males produced at a higher temperature, and thus presenting a higher ratio of antenna to tibia, did not find the pheromone more often, they flew a little more actively than those produced at lower temperature, when facing the sex pheromone. Nevertheless, the causes and implications of sexual dimorphism in *A. aphidimyza* are thus still to explore.

Keywords

Antennae size, *Aphidoletes aphidimyza*, males, mating, olfactometry, sexual dimorphism, sexual selection

Introduction

Sexual dimorphism may be shaped in two non-exclusive ways: natural selection and sexual selection (Land, 1980; Hedrick & Temeles, 1989; Shine, 1989). Natural selection appears if individuals composing a population present variable inheritable traits inducing fitness differences in a given environment (Endler, 1986; Freeman & Herron, 2007). It may impact body size and traits related to a different exploitation of the environment by the two sexes (Hedrick

& Temeles, 1989; Shine, 1989; Ricklefs & Miller, 2005). Natural selection can induce the regression of some conspicuous traits in one sex, due to hazard it engenders (Wallace, 1889). Sexual selection acts on traits that are useful to obtain a mate, or a better-quality mating partner, and can explain the appearance of some sexual dimorphic traits that cannot have evolved by natural selection as they seem unfavourable to survive (Darwin, 1871a; Futuyma, 2005; Smith & Smith, 2009; Shuker, 2010; Shuker, 2014). The sexual selection can materialise in two non-exclusive ways: the intrasexual selection and the intersexual selection (Hunt *et al.*, 2009; Cézilly & Allainé, 2016). The intrasexual selection occurs between individuals of the same sex, generally males, and the members of the other sex reproduce with the winners of this competition to mate. The intersexual selection involves individuals of the two sexes and the choice, generally by the female, of the most attractive mate among individuals of the opposite sex (Bateman, 1948; Trivers, 1972; Thornhill & Alcock, 1983; Ricklefs & Miller, 2005; Freeman & Herron, 2007; Smith & Smith, 2009). Both intrasexual and intersexual selections can lead to sexual dimorphism (Moore, 1990; Berns, 2013).

Sexual dimorphism at the level of the antennae is frequent in insects. These sexually dimorphic antennae can be produced by the natural selection, for example when females have to find a host by the scent. In this case, antennae of females are more specialized than those of males (Chapman, 1982; Chapman, 1998). However, antennae of males are generally more complex than those of females (Sivinski, 1997; Chapman, 1998) and many authors report sexually dimorphic antennae produced by sexual selection. Indeed, intrasexual selection can favour larger antennae in males for them to better perceive sexual signals and thus find females faster, which has rarely been studied (Darwin, 1871a; Chapman, 1982; Johnson *et al.*, 2017). Moreover, intersexual selection can lead to oversized antennae in males if females preferentially choose mates with more developed antennae (Jiménez-Pérez & Wang, 2004; Koshio *et al.*, 2007; Johnson *et al.*, 2017). This preference of the female for long antennae in males could be arbitrary (Fisher's runaway process – Fisher, 1915; Cézilly & Allainé, 2016) or long antennae could be an indicator trait of the quality of the male (good genes hypothesis), especially if they are costly to produce and maintain (handicap hypothesis) (Zahavi, 1975; Smith & Harper, 2003; Andersson & Simmons, 2006; Shiel *et al.*, 2015; Johnson *et al.*, 2017). Sexually dimorphic antennae that are larger in males could thus have evolved by sexual selection.

Aphidoletes aphidimyza is a gall midge preying on aphids at the larval stage and feeding on honeydew and nectar at the adult stage (Kuo, 1982; Markkula & Tiittanen, 1985; Choi *et al.*, 2004b; Watanabe *et al.*, 2014; Dumont *et al.*., 2015). This species can be sexed easily with the naked eye thanks to the antennal sexual dimorphism: males have feathery curved antennae as long as their body whilst females have short straight antennae (Markkula & Tiittanen, 1985; Zhang & Yang, 2008; Tabadkani *et al.*, 2012b; Tabadkani *et al.*, 2012c; Tabadkani *et al.*, 2013). *Aphidoletes aphidimyza* presents a nocturnal mating behaviour, which is rather complex compared to other gall midges (Harris, 1973; Harris & Foster, 1999; van Lenteren *et al*., 2002; van Lenteren & Schettino, 2003). Females, suspended in a spider web by the front legs, call for a mate by emitting a sex pheromone – the (2R,7S)-diacetoxytridecane, identified by Choi *et al.* (2004a). Attracted males land in the spider web and move to a female to mate while wing-fanning. During the copulation, the male extends its wings then folds them against its body. Females are supposed to be monoandrous as they stop to emit sex pheromone and refuse to mate again after a first mating (van Lenteren *et al*., 2002; van Lenteren & Schettino, 2003). The ratio of the length of right antenna to the length of right hind tibia is important for males of *A. aphidimyza* to get to females (Nicolas & Hance, in prep.a) and the environmental temperature during their development influences this ratio. Males produced at a higher temperature are globally smaller, with more symmetrical antennae and a ratio of antenna to tibia higher than males produced at a lower temperature (Nicolas & Hance, in prep.b).

The difference of antennae between males and females of *A. aphidimyza* is more probably due to sexual selection than to natural selection. Indeed, males and females have no reason to use their environment differently, except that females have to find a spider web for mating and a site to lay eggs. However, males have longer antennae than females, which suggests that these ecological differences between them are not the main drivers of the sexual dimorphism in antennae. Moreover, producing and bearing these extravagant antennae must be costly (Emlen, 2001; Symonds *et al.*, 2011), which should not be favoured by the natural selection. Contrariwise, it can represent an advantage for the sexual selection. Indeed, a compensation could be obtained by males with well-developed antennae, which could preferentially be chosen by females to mate, and so would be favoured by intersexual selection (Jiménez-Pérez & Wang, 2004; Koshio *et al.*, 2007; Johnson *et al.*, 2017). Oversized antennae could potentially impede flight and thus represent a handicap for the mobility. Furthermore, as males of *A. aphidimyza* have to locate females, which mate just once and produce a sexual pheromone, it is probable that their more developed antennae are promoted by intrasexual selection (Darwin, 1871a; Cézilly &

Allainé, 2016; Johnson *et al.*, 2017). Males of *A. aphidimyza* with longer antennae and especially with a higher ratio of antenna to tibia could thus more easily locate and reach a source of emission of pheromone and be accepted readily by a mate, particularly if they can fly properly. Males produced at a higher temperature should thus be favoured, compared to males produced at a lower temperature, as they have a higher antenna to tibia ratio (Nicolas & Hance, in prep.a).

In order to study the effect of differences in morphology (induced by different developmental temperatures: 18 °C and 28 °C) on the reproduction of males of *A. aphidimyza*, two kinds of experiments (mating and olfactometry) were performed. Firstly, in mating experiments, two males (produced at low and high temperatures) and one female (produced at intermediate temperature) were placed in a cage. All the individuals were removed once a mating occurred and the antennae, tibia and wings of males were measured and compared between mated and not mated males. Secondly, in order to determine if antennae size can be a handicap to fly, the capacity of males, reared at low or high temperatures, to reach a source of sex pheromone at two air flow speeds in an olfactometer was studied. Movements of males were then analysed in relation to their morphology and the air flow speed.

Materials and methods

Insects rearing

Individuals of *Aphidoletes aphidimyza* were bought from Koppert B.V. (Rodenrijs, Netherland) and then reared following Nicolas & Hance (in prep.b) inspired by Le Goff *et al.* (2016) and Kohandani *et al.* (2017). The aphid preys, *Myzus persicae*, initially obtained from Viridaxis S.A. (Gosselies, Belgium), were reared on sweet pepper (*Capsicum annuum*). Every two days, three plants infested with preys were taken from their mass rearing cage and put in the mass rearing cage of the predator to obtain eggs. After two days, three plants with aphids and eggs of the predator were recovered and each of these plants was placed in a different climatic room. The relative humidity in the climatic rooms was between 50 and 60 % with a light of roughly 20,000 lumens between 6 p.m. and 8 a.m. The reverse photoperiod was useful to perform experiments in the daytime, despite the nocturnal habits of *A. aphidimyza*. In each climatic room, plants were bended above a harvest box with sand, to recover larvae of *A. aphidimyza* ready for the pupation. Every two days, the harvest box with sand was replaced by another one with new sand. The previous box was sieved two days later, to collect the pupae, then put back in the right room.

The temperature conditions in the climatic rooms (constant day and night) were:

- Low temperature (low t°): 17.6 ± 1.0 °C.
- Intermediate temperature (intermediate t°): 22.6 ± 1.0 °C.
- High temperature (high t°): 27.8 ± 1.0 °C.

The pupae of *A. aphidimyza* obtained at the different temperatures were isolated before emergence of adults in microtubes of 1.5 ml with a piece of cotton swab soaked in a solution of ¼ of the volume of white sugar and ¾ of water, to feed the emerged insects.

Mating experiments

These experiments aimed at defining the phenotypic traits of males of *A. aphidimyza* that can explain their mating success. As in Nicolas & Hance (in prep.a.), a glass cage (30 by 30 by 40 cm^3) with one wall partially made of plexiglass and a small surface of fine mesh (to ventilate) was used for the experiments. Instead of a spider web, as used by individuals into the wild, fine mesh was attached to the ceiling of the cage for insects to hang to mate. The experiments were performed in the same climatic room as the rearing at intermediate t°, from 12 to 6 p.m., in the dark with a 40 W lighted red bulb. The red light was used to see the insects without disturbing their scotophase, since Diptera are not supposed to see red light (Schaefer & Wilkinson, 2004).

For each replicate, one fed virgin female less than 72 hours old from the intermediate t° was put into the cage, 15 minutes before males to have the time to hang and to begin emitting sex pheromone. Fifteen minutes after the female, two fed virgin males (one from low t° and the other from high t°), both less than 72 hours old, were released in the cage. Insects were then observed until a mating occurred (or half an hour had elapsed). When a mating was observed, all the individuals were retrieved and stored in a -20 °C freezer, separated between mated and not mated males. If no mating was observed during 30 minutes, all the insects were collected and replaced by new ones.

The two males used together in a same replicate, identified by mating status (mated or not mated), were compared by pair for the phenotypic traits measured (as explained hereafter in the measured phenotypic traits part). In this way, it was determined whether the mated male was smaller or larger than the not mated one for each parameter of each replicate. The number of mated males that were the smallest or the largest of their replicate for each phenotypic trait was thus counted for all replicates (N = 19).

As the rearing temperatures used to produce insects were the same as the ones used by Nicolas & Hance (in prep.b), morphological differences between males produced at low temperature and at high temperature must be very marked. It can thus reasonably be assumed that the smallest male of the replicate came from the high t° rearing at while the largest male came from the low t° rearing at.

Olfactometry

These experiments, inspired by Nicolas *et al.* (in prep.b), were performed to relate the behaviour of males of *A. aphidimyza* facing the sex pheromone carried by air flows of different speeds to the morphological differences resulting from rearing at different temperatures, especially to determine if antennae can be a handicap to fly. One fed virgin male (different from males of the mating experiments), less than 72 hours old, was used for each replicate. The experiments were performed with a Y-shaped glass olfactometer of 25 mm ID with a central branch of 20 cm and side arms of 18 cm at 60° (device inspired by Choi *et al.* (2004a), see in Nicolas *et al.* (in prep.b) for a diagram). All the experiments were performed between 20 °C and 23 °C, between 12 to 6 p.m., in the dark under a red light (two 25 W red bulbs). The red light was chosen because insects are not sensitive to this wavelength (Schaefer & Wilkinson, 2004) and thus the nocturnal behaviour of males was not disturbed. The smells were carried by a flow of ultrapure synthetic air blown trough a charcoal filter to the base of each arm of the Y olfactometer and then toward the central branch were the insect was released. One ng of the synthetic female sex pheromone – (2R,7S)-diacetoxytridecane, prepared according to the procedure of Choi *et al.* (2004a) – dissolved in 100 µL of pentane was used at the base of one arm and 100 µL of pentane alone was used at the base of 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 for pentane to evaporate before the use. The filter papers were then inserted in the olfactometer at 2 cm of the orifice of the arms. A line was traced under the olfactometer at 6 cm of the beginning of the arms to determine the end of the test, when a male had crossed the line. The position of the olfactometer and the arm where sex pheromone and control were placed were rotated.

To begin a replicate, a small glass jar (just fitting to the entry of the olfactometer) containing a male of *A. aphidimyza* was introduced in the central branch of the olfactometer. One arm of the olfactometer was provided with the female sex pheromone while the other arm was not. The times the insect took to enter the olfactometer (by exiting the jar) and to cross the line under the

olfactometer (end of the test) were recorded (see in Nicolas *et al.* (in prep.b) for an illustration). If the insect did not go out from the glass jar after 5 minutes or if it did not cross the line within 5 minutes after exiting the jar, the replicate was stopped. All the males (N = 180) were stored in a -20 °C freezer after experiments, for subsequent measurements. After one replicate, whether the male had crossed the line or not, the apparatus was cleaned and new sources of smell and insects were used for the next test.

The capacity of males of *A. aphidimyza* to perceive and to respond to the sex pheromone was studied according to two factors:

- Rearing temperature: males produced at low t° (17.6 °C) and at high t° (27.8 °C), inducing marked morphological differences between them (Nicolas *et al.*, in prep.b).
- Air flow speed in the olfactometer: slow flow (0.28 mL/s) and fast flow (2.78 mL/s).

Measured phenotypic traits

To measure phenotypic traits of males, the insects were spread in a Hanks' balanced salts solution (98 g/l), with the structures to measure well flat, and pictures were taken. Afterwards, the following structures were measured with the ImageJ software for Mac:

- Length of both antennae. The total length of the flagellum (12 flagellomers), without scape and pedicel, was measured for the mating experimentations. Only the first seven flagellomers (because of breakage of last flagellomers when males were collected in the olfactometer) were measured for the olfactometry part.
- Right hind tibia length.
- Right wing area.

For each parameter, the mean of three measures was taken, in order to diminish the variability related to the measurement (as suggested by Palmer & Strobeck, 1986, by Merilä & Björklunds, 1995 and by Blanckenhorn *et al.*, 2003).

The symmetry of antennae was estimated by the absolute value of the difference between right antenna length and left antenna length (|RA-LA|).

The ratio of the length of the right antenna divided by the length of the right hind tibia (ratio of right antenna to tibia) was calculated because it discriminates between males capable to obtain a mate (Nicolas & Hance, in prep.a) and because it varies with the temperature of development (Nicolas & Hance, in prep.b).

Statistical analyses

Statistical analyses were performed using GraphPad Prism version 5.0 software for Mac, with a significance level (P) set at $\alpha = 0.05$ and under two-tailed hypothesis, unless otherwise stated. The margin error is standard error (SE).

Mating experiments

A binomial test was used to determine, for all the phenotypic traits measured, whether the proportion of mated males that were the largest of their replicate in the total number of repetitions was different from a random repartition (one-tail sign test, 50/50 repartition).

A Student t-test for paired samples was performed to know if the measured parameters were different according to the mating status of the male in each replicate.

Olfactometry

A Fisher's exact test was applied to determine whether the number of males that entered the olfactometer or that crossed the line, among all the males, was influenced by the rearing temperature or the air flow speed.

The subsequently presented analyses were performed using JMP version 13.2.0 software for Mac.

A binomial test was used to determine whether the proportion of males that went into the arm of the olfactometer with the pheromone, among males that crossed the line, was different from a random distribution (sign test, 50/50 repartition, under one-tailed hypothesis). A Fisher's exact test was applied to determine whether the number of males that went into the arm with the pheromone, among males that crossed the line, was influenced by the rearing temperature or the air flow speed.

After a $\log_{10}$ transformation of the time to enter the olfactometer and the time to cross the line traced under the olfactometer, multivariate correlations were estimated by the restricted maximum likelihood (REML) method, for these two transformed variables and the antennae length, the tibia length and the wing area of the males that crossed the line.

Results

Mating experiments

Results of mating experiments indicate that the number of mated males presenting the longer antennae of their replicate cannot be considered as different from the number of mated males presenting the smaller antennae or from a random repartition (binomial tests, TABLE 1). The analyses concerning the symmetry of antennae (|RA-LA|), the ratio of right antenna to tibia (RA/tibia), the right hind tibia length and the right wing area come to the same conclusion.

Concerning the analysis of the results of measurements for the antennae lengths, the antennae symmetry, the ratio of right antenna to tibia, the right hind tibia length and the right wing area, no significant difference appeared between mated males and not mated males (paired student t-tests, TABLE 1). The mean length of antennae is almost the same for the two sides of a same male.

TABLE 1. Counts (first line) and results of the associated binomial tests (second line), means (± SE) for mated and not mated males (third and fourth lines) and results of the paired student t-tests associated to means (fifth line) for the morphological parameters measured and calculated in males (lengths of the right and left antennae, symmetry of antennae, ratio of the right antenna length to the right hind tibia length, length of the right hind tibia and area of the right wing).

	Right antenna (RA)		**Left antenna (LA)**		**|RA-LA|**		**RA/tibia**	
	Small	**Large**	**Small**	**Large**	**Small**	**Large**	**Small**	**Large**
Number of mated males	10	9	9	10	10	9	10	9
Binomial test (sign test, N = 19)	NS P = 0.500		NS P = 0.500		NS P = 0.500		NS P = 0.500	
Mated males	2.76 ± 0.05 mm		2.75 ± 0.05 mm		0.03 ± 0.01 mm		3.10 ± 0.04	
Not mated males	2.78 ± 0.05 mm		2.78 ± 0.05 mm		0.03 ± 0.00 mm		3.07 ± 0.03	
Paired t-test (df = 18)	NS P = 0.867 t = 0.170		NS P = 0.753 t = 0.319		NS P = 0.794 t = 0.266		NS P = 0.576 t = 0.570	

	Tibia		Wing	
	Small	**Large**	**Small**	**Large**
Number of mated males	8	11	11	8
Binomial test (sign test, N = 19)	NS P = 0.324		NS P = 0.324	
Mated males	0.89 ± 0.02 mm		1.31 ± 0.05 mm	
Not mated males	0.91 ± 0.02 mm		1.13 ± 0.05 mm	
Paired t test (df = 18)	NS P = 0.656 t = 0.454		NS P = 0.723 t = 0.361	

Olfactometry

There is a relation between the rearing temperature and the fact that males entered the olfactometer (N_{total} = 180, $N_{low\ t°}$ = 135, $N_{high\ t°}$ = 45, Fisher's exact test: P = 0.006). The males reared at high t° exited the jar proportionally more often (in 82.2 % of cases) than the males produced at low t° (in 59.3 % of cases) (FIGURE 1, A). Similarly, there is a relation between the rearing temperature and the fact that males crossed the line traced under the olfactometer (Fisher's exact test: P = 0.035). The males reared at high t° crossed the line proportionally more often (in 53.3 % of cases) than the males produced at low t° (in 34.8 % of cases) (FIGURE 1, B).

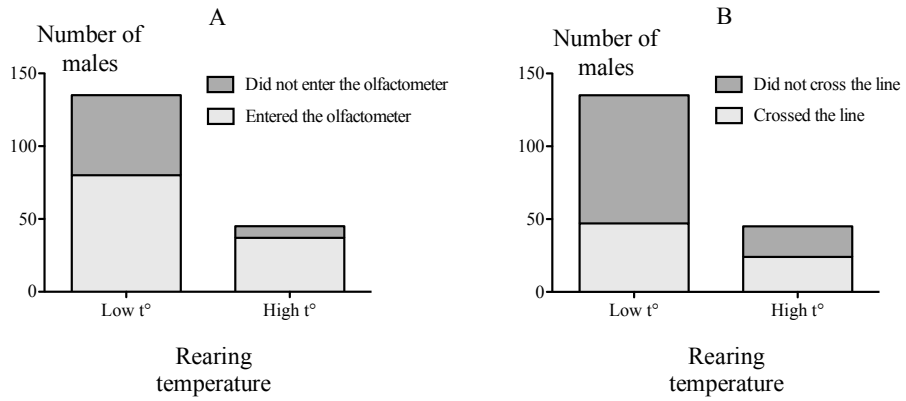


FIGURE 1. Number of males that entered the olfactometer or not (A) and that crossed the line under the olfactometer or not (B), according to the rearing temperature.

There is no relation between the air flow speed in the olfactometer and the fact that males entered the olfactometer (N_{total} = 180, $N_{slow\ flow}$ = 108, $N_{fast\ flow}$ = 72, Fisher's exact test: P = 0.633) (FIGURE 2, A). Similarly, there is no relation between the air flow speed and the fact that males crossed the line traced under the olfactometer (Fisher's exact test: P = 0.534) (FIGURE 2, B).

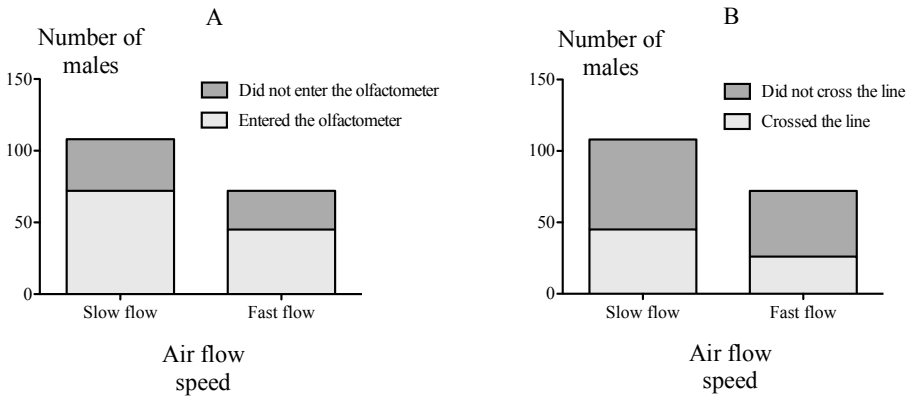


FIGURE 2. Number of males that entered the olfactometer or not (A) and that crossed the line under the olfactometer or not (B), according to the air flow speed.

Males that crossed the line traced under the olfactometer (N = 71) went more often into the arm with the sex pheromone (51 males) than into the arm with the pentane alone (20 males) (sign test: N = 71, P = 0.000). On the other hand, there is no relation between the rearing temperature of males or the air flow speed in the olfactometer and the fact that males went into the arm of the olfactometer containing the sex pheromone (Fisher's exact test: P = 0.560 for rearing temperature and P = 0.276 for air flow speed).

Antennae size, tibia length and wing area are very correlated with each other, but the $\log_{10}$ of the time to enter the olfactometer and of the time to cross the line are not correlated with each other or with these morphological parameters (TABLE 2).

TABLE 2. Values of the multivariate correlations calculated between the time to enter the olfactometer or the time to cross the line, after a $\log_{10}$ transformation, and the antennae lengths, the tibia length and the wing area.

	$\log_{10}$ of the time to enter the olfactometer	**$\log_{10}$ of the time to cross the line**
Right antenna length	-0,363	-0,155
Left antenna length	-0,394	-0,138
Tibia length	-0,306	-0,142
Wing area	-0,230	-0,214

Discussion

In the olfactometry experiments, a higher proportion of males entered the olfactometer and crossed the line when they were reared at a higher temperature rather than when they were reared at a lower temperature. The proportions are higher than those of other similar experiments (Nicolas *et al.*, in prep.b) (TABLE 3). This could be explained by the facts that males of a higher temperature present a ratio of antenna to tibia higher than those of a lower temperature (Nicolas & Hance, in prep.b) and that this ratio is important for males of *A. aphidimyza* in the context of reproduction (Nicolas & Hance, in prep.a). The males produced at a higher temperature, and thus presenting a higher ratio of antenna to tibia, flew more actively than those produced at a lower temperature, when facing the sex pheromone. The importance of the antennae size compared to the body size seems to be confirmed and can give an idea of what could happen in the context of climate change, as higher temperatures are expected in the next decades (Wigley & Raper, 2001; IPCC, 2013; Lhotka *et al.*, 2018). Moreover, as males of high temperature are also smaller, the experiments can be linked to those of Zamudio *et al.* (1995) showing that small males, produced at a higher temperature, are more vigorous than others produced at a lower temperature. Contrariwise, the fact of crossing the line could be interpreted as a choice of an arm of the olfactometer by the male. However, it is not possible, in this experiment, to distinguish between a real choice and a constraint on the male induced by the experimental conditions, or even differences in males' temperament.

TABLE 3. Percentages of males that entered the olfactometer and that crossed the line, according to their rearing temperature.

	Rearing temperature		
	Low t° (18 °C) N = 135	**High t° (28 °C) N = 45**	**Nicolas *et al.* (in prep.b) (23-25 °C) N = 78**
Entered the olfactometer	59 %	82 %	35 %
Crossed the line	35 %	53 %	27 %

Contrary to the rearing temperature, no relation could be highlighted between the air flow speed in the olfactometer and the fact that males entered the device or crossed the line. The sizes of the antennae and of the body do not represent a handicap or a help for males of *A. aphidimyza* to move into the olfactometer, which is surprising considering the higher air flow speed (Symonds *et al.*, 2011).

Males of the olfactometry experiments went more often into the arm with the sex pheromone than into the control arm, like in Nicolas *et al.* (in prep.b), which was obviously expected. Contrary to previous experiments (Nicolas & Hance, in prep.a), this is not explained by differences of the RA/T ratio. However, in the experiments of Nicolas & Hance (in prep.a), males that competed for a same female were taken randomly in a rearing at a same temperature and were measured after mating. Here, the rearing temperature was used to obtain these phenotypic differences. In the same time, we observed in the olfactometry experiments that males reared at 18 °C flew less actively than males reared at 28 °C. It is thus possible that a low developmental temperature induces a lesser activity, which is somewhat compensated by changes in morphology that give the same access to females as for males reared at high temperature (and thus with a high flying activity).

The times the males took to enter the olfactometer or to cross the line cannot be explained by the analysed parameters either. The prediction was that males with longer antennae or with a higher ratio of antenna to tibia compared to other males (intrasexual selection, as in Johnson *et al.*, 2017) should find the arm with the pheromone more easily, but this is not the case. Maybe it is not the length of antennae but their efficiency that is important for *A. aphidimyza* males to find and reach the source of pheromone. Even though it is difficult to implement because of the very small size of these structures, sensilla number and operationality could be assessed to complete future analyses, as these parameters can vary with temperature (Bourdais *et al.*, 2006) and are known to influence the reproductive behaviour (Ljungberg *et al.*, 1993; Zhang & Yang, 2008; Hazaa, 2010; Yan *et al.*, 2014).

No significant difference could be highlighted between mated and not mated males in mating experiments. There is no choice of the female based on the phenotypic traits of males, in the conditions under which the experiments were done. A difference in the ratio of antenna to tibia had still been observed between mated and not mated males in comparable experiments (Nicolas & Hance, in prep.a) and the differences of morphologies between the two males used in a same replicate have been accentuated by the different temperatures of development, as suggested in Nicolas & Hance (in prep.b). Maybe the use of a bigger cage for the experiments could improve the results, by moving the males away from the female calling, which would be harder to find for males with shorter antennae or lower ratio of antenna to tibia.

Contrary to what was expected, males with longer antennae or with a higher ratio of antenna to tibia did not reach a source a pheromone and were not

accepted by a mate more easily than other males. Males developed at a higher temperature have not been favoured compared to others, even though males reared at a higher temperature flew more actively than those coming from a lower temperature, in the olfactometry experiments. The importance of the ratio of antenna to tibia for mating (Nicolas & Hance, in prep.a) has not been confirmed by these mating and olfactometry experiments. The results of these investigations on the causes and implications of sexual dimorphism in *A. aphidimyza* are not really conclusive; more experiments are needed to strengthen the conclusions.

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6. Summary

In the ARTICLE 4 (Nicolas & Hance, in prep.b), the development of individuals and the morphology of males have been studied according to their developmental temperature. Characteristics of pupae, some parameters linked to emergence of adults from pupae and morphology of adult males have been studied for individuals reared at three different temperatures. As expected, pupae and adults reared at a higher temperature were smaller and developed faster than those reared at a lower temperature. Contrary to what was predicted, the sizes of body parts (antennae and wings) relative to body size, for males, have been affected differently by these different temperatures, even though the absolute size of these structures evolved globally in the same direction as the body size. A negative allometry has been observed for the size of antennae whilst a positive allometry has been observed for the size of wings, relative to body size, with the changes of developmental temperature.

In the ARTICLE 5 (Nicolas *et al.*, in prep.a), the reproductive behaviour of males has been studied according to their differences of morphology engendered by different developmental temperatures. Mating experiments have been performed with males produced at different temperatures to define the

impact of the morphology of males of *A. aphidimyza* on their mating success. Olfactory experiments have been conducted to relate the behaviour of males facing the sex pheromone, carried by different air flow speeds, to their morphology, especially to determine if antennae size could be a handicap to fly. The supposition that males of *A. aphidimyza* with longer antennae and especially with a higher antenna/tibia ratio should more easily locate and reach a source of pheromone and should be accepted easily by a mate, particularly if they can fly properly, was not confirmed. Despite their higher antenna to tibia ratio, males produced at a higher temperature were not favoured compared to males produced at a lower temperature.

Even though the effects of the temperature on the morphology of males of *A. aphidimyza* are better known thanks to this research, the implications this can have on the fitness in relation to reproduction remain unclear. Despite the use of males with very different phenotypes, produced by different rearing temperatures, the prediction of the beginning of the thesis that the access to a mating partner should be facilitated for males by longer and more symmetric antennae has not been validated.

The results of this chapter can be summarised as follow:

- Variations in phenotypic traits of males, especially in antennae and antenna to tibia ratio, have been produced using different developmental temperatures (ARTICLE 4: Nicolas & Hance, in prep.b).
- No fitness differences between these variants of phenotypic traits have been highlighted in connection with the competition to obtain a mate (ARTICLE 5: Nicolas *et al.*, in prep.a).
- The inheritance of phenotypic traits has not been studied in ARTICLE 4 (Nicolas & Hance, in prep.b) and ARTICLE 5 (Nicolas *et al.*, in prep.a) but conclusions can be transposed to inheritable differences of phenotypic traits as the selective pressure will be the same whether variations of traits were inheritable or not (see point 3. just above).

The hypothesis that antennal sexual dimorphism could be due to a better access of males of *A. aphidimyza* with longer and more symmetric antennae to a mate is not proved by the experiments of this CHAPTER IV. The supposition that intrasexual and intersexual selection shaped the impressive antennae of males of *A. aphidimyza* is thus not consolidated.

CHAPTER V. GENERAL DISCUSSION

As the results of the four articles have already been discussed in the articles themselves and in the summaries at the end of CHAPTER III and CHAPTER IV, this general discussion will focus on what emerges from pooled results, to answer the main question of this thesis: what played a leading role in the appearance and maintenance of antennal sexual dimorphism in *Aphidoletes aphidimyza*?

1. Morphology and influence of temperature

Globally, larger males (body size being estimated by tibia size) present longer antennae and larger wings, whatever the developmental temperature is.

At a developmental temperature close to the optimum, there were positive linear relations between the size of the measured phenotypic traits (antennae, wing and tibia) in males of *A. aphidimyza* (ARTICLE 2: Nicolas *et al.*, in prep.b).

The temperature has an impact on the size of the different traits and also on the relations between them. When the developmental temperature was higher, the individuals produced were globally smaller (as indicated by their smaller tibia), had smaller antennae and wings, but the antennae size increased and the wing size decreased relative to body size. The antennae size presents a negative allometry and the wing size a positive allometry to the body size according to temperature variations (ARTICLE 4: Nicolas & Hance, in prep.b). The results on the absolute sizes are in accordance with the temperature-size rule, which states that development at higher temperatures engenders smaller adult body sizes (Ray, 1960; Atkinson, 1994; Atkinson & Sibly, 1997; Davidowitz & Nijhout, 2004; Kingsolver & Huey, 2008). Concerning the relative sizes, larger individuals seem to need larger wings to cope with their body size. A trade-off between wings and antennae could explain these traits related to the body size vary inversely and may indicate a morphological cost to produce oversized antennae (like in Kawano (1997) reporting trade-off between mandibles and wings).

2. Reproductive behaviour according to morphology

Males of *A. aphidimyza* with longer antennae compared to their body (evaluated by tibia size) have a higher probability to gain access to a mate than others. Indeed, in some mating experiments, the antenna to tibia ratio was higher for mated than for not mated males (ARTICLE 3: Nicolas & Hance, in prep.a). Moreover, the males produced at a higher temperature and presenting a higher antenna to tibia ratio flew more actively in olfactometry experiments than those produced at a lower temperature, even though they did not find the pheromone more often (ARTICLE 5: Nicolas *et al.*, in prep.a). This antenna to tibia ratio thus seems to play a more important role in intersexual selection than in intrasexual selection, a greater ratio improving the mating success of males of *A. aphidimyza*.

The most likely processes to lead to this preference of females for mates with long antennae compared to their body are the Fisher's runaway process and the good genes model:

- According to the Fisher's runaway process, females would prefer, for no apparent reason, small-bodied males with long antennae. If the preference of females and this kind of male morphology are inheritable, the female descendants will also prefer mates presenting this kind of morphology and the male descendants will be small-bodied with long antennae. The fitness of the descendants will thus be improved from generation to generation, reinforcing the trait "long antennae relative to body size", in case of directional selection (Fisher, 1915; Fisher, 1930; Smith, 1991; Ryan, 1998; Futuyma, 2005; Freeman & Herron, 2007; Cézilly & Allainé, 2016).
- In accordance with the good genes model, the fact that males are small-bodied with long antennae would indicate their good genetic quality, which would be advantageous for their offspring. The small-bodied males with long antennae could be, for example, handicapped to fly, because of their long costly antennae relative to their small size, according to the Zahavi's handicap principle. Females could prefer these small-bodied males with long antennae because if they can produce these costly antennae despite their small body or survive with this handicap, it is a clue of their superior genotype, which is advantageous to transmit to the progeny (Zahavi, 1975; Thornhill & Alcock, 1983; Ryan, 1998; Smith & Smith 2009; Cézilly & Allainé, 2016).

However, no influence of the impressive antennae of males of *A. aphidimyza* on their flying capacity was highlighted in the experiments, even in relation to the body size (evaluated by tibia size). The males reached the source of sex pheromone as easily in slow air flow than in fast air flow in olfactometry experiments, whatever their rearing temperature and thus their morphology were (ARTICLE 5: Nicolas *et al.*, in prep.a). Perhaps experiments in a larger device, possibly in the presence of predators of *A. aphidimyza* or with an even faster air flow, could give more indications on the handicap or the advantage – like enhanced detection of sex pheromone (Johnson *et al.*, 2017) or better lift and stability (Symonds *et al.*, 2011) – long antennae can constitute for males. The centre of gravity of males presenting antennae of different sizes should also be located and the influence of these potential differences on the flying capacity could be estimated (as the position of the centre of gravity is known to have an impact on the flying ability – Berwaerts *et al.*, 2002).

These long antennae in males, certainly costly to produce with a small body, as it could be indicated by the allometries in the ARTICLE 4 (Nicolas & Hance, in prep.b), could thus inform on the quality of the male bearing them. However, they seem not to constitute a handicap to fly. In the light of these results, the good genes model and the Fisher's runaway process remain most appropriate to explain the preference of females of *A. aphidimyza* for males with long antennae relative to their size. Nonetheless, the cost to produce long antennae in relation to the body size is the most likely explanation in the good genes model, the handicap to fly being dismissed until proven otherwise.

In order to better understand the roles of the Fisher's runaway process and of the good genes model on the morphology of males of *A. aphidimyza*, experiments could be performed to obtain an indication on the cost of long antennae.

The nutrition directly influences insect growth rate, which influences adult body size (Chapman, 1998; Davidowitz & Nijhout, 2004; Berg & Merritt, 2009; Chown & Gaston, 2010; Martel *et al.*, 2011). The prediction may be that males reared with different amounts of aphids could present different morphologies, especially in case of shortage. It could be interesting to analyse the allometries of antennae and wing to body size to see if males can fully develop their impressive antennae when less aphids are available for their development. This would give a better indication on the costs to produce antennae comparatively to wings and body size and on potential trade-offs between these structures. An evaluation of the energy reserves (glucose and lipids), as they may be positively correlated with the mating success of males (Blanckenhorn *et al.*, 2003), could advantageously complement the analyses.

The body could also be weighed, in addition to the measurement of antennae, wings and tibias, to calculate the wing loading – body weight to wing area, Danforth (1989) – at the different rearing conditions and thus obtain a better indication of the flying capacity of individuals.

In future experiments, an assessment of the capacity of males to copulate several times, the number of spermatozoa transmitted at each mating as well as the quantity and the quality of spermatozoa produced, according to the males' phenotypic traits, would give important information on the trade-offs between reproduction and morphology.

3. Causes of sexual dimorphism in *Aphidoletes aphidimyza*

The results can be recapped according to the reasoning proposed in the CHAPTER II, point 4.2.3.:

- Phenotypic traits of males of *A. aphidimyza*, especially antennae, are naturally variable (ARTICLE 2: Nicolas *et al.*, in prep.b).
- The access of males of *A. aphidimyza* to a mate is related to their antenna to tibia ratio, males with a higher ratio having a higher probability to mate (ARTICLE 3: Nicolas & Hance).
- Even though the inheritance of phenotypic traits has not been studied, conclusions can be transposed to inheritable differences of phenotypic traits because the selective pressure will be the same for variations of traits inheritable or not (CHAPTER IV, point 3.).

Consequently, all the conditions seem met for the sexual selection to act and lead to evolution (unless sexual selection released in the rearing conditions, as the conditions are stable). The males of *A. aphidimyza* with a higher antenna to tibia ratio should thus become prevalent in the population over the time.

Larger wings and antennae have been observed, as expected, in the larger males of *A. aphidimyza*, with the relative size of antennae and wings compared to body size that vary inversely with temperature. Contrariwise, the prediction that longer and more symmetric antennae could facilitate the access of males to a mate has not been confirmed. It is rather a high antenna to tibia ratio that improved the reproductive success of males *of A. aphidimyza*. The female would thus evaluate the disproportion of antennae relative to the body size. Maybe the advantage of long antennae in males is not only brought by the length of antennae themselves but also by the presence of sensilla on antennae, known to impact the reproductive behaviour (Ljungberg *et al.*, 1993; Zhang & Yang, 2008; Hazaa, 2010; Yan *et al.*, 2014); so, it would be interesting to investigate that point.

No firm conclusion can be drawn concerning the prediction that intrasexual or intersexual selection or both have shaped the striking antennal sexual dimorphism in *A. aphidimyza*. The antenna to tibia ratio influenced the access of males to a mate in some experiments, but the absolute length of antennae did not. This antenna to tibia ratio, which is higher for mated males than for not mated males, gives a partial but not a definitive answer to this questioning about the factors that may cause sexual dimorphism in *A. aphidimyza*. Further experiments are needed to understand how and why the antennal sexual dimorphism appears in *A. aphidimyza* and to fully understand the role of the antenna to tibia ratio.

4. Implications for the use of *Aphidoletes aphidimyza* in biological control

The experiments for this thesis were performed with a strain of *A. aphidimyza* coming from the company Koppert, which is specialized in the mass rearing and commercialisation of insects for biological control. It is thus not a wild strain. This could have implications on the functional morphology. Indeed, the needs of insects living in a climatic room or into the wild are probably not the same, and it would impact the genetic diversity and the morphology of males (Hopper *et al.*, 1993). For example, as industrial rearing is done at high density, the pressure to find a mate is probably reduced. However, working with a wild strain, with more genetic diversity, would have been difficult for the following reasons: difficulties to catch insects alive, and to identify and rear the species, problems of stability over the time, and maintenance of genetic diversity. Moreover, as the strain is commercially produced by Koppert for aphid control, experiments with this strain are useful to obtain information of what could happen, in current conditions, in biological control programs. Our results showed that a variability between individuals still remains for most morphological indicators (ARTICLE 2) and that the susceptibility to environmental conditions (temperature) still differs between individuals (ARTICLE 4). Furthermore, with less genetic diversity than into the wild, the differences observed between males in the experiments have probably just been underestimated; so, the conclusions that were drawn are still reliable. Contrariwise, this lower genetic diversity in reared strains, in addition to the adaptation of the strain to constant production conditions, could reduce the performances of the species used in biological control (Hopper *et al*., 1993).

The effects of temperatures, as observed in the ARTICLE 4 and in the ARTICLE 5, give information about what could happen if the individuals produced for biological control were reared at those different temperatures. The reproduction of individuals as well as the temperature are crucial aspects of rearing (Cohen, 2001). The size of females of *A. aphidimyza* was not assessed, but the size of pupae (without distinction of the gender) and of males were. An increase of the size of pupae and of males has been observed at a lower development temperature; so, it should be logical that females' size increases too. Since, in insects, females' body size is generally related to its fecundity – bigger females are more fecund than smaller ones (Honěk, 1993; Stillwell *et al.*, 2010) – females obtained at a lower temperature should be more fecund, which is interesting for their use in biological control. This point should be investigated in *A. aphidimyza*. However, the production of individuals for biological control will take more time at low than at high temperature – the development time of *A. aphidimyza* increases when temperature decreases (ARTICLE 3: Nicolas & Hance, in prep.a) – and a cost-benefit analysis has thus to be conducted to determine the optimal temperature for a commercial production.

A last point is the efficiency of *A. aphidimyza* under conditions of high temperatures, as what can be recorded under greenhouses in the summer. It appeared, in the olfactometry analyses, that individuals reared at high temperature, even if smaller, seem to be more active that those reared at low temperature. This observation should be confirmed for females and its consequences on their foraging capacity should be investigated.

5. Perspectives

In this study, antennae and wing sizes have sometimes been divided by the tibia size (as a proxy of the body size), to remove the body size component of variation, which is a common application of ratios in biology, even though there is no consensus on the best way of working (Atchley *et al.*, 1976; Daly, 1985; Packard & Boardman, 1988; Packard & Boardman, 1999; Mongiardino *et al.*, 2015). Another solution could consist in log-transforming the ratios before using them (Mongiardino *et al.*, 2015). Moreover, some other analyses could be performed to confirm the results presented. For example, ANCOVA could be used instead of ANOVA, even though the conclusions are the same in the majority of cases, and results of ratio analyses are supposed to be robust when the treatment effect is very marked, which is the case here (Atchley *et al.*, 1976; Packard & Boardman, 1988; Packard & Boardman, 1999). Furthermore, the statistical analyses performed do not concern the use of ratios for physiological

or phylogenetical purposes, which seem quite sensitive to bias (Packard & Boardman, 1988; Mongiardino *et al.*, 2015).

The movement pattern of males of *A. aphidimyza* facing the sex pheromone, according to the rearing temperature, has been observed in olfactometry experiments (ARTICLE 5: Nicolas *et al.*, in prep.a). A higher proportion of males entered the olfactometer and crossed the finishing line when they were reared at a higher temperature, rather than when they were reared at a lower temperature. Males reared at low temperature flew less actively, at intermediate temperature, than males reared at high temperature. It would be interesting to further study the impact of temperature on the behaviour of males, in order to differentiate a real choice from constraints induced by the experimental conditions, or from differences of activity produced by developmental temperature, or even from differences in males' temperament. The real motivation would give more indications on the intersexual selection.

Finally, in order to gain a more global view of the causes of sexual dimorphism, it would also be interesting to focus on other proximate causations of long antennae in males of *A. aphidimyza*. For example, study the developmental mechanisms leading to antennal sexual dimorphism (intern stimuli) in addition to the effect of temperature on the development (extern stimuli). The ultimate causations could also be investigated further, for example by studying the evolution of antennal sexual dimorphism in species related to *A. aphidimyza* (phylogeny), rather than only selective forces operating at adulthood (function of long antennae) (Tinbergen, 1963; Freeman & Herron, 2007; Tammaru *et al*., 2010; Hladký & Havlíček, 2013). For example, many species of gall midges have sexually dimorphic antennae (Gagné, 1994), but a difference of antennae size between males and females is quite rare in other families of Diptera, although observable in *Prochyliza xanthosoma* (Piophilidae) (Bonduriansky & Rowe, 2005). The influence of the antenna to tibia ratio on the mating success could also be evaluated in this species, to compare the results to those obtained with *A. aphidimyza*.

As often, Science answered a question with another question...

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"Je vous souhaite de souhaiter.
Je vous souhaite de désirer. Le bonheur, c'est déjà vouloir. Comme en droit pénal, l'intention vaut l'action. Le seul fait de rêver est déjà très important.
Je vous souhaite des rêves à n'en plus finir. Et l'envie furieuse d'en réaliser quelques-uns.
Je vous souhaite d'aimer ce qu'il faut aimer, et d'oublier ce qu'il faut oublier.
Je vous souhaite des passions.
Je vous souhaite des silences.
Je vous souhaite des chants d'oiseaux au réveil et des rires d'enfants.
Je vous souhaite de respecter les différences des autres parce que le mérite et la valeur de chacun sont souvent à découvrir.
Je vous souhaite de résister à l'enlisement, à l'indifférence et aux vertus négatives de notre époque.
Je vous souhaite enfin de ne jamais renoncer à la recherche, à l'aventure, à la vie, à l'amour, car la vie est une magnifique aventure et nul de raisonnable ne doit y renoncer sans livrer une rude bataille.
Je vous souhaite surtout d'être vous, fier de l'être et heureux, car le bonheur est notre destin véritable."

(Brel, 1968)