Chiara De Pasqual

On the Maintenance of Intrapopulation Genetic Variation

The Interplay Between Sexual Selection, Chemical Communication, and Fitness in the Wood Tiger Moth (*Arctia plantaginis*)

UNIVERSITY OF JYVÄSKYLÄ

FACULTY OF MATHEMATICS
AND SCIENCE

Chiara De Pasqual

On the Maintenance of Intrapopulation Genetic Variation

The Interplay Between Sexual Selection, Chemical Communication, and Fitness in the Wood Tiger Moth (*Arctia plantaginis*)

Esitetään Jyväskylän yliopiston matemaattis-luonnontieteellisen tiedekunnan suostumuksella
julkisesti tarkastettavaksi yliopiston vanhassa juhlasalissa S212
joulukuun 2. päivänä 2022 kello 12.

Academic dissertation to be publicly discussed, by permission of
the Faculty of Mathematics and Science of the University of Jyväskylä,
in building Seminarium, auditorium S212, on December 2, 2022 at 12 o'clock noon.

JYVÄSKYLÄN YLIOPISTO
UNIVERSITY OF JYVÄSKYLÄ

JYVÄSKYLÄ 2022

Cover picture by Sofia De Pasqual.

Ai miei giallo-blu

ABSTRACT

The maintenance of intrapopulation phenotypic trait variation often is the result of the interplay between multiple selective forces. Genetic associations between color traits and life-history traits can create context-dependent advantages and affect intrapopulation dynamics. In the polymorphic wood tiger moth (*Arctia plantaginis*), the two male hindwing phenotypes (genotypes: WW/Wy = white, yy = yellow) show morph-linked advantages that are context-dependent across ecological contexts. Although female hindwing coloration spans continuously from yellow to red they genetically bear the same male color alleles. In this thesis, I investigated the potential contribution of sexual selection, chemical communication, and fitness of the different genetic color morphs (i.e., WW, Wy, yy) in maintaining the two male color morphs. Mate choice and mate recruitment were affected by context-dependent advantages. yy females were more attractive to males in high-density scenarios because of their early calling strategy that likely allowed them to outcompete WW and Wy females. WW females were, instead, more attractive when females were simultaneously calling, perhaps due to their longer calling duration. yy males were faster to reach females than white (WW, Wy) males in low-density scenarios, while white males and males with denser antennae (i.e., higher lamellae count) located females faster in high-density scenarios. At the reproductive level, males and females showed genotype-specific (dis)advantages. Wy females benefit from higher likelihood of reproductive output among female genotypes leading to strong heterozygote advantage. yy males had the lowest reproductive output among male genotypes while pairs with yellow females suffered the lowest mating success. The presence of one or two copies of the *yellow* allele affected several life-history traits, from pre- to post-copulatory stages. Thus, the color locus has pleiotropic effects on several life-history traits, offering a mechanism of balancing selection that enables the persistence of genetic and phenotypic variation in natural populations.

Keywords: Balancing selection; heterozygote advantage; pleiotropy; receiver antenna variation; signaling strategy variation; wood tiger moth.

Chiara De Pasqual, University of Jyväskylä, Department of Biological and Environmental Science, P.O. Box 35, FI-40014 University of Jyväskylä, Finland

TIIVISTELMÄ

De Pasqual, Chiara
Seksuaalisen valinnan ja kemiallisen viestinnän vaikutus kelpoisuuteen ja geneettisen muuntelun säilymiseen täpläsiilikkäällä, *Arctia plantaginis*
Jyväskylä: Jyväskylän yliopisto, 2022, 54 p.
(JYU Dissertations
ISSN 2489-9003; 579)
ISBN 978-951-39-9244-6 (PDF)
Diss.

Populaation geneettistä muuntelua ylläpitävät useiden valintapaineiden yhteis-vaikutukset. Ominaisuuksien geneettinen kytkeytyminen elinkierto-piirteiden välillä ja niiden kelpoisuusvaikutukset voivat olla tilanteesta riippuen joko positiivisia tai negatiivisia. Nämä kytkeytymiset vaikuttavat siten geneetisen muuntelun dynamiikkaan. Polymorfisella täpläsiilikäs perhosella (*Arctia plantaginis*) koiraiden takasiipien kaksi värimuotoa (genotyypit: WW/Wy = valkoinen, yy = keltainen) ovat kytkeytyneet useisiin elinkierto-piirteisiin. Naaraat kantavat samoja alleeleja, mutta naaraiden takasiipien väritys vaihtelee tasaisesti keltaisesta punaiseen. Tässä tutkielmassa perehdyin näiden värimuotojen (WW, Wy, yy) välisiin eroihin seksuaalivalinnassa, kemiallisessa viestinnässä ja kelpoisuudessa sekä tutkin geenikytkentöjen roolia populaation polymorfisen muuntelun säilymisessä. Värimuotojen välillä oli eroja kumppaneiden houkuttelussa ja valinnassa: yy-naaraat houkuttelivat koiraita tehokkaammin korkeammissa koirastiheyksissä. Tämä johtui todennäköisesti erilaisista naaraiden strategioista: yy-naaraat aloittavat houkuttelun aiemmin kuin WW ja Wy genotyyppien naaraat ja saivat näin kilpailuedun. Toisaalta WW-naaraat pystyivät jatkamaan houkuttelua pidempään kuin yy-naaraat. Keltaiset (yy) koiraat löysivät naaraat valkoisia (WW, Wy) koiraita nopeammin matalammissa tiheyksissä, kun taas valkoiset koiraat sekä koiraat, joiden tuntosarvien aistinsolujen tiheys oli korkea, löysivät naaraat nopeammin korkeammissa tiheyksissä. Värigenotyyppien välillä oli myös lisääntymiseen liittyviä eroja, mikä viittaa voimakkaaseen heterotsygootin etuun: Wy-naaraiden lisääntymismenestys oli korkeampi kuin WW ja yy naaraiden. yy-naaraiden pariutumistodennäköisyys oli heikoin. Myös keltaisilla koirailla oli heikoin lisääntymismenestys. Keltaisen y-alleelin läsnäolo vaikutti siis negatiivisesti useisiin elinkierto-ominaisuuksiin. Värityksen määräävien alleelien pleiotrooppiset vaikutukset johtavat stabiloivaan valintaan, joka voi mahdollistaa genotyyppisen ja fenotyyppisen muuntelun säilymisen populaatiossa.

Avainsanat: Heterotsygootin etu; pleiotropia; polymorfia; stabiloiva valinta.

Chiara De Pasqual, Jyväskylän yliopisto, Bio- ja ympäristötieteiden laitos PL 35, 40014 Jyväskylän yliopisto

Author's address   Chiara De Pasqual
Department of Biological and Environmental Science
P.O. Box 35
FI-40014 University of Jyväskylä
Finland
chiara.depasqual@jyu.fi

Supervisors   Academy Professor Johanna Mappes
Organismal and Evolutionary Biology Research Program
P.O. Box 4
FI-00014 University of Helsinki
Finland   &
Department of Biological and Environmental Science
P.O. Box 35
FI-40014 University of Jyväskylä
Finland

Professor Astrid T. Groot
Institute of Biodiversity and Ecosystem Dynamics (IBED)
P.O. Box 94240 University of Amsterdam
Amsterdam 1098 XH
The Netherlands

Assistant professor Emily Burdfield-Steel
Institute of Biodiversity and Ecosystem Dynamics (IBED)
P.O. Box 94240 University of Amsterdam
Amsterdam 1098 XH
The Netherlands

Reviewers   Professor Maya Evenden
B-428 Biological Sciences – Botany Wing
11355 – Saskatchewan Drive
T6G 2E9 University of Alberta
Canada

Associate Professor John Fitzpatrick
Zoologiska institutionen: Etologi
106 91 Stockholm University
Sweden

Opponent   Associate Professor Matthew Symonds
School of Life & Environmental Sciences
221 Burwood Highway
Burwood VIC 3125
Deakin University
Australia

CONTENTS

ORIGINAL PAPERS

LIST OF ORIGINAL PUBLICATIONS

The thesis is based on the following original papers, which will be referred to in the text by their Roman numerals I-IV.

I   De Pasqual, C., Groot, A.T., Mappes, J., Burdfield-Steel, E., 2021. Evolutionary importance of intraspecific variation in sex pheromones. *Trends in Ecology and Evolution* 36: 848-859.

II   De Pasqual, C., Selenius, E., Burdfield-Steel, E., Mappes, J., Morph-linked pheromone signaling and male recruitment in a polymorphic moth. Manuscript.

III   De Pasqual, C., Groot, A.T., Selenius, E., Ottocento, C., Galarza, J.A., Brien, M.N., Burdfield-Steel, E., Mappes, J., The role of morph-specific calling behavior and pheromone blend on female attractiveness in a polymorphic moth species. Manuscript.

IV   De Pasqual, C., Suisto, K., Kirvesoja, J., Gordon, S., Ketola, T., & Mappes, J., 2022. Heterozygote advantage and pleiotropy contribute to intraspecific color trait variability. *Evolution* 76: 2389-2403.

Table of author contribution to the original publications.

Study	I	II	III	IV
Original idea	EBS,JM,CDP	CDP,JM	CDP,JM	JM,SG,CDP
Study design	-	JM,CDP,ES	CDP,JM,AG,EBS	CDP,JM,SG
Data collection	CDP,AG,EBS	CDP,ES	CDP,CO,ES, JM,JG,MB	KS,JK,CDP, JM,SG
Data analysis	-	CDP,ES,EBS	CDP,ES	CDP,TK,SG
Writing	CDP,EBS, AG,JM	CDP,ES, JM,EBS	CDP,AG,EBS, JM,ES,CO,JG,MB	CDP,JM,SG, TK,KS,JK

CDP = Chiara De Pasqual, JM = Johanna Mappes, EBS = Emily Burdfield-Steel, AG = Astrid Groot, ES = Eetu Selenius, CO = Cristina Ottocento, SG = Swanne Gordon, TK = Tarmo Ketola, JG = Juan Galarza, MB = Melanie Brien, KS = Kaisa Suisto, JK = Jimi Kirvesoja

1 INTRODUCTION

1.1 How is trait variation maintained within natural populations?

A fundamental question in evolutionary biology is how phenotypic and genetic variation is maintained within populations. Specifically, the persistence of multiple forms of a trait has represented a long-standing conundrum in evolutionary biology, and it is destined to persist. According to theory, a trait should converge on an optimum due to selection (Cardé and Baker 1984, Endler 1988). However, a wealth of traits shows variation within populations, (e.g., body size, color pattern, or behavior), suggesting that the complexity of nature has possibly led to many optima for a trait (i.e. polymorphism). Polymorphism is defined as the occurrence of two or more distinct morphs within a single interbreeding population in such proportions that the rarest morph cannot be maintained just by simple mutation (Ford 1945, Huxley 1955). This thesis focuses on genetic polymorphism, in which the diverse morphs are determined only by genetic factors, to be distinguished from polyphenism, when the interplay between environmental cues and the genetics of the trait determines the morphs to be expressed. Studying how phenotypic variability is maintained within populations allows us to expand our understanding of the selective forces that can generate and/or maintain genetic diversity, for example through correlations between traits that favor individuals in different ecological contexts. In addition, if a specific trait variant increases the fitness of the individual, and it is heritable, there is potential for evolution to occur and speciation.

The maintenance of polymorphisms depends both on the regimes of selection that affect the trait and on the underlying genetic architecture of the trait. Genetic correlations between traits can be maintained in different ways. For example, through pleiotropy, when a single locus affects two or more seemingly unrelated phenotypic traits. Alternatively, genetic correlations can be maintained through physical linkage between sets of loci (McKinnon and Pierotti 2010, Wellenreuther *et al.* 2014). Such "simple" genetic arrangements are needed for the polymorphism to be maintained across generations and avoid the risk of

recombination during reproduction (Llaurens *et al.* 2017, Jamie and Meier 2020). However, a trait that is determined by a "simple" genetic basis can still have profound impacts on the fitness of individuals. This is because genes are always part of complex metabolic and genetic networks and their expression, or lack thereof, can impact multiple traits (Wright 1982, McKinnon and Pierotti 2010, Wellenreuther *et al.* 2014). One of the most iconic examples of color trait affecting an array of unrelated life history traits, is the side-blotched lizard *Uta stansburiana*. The genetically expressed yellow, orange, and blue color throat in males and females carry pleiotropic effects on the behavior, physiology, and life-history traits of the three color morphs in such a way that it has created a stable polymorphism (Sinervo and Lively 1996, Sinervo *et al.* 2000, Svensson *et al.* 2001).

As the side-blotched lizard, and many other, examples suggest, some form of balancing selection is required to maintain standing genetic variation, i.e., the presence of more than one allele at a given locus, in the population (Ford 1945, Huxley 1955, Hedrick *et al.* 2014, Wellenreuther *et al.* 2014, Mérot *et al.* 2020). Mechanisms as heterozygote advantage (Krüger *et al.* 2001, Johnston *et al.* 2013, Hedrick *et al.* 2014), disassortative mating (Hedrick *et al.* 2016), or negative frequency-dependent selection (Sinervo *et al.* 1996, Hughes *et al.* 2013) allow genetic variation to persist within populations, and ultimately lead to balanced polymorphisms. An endless number of traits can be polymorphic within populations. This thesis focuses on the color trait. Color morphs have long been of interest to evolutionary biologists for different reasons. First, coloration as a trait plays fundamental roles in a variety of ecological and physiological processes, such as sexual selection and thermoregulation (Graham *et al.* 1980, Kemp and Macedonia 2007, Cuthill *et al.* 2017, Endler and Mappes 2017). Therefore, color morphs are the target of multiple selective pressures and allow for investigations on many ecological levels, from interspecific (e.g., predator-prey interactions), intraspecific interactions (e.g., sexual selection), to how the environment contributes to maintain multiple color morphs. Second, color morphs are easy-to-follow, and to detect, phenotypic traits. This means that the effect of selection on phenotypic traits of a population can be investigated even if the genetic basis of the color trait is not known, or molecular tools are not available (Ford 1945). Lastly, color morphs are usually characterized by simple genetic basis, thus highly heritable and can have potential role in speciation events (Gray and McKinnon 2006, McKinnon and Pierotti 2010; Wellenreuther *et al.* 2014).

This thesis investigates how the interplay of sexual selection, chemical communication, and fitness of the different wood tiger moth color morphs can affect intrapopulation dynamics and, ultimately, contribute to maintaining its phenotypic variation. I will explore this across the whole reproductive sequence, from mate attraction to reproductive output (Fig. 1). In other words, I will explore whether females and males of three genetic color morphs (referred to as WW, Wy, yy) have specific advantages in reproduction through female attractiveness, male perception, mating probability, and fitness of the three color morphs. Is the color morph bearing diverse alleles (i.e., Wy) at advantage over the others, hence both color alleles are passed to the next generation? Or are the different color

morphs favored at different stages of the reproductive sequence? At the end of this story, we may be able to add a piece to the jigsaw puzzle of polymorphism maintenance.
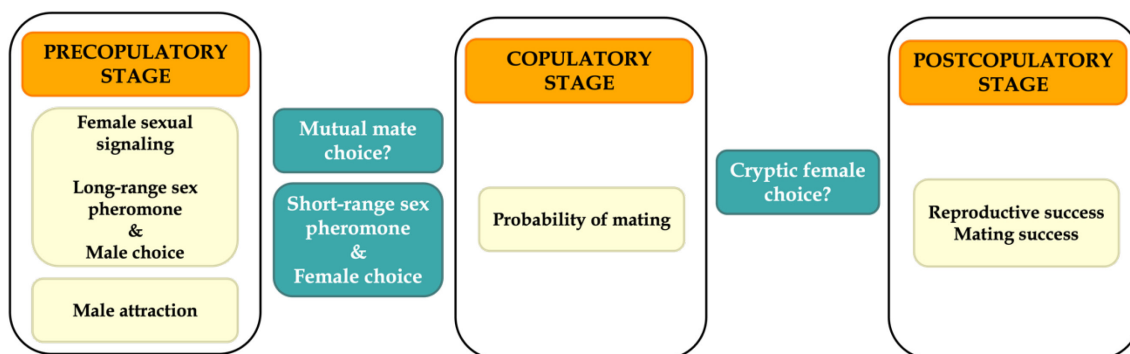

FIGURE 1   Schematic illustration of the different stages of the reproductive sequence considered in this thesis (in orange). For each reproductive stage the traits studied are listed. Potential mechanisms that can take place between two reproductive stages which can potentially affect the "next" reproductive stage but were not studied in this thesis, are reported in green.

1.2   Sexual selection across the reproductive sequence

In his seminal work, Darwin proposed a selective force that he named 'sexual selection' because he was puzzled by the existence of exaggerated sexual traits that could not be explained by natural selection alone (Darwin 1859). Since its inception, the field of sexual selection has broadened to consider a wealth of taxa, sexual traits, and mechanisms of sexual competition that go beyond the initial 'female choice and male-male competition' dichotomy (Andersson 1994). Sexual selection has been recently defined by Shuker and Kvarnemo (2021) as '*any selection that arises from fitness differences associated with non-random success in the competition for access to gametes for fertilization*'. This definition suggests that sexual selection can still continue once an individual has acquired a mate, and has mated (Eberhard and Cordero 1995, Shuker and Kvarnemo 2021).

Mate choice is typically based on the variation in sexual traits and individual quality (Darwin 1859, Andersson 1994, Johansson and Jones 2007). Sexual selection and mate choice are dynamic processes; the individuals involved in the selection process can alternate in their role of signalers and receivers under the strength of mutual mate choice (Svensson 1996). In moths, females emit very minute quantities (ng or less) of sex pheromone that spread in the environment (Greenfield 1981). Males are equipped with sensitive receiving structures (i.e., antennae) and use them to locate the signaling female. Thus, in the first step of reproduction, female moths are typically the signalers and males are the receivers. The small quantities of pheromone released by females may result in sexual selection on male's antennae (Greenfield 1981). Typical of systems

characterized by scramble competition, males with traits that allow them to be more competitive and reach a female faster than others will have a selective advantage (Herbestein *et al.* 2017). Once a female has successfully attracted a male, the role of signaler and receivers may swap between sexes. Males can become signalers and females receivers. Male moths typically release short-range sex pheromones from specific scent structures (androconia or hairpencils) (Birch 1990), and females may use this trait to perform female choice (Greenfield 1981, LaMunyon and Eisner 1993). Sexual selection and mate choice are thus not static, one-way processes. As a result of female choice, a successful attraction event may not result in a copulation event. Males can be rejected by the female, for example based on a suboptimal amount of male sex pheromone (Iyengar and Eisner 1999), or females may not be able to perform female choice, if, for example, too many males are attracted, and male-male competition hampers female choice (Wong and Candolin 2005).

In some cases, the outcome of female choice and sexual selection is not yet defined at the copulation stage, as additional mechanisms can continue to affect female choice after mating. By means of cryptic choice, females can manipulate offspring paternity through postcopulatory mate selection (Eberhard and Cordero 1995), for example when females mate multiple times, but fertilize their eggs with the bigger spermatophore (LaMunyon and Eisner 1993). Finally, a mating event may not lead to any offspring, if a female suffers from an inability to produce viable eggs. For these reasons, focusing our attention on the multiple steps of the reproductive sequence can give us a more meaningful insight on the ecology and dynamics of a species.

1.3 Traits that can affect reproductive fitness

1.3.1 Chemical communication channels are not expected to vary within populations

Sex pheromones are most often mixtures of chemical compounds involved in intraspecific communication. They are released by an individual and stimulate a behavioral response in the receiver (Karlson and Lüscher 1959). Interactions between species have been traditionally considered the predominant selective force molding sex pheromone variation and its evolution (Cardé *et al.* 1977, Cardé and Baker 1984, Ando *et al.* 2004). To increase species recognition and avoid cross-species attraction, sex pheromone are species-specific blends to maintain reproductive isolation (Roelofs and Comeault 1969, Cardé *et al.* 1977, Cardé and Baker 1984). While pheromone blends are typically species-specific, there are costs and biosynthetic limitations to producing different and unique sex pheromone compounds (Tillman *et al.* 1999). Closely related, or sympatric species often use different ratios of shared compounds which have gone through simple chemical modifications rather than becoming completely different compounds (Roelofs and Comeault 1969, Cardé *et al.* 1977, Symonds and Elgar 2007).

Consequently, males and females are expected to be finely tuned to locate each other, with female pheromone signals experiencing stabilizing selection (Linn and Roelofs 1983, Cardé and Baker 1984). However, if variation in signals and responses are not expected, it is difficult to explain why some signalers are more preferred than others when it comes of sexual signaling. For instance, some *Chloridea* (*Heliothis) virescens* females are found to constantly fail in recruiting males, and yet remain in the population (van Wijk *et al.* 2017). In addition, *Agrotis ypsilon* males' perception of constituent ratios of the female pheromone blend can be shifted in the presence of host plant volatiles (Hoffmann *et al.* 2020). These two cases introduce us to the complex dynamism that can affect chemical communication systems. Physiological changes, genetic, and environmental factors can contribute to within- and between-individual variation at the population level.

1.3.2 Variation in sex pheromones and sexual signaling

Sex pheromone release initiates mate attraction in moths. Thus, sex pheromones not only function as species recognition signals, but are also signals involved in mate recognition and assessment (Johansson and Jones 2007). To make mate choice possible, pheromone signaling must vary between individuals and should honestly reflect the quality of an individual (Johansson and Jones 2007). Pheromones can be costly signals to produce and maintain, and females that experience prolonged and increased pheromone signaling can suffer from reduced fitness (Harari *et al.* 2011, Blankers *et al.* 2021). To understand how widespread pheromone signaling variation is, we may need to think that individuals in nature, are virtually always exposed to multiple selective pressures, for example predators, pathogens, or abiotic factors which can interact with the physiology of the individual. Thus, individuals in a population can be affected differently from conspecifics by their surroundings, starting already during development (Groot *et al.* 2010, Balmer *et al.* 2018). Therefore, populations will always experience some degree of variation in sexual signaling.

Understanding the causes and consequences of pheromone signaling variation go beyond the simple awareness that it exists. It can also shed some light on how intrapopulation dynamics are affected by the signal variation, through differences in female attractiveness. Likely to outcompete younger and more fecund females, the strategy of pheromone signaling can change within the life span of an individual. Older females can release pheromone earlier in the night compared to younger females (Pham *et al.* 2021) or they can increase the time spent calling (Umbers *et al.* 2015) in an attempt to outcompete younger females. *Utetheisa ornatrix* moth females, instead, aggregate in response to conspecifics pheromone signaling, likely to optimize their calling effort (Lim and Greenfield, 2008). These signaling strategies can affect male perception and mate location, and ultimately, the fitness of the individual.

1.3.3 Variation in the receiver perception

Once male moths perceive sex pheromones, they typically perform a zig-zag upwind flight to reach the pheromone source (Linn and Roelofs 1983, Baker *et al.* 1985, Cardé 2016). Male antennae are impressive biological receptor organs. Males of the emperor moth (*Saturnia pavonia*) can smell the female pheromone from about 11 km away (Regnier and Law 1968). The antenna directly protrudes from the male's head through the basal segment. To this segment follows the pedicel, which contains a collection of sensory cells, and the flagellum, the rest of the antenna pseudosegments. The functional unit on the antennae responsible for detecting and activating the male response is the sensillum trichodeum. Once the male's antenna encounters pheromone compounds, the chemical molecules enter the sensillum through pores on the surface and the signal is transferred to the central nervous system (Leal 2005). This triggers the male's upwind flight (Baker and Hansson 2016).

Males have typically more elaborate antennae than females, as they need to be more sensitive to chemical signals (Symonds *et al.* 2012, Elgar *et al.* 2019). Since first theorized by Darwin (1871), traits that increase a male's ability to mate and gain reproductive success are likely under sexual selection. This reasoning is well suited for structures like male antennae that need great sensitivity to pick up the minute quantities of sex pheromone released by a female (Greenfield 1981, Svensson 1996, Elgar *et al.* 2019). Phylogenetic analyses indicate that more elaborate, or longer, antennae have evolved in species with lower males abundance or with females releasing pheromone compounds with lower molecular weights, but they also suggest that other factors have contributed to shape their complex structures (Symonds *et al.* 2012). Male antennae have also evolved in response to the mating system that characterizes their species, with elaborate antennae more frequently found in monandrous species, where it is particularly important for males to quickly detect and locate a virgin female (Johnson *et al.* 2022).

In a phylogenetic analysis, Symonds et al., (2012) found that elaborate antennae are typically found in species with larger males. The authors suggested there are costs associated with the development of more elaborate antennae. Species with smaller body size may bear higher costs of elaborate antennae than bigger species (Symonds *et al.* 2012). If costs associated with antennal development have been proposed between species, similar costs should also be expected to affect antennal variation between males of the same species. This could be due to the high costs of generating neuron tissue during individual development (Elgar *et al.* 2019). Antennal development is therefore expected to be costly for males (Symonds *et al.* 2012, Johnson *et al.* 2017, Pham *et al.* 2022) suggesting that natural variation in these structures probably exists within populations. Morphological variation likely affects males' ability to locate females, and subsequently their reproductive success. Nevertheless, the effect of antennal variation on males' ability to perceive and locate a female, and their effect on scramble competition, has been seldom investigated (Jayaweera *et al.* 2017, Elgar *et al.* 2019). In addition to variation in morphological structures in

antennae, the olfactory system is, in general, characterized by a high level of plasticity that can also affect male's perception during adult life (Anton and Rössler 2020).

Thus, investigating potential associations between genetic color morphs, and variation in male antennal morphology and female pheromone signaling may help to advance our understanding of how genetic architecture and behavior could affect species' dynamics and potentially maintain genetic variation within populations.

1.3.4 Heterozygote advantage and pleiotropic effect

Genetic color morphs are expressed through different allele combinations at the same locus. Genes are always part of complex metabolic and genetic networks, and their expression, or lack thereof, will impact many traits (Wright 1982, McKinnon and Pierotti 2010). This may create patterns of genetic correlation, for example between color and other traits, and lead to morph-specific fitness advantages. These correlations between traits can be expressed through genetic linkage, for example chromosomal inversions, or be physically unlinked and expressed through pleiotropic effects (i.e, when a single gene influences two or more characters) (Sinervo and Svensson 2002, McKinnon and Pierotti 2010, Llaurens, *et al.* 2017). A stable polymorphism is most often determined by a simple genetic basis or genes in tight linkage, which has a reduced risk of recombination when passed to the next generation than multi-locus traits (Ford 1945, Lewontin and Kojima 1960, Llaurens *et al.* 2017, Jamie and Meier 2020). Together with a simple genetic basis for limited recombination, examples of balanced polymorphisms are often characterized by high heterozygosity (Llaurens *et al.* 2017). When individuals that are heterozygous (i.e., characterized by two different alleles) at a locus have a higher fitness compared to homozygous individuals, both alleles can be maintained in the population (Fisher 1922, 1930, Hedrick 2012). If the recessive allele carries deleterious mutations, the deleterious effect determines a fitness disadvantage when two copies are present in homozygote recessive individuals, but it is masked in heterozygote individuals. This reinforces heterozygosity in the population (Llaurens *et al.* 2017).

First theorized by Fisher (1922), heterozygote advantage is a mechanism that has interested researchers for nearly a century (e.g., Fisher 1930, Ford 1945, Hedrick 2012). Cases of heterozygote advantage are often coupled with other selective mechanisms, suggesting that the focal trait is likely to affect other genes and life-history traits. The polymorphic color coat of wolves (*Canis lupus*) in Yellowstone National Park is maintained by heterozygote advantage coupled with weak selection and a strong contribution of disassortative mating (Coulson *et al.* 2011, Hedrick *et al.* 2014, Hedrick *et al.* 2016). As expected, the locus correlated to the color trait has pleiotropic effects on seemingly unrelated traits (Wright 1982, McKinnon and Pierotti 2010). In some of the most emblematic examples, single genes affect multiple life-history traits. In *Drosophila melanogaster*, the lack of the *yellow* gene expression alters the morphological and structural portion on the leg section that males use to grab the female, and

disrupts body pigmentation, male courtship behavior, and mating success (Bastock 1956; Wilson *et al.* 1976, Massey *et al.* 2019).

When the color trait affects sex related traits, such as reproductive output or mating success, it can affect intrapopulation dynamics, for example through potential sexual conflict if males and females have different advantages.

1.4 Relevance of the topic outside evolutionary biology

One may wonder what is the benefit of researching the mate preference and reproductive output of a colorful moth species that is neither a threat to agriculture nor considered as a threatened species?

First and foremost, understanding the mechanisms that maintain trait variation helps us to expand our understanding of how nature works. Traits are molded by many mechanisms and selective pressures caused by the huge variety of habitats, environmental conditions, and biotic factors. Trait variation can, in turn, affect populations and species dynamics. Furthermore, understanding how selection and maintenance of trait variation works can be helpful both to understand the full impact of environmental changes on biodiversity and how genetic diversity can instead originate.

Moreover, the role of an evolutionary biologist is to study how a signal can be disrupted, and how this affects the ecology of the species. Humans have profound impacts on natural systems and continuously challenge other species. For example, urbanization alters many environmental features (e.g., light, noise) and affects traits involved in sexual communication (van Geffen *et al.* 2015, Heinen-Kay *et al.* 2021). Anthropogenic selective pressures can also promote sex pheromone evolution. To reduce our impact on ecosystems, alternative methods to chemical pesticides are being used to control for pest damage in agriculture. Techniques such as pheromone trapping, and mating disruption, are commonly used as an environmentally friendly alternative to pesticides but the continuous use of artificial pheromone lures exert selective pressure on pest species which acts to change their sexual signal and can lead to the evolution of resistance to artificial lures (Evenden 2016; Evenden and Haynes 2001). Understanding selection pressures acting on pheromone signals and receivers can help us fight evolution with evolution.

1.5 The wood tiger moth as model species

The wood tiger moth (*Arctia plantaginis*) is a compelling study species to investigate how genetic variation can be maintained through different mechanisms and selective pressures in different ecological contexts. The male hindwing coloration is determined by a simple genetic basis (Suomalainen 1938, Nokelainen *et al.* 2022, Brien *et al.* 2022): a one locus-two allele polymorphism

(dominant W allele and recessive y allele), which phenotypically translates into white (genotype: WW, Wy) and yellow (genotype: yy) hindwings in males (Fig. 2). Besides being polymorphic (and sexually dimorphic), the wood tiger moth is also an aposematic species, thus the color locus can be affected both by interactions between conspecifics (i.e., sexual selection) and between species (i.e., natural selection). The effect of these selective pressures can, at times, be conflicting and favor different color morphs differently. In almost 20 years of experiments, studies have indeed shown that the interplay between environmental factors and the genetic architecture of the color locus leads to morph-associated advantages. The white and yellow morphs are differently protected against avian and terrestrial predators (Nokelainen *et al.* 2012, 2014, Rojas *et al.* 2017, 2019, Winters *et al.* 2021). Yellow males are generally better protected against predators (Nokelainen *et al.* 2012, 2014, Rojas *et al.* 2017), although the predation pressure on the two color morphs can change in relation to the predator community (Nokelainen *et al.* 2014) and with the light environment (Nokelainen *et al.* 2021), leading to context-dependent morph advantage. Under sexual selection, white males have usually higher mating success (Nokelainen *et al.* 2012, Gordon *et al.* 2018, De Pasqual, *et al. in prep.*) although it is context-dependent. While females generally prefer to mate with white males (Nokelainen *et al.* 2012, Gordon *et al.* 2018), their choice and preference is thought to be flexible and affected by the male morph frequency as males of either morph have a reproductive advantage when represent the most common morph (Gordon *et al.* 2015). Male morphs also differ in their reproductive output, with yellow males being generally less successful than white males (Gordon *et al.* 2018, IV). While previous studies have shown that different male color morphs have different fitness advantages, much less is known on the female side. This is because female hindwing coloration varies from yellow to red (Hegna *et al.* 2015), and although it was suspected for long time, only recently it has been confirmed that they carry the same color alleles as the males, and pass them to their sons (Suomalainen 1938, Brien *et al.* 2022, Nokelainen *et al.* 2022) (Fig. 2). If the color alleles affect the fitness of males, then they may have an effect on females too, and females can therefore contribute to the maintenance of the male hindwing coloration.

The wood tiger moth is also a suitable species because it can be easily reared in a laboratory setting. This allows us to work at different experimental scales, from small scale, e.g., plastic containers where behavior can be scrutinized in detail and confounding factors from the environment can be minimized, to larger scales. In this thesis, I have also performed experiments at a semi-field scale, i.e., a 30 x 20 x 3 m enclosure, where moths could behave more naturally as they could easily spread out in the larger environment but at the same time I was sure of their occurrence and genotypic frequency. Finally, I also tested sexual selection in a completely natural field setup, where I tested laboratory reared females with wild males. In this large scale setup, I accepted that I could not control for some variables, for example experimental females competing with naturally occurring females, and the number and genotype of males in the surrounding area. However, having both the semi-field and field setup allowed

me to gain insights into the potential effect of (higher- and lower-) population density, and into the repeatability of the moths behavior. By testing potential associations between fitness related traits of the three color morphs in different environment I could test for context-dependent fitness advantages.
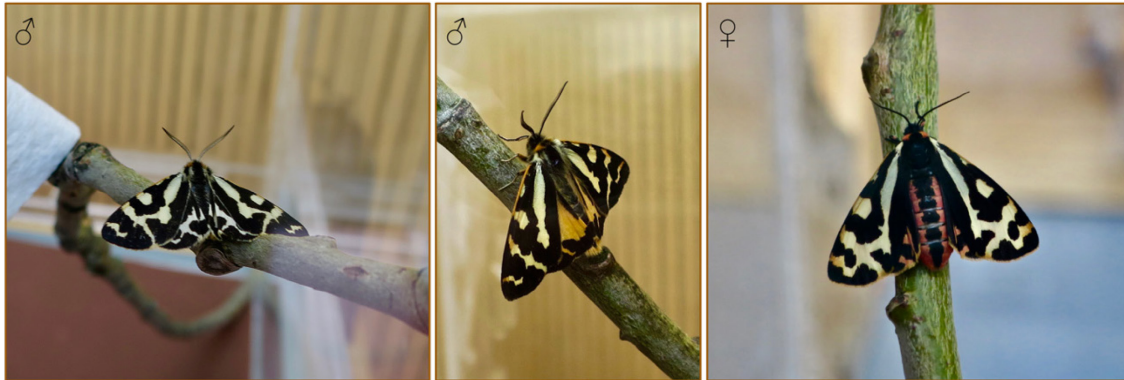

FIGURE 2   Wood tiger moth hindwing coloration. Males have either white (genotype: WW, Wy) or yellow (genotype: yy) hindwings, whereas females have red hindwing and body coloration. Although females do not phenotypically express the male white and yellow coloration, they genetically bear the color alleles resulting in WW, Wy, and yy color genotypes. (Photos: Chiara De Pasqual).

1.6   Aim of the thesis

The maintenance of phenotypic variability within natural populations is often the result of the interplay between multiple mechanisms and selective forces that are acting on the same trait. In this thesis, I investigated the potential contribution of sexual selection, chemical communication, and reproductive fitness of different genetic color morphs in maintaining intrapopulation phenotypic variation. The studies included in this thesis are based on two main hypotheses. The first hypothesis tested for heterozygote advantage across the reproductive sequence. As already introduced, heterozygote advantage is a powerful mechanism to maintain genetic variability within populations (Ford 1945, Hedrick 2012). In addition, Gordon *et al.* (2018) showed that wood tiger moth males originated from "mixed-morph lines" (i.e., more heterozygous) had higher mating success. Thus, I hypothesized that Wy females were more attractive than the other two genotypes, that Wy males located females faster than the other two genotypes (II), and both Wy males and females had higher reproductive output (IV). Given that previous studies have shown that morph-specific advantage can be affected by the interaction with the environment, the second hypothesis tested for context-dependent fitness advantages across the reproductive sequence.

In study I, I reviewed relevant and recent literature investigating sources of variation in chemical communication channels in insects, both in signalers (i.e., sex pheromone) and receivers. While pheromone diversity varies greatly between species in response to heterospecific interactions, sex pheromones can

have a role in mate choice and assessment and thus potentially evolve also under sexual selection (Johansson and Jones 2007, Symonds and Elgar 2007, Steiger and Stökl 2014). The aim was to build an overview of the types of selective pressures that can affect both signaler and receiver's chemical communication. This allowed me to understand how the variation in the ability to signal and to perceive sex pheromones can affect intrapopulation dynamics (e.g., signaler's attractiveness and mating success) and have potential evolutionary consequences for species.

In study II, I investigated potential associations between color morph related variation in sex pheromone signaling and male antenna morphology in the wood tiger moth. Increasing evidence shows that variation in the quality or quantity of pheromone blends and/or variation in pheromone calling strategies can affect mate recruitment. However, very little is known about the role of male antennal variation in affecting males' ability to locate and reach females. I investigated how variation in these two traits can affect mate recruitment and mate choice. I tested for associations between genetic color morph and pheromone attractiveness of females of different color genotype, and for potential "strategies" of differently attractive females. In addition, I investigated whether variation in male antenna morphology could affect males' ability and "speed" to reach the female.

In study III, I investigated the potential mechanisms behind different female attractiveness. Even though there was an indication that the advantage of the more attractive females was due to a strategy of early calling, as females can adjust their calling behavior to outcompete conspecifics or lower the risk of dying unmated (Umbers *et al.* 2014, Pham *et al.* 2021), female calling behavior in study II was not directly observed and controlled. In addition, differences in female attractiveness can be linked to qualitative or quantitative differences in the pheromone blend. Thus, I tested for the potential role of blend composition (quality), titer (quantity), or female calling behavior in explaining the higher attractiveness of yy genotype female of study II. In addition, I tested for potential costs associated to their higher attractiveness.

In study IV, I investigated the effect of genotype variation (i.e, genotype color morphs) on mating delay, copulation probability, and reproductive output of the different color genotypes. In addition to investigating potential Wy advantage at the reproductive output, I investigated whether carrying one or two copies of the *yellow* allele would affect traits differently across the reproductive sequence. This is because the gene expressing the wood tiger moth hindwing coloration belongs to the *yellow* family, which has been shown to have pleiotropic effects on many life-history traits within a species (Massey *et al.* 2019, Connahs *et al.* 2022).

2   METHODS

In this thesis, I used a variety of methods to investigate the potential role of sexual selection and chemical communication to maintain phenotypic trait variation. Below, I included relevant aspects of the study species' biology and laboratory rearing, and main features of the methods used for collecting behavioral, chemical, and life-history trait data. Further details of the methods are included in the respective thesis chapters.

2.1   Study species

The wood tiger moth is a capital breeder; adults do not feed thus cannot acquire further resources for reproduction. Thus, at the larval stage, they may incur resource allocation trade-offs between development and adulthood. As the species overwinters as larvae, they spend most of their life cycle in this stage and live as adults for only a few days (Ojala *et al.* 2007). In Finland, adults eclose between mid-June and mid-July, depending on the latitude, and typically have one generation per year (i.e., univoltine). This species is both diurnal and crepuscular. It flies during daytime hours (Rojas *et al.* 2015), but individuals show active sexual behavior (i.e, pheromone calling and male searching activity) between 5.00 p.m. and 12.00 a.m., with mating activity that can extend into the night (Nokelainen *et al.* 2012, Gordon *et al.* 2015) and a mating peak in laboratory around sunset (~ 10.00 – 11.00 p.m.) (Pers. Obs). Females of the Erebidae (formerly Arctiidae) family typically release long chains of unsaturated hydrocarbons derived from linoleic or linolenic acid precursors acquired through their larval diet (Millar 2000). Females attract males by releasing a sex pheromone, and if a suitable male is attracted, they engage in a copulation event. Females lay, on average, 250 eggs within a few days from the copulation event. Polyphagous larvae hatch after about seven days and they feed on a variety of plants (e.g., *Plantago* sp., *Taraxacum* sp., and *Rumex* sp.), grow until about the third instar, overwinter as larvae, then pupate the following spring, hatch into adults, and the cycle starts again.

2.2 Moth rearing and stock maintenance

Except for the wild males caught in the field experiment, all the moths used in this thesis were reared in the laboratory. The laboratory stock was established in 2013 and maintained at the Department of Biological and Environmental Science, University of Jyväskylä (Finland). The greenhouse was kept at a constant 20 - 25 °C temperature and used natural light. During stock maintenance, one male and one female were paired in transparent plastic boxes (13 H x 7 W x 9 L cm) with mesh on the lid for aeration. Three color genotype lines have been established in the stock for experimental purposes, with moths paired to produce offspring with known genotype (i.e., WW, Wy, yy). To avoid high levels of inbreeding, wild individuals were annually introduced in the laboratory population, and matings were also planned between individuals of the three genotypes lines. Under laboratory conditions the wood tiger moth can produce up to three generations per year. For each mating pair, eggs were counted four days after the female had started oviposition, and larvae were counted 14 days after hatching and divided into containers of 30 larvae each. The hatching success was calculated as the number of larvae hatched over the number of eggs laid. The pupal stage lasts seven to ten days, depending on the time of the year, before eclosing as adults. Adults were kept in a cooler cabinet (7 °C) until used for the experiments, and generally were taken out in room temperature around 12.00 p.m. on the day of the experiment to acclimatize.

2.3 Literature review (I)

To understand the potential selective pressures that can introduce variation in sex pheromone signals and receiver perception, the ecological relevance of trait variation within populations, and its potential evolutionary implications, I reviewed the most relevant scientific literature on insects and compiled the findings in a review. I searched for studies that described different sources of pheromone variation and receiver variation, such as developmental trade-offs, physiological, and environmental causes. I then looked at how signaler and receiver variation could influence intraspecific and intrapopulation dynamics. For example, I focused on studies showing how such variation affected individual mating success, helped signalers avoid sexual conflicts or mating with inbred individuals. Finally, I explored how pheromone differentiation driven by ecological changes (e.g., host plant shift) or by intrinsic factors (e.g., shift in mating preferences) may start the speciation process.

2.4 Precopulatory stage: Behavioral studies (II, III)

2.4.1 Female attractiveness in the field and in semi-field setup (II, III)

Potential associations between female color genotype, sex pheromone variation, and potential female calling strategies (e.g., speed and duration of attraction) were tested with two experimental setups. Females of different genotypes were housed singly in bucket traps from where they could signal for males, but not be seen or made physical contact with, as the housing cage was covered with fine mesh.

In the field, two traps with females of different genotypes were placed approximately 10 m apart in each of the six locations chosen within Tornio municipality, northern Finland (65.8473 °N, 24.1520 °E) (Fig. 3a, b, c). Two female genotypes were randomly assigned to a location on the first day of the experiment and carefully rotated between locations in the following nights (II). In the semi-field setup, nine females (three per genotype) were simultaneously tested over one night, by placing them in a three-by-three grid of a 30 x 20 x 3 m enclosure, at the Konnevesi Research Station (62.6278 °N, 26.2939 °E), Jyväskylä municipality, Central Finland (Fig. 3a, d, e). In both setups, females were tested over one night and replaced with new ones for the following night. The enclosure setup was used both to test the attractiveness of females that followed their natural onset of calling behavior (II), and of females that were simultaneously calling (III). While the field traps were checked only in the following morning, the enclosure traps were checked with 1.5 – 2 hour interval, starting at 6 p.m. until moth activity ceased for the day (i.e., 11.00 p.m. – 12.00 a.m.). Males released in the enclosure setup were individually marked with a four-dots, two-color system on the underside of their wings to allow individual identification once trapped. In both experimental setups we tested the likelihood of attracting at least one male and the proportion of males attracted by each female color genotype. Furthermore, we tested for the speed (i.e., daily attractiveness in both setups) and duration (in the enclosure setup) of female attractiveness. Finally, we considered the partial (i.e, color) and full (i.e., genotype) allele combination of the males attracted by each female genotype, to test for any sign of disassortative attraction.
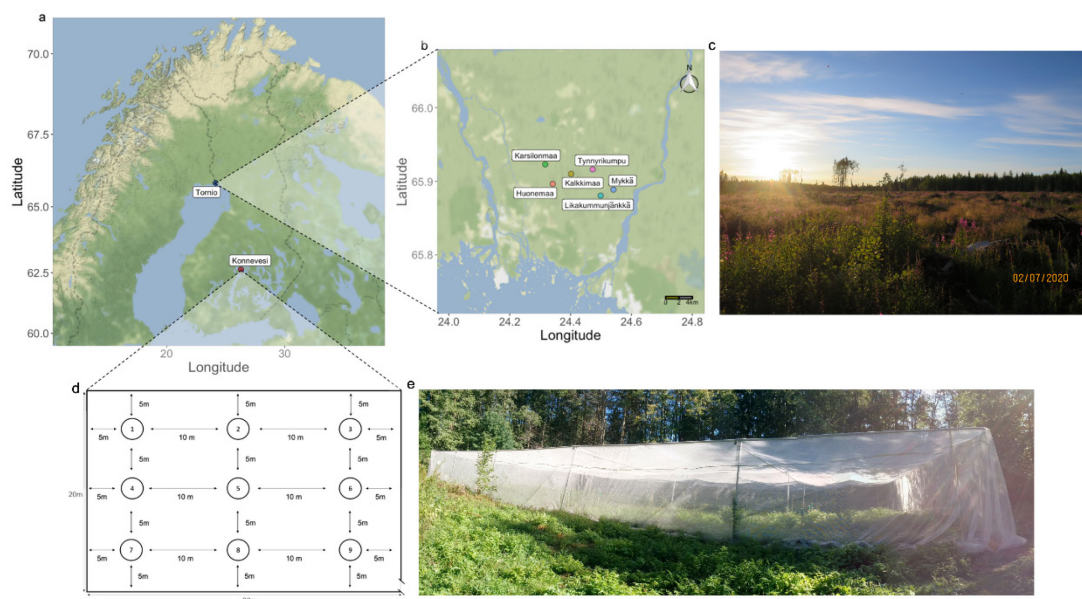
FIGURE 3   Field and semi-field experimental setups. a) map showing the two locations of the experiment, Tornio and Konnevesi. b) zoomed in map with the six field locations in Tornio municipality and c) an example of a location from Karsilonmaa site. d) schematic drawing of the three-by-three grid trap position of the e) enclosure. (Photos: Chiara De Pasqual).

2.4.2 Onset and duration of pheromone releasing behavior (III)

Female calling behavior was observed with a 15 min interval during 24 hours for a period of seven days per female. A one-day old female was individually housed in a transparent jar with a small, moistened sponge at the bottom. Before starting the behavioral observations, female's antennae were cut off to prevent females from perceiving their own pheromone and their behavior to be affected. At every interval it was marked whether the female was actively extruding the gland (i.e, calling) or not. The onset of the calling behavior per female per day was marked as the time when calling commenced. The duration of calling was instead calculated as the total time (in min) of calling per female per day.

2.4.3 Male order of arrival to traps (II)

Males' ability to locate the females was tested through potential associations with male color genotype and antennae features. After one night of capture in the field, or at two-hour interval in the enclosure, traps were emptied, and males recorded for their color genotype or morph. In addition, one antenna was excised and stored in ethanol until photographed (Fig. 4a). For each antenna, the area, the length, and the number of lamellae were measured.
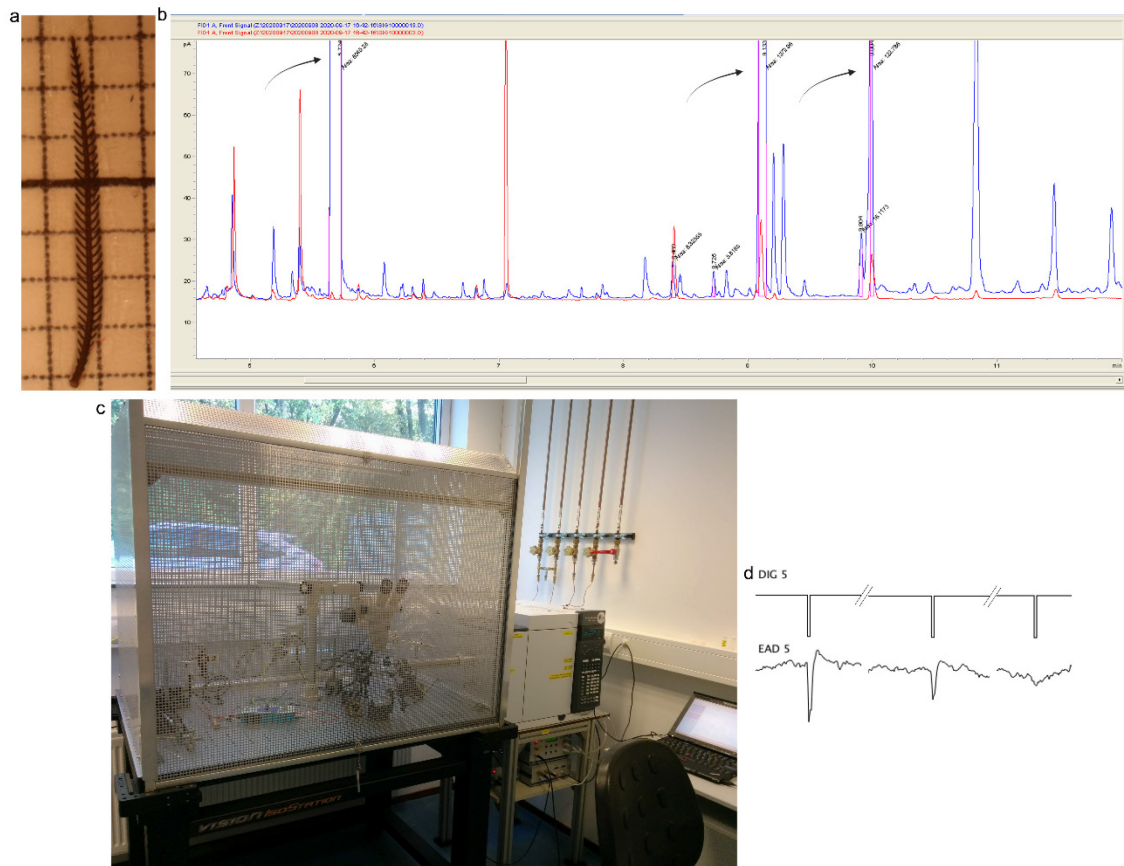
FIGURE 4   Electrophysiology and sex pheromone. a) excised male antennae were used to test for the role of antennae variation in males' ability to perceive females (see section 2.4.3) and for electrophysiological testing (c, d). b) overlaid chromatograms of female gland extract (blue trace) and mixture of reference compounds (red trace). Arrows indicate, in order, the peak of n-pentadecane (internal standard), 3Z,6Z,9Z-21:H the major compound, and 1,3Z,6Z,9Z-21:H the second major compound. c) setup used for GC-EAD analyses in a Faraday cage, with gas chromatography (GC) and computer for reading the antennal responses on the right. d) EAD of male antennal response to, in order, the gland extract, the positive control (3Z,6Z,9Z-21:H), and the negative control (hexane). (Photos: Chiara De Pasqual).

2.5   Analyses of pheromone extracts and antennal responses (III)

2.5.1 Pheromone extractions (III)

Female sex pheromone was extracted by excising the gland located at the tip of the female abdomen with surgical scissors. The gland was then soaked for ~ 45 min in 50 µl of hexane containing 200 ng of the n-pentadecane internal standard used as a known quantity to later quantify the pheromone compounds. The female was killed immediately after gland excision to avoid unnecessary suffering. Once the gland tissue was discarded, the pheromone extraction was stored in the -20 °C freezer until analysis. The pheromone extract was concentrated to 5 µl, of which 2 µl of extract topped with 1 µl of octane were

injected in the gas chromatography (GC). The putative sex pheromone compounds were identified by comparison to a mixture of reference compounds and quantified by integrating the area under the peak using Agilent ChemStation software (v. B.04.03) (Fig. 4b).

2.5.2 Tentative identification of behaviorally active pheromone compounds (III)

Electrophysiological studies were performed to detect physiologically active compounds that were likely to be behaviorally active. Male antennal responses to female sex pheromone compounds were investigated by using gas chromatography coupled with electroantennographic detection (GC-EAD) (Fig. 4c). One antenna was excised from the male's head and mounted between two micro glass capillaries containing 0.1 M KCl 1% PVP Ringer solution completed with two silver electrodes, the recording and the reference electrode. Aliquots (1-2 µl) of gland extracts were injected in a cold on-column injector. The GC effluent further split in 1:1 between the FID and the EAD. The GC effluent moved through the transfer line into a glass tube where it was continuously mixed with a humidified airstream. The antenna was positioned at the exit of the glass tube. The electrodes were connected to an amplifier and both the antenna responses, and the FID data were connected to a software for data visualization. Once a biologically active compound elicited antennal response, a depolarization event was recorded on the screen with corresponding peak on the FID. Tentative identifications of the behaviorally active compounds were carried out by collaborators at Pherobank B.V. (Wijk bij Duurstede, the Netherlands). The compounds were subsequently tested both separately and in blends of different compounds and ratios to confirm they elicited male attraction. They were loaded in red rubber septa and housed in bucket traps tested both in the field in Tornio municipality and in the Konnevesi Research Station enclosure. The active pheromone compounds were also used for later quantification of the female pheromone extracts to link pheromone blend variation with female attractiveness.

2.5.3 Electrophysiology detection (EAD) (III)

Male antennal responses to the whole pheromone bouquet were tested through electroantennography detection (EAD), a technique to measure antennae responses to given odors. The antenna preparation followed the same protocol described in 2.5.2. The test stimulus was deposited on small paper filter and placed in a glass Pasteur pipette after hexane evaporation. The test stimulus was injected into the continuous airstream and the antenna depolarization was recorded through the GcEad/2014 software (Fig. 4d). The same software was then used to integrate the amplitude (mV) of the antennal response. Each male antenna was tested with pheromone extracts of all three female genotypes, preceded and followed by puffs of positive (i.e., pheromone major compound) and negative (i.e., hexane) controls.

2.6   Copulatory and postcopulatory stage (IV)

Potential differences in mating probability and reproductive output of the three color genotypes were tested by means of a mating experiment and the life-history traits collected during stock maintenance, respectively. The mating experiment was carried out by following a subset of the individuals, paired during stock maintenance, over eight hours of observation trials. For each pair, copulation probability (i.e., whether they mated or not), mating delay, and longevity after one copulation event, were observed.

Life-history traits, such as fecundity, fertility, hatching success, oviposition, and hatching day (i.e., the number of days it took to lay the first egg or for the first larvae to hatch, respectively) were collected by using the 19 generations (or seven years) of data collected through stock maintenance.

3 RESULTS AND DISCUSSION

3.1 Pleiotropic effect of the color locus

3.1.1 Precopulatory stage (II, III)

Mate choice and sexual selection starts by attracting, or finding, individuals of the opposite sex. Associations between the color genotype and both variation in female pheromone signaling and males' ability to reach females were found to affect both sexes. Females were characterized by skewed attractiveness, with a few females exhibiting greater attractiveness to males than others. Across all experiments, only 18 females out of the 179 tested caught more than 10% of the total number of males attracted in one night. When the onset of calling behavior followed females' natural rhythms, the most attractive females typically belonged to the yy genotype (II). Conversely, when the onset of calling was standardized, and females were synchronously calling, the most attractive females typically belonged to the WW genotype (III). The higher attractiveness of yy females was likely due to a combination of early calling behavior (which could outcompete other females) and a higher population density (II, III) (Fig. 5). Since yy females typically suffer from lower mating success compared to white females (IV), they may start calling earlier than WW and Wy females in an attempt to increase their reproductive output. However, higher yy attractiveness was typically limited to few females which suggests that it is a costly strategy, otherwise it would be more common in the population. There may be two reasons behind this. First, higher female attractiveness may trade-off with the resources available for investment in offspring, thus attractive females suffer a particularly low fitness. Males attracted to these females would then incur in the risk of reproductive failure, thereby generating potential sexual conflict between the two sexes. Second, the more attractive females end up having a reduced probability of mating due to male harassment. In these experiments, moths could not engage in copulation, thus it was not possible to observe whether more attractive females had higher chances of copulating when attracting one or many males.
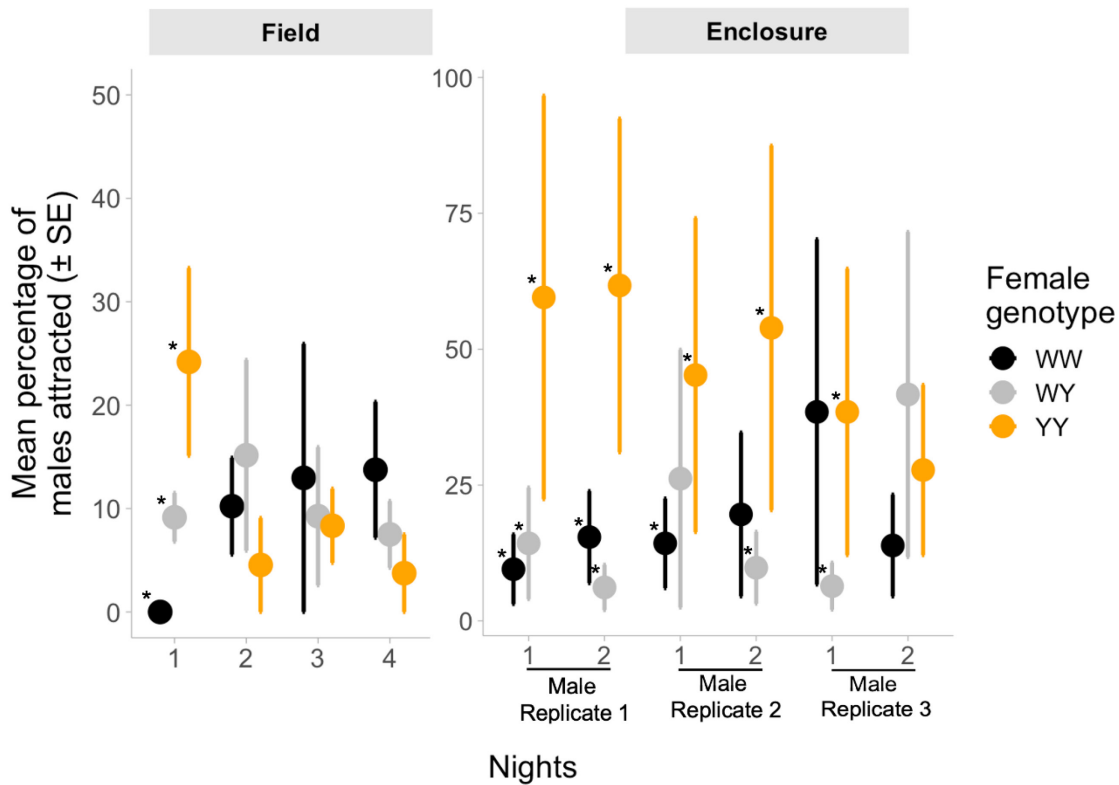
FIGURE 5   Percentage of males attracted by female genotype by night of experiment. Significant differences, by night, between genotypes are denoted with asterisks (II).

While yy females seemed to be favored at higher population density, male morphs' ability to locate and reach females was affected in a density-dependent way. White (WW and Wy) males reached females faster in the higher density scenario (i.e., the enclosure), while the opposite was found for yellow males, which located females faster than white males in the field (II) (Fig. 6). This suggests that males may be affected by different population densities (or some other environmental factor) and characterized by morph-specific behavioral strategies. The differences between morphs in the two experimental scenarios may hint at a role for intrasexual competition that affects male morphs differently, with white males outcompeting yellow males. Alternatively, the two male color morphs may be characterized by different behavioral strategies. Yellow males typically have a narrower daily flight activity period and tend to stay closer to females than white males. White males have a broader daily flight activity period and a tendency to disperse more during the day (Rojas *et al.* 2015, Gordon *et al.* unpublished). The less explorative and "energy-saving" behavior of yellow males in denser populations, has also been shown in a mating experiment. When the probability of mating for each of the three color genotypes was tested in a high density scenario, yy males only mated during the second night of observations, while white males typically mated already during the first night (De Pasqual *et al.* in prep).
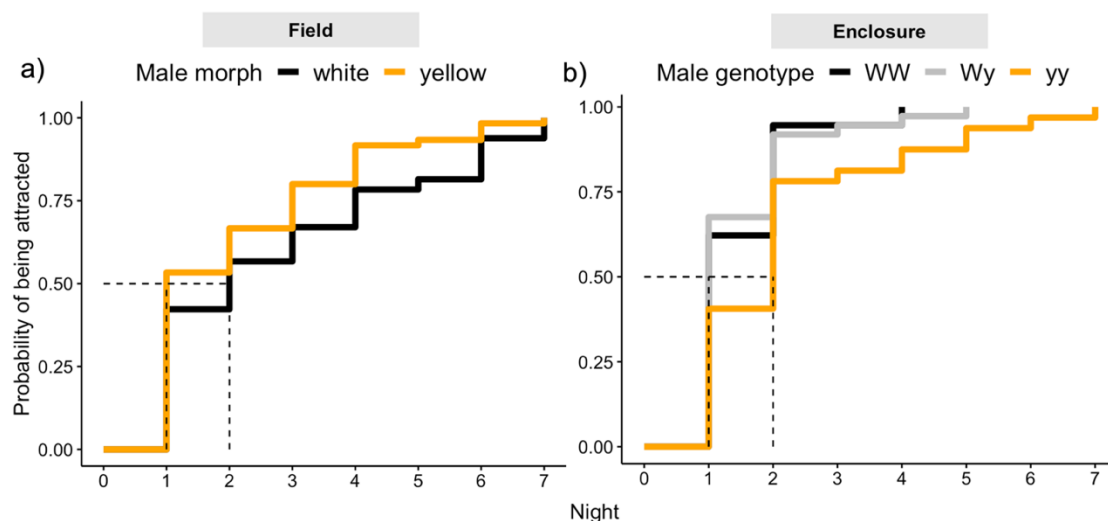
FIGURE 6   Male morphs' ability to locate and reach females was affected by the experimental setup. a) yellow males reached females faster in the lower density scenario (i.e., the field) while white (WW and Wy) males reached females faster in the higher density population (i.e., the enclosure) (II).

Altogether, the combined effect of genotype-related pheromone signaling strategies and the different abilities of male morphs to locate females affected mate recruitment (II). These genotype-specific differences at the precopulatory level (II) add to previously investigated morph-specific traits contributing to sexual and natural selection (e.g., Nokelainen *et al.* 2012, Gordon *et al.* 2015, 2018, Rojas *et al.* 2015, Winters *et al.* 2021). Finally, in higher density populations, we found that yy females generally attracted males faster than white females and that white (WW or Wy) males located females faster than yellow males. This may indicate disassortative attractiveness, when individuals with dissimilar genotype color alleles locate each other faster than other allele combinations. If this is followed by a successful mating event, then both color alleles have increased chances of being maintained within the population.

3.1.2 Copulatory and postcopulatory stage (IV)

Complex polymorphisms within populations are often the result of balancing selection. When a polymorphic trait is expressed through one locus with two alleles, with one dominant over the other, balancing selection can be expressed through heterozygote advantage. At the postcopulatory stage, egg clutches laid by Wy wood tiger moth females were more likely to hatch into larvae, leading to higher larval survival and hatching success than in homozygote females (IV) (Fig. 7). This was likely due to a genetic advantage of the successful combination of W and y alleles, rather than to maternal or paternal effects in inheritance (IV). This finding adds to a few other known cases of heterozygote advantage associated with the color locus in wild populations that are characterized by stable color polymorphisms (Krüger *et al.* 2001, Hedrick *et al.* 2014, Jay *et al.* 2021). In the wood tiger moth, heterozygote advantage may be context-dependent: heterozygote females benefit from higher reproductive output (IV), while heterozygous males have a mating advantage due to either female choice or male-male competition

(IV, Gordon *et al.* 2018) and are better defended against avian predators (Winter *et al.* 2021).

Because genes are part of complex metabolic and genetic networks, their expression is likely to affect multiple traits (Wright 1982, McKinnon and Pierotti 2010). When one gene affects seemingly unrelated traits it is said to have a pleiotropic effect. Genes of the *yellow*-family are well known for having pleiotropic effects on life-history traits and behavior (Bastock 1956, Massey *et al.* 2019, Connahs *et al.* 2022) and these genes are conserved across insects (Ferguson *et al.* 2011). Male coloration in the wood tiger moth is likely to be regulated by a *yellow*-family gene (Brien *et al.* 2022), and although the exact genetic mechanism behind its expression is not yet known, it seems to carry pleiotropic effects in the wood tiger moth as well, especially because the effects are expressed in females that do not express the yellow phenotype. Especially when expressed in recessive homozygote individuals (i.e, yy) the *yellow* allele seemed to have a deleterious effect on reproductive fitness. yy males had the lowest reproductive output, while yy females suffered reduced mating success and their offspring were less likely to reach adulthood (IV). The *yellow* allele may be maintained in the population through higher yy male survival against predators (Nokelainen *et al.* 2012, 2014, Rojas *et al.* 2017), and via heterozygote (Wy) individuals (IV, Gordon *et al.* 2018, Winters *et al.* 2021). In addition, the expression, or lack thereof, of the *yellow* gene has general effects on behavior. Loss of its expression in *D. melanogaster* reduces mating success due to changes in courtship behavior and structural changes in the portion of the leg used to grasp the female (Bastock 1956, Massey *et al.* 2019). In contrast, in *Bicyclus anynana*, the *yellow* gene needs to be suppressed in males to properly perform courtship behavior (Connahs *et al.* 2022). In the wood tiger moth, individuals that do not express the *yellow* allele, that is, yy individuals, also seem to have their behavior affected: yy females seem to start pheromone release earlier than white females, and yy males have narrower daily flight activity (Rojas *et al.* 2015), tend to stay closer to females in the field (Gordon *et al.* unpublished), seem to have density-dependent behavioral patterns (II), and tend to mate later than white males (De Pasqual *et al.* in prep.).
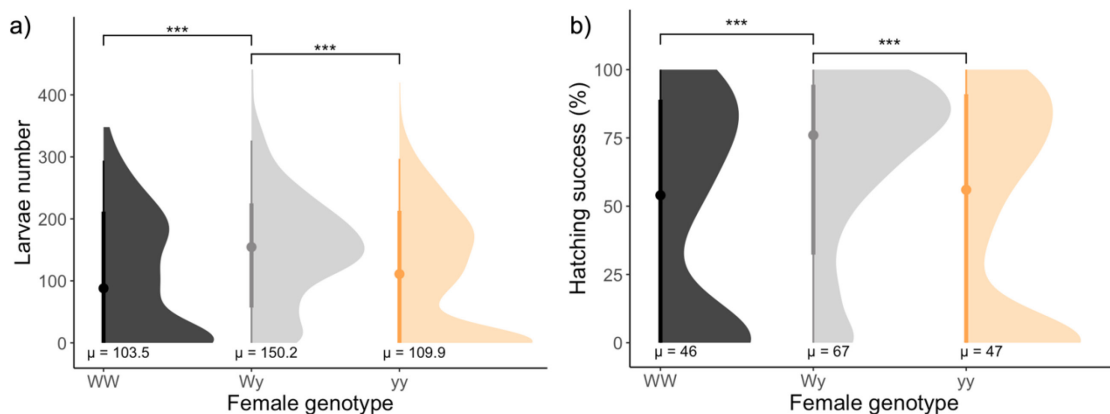

FIGURE 7   Differences in fertility (i.e., number of larvae) and hatching success of the three color genotypes in females. Wy females had higher fertility and hatching success compared to WW and Wy females.

3.2 Signaler and receiver variation

3.2.1 Intrapopulation pheromone communication channels variation (I)

Although chemical communication is considered the oldest form of communication (Wyatt 2014), the lack of human's ability to perceive sex pheromones and the challenges posed in analyzing pheromone extracts in minute quantities (ng or less) have likely held back our appreciation for differences between individuals (I).

We found that the increasing volume of studies showing a high degree of variation in both signalers and receivers suggest that pheromone communication channels are not only molded by interspecific interactions but by multiple selection forces (I). In addition, we suggested that the sources of variation, both in signalers and receivers, arise from a combination of context-dependent, condition-dependent, or genetic mechanisms (I). Such variation may challenge the traditional view that pheromone blends should be under strong stabilizing selection and males tuned to specific blend ratios (Baker *et al.* 1981, Linn *et al.* 1983, Cardé and Baker 1984, Linn and Roelofs 1985).

To understand the implications of pheromone signals and receivers' variation, we looked at how sources of variation could directly affect, or interact to affect, intrapopulation dynamics. For example, vicinity to conspecifics, host plant volatiles, physiological, and genetic mechanisms can affect signaler attractiveness, receiver perception, and, in general the fitness of the individual (van Wijk *et al.* 2017, Xu *et al.* 2019, Zakir *et al.* 2017). If variation in signals and responses increases the fitness of the individual, and variation is heritable, then chemical communication systems can evolve and may be drivers of species evolution. However, understanding whether trait variation has preceded or followed an evolutionary event is by default very challenging. This may be particularly true in chemical communication channels since, at least in the moth species studied so far, the genomic regions involved in signaling and variation are typically unlinked, thus the lack of genetic correlation favors the evolution of complex traits (Groot *et al.* 2016). Therefore, it is very difficult to point at clear examples in which chemical communication channel variation may have led to species evolution, but it has been proposed in some cases. For example, changes in mating signals following host plant shifts have been proposed in two parental species of flea beetles (*Altica fragaria* and *A. viridicyanea*) (Xue *et al.* 2016). Alternatively, changes in the chemical communication channels have been proposed as drivers of population divergence, in the *Eriocrania semipurpurella-sangii* species complex (Lassance *et al.* 2019). Variation and novelty in sex pheromone blends can be introduced, for example, through genomic duplication or amino acid substitutions (Buček *et al.* 2015), while the switch to new preferences can take place in odorant receptors in the peripheral or central nervous system (Koutroumpa *et al.* 2016, van Schooten *et al.* 2020). While cases of evolution of signals and response may be difficult to pin-point in natural scenarios, tangible examples can be observed when applying techniques

such as synthetic pheromone lures for mating disruption. The constant selective pressure exerted by the continuous emission of the pheromone blend from the dispensers can result in changes of the female pheromone blend or male responses (Evenden and Haynes 2001, Mochizuki *et al.* 2002, Tabata *et al.* 2007). The combination of examples from natural and more "artificial" scenarios (i.e., synthetic lures) show the importance of considering variation in signals and responses and their implications for intrapopulation dynamics and species evolution. After all, in nature, populations always experience some level of variation because individuals are simultaneously affected by several external factors which interact with the physiological state of the individual.

3.2.2 Female signaling strategies (I, II, III)

As introduced in 3.2.1, calling behavior and chemical blends are two key drivers of sex pheromone signaling variation and can be non-mutually exclusive sources of skewed female attractiveness. While pheromone blend quantity or quality can affect female attractiveness and be involved in mate choice (Ruther *et al.* 2009, Harari *et al.* 2011, van Wijk *et al.* 2017), we did not find any correlation between the pheromone quantity or quality of the three color genotypes and female attractiveness (III).

The skewed genotype attractiveness was instead associated with female calling behavior (II, III). Traps baited with yy females were more likely to host males earlier in the night compared to the other two female genotypes (II). The earlier onset of calling behavior of a subset of yy females could explain males arriving earlier in traps which potentially allow them to outcompete conspecifics (III, Pham *et al.* 2020, 2021) (Fig. 8). However, calling earlier may come with costs. yy females decreased the time they spent calling as they aged (III). Producing and maintaining sex pheromone signaling can indeed be costly (Harari *et al.* 2011, Umbers *et al.* 2015, Blankers *et al.* 2021). Temporal differences in calling behavior have been shown to affect species dynamics, such as outcompeting younger and more fecund females or maintaining reproductive isolation between strains (Pashley *et al.* 1992, Schöfl *et al.* 2009, Pham *et al.* 2021). For the wood tiger moth, we can hypothesize that WW and yy females may have an attraction advantage at different population densities. With their earlier onset of calling, yy females may benefit in denser populations while the longer duration of calling among WW females may be beneficial to them in sparser populations. Finally, since calling duration decreased with age in yy females, and increased with age in WW and Wy females (III), and while females of different ages are likely to co-occur in the field, females may benefit from being close by. This leads to the question: should females "compete" or "collaborate" with each other during mate attraction? It may sound reasonable for females to move closer to others, to optimize the calling effort, and increase the chances of attracting males (Lim and Greenfield 2008). In the field, it is not uncommon to find small aggregations of wood tiger moth females which indeed suggests that they may move close to each other. On one hand, it may be advantageous for high quality females to move away from potential competitors, on the other hand it may be

advantageous for high and low quality females to stay in close proximity as high quality females may start copulating faster than when alone (van Wijk *et al.* 2017). These differences in female calling behavior and potential strategies may thus play an important role in intraspecific dynamics and mate choice within populations. In the specific case of the wood tiger moth, homozygote females (i.e., WW, yy) can potentially outcompete the more fertile Wy females (IV) by trying to secure a male faster than Wy females.
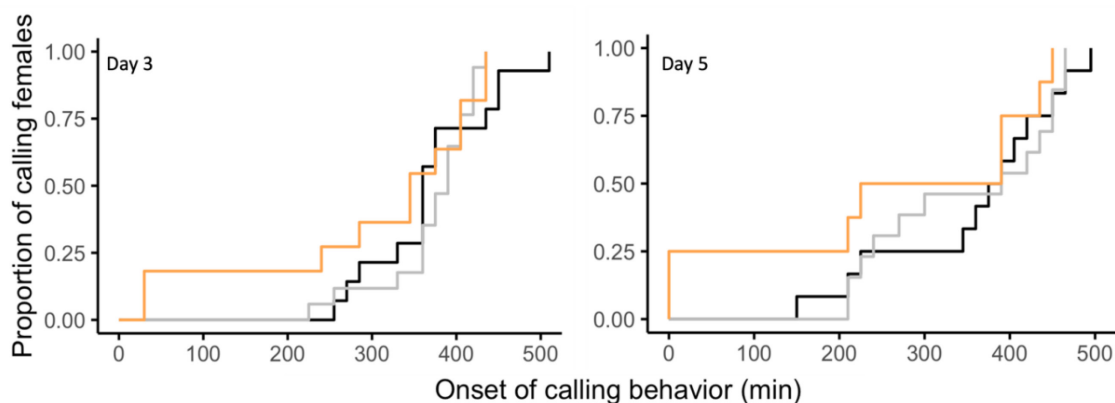

FIGURE 8   Cumulative proportion of calling females per genotype on day three and five. A subset of yy females is calling earlier than WW and Wy females (III).

3.2.3 Variation in male antennae (I, II)

As well as signalers showing variation in their sexual signaling, the receiver's ability to perceive sexual signals is also likely to vary between individuals (I, Anton and Rössler 2021). Through plasticity or modulation, the olfactory system can adjust in response to the environment and affect the receiver's perception (Anton and Rössler 2021). During development, individuals can adjust their investment in receptor organs according to the environment in which they are developing (Johnson *et al.* 2017). Furthermore, sublethal doses of pesticide can increase peripheral sensitivity of males to favor mating in *Spodoptera littoralis*, a crop pests species (Lalouette *et al.* 2016). Alternatively, changes at the physiological level, for example after mating, can make males of some species less sensitive to sexual signals and to cues for mating sites (Kromann *et al.* 2015, Sollai *et al.* 2018). Variation in the olfactory system due to context-dependent or condition-dependent factors is likely to lead to differences in the receiver's sensitivity and responses (I, Anton and Rössler 2021).

Despite the self-evident important role of male antennae to detect and direct males towards receptive females, the effect of antennal natural variation on how quickly and efficiently males can locate females is seldom investigated (Jayaweera and Barry 2017, Elgar *et al.* 2019). Antennal variation is expected among males since these are likely costly structures to develop (Symonds *et al.* 2012, Johnson *et al.* 2017, Pham *et al.* 2022). Typically, males with bigger antennae, thus with more odorant receptors, should be favored in locating females faster (Symonds *et al.* 2012, Elgar *et al.* 2019). This can have remarkable repercussions at

the intrapopulation level in terms of male-male competition and males that will eventually mate. Wood tiger moth males with denser antennae (i.e., higher number of lamellae) reached females faster in the enclosure setup (II) (Fig. 9). Since the enclosure represented a high-density population environment, having a higher number of lamellae (i.e., higher number of receptors) could make males more competitive in scramble competition. Denser antennae may be a trait under sexual selection, as it improves pheromone perception in a higher density scenario. In addition, it is a trait that may indicate higher quality males due to the costs of producing bigger sensory organs (Johnson *et al.* 2017). Because males and females were not allowed to engage in copulation, this experimental setup allowed us to test the male's ability to locate and reach females (II). Future investigations are required to understand whether there are costs for wood tiger moth males associated with developing more sensitive antennae, for example in male reproductive ability.
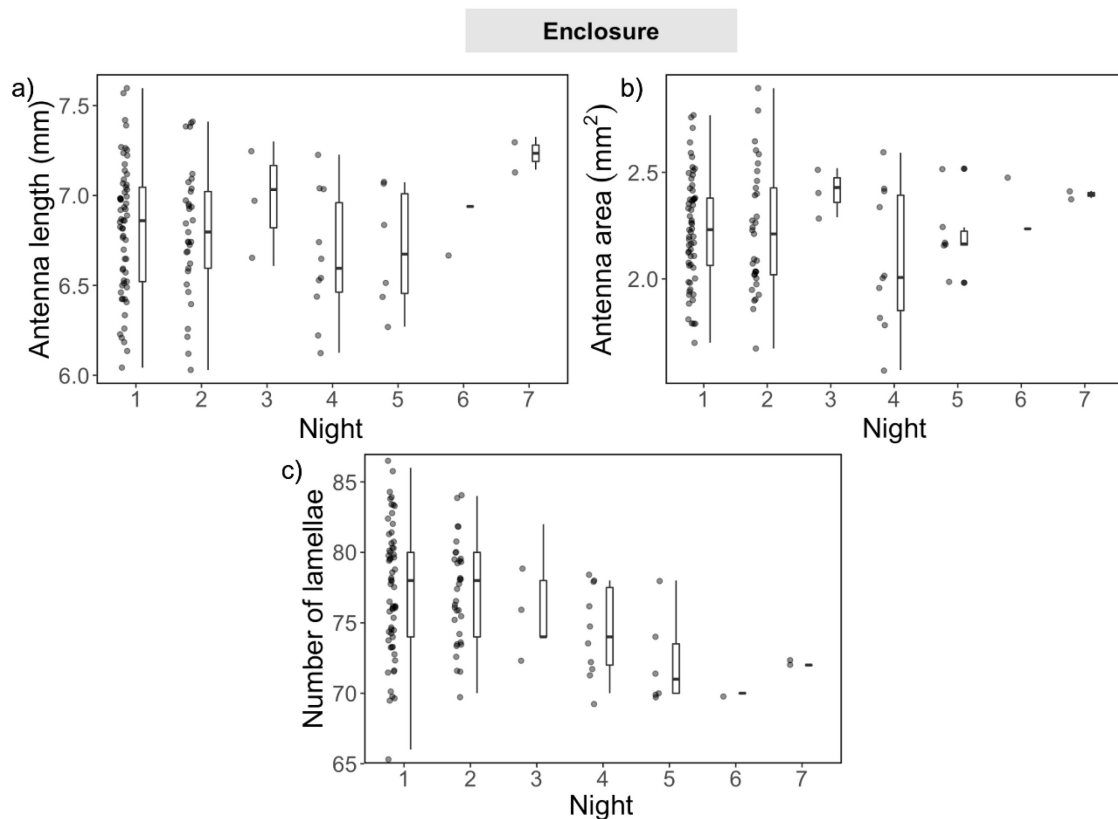

FIGURE 9   In the enclosure experiment, a) the length and b) area of the male antennae did not affect their order of arrival to females whereas c) males with a higher number of lamellae reached the females faster than males with fewer (II).

4 CONCLUSIONS AND FUTURE DIRECTIONS

The question of how phenotypic variability is maintained within populations has puzzled evolutionary biologists for a long time. Alternative color morphs are expected to be maintained within populations through some form of balancing selection, either heterozygote advantage, negative frequency-dependent selection, or disassortative mating (Wellenreuther *et al.* 2014, Llaurens *et al.* 2017). Despite polymorphisms typically having a simple genetic architecture (i.e., one or few tightly linked loci), the expression of these loci can have profound pleiotropic effects on traits that do not seem necessarily related (Wright 1982, McKinnon and Pierotti 2010). These can lead to many, and sometimes contrasting, selective forces affecting an individual's fitness and, ultimately, lead to complex polymorphisms. In this thesis, I found several genotype-specific behavioral and reproductive traits associated with the color morph. Carrying one or two copies of the *yellow* allele affected a variety of traits. At the precopulatory stage, yy females were more attractive to males than WW and Wy females (II). White (WW or Wy) males located females faster than yellow in a higher density scenario, while yellow males located females faster than white (WW or Wy) males in a lower density scenario. At the postcopulatory stage, yy individuals suffered a lower reproductive output and yy females also a lower mating success. Heterozygote (Wy) females were more fertile and showed a higher hatching success than WW and yy females. (IV). Through heterozygote advantage, both colour alleles could be maintained in the population under balancing selection. This would result in an expected frequency of the color phenotypes of 3:1 white:yellow color morphs, led by the W allele dominance. However, the expected ratio of the two color morphs is rarely found in natural populations (Hegna et al., 2015). Across the wood tiger moth's geographic distribution, populations have varying frequencies of white and yellow males. While in Central Finland, the populations are somewhat close to the 3:1 ratio, many other populations deviate from the expected ratio. Some of these populations have high frequency of yy individuals despite the reproductive disadvantages (Hegna et al., 2015; IV). This suggests that other selective forces are at play, for example context-dependent fitness advantages. Context-dependent advantages of the two

male color morphs have been shown under multiple selective forces (Nokelainen *et al.* 2012, 2013, 2014, 2022, Galarza *et al.* 2014, Gordon *et al.* 2015, 2018, Rojas *et al.* 2015, 2017, 2019, Winters *et al.* 2021). Here, I found that morph-specific advantages also extend to the pre- and post-copulatory level both in males and females, and is context-dependent. yy females were most attractive in higher population densities due to their early calling behavior (II, III). Male traits affected their ability to locate females in a context-dependent way, led by antennal variation and perhaps by different behavioral strategies (II). Finally, Wy and yy females had opposing reproductive advantages (IV).

The findings in this thesis have also created several questions and avenues for future investigations.

(1) The finding that the higher attractiveness was typically limited to few yy females (II), opens up the question of whether the higher female attractiveness comes with a fitness cost, which could explain why the yy female strategy of early calling, seen in a proportion of the yy females, is not more common in the population. Future investigations should follow closely what happens when a female attract males. Male harassment or different resource allocation during development have been shown to impact the fitness of the individual (Graham *et al.* 1980, Gilchrist and Rutowski 1986). There is the risk that a female attracting many males may fail to mate due to high male competition that results in a lack of copulation attempts. Alternatively, allocating more resources into pheromone signaling during development may trade-off with the resources available for investment in offspring. Either scenario would negatively affect female fitness and offer potential explanations for why only a few females within the population are very attractive.

(2) We found that females of the three color genotypes were characterized by different patterns of calling behavior. Some yy females called earlier than others and, in general, decreased their calling duration as they aged, while WW and Wy females increased their calling duration with age (III). Since female calling strategy changed with age, and in the field females of different ages coexist, this led me to hypothesize that females may employ strategies to increase their chances of attracting a male, particularly regarding whether they should "move away" from other calling females or "aggregate". By moving closer or far away from other signalers, females can increase their chances of attracting a conspecific and mating (Lim and Greenfield 2008, van Wijk *et al.* 2017, Borshagovski *et al.* 2019). In the case of the wood tiger moth, it could be particularly advantageous for WW and yy females to be close to each other (III).

(3) While we know quite a lot about morph-specific advantages under different selective pressures (e.g., Nokelainen *et al.* 2012, 2013, 2014, 2021, Gordon *et al.* 2015, 2018, Rojas *et al.* 2015, 2017, 2019, Rönkä *et al.* 2018, 2020) there is still a lot to explore on the male side, especially under sexual selection. Interesting avenues to explore are, for example, the potential roles of visual signals vs male

sex pheromone in female choice, and if and how mutual mate choice affects the mating outcome. For example, if wing luminance or male sex pheromone could represent traits involved in female choice (Iyengar *et al.* 2001, Constanzo and Monteiro 2007, Papke *et al.* 2007).

Besides these potential new avenues of research, this thesis has also shown that the experimental design and the traits we decide to measure can affect how we advance our understanding of the species. For example, by using different experimental scales (i.e., field and enclosure setups), I found that males benefit from context-dependent advantages, and this can have further implications for population fitness. In addition, by studying how genetic variation affects reproductively relevant traits from the precopulatory to the postcopulatory stage we have revealed stage-specific (dis)advantages of the genotypes. While this thesis is by no means complete in its exploration of the pre- to post-copulatory stage, if I had only focused on the precopulatory stages I would have missed out on finding that Wy females actually have a postcopulatory advantage. Finally, in a world where molecular biology and bioinformatics have become the predominant tools in science, we should not forget that behavioral investigations can also reveal important aspects of the ecology of a species. The wood tiger moth is becoming an emerging study system to study complex polymorphisms in nature. In that respect it resembles some other famous polymorphisms such as ruffs (*Philomachus pugnax*) and side-blotched lizards (*Uta stansburiana*) (Sinervo and Lively 1996, Küpper *et al.* 2015). Complex interactions between the genetic architecture of the polymorphism, behavior, and fitness related traits make polymorphic species attractive systems to investigate how genetic variation is generated and maintained within populations. Furthermore, we should not forget that individuals are also affected by the environment they live in. Thus, the polymorphism becomes even more complex if morphs have advantages at different densities or thrive under different environmental conditions. Thus, we need to understand how both the genetic architecture and the ecology of the species are interrelated to understand how complex polymorphisms are maintained. While this thesis has left us with many more questions and avenues to explore, we can still conclude that nature is complex and will never cease to amaze us.

Acknowledgements

A few key things have kept me going throughout this journey; good food, listening in repeat to "you made it through another day" verse, and you people. Each and every one of you have been fundamental to compile more than four years into these few pages. And also, you, not mentioned here. If you are reading this section and feel left out, do not be surprised. I forgot to thank lab members during my PhD seminar test run, so I am not surprised if I did it here as well. Sorry to you in advance!

I have had three amazing, truly caring, and supportive supervisors. Three powerful personalities from whom I have learned a lot. First and foremost a *huge* thank you goes to Johanna. You believed in me already seven years ago, when I looked as lost as someone that just landed on a foreign planet and still warmly welcomed me in your research group. I will always be grateful for the things I have learned that shaped both my scientific- and personal-side. I am also very grateful to your overwhelming enthusiasm, your eagerness to discussion, and brilliant curiosity. Thank you for all the times you were enthusiastic for me when I had lost faith, for allowing me to make my mistakes (and refraining yourself most of the times from saying "I told you so"), and to provide guidance every time I needed it. Thank you also for all the food, dinners, sauna, berry, and mushroom picking. You are not only an example as supervisor, but there is also so much to learn from you in all aspects. Astrid, what struck me when we first met was your energy and true passion for science. So much energy that even paragraphs and written words struggled to have a grip on the word sheet. But this has thought me that there is always room for improvement, and good results are achieved through perseverance. Thank you for teaching me so much about pheromones and for your guidance through many experiments. And thank you for always making time for one more meeting and one more discussion in your busy schedule. Thank you Emily, for always being there and for being so kind all the time, from offering a place to stay at the beginning, to cheering for the smallest of my achievements, and to promptly defuse my disappointment when failures would hit me. You were also very patient with teaching me how to turn long and convoluted phrases, that are only good to lose the reader, into short and crispy sentences, to keep the reader's attention. …Well, I am still working on it…

I am very grateful to Associate Professor Matthew Symonds for accepting to be my honorable opponent, even if it meant visiting Finland in the cold and dark month of December. Thank you to Associate Professor John Fitzpatrick and Professor Maya Evenden for accepting to pre-examine my thesis. I am very grateful for your thorough reviews and constructive feedback to my work. A special thank you goes to my follow-up group members, Carita and Lutz. You were always very kind, and truly interested in my progress and to my well-being. Jari Haimi, thank you for simply sparkling positive encouragement at the very end of this journey when dealing with formatting and fonts. Thank you Nina Pekkala for guiding me through this last phase in a very kind and supporting way. In my PhD, I have spent a considerable amount of time running

experiments in Konnevesi, and this was possible thanks to the Konnevesi staff which was always very willing to assemble and disassemble the big outdoor enclosure, happy to create the path in the enclosure when the vegetation would reach my shoulders, and for supervising me from a distance while I (tried to) fix the holes in the tent.

A PhD thesis shapes up thanks also to a very supporting and enthusiastic lab. The *plantaginis* group with its past and present members Kaisa, Ossi, Bibi, Swanne, Anne, Janne, Jimi, Alma, Melanie, Cristina, Liam, Zowi, Eetu, Sandra, Elisa, Sari, Juan, Sanni, have simply been fundamental to my work. Kaisa, Jimi, and Alma, you were amazing in making sure I always had the moths I needed, supported me with the pedigree, and helped me to find my way around in Jyväskylä and Helsinki. Ossi thank you for coming to the rescue in Likakummunjankka. I am sure you would have just told the perfect story had I become the protagonist of 1000 Ways to Die TV show. Cris, simply thank you for sharing with me the whole journey. It was vital to my mental health to know I could always find support in you. You always listened to my rants and had the ability to calm me down when things would go very wrong. I could also always count on you in running a few more trials and in making memorable new adventures in Amsterdam. Destiny brought us to meet in Jyväskylä even if we are from the same small town in Verona. I wish you strength in your last stretch, you are so close, and you will simply do great! Over the years I have had several office mates, but one in particular was just the office mate everyone should have once in their lifetime; Liam, O'Liam, so much frustration and anger, was let out with you. You always had one minute (well, *many* minutes) to listen to me and to offer a wise word in return. And that was the best therapy I could wish for. Eetu, Liisa, and Matleena you have been awesome students. Thank you for trusting me with your theses. It was an honor to have such talented, smart, and patient students. I am still sorry for all the knee- and back- hurting for hours and hours spent on the floor, and, to you Eetu, for also the many sleepless nights observing moths. Eetu, I am also very happy you made it out of the field that one night where the threat of mosquitos crossed paths with a big bird. And for the home-made lab. Thank you Teemu for your endless enthusiasm, for always agreeing to help, and for marking those moths when my hand was broken. And thank you Tuuli, for digging down those poles in Konnevesi. They have been there ever since, and I believe we have now marked the territory for good.

Chloe, Justine, Cris, Annis, Vitalja, Nadja, Christina, the EMPSEB committee. With you, I have learned that nothing is impossible, and with team effort one can go far and big. We took on a huge task, not knowing the half of the sweat and time we would have to put in, but we outdid it! I am so grateful to Annis, Cris, and Vitaljia for alleviating the painful writing period with the writing club over another missed summer. Not only I felt supported, but sharing the same struggle made me go through that period a thousand times better.

I had the pleasure to interact daily with an awesome group of scientists at the Department. Thank you, among others, Matteo, Justine, Chloé, Mariana, Neda, Ilze, Sara H., Sara C., Tarmo, Ilkka, Aigi, Jannis, Matthieu, Emily, Andrés, Cecile, Heli, for Friday's 10 a.m. coffee breaks, floorball, and the after-lunch table

football challenge. Thank you also to the PhD students cohort. It was nice to hang out together during Sauna and Support gatherings which constantly reminded me that my struggle is often a shared struggle, and we can learn from each other.

During these years, I have also crossed path with friends I have shared more than just university-related activities. Thank you Tiffany and Anton for so many adventures together in National Parks, grilling, sliding down slopes, and walking on a barely frozen lake. Tiff, thank you for endless chats. I think running a half marathon will remain a dream. We had to abort the mission rather soon as we realized we could not run one in parallel to the PhD's marathon, but I am afraid by now our knees will decide for us. I am, however, proud of our training sessions with Miriam's cakes, indian food, and sushi. Thank you Didi for our podcasts full of laughs, stories, and long-distance support.

It is a shame the pandemic stopped my visits to Amsterdam, I always had tons to learn, and I had so much fun during my visits, even when they involved taking a train at 5 a.m. to analyze more samples. Every visit was a memorable experience thanks to an amazing bunch; Ke, Elise, Naomi, Federica, Laura, Dennis, Thomas, Reneé, Peter. Peter, I especially enjoyed my time in the lab with you. Thank you for your energy and for optimizing every setup even when we did not really have the time or enough moths to work with. Thank you to Frans, Henk, and the Pherbank group for your patience over the years, for many last minute meeting while I was in the field, and for agreeing to always ship one more set of lures. Science brings people to constantly move away, but it is not always for the worst when it brings you to meet a vibrant and smart group of people such as the ESB group. Deryk, Nora, Katja, Patrick, Ina, Jonna, Pierre, Beatriz, Rose, Edward, Purabi, Teresa, Carly, thank you for interesting weekly meetings, chats, and discussions.

I could not have achieved any of this without the support of friends and family. Sara, a true friend, always ready to listen and support me from thousands of km away. Memorable are our hours-long videocalls. Thank you, Simone, for being so patient in those occasions. Vale and Mary, I am so glad for *that* lab course a few days before Christmas that brought us to found the Adventure Girls trio. To our many trips mixed with spiders, deers, AWOL cormorants, and birds that helped along the way, and to many more adventures together. Grazie alla mia famiglia, alla quale non credo nemmeno di poter iniziare ad esprimere la mia gratitudine per il vostro supporto incondizionato, nonostante le poche e corte occasioni passate assieme ultimamente. Grazie per avermi sempre offerto un appoggio e mai negato un consiglio critico che mi ha sempre aiutato a vedere le cose con occhi diversi. And last, but not least, Mikael. I truly could not have made it without such a supportive partner, always there to patiently listen to my frustration, and bring light when I was (hilariously) miserable at the end. Thank you for your patience, pragmatism, and for being (*so much*) balanced all the time. You kept me grounded. GP2, R., R., H., and B. also immensely helped in this journey. Thank you for the many laughs, confused pronunciations, twists, great adventures, and for showing me the wonder of nature which greatly helped to distract my mind from work. No two hikes in the forest are ever the same when you are around.

RIASSUNTO (RÉSUMÉ IN ITALIAN)

Mantenimento della variazione genetica nelle popolazioni: Interazione tra selezione sessuale, comunicazione chimica, e fitness nella wood tiger moth (*Arctia plantaginis*)

In natura esistono numerose specie le cui popolazioni sono caratterizzate da individui che presentano variazione fenotipica, ad esempio la colorazione delle ali di farfalla o il piumaggio degli uccelli può differire tra individui. Di conseguenza, il tratto viene definito polimorfico e la popolazione definita polimorfica per quel tratto. Studiare come la variazione fenotipica sia mantenuta nelle popolazioni naturali scaturisce da un'incongruenza con le predizioni teoriche. Se per effetto della selezione naturale il tratto dovrebbe convergere verso un optimum, e quindi essere presente in una sola variante, perché ci sono popolazioni che presentano varietà a livello di fenotipo? Sta diventando sempre più chiaro che è possibile mantenere diverse varianti di un tratto attraverso l'effetto sinergico della base genetica che correla i due tratti e delle pressioni selettive che agiscono sui tratti. Difatti, se due tratti sono associati a livello genetico, l'effetto dell'agente selettivo (ad esempio, un predatore, o un patogeno) su un tratto avrà conseguenze anche sul tratto associato. Perciò, correlazioni genetiche tra diversi tratti fanno in modo che la variazione fenotipica possa persistere generazione dopo generazione. Affinché sia possibile, i due tratti devono essere o "fisicamente" collegati[1] a livello cromosomico ed essere ereditati assieme, oppure l'espressione di un tratto ne influenza altri tramite una forma di collegamento "incompleto". In questo caso, il prodotto espresso da un gene (per esempio un enzima) influenza l'espressione di un gene che non sembra direttamente collegato al primo, e causano un effetto sulla fitness dell'individuo. Per esempio, nel moscerino della frutta (*Drosophila melanogaster*) è stato dimostrato che la mancanza di espressione del gene che conferisce la colorazione marrone/nera della cuticola che riveste il corpo influisce negativamente anche sul comportamento dei maschi. I maschi non sono più in grado di eseguire correttamente la danza corteggiatrice e la loro capacità di accoppiarsi viene ridotta a seguito di modifiche a livello della cuticola della porzione di zampa di solito usata per afferrare le femmine, che così riescono a sottrarsi più facilmente all'accoppiamento.

In molte specie di falene, le femmine rilasciano feromoni[2] nell'aria allo scopo di attirare maschi. Siccome questi animali sono molto piccoli, e devono essere in grado di attirare un individuo con successo, per molto tempo si è ipotizzato che tutte le femmine di una popolazione fossero caratterizzate da una miscela identica di feromoni e che non esistessero strategie diverse di emissione.

---

[1] Due geni si definiscono "fisicamente" collegati (physical linkage) quando sono localizzati sullo stesso cromosoma e vengono perciò ereditati assieme senza che avvenga ricombinazione genetica durante l'evento riproduttivo.

[2] I feromoni sono composti chimici, di solito presenti in miscele di due o più composti, che vengono emessi da un individuo con lo scopo di attrarre uno o più individui del sesso opposto.

Questo perché i maschi devono essere in grado di localizzare le femmine facilmente, ed è a vantaggio di entrambi i sessi essere in grado di trovarsi in maniera rapida e senza margine di errore. Ciò nonostante, produrre e rilasciare feromoni può alle volte richiedere un sostanziale impiego di risorse, per cui è probabile che alcune femmine rilascino meno feromone, oppure anticipino/ritardino il rilascio durante la notte. C'è un numero sempre crescente di studi che infatti dimostra come usando diverse strategie di rilascio, le femmine possano influenzare la scelta dei maschi. Allo stesso modo, anche i maschi possono differire nella loro capacità di percepire e localizzare le femmine, per esempio in relazione alla dimensione delle loro antenne. Se esistono differenze nei feromoni rilasciati dalle femmine, per esempio femmine che producono più feromone sono gialle, o nella capacità dei maschi di localizzare femmine, e queste differenze sono associate alle diverse colorazioni delle ali, ci possono essere ripercussioni sugli individui che si accoppieranno, e quindi sulle colorazioni che vengono trasmessi alla generazione successiva.

Quando un tratto viene espresso attraverso molte varianti suggerisce che la base genetica che li caratterizza è composta da diversi alleli[3]. Gli individui che portano alleli diversi nello stesso tratto vengono definiti eterozigoti, da distinguersi dagli individui che portano alleli dello stesso tipo, definiti omozigoti. Popolazioni polimorfiche sono tipicamente caratterizzate da alti livelli di individui eterozigoti. Questo permette a diversi alleli, e quindi a diverse forme del tratto, di essere mantenuti nella popolazione. Inoltre, la presenza di individui eterozigoti da anche la possibilità ad alleli che sono deleteri, quando presenti in multiple copie in individui omozigoti, di essere mantenuti nella popolazione perché mascherati quando sono portati nel corredo genetico di individui eterozigoti. Questo può portare ad individui eterozigoti ad avere particolare vantaggio di fitness, attraverso un meccanismo chiamato vantaggio dell'eterozigote. Allo stesso tempo, lo svantaggio creato da gruppi di alleli deleteri, e di conseguenza ridotta capacità riproduttiva, può indurre competizione tra individui, per esempio per evitare di accoppiarsi con individui incapaci di produrre prole in grado di raggiungere lo stadio adulto.

Questa tesi ha l'obiettivo di investigare eventuali associazioni tra la variazione del tratto del colore e tratti che sono coinvolti nella fitness riproduttiva dell'individuo. Lo scopo è di capire più a fondo come la variazione di questi tratti possa influenzare le dinamiche riproduttive tra individui e potenzialmente mantenere la variabilità genetica e fenotipica nelle popolazioni. Ho scelto di studiare potenziali associazioni attraverso l'intera sequenza riproduttiva, ovvero dal reclutare il sesso opposto (stadio pre-copulativo), allo stadio di accoppiamento, fino alla produzione di prole quindi allo stadio post-copulativo. Questo perché non è detto che una volta che due individui entrino in contatto, e si accoppino, poi riescano a riprodursi con successo. A questo scopo, ho utilizzato la wood tiger

---

[3] Un allele è una forma alternativa, o versione, di un gene. Individui diploidi, ovvero che hanno due copie dello stesso cromosoma, ereditano una copia del cromosoma dalla madre e una copia dal padre. Se queste due copie sono uguali, l'individuo si dice omozigote per il gene, mentre se presenta copie diverse, viene definito eterozigote.

moth (*Arctia plantaginis*), una specie di falena che presenta una colorazione polimorfica, che nei maschi si traduce in ali bianche e gialle. La colorazione è determinata geneticamente da due alleli. La colorazione bianca è determinata dall'allele dominante[3] W e può essere espressa sia da individui che portano due copie dell'allele (omozigoti WW) o individui eterozigoti (Wy), mentre i maschi gialli sono omozigoti per gli alleli recessivi[4] (yy). Nonostante le femmine presentino una colorazione che va dal giallo al rosso, portano gli stessi alleli dei maschi (W e y) che però non vengono espressi. Studi precedenti sulla specie hanno dimostrato che la colorazione delle ali è associata a diversi vantaggi, per esempio i maschi gialli hanno un minor rischio di essere predati mentre i maschi bianchi hanno generalmente un vantaggio a livello riproduttivo. Nonostante ciò, se le condizioni cambiano, questi vantaggi possono venire meno. Ad esempio, in ambienti meno luminosi (come nella foresta) i maschi gialli vengono predati più spesso dei maschi bianchi. Quindi considerare da prima a dopo della copulazione potrebbe svelare vantaggi che dipendono dallo stadio della sequenza riproduttiva. Per studiare ciò, ho utilizzato approcci multipli. Innanzitutto, ho condotto una revisione degli studi recentemente pubblicati che riguardano l'effetto della variazione sia nei feromoni rilasciati dalle femmine sia delle strutture ricettive dei maschi ed il loro effetto sulla probabilità di accoppiarsi. Per studiare eventuali associazioni tra i tratti femminili (feromoni) e maschili (antenne) ed il colore delle ali, ho testato l'attrazione delle femmine di diverso genotipo sia in condizioni naturali che semi-naturali (ovvero in una grande tenda), mettendo femmine in trappole in cui rilasciando feromone avrebbero intrappolato eventuali maschi attratti. Successivamente, ho testato se la differenza in attrazione fosse dovuta alla quantità o alla composizione dei feromoni, oppure legata a diverse strategie di rilascio. Per quanto riguarda i tratti dei maschi, ho tagliato una antenna dei maschi trovati nelle trappole per determinare se la morfologia delle antenne correlasse con la loro capacità di localizzare le femmine. Infine, per testare se le diverse colorazioni sono associate a differenze nella probabilità di accoppiarsi, ho accoppiato un maschio ed una femmina e testato la frequenza con cui si sono accoppiati. Infine, ho contato il numero di uova prodotte da ciascuna coppia e la percentuale di uova che si è schiusa in larve come indice di fitness riproduttiva. Nei paragrafi successivi riporto i risultati principali di questi esperimenti.

Dalla revisione di studi è emerso che molte sono le forze selettive che agiscono sia sui feromoni che sulla capacità dei maschi di percepire i feromoni; quindi, sia maschi che femmine sono soggetti a cambiamenti nei loro tratti. Queste forze selettive possono essere racchiuse in tre categorie. Possono essere dovute dal contesto, ovvero dalla vicinanza di conspecifici[4] o dei composti volatili che vengono rilasciati dalle piante su cui si sviluppano, dalla condizione dell'individuo, che può essere legata all'età, oppure da meccanismi genetici. Di conseguenza, gli individui possono essere più o meno attrattivi e ciò può avere conseguenze a livello delle dinamiche di popolazione. Ad esempio, gli individui possono usare questi segnali per evitare di accoppiarsi con consanguinei o con

---

[4] In biologia, detto di organismi che appartengono alla stessa specie.

individui che hanno una fitness ridotta, come per esempio quelli che si sono recentemente accoppiati. Infine, l'effetto della variazione in questi segnali può estendersi ad influenzare la storia evolutiva della specie. Una volta capito quanto importante potesse essere l'effetto della variazione in questi segnali, ho studiato i potenziali effetti quando associati alla colorazione delle ali, usando la wood tiger moth.

Ho trovato che la capacità di attrazione delle femmine della wood tiger moth differisce molto tra individui, anche se solitamente le femmine più attrattive appartengono al genotipo giallo (yy). Queste femmine solitamente sono più attrattive quando ci sono molti maschi nelle vicinanze e ciò è dovuto all'abilità delle femmine del genotipo giallo (yy) di anticipare le femmine degli altri due genotipi (WW e Wy) emettendo feromone prima nella notte. La differenza sembra risiedere solo a livello di strategia di rilascio delle femmine dal momento che né la quantità né la composizione dei feromoni è associata con quanto attrattive sono le femmine. Per quanto riguarda i maschi, maschi bianchi e gialli godono di vantaggi che dipendono dal contesto sperimentale. In particolare, i maschi gialli sono in grado di localizzare le femmine più rapidamente quando la popolazione dei maschi è meno densa, mentre i maschi bianchi sono più veloci quando la popolazione è più densa. Inoltre, quando la popolazione è più densa, i maschi con antenne che ospitano un numero maggiore di lamelle (che accoglie i recettori per percepire i feromoni), raggiungono le femmine più velocemente.

Infine, mentre gli individui dei tre genotipi hanno tutti la stessa probabilità di accoppiarsi, le coppie formate da femmine del genotipo giallo (yy) e maschi bianchi (WW o Wy) tendono ad accoppiarsi più velocemente delle altre coppie. Ciò nonostante, le femmine del genotipo giallo (yy) non godono di una più elevata probabilità di avere prole. Queste femmine, infatti, hanno una fitness riproduttiva particolarmente bassa e falliscono di più delle femmine bianche (WW e Wy) in avere prole. Anche i maschi gialli soffrono particolarmente nel produrre prole. Contrariamente al ridotto successo degli individui del genotipo giallo (yy), femmine eterozigoti (Wy) hanno un vantaggio riproduttivo rispetto alle femmine del genotipo WW e yy. Ciò significa che entrambi gli alleli (W e y) hanno elevata probabilità di essere passati alla generazione successiva e mantenuti a livello della popolazione. Questo suggerisce anche che le femmine, che non esprimono la colorazione dei maschi perché tendenzialmente sono rosse, possono ricoprire un ruolo particolarmente importante nel mantenere la colorazione polimorfica dei maschi.

I risultati di questa tesi dimostrano come la base genetica e le forze di selezione che agiscono su questi tratti associati siano importanti per mantenere sia la variazione fenotipica che genotipica dei tratti e di conseguenza mantenere le popolazioni polimorfiche. Complesse interazioni tra la base genetica dei tratti, il comportamento degli individui, l'effetto della variazione dei tratti, e l'ambiente circostante possono bilanciare il vantaggio tra alleli diversi, e quindi mantenere complessi polimorfismi. È quindi fondamentale capire sia come l'architettura genetica che l'ecologia della specie interagiscano tra di loro per capire come complessi polimorfismi siano mantenuti in natura.

REFERENCES

Andersson, M. 1994. *Sexual selection*. Princeton, NJ: Princeton University Press.

Anton, S., & Rössler, W. 2021. Plasticity and modulation of olfactory circuits in insects. *Cell Tissue Res.* 383: 149–164. https://doi.org/10.1007/s00441-020-03329-z

Baker, T. C., Meyer, W., & Roelofs, W. L. 1981. Sex Pheromone Dosage and Blend Specificity of Response by Oriental Fruit Moth Males. *Entomol. Exp. Appl.* 30: 269–279. https://doi.org/10.1111/j.1570-7458.1981.tb03110.x

Baker, T. C., & Hansson, B. S. 2016. Moth sex pheromone olfaction: flux and flexibility in the coordinated confluences of visual and olfactory pathways. In *Pheromone communication in moths: evolution, behavior, and application.* (Allison, J., & Cardé, R. T., eds). pp. 139-171. University of California press.

Baker, T. C., Willis, M. A., Haynes, K. F., & Phelan, P. L. 1985. A pulsed cloud of sex pheromone elicits upwind flight in male moths. *Physiol. Entomol.* 10: 257-265.

Balmer, A. J., Brakefield, P. M., Brattström, O., & van Bergen, E. 2018. Developmental plasticity for male secondary sexual traits in a group of polyphenic tropical butterflies. *Oikos* 127: 1812–1821. https://doi.org/10.1111/oik.05291

Bastock, M. 1956. A Gene Mutation Which Changes a Behavior Pattern. *Evolution* 10: 421. https://doi.org/10.2307/2407002

Birch, M. C., Poppy, G. M., & Baker, T. C. 1990. Scents and eversible scent structures of male moths. *Annu. Rev. Entomol.* 35: 25-54.

Blankers, T., Lievers, R., Plata, C., van Wijk, M., van Veldhuizen, D., & Groot, A. T. 2021. Sex pheromone signal and stability covary with fitness. *R. Soc. Open Sci.* 8: 210180. https://doi.org/10.1098/rsos.210180

Borshagovski, A. M., Baudry, G., Hopkins, J., & Kaitala, A. 2019. Pale by comparison: Competitive interactions between signaling female glow-worms. *Behav. Ecol.* 30: 20–26. https://doi.org/10.1093/beheco/ary161

Brien, M. N., Orteu, A., Yen, E. C., Galarza, J. A., Kirvesoja, J., Pakkanen, H., Wakamatsu, K., Jiggins, C. D., & Mappes, J. 2022. Colour polymorphism associated with a gene duplication in male wood tiger moths. *BioarXiv.* https://doi.org/10.1101/2022.04.29.490025

Buček, A., Matoušková, P., Vogel, H., Šebesta, P., Jahn, U., Weißflog, J., Svatoš, A., & Pichová, I. 2015. Evolution of moth sex pheromone composition by a single amino acid substitution in a fatty acid desaturase. *PNAS.* 112: 12586–12591. https://doi.org/10.1073/pnas.1514566112

Cardé R. T., 2016. Moth navigation along pheromone plumes. In *Pheromone communication in moths: evolution, behavior, and application.* (Allison, J., & Cardé, R. T., eds). pp. 173-189. University of California press.

Cardé, R. T., & Baker, T. C. 1984. Sexual Communication with Pheromones. In *Chemical ecology of insects*. Springer, Boston, MA.

Cardé, R. T., Cardé, A. M., Hill, A. S., & Roelofs, W. L. 1977. Sex pheromone specificity as a reproductive isolating mechanism among the sibling speciesArchips argyrospilus andA. mortuanus and other sympatric tortricine moths (Lepidoptera: Tortricidae). *J. Chem. Ecol.* 3: 71–84.

Connahs, H., Tan, E. J., Ter, Y., Dion, E., Matsuoka, Y., Bear, A., & Monteiro, A. 2022. The yellow gene regulates behavioural plasticity by repressing male courtship in Bicyclus anynana butterflies. *Proc. R. Soc. B.* 289: 20212665. https://doi.org/10.1098/rspb.2021.2665

Costanzo, K., & Monteiro, A. 2007. The use of chemical and visual cues in female choice in the butterfly Bicyclus anynana. *Proc. R. Soc. B.* 274: 845-851. https://doi.org/10.1098/rspb.2006.3729

Coulson, T., MacNulty, D. R., Stahler, D. R., VonHoldt, B., Wayne, R. K., & Smith, D. W. 2011. Modeling effects of environmental change on wolf population dynamics, trait evolution, and life history. *Science* 334: 1275–1278. https://doi.org/10.1126/science.1209441

Cuthill, I. C., Allen, W. L., Arbuckle, K., Caspers, B., Chaplin, G., Hauber, M. E., Hill, G. E., Jablonski, N. G., Jiggins, C. D., Kelber, A., Mappes, J., Marshall, J., Merrill, R., Osorio, D., Prum, R., Roberts, N. W., Roulin, A., Rowland, H. M., Sherratt, T. N., … Caro, T. 2017. The biology of color. *Science* 357: eaan0221. https://doi.org/10.1126/science.aan0221

Darwin C. 1859. *On the origin of species by means of natural selection.* Murray, London.

Darwin C. 1871. *The descendent of man, and selection in relation to sex.* Murray, London.

De Pasqual, C., Selenius, E., Hanninen, M., Kartano, L., & Mappes, J., Color morph specific mating advantage maintained in different densities of a polymorphic moth species. *In prep.*

Eberhard, W. G., & Cordero, C. 1995. Sexual selection by cryptic female choice on male seminal products-a new bridge between sexual selection and reproductive physiology. *Trends Ecol. Evol.* 10: 493-496. https://doi.org/10.1016/S0169-5347(00)89205-8

Elgar, M. A., Johnson, T. L., & Symonds, M. R. E. 2019. Sexual selection and organs of sense: Darwin's neglected insight. *Anim. Biol.* 69: 63–82. https://doi.org/10.1163/15707563-00001046

Endler, J. A. 1988. Frequency-dependent predation, crypsis and aposematic coloration. *Philos. Trans. R. Soc.* 319: 505–523. https://doi.org/10.1098/RSTB.1988.0062

Endler, J. A., & Mappes, J. 2017. The current and future state of animal coloration research. *Philos. Trans. R. Soc.* 372: 20160352 https://doi.org/10.1098/rstb.2016.0352

Evenden, M. L., 2016. Mating disruption of moth pests in integrated pest management: a mechanistic approach. In *Pheromone communication in moths: evolution, behavior, and application.* (Allison, J., & Cardé, R. T., eds). pp. 365-393. University of California press.

Evenden, M. L., & Haynes, K. F. 2001. Potential for the evolution of resistance to pheromone-based mating disruption tested using two pheromone strains of the cabbage looper, Trichoplusia ni. *Entomol. Exp. Appl.* 100: 131-134.

Ferguson, L. C., Green, J., Surridge, A., & Jiggins, C. D. 2011. Evolution of the insect yellow gene family. *Mol. Biol. Evol.* 28: 257–272. https://doi.org/10.1093/molbev/msq192

Fisher, R. A. 1922. On the dominance ration. *Proc. R. Soc. Edinb.* 42, 321-341.

Fisher, R. A. 1930. The evolution of dominance in certain polymorphic species. *Am. Nat.* 64: 385-406

Ford, E. B. 1945. Polymorphism. *Biol. Rev.* 20: 73–88. https://doi.org/10.1111/J.1469-185X.1945.TB00315.X

Galarza, J. A., Nokelainen, O., Ashrafi, R., Hegna, R. H., & Mappes, J. 2014. Temporal relationship between genetic and warning signal variation in the aposematic wood tiger moth (Parasemia plantaginis). *Mol. Ecol.* 23: 4939–4957. https://doi.org/10.1111/MEC.12913

Gilchrist, G. W., & Rutowski, R. L. 1986. Adaptive and incidental consequences of the alba polymorphism in an agricultural population of Colias butterflies: female size, fecundity, and differential dispersion. *Oecologia* 68: 235-240.

Gordon, S. P., Burdillat, S., & Mappes, J. 2018. Phenotype-dependent mate choice and the influence of mixed-morph lineage on the reproductive success of a polymorphic and aposematic moth. *Evol. Ecol.* 32: 427–441. https://doi.org/10.1007/s10682-018-9944-5

Gordon, S. P., Kokko, H., Rojas, B., Nokelainen, O., & Mappes, J. 2015. Colour polymorphism torn apart by opposing positive frequency-dependent selection, yet maintained in space. *J. Anim. Ecol.* 84: 1555-1564. https://doi.org/10.1111/1365-2656.12416

Graham, S. M., Watt, W. B., & Gall, L. F. 1980. Metabolic resource allocation vs. mating attractiveness: Adaptive pressures on the "alba" polymorphism of Coliasbutterflies. *PNAS* 77: 3615-3619. https://doi.org/10.1073/pnas.77.6.3615

Gray, S. M., & McKinnon, J. S. 2007. Linking color polymorphism maintenance and speciation. *Trends Ecol. Evol.* 22: 71–79. https://doi.org/10.1016/j.tree.2006.10.005

Greenfield, M. D. 1981. Moth Sex Pheromones: An Evolutionary Perspective. *Fla. Entomol.* 64: 4. https://doi.org/10.2307/3494597

Groot, A. T., Claßen, A., Staudacher, H., Schal, C., & Heckel, D. G. 2010. Phenotypic plasticity in sexual communication signal of a noctuid moth. *J. Evol. Biol.* 23: 2731–2738. https://doi.org/10.1111/j.1420-9101.2010.02124.x

Groot, A. T., Dekker, T., & Heckel, D. G. 2016. The Genetic Basis of Pheromone Evolution in Moths. *Annu. Rev. Entomol.* 61: 99–117. https://doi.org/10.1146/annurev-ento-010715-023638

Harari, A. R., Zahavi, T., & Thiéry, D. 2011. Fitness cost of pheromone production in signaling female moths. *Evolution* 65: 1572–1582. https://doi.org/10.1111/j.1558-5646.2011.01252.x

Hedrick, P. W. 2012. What is the evidence for heterozygote advantage selection? *Trends Ecol. Evol.* 27: 698–704. https://doi.org/10.1016/j.tree.2012.08.012

Hedrick, P. W., Smith, D. W., & Stahler, D. R. 2016. Negative-assortative mating for color in wolves. *Evolution* 70: 757–766. https://doi.org/10.1111/EVO.12906

Hedrick, P. W., Stahler, D. R., & Dekker, D. 2014. Heterozygote advantage in a finite population: Black color in wolves. *J. Hered.* 105: 457–465. https://doi.org/10.1093/jhered/esu024

Hegna, R. H., Galarza, J. A., & Mappes, J. 2015. Global phylogeography and geographical variation in warning coloration of the wood tiger moth (*Parasemia plantaginis*). *J. Biogeogr.* 42: 1469–1481. https://doi.org/10.1111/jbi.12513

Heinen-Kay, J. L., Kay, A. D., & Zuk, M. 2021. How urbanization affects sexual communication. *Ecol. Evol.* 11: 17625–17650. https://doi.org/10.1002/ece3.8328

Herberstein, M. E., Painting, C. J., & Holwell, G. I. 2017. Scramble competition polygyny in terrestrial arthropods. In *Advances in the Study of Behavior* (Naguib, M., Podos, J., Simmons, L. W., Barrett, L., Healy, S. D., Zuk, M. eds). 49: 237–295. Elsevier, Cambridge, Massachussetts. https://doi.org/10.1016/BS.ASB.2017.01.001

Hoffmann, A., Bourgeois, T., Munoz, A., Anton, S., Gevar, J., Dacher, M., & Renou, M. 2020. A plant volatile alters the perception of sex pheromone blend ratios in a moth. *J. Comp. Physiol. A Neuroethol. Sens. Neural Behav. Physiol.* 206: 553–570. https://doi.org/10.1007/s00359-020-01420-y

Hughes, K. A., Houde, A. E., Price, A. C., & Rodd, F. H. 2013. Mating advantage for rare males in wild guppy populations. *Nature* 503: 108–110. https://doi.org/10.1038/nature12717

Huxley, J. 1955. Morphism and evolution. *Heredity* 9(1), 1-52.

Iyengar, V. K., & Eisner, T. 1999. Female choice increases offspring fitness in an arctiid moth (Utetheisa ornatrix). *PNAS.* 96: 15013-15016 https://doi.org/10.1073/pnas.96.26.15013

Iyengar, V. K., Rossini, C., & Eisner, T. 2001. Precopulatory assessment of male quality in an arctiid moth (Utetheisa ornatrix): hydroxydanaidal is the only criterion of choice. *Behav. Ecol. Sociobiol.* 49: 283-288. https://doi.org/10.1007/s002650000292

Jamie, G. A., & Meier, J. I. 2020. The Persistence of Polymorphisms across Species Radiations. *Trends Ecol. Evol.* 35:795–808. https://doi.org/10.1016/j.tree.2020.04.007

Jay, P., Chouteau, M., Whibley, A., Bastide, H., Parrinello, H., Llaurens, V., & Joron, M. 2021. Mutation load at a mimicry supergene sheds new light on the evolution of inversion polymorphisms. *Nat. Genet.* 53: 288–293. https://doi.org/10.1038/s41588-020-00771-1

Jayaweera, A., & Barry, K. L. 2017. Male antenna morphology and its effect on scramble competition in false garden mantids. *Die Naturwissenschaften* 104: 1-9. https://doi.org/10.1007/S00114-017-1494-0

Johansson, B. G., & Jones, T. M. 2007. The role of chemical communication in mate choice. *Biol. Rev.* 82: 265–289. https://doi.org/10.1111/j.1469-185X.2007.00009.x

Johnson, T. L., Elgar, M. A., & Symonds, M. R. E. 2022. Movement and olfactory signals: Sexually dimorphic antennae and female flightlessness in moths. *Front. Ecol. Evol.* 836. https://doi.org/10.3389/fevo.2022.919093

Johnson, T. L., Symonds, M. R. E., & Elgar, M. A. 2017. Anticipatory flexibility: Larval population density in moths determines male investment in antennae, wings and testes. *Proc. Royal Soc. B.* 284: 20172087 https://doi.org/10.1098/rspb.2017.2087

Johnston, S. E., Gratten, J., Berenos, C., Pilkington, J. G., Clutton-Brock, T. H., Pemberton, J. M., & Slate, J. 2013. Life history trade-offs at a single locus maintain sexually selected genetic variation. *Nature* 502: 93–95. https://doi.org/10.1038/nature12489

Karlson, P., & Lüscher, M. 1959. 'Pheromones': a new term for a class of biologically active substances. *Nature.* 183: 55–56.

Kemp, D. J., & Macedonia, J. M. 2007. Male mating bias and its potential reproductive consequence in the butterfly Colias eurytheme. *Behav. Ecol. Sociobiol.* 61: 415-422. https://doi.org/10.1007/s00265-006-0269-y

Koutroumpa, F. A., Groot, A. T., Dekker, T., & Heckel, D. G. 2016. Genetic mapping of male pheromone response in the European corn borer identifies candidate genes regulating neurogenesis. *PNAS.* 113: E6401–E6408. https://doi.org/10.1073/pnas.1610515113

Kromann, S. H., Saveer, A. M., Binyameen, M., Bengtsson, M., Birgersson, G., Hansson, B. S., Schlyter, F., Witzgall, P., Ignell, R., & Becher, P. G. 2015. Concurrent modulation of neuronal and behavioural olfactory responses to sex and host plant cues in a male moth. *Proc. Royal Soc. B.* 282: 20141884 https://doi.org/10.1098/rspb.2014.1884

Krüger, O., Lindström, J., & Amos, W. 2001. Maladaptive mate choice maintained by heterozygote advantage. *Evolution* 55: 1207-1214. https://doi.org/10.1554/0014-3820(2001)055[1207:mmcmbh]2.0.co;2

Küpper, C., Stocks, M., Risse, J. E., dos Remedios, N., Farrell, L. L., McRae, S. B., Morgan, T. C., Karlionova, N., Pinchuk, P., Verkuil, Y. I., Kitaysky, A. S., Wingfield, J. C., Piersma, T., Zeng, K., Slate, J., Blaxter, M., Lank, D. B., & Burke, T. 2015. A supergene determines highly divergent male reproductive morphs in the ruff. *Nat. Genet.* 48: 79–83. https://doi.org/10.1038/ng.3443

Lalouette, L., Pottier, M. A., Wycke, M. A., Boitard, C., Bozzolan, F., Maria, A., Demondion, E., Chertemps, T., Lucas, P., Renault, D., Maibeche, M., & Siaussat, D. 2016. Unexpected effects of sublethal doses of insecticide on the peripheral olfactory response and sexual behavior in a pest insect. *Environ. Sci. Pollut. Res.* 23: 3073–3085. https://doi.org/10.1007/s11356-015-5923-3

LaMunyon, C. W., & Eisner, T. 1993. Postcopulatory Sexual Selection in an Arctiid Moth (Utetheisa ornatrix). *PNAS.* 90: 4689-4692. https://doi.org/10.1073/pnas.90.10.4689

50

Lassance, J. M., Svensson, G. P., Kozlov, M. v., Francke, W., & Löfstedt, C. 2019. Pheromones and Barcoding Delimit Boundaries between Cryptic Species in the Primitive Moth Genus Eriocrania (Lepidoptera: Eriocraniidae). *J. Chem. Ecol.* 45: 429–439. https://doi.org/10.1007/s10886-019-01076-2

Leal, W. S. 2005. Pheromone Reception. In *The chemistry of pheromones and other semiochemicals II* (Shulz, S. ed.) Springer Science & Business Media. 1–36. https://doi.org/10.1007/b98314

Lewontin, R. C., & Kojima, K.-I. 1960. The Evolutionary Dynamics of Complex Polymorphisms. *Evolution* 14: 458-472.

Lim, H., & Greenfield, M. D. 2008. Female arctiid moths, Utetheisa ornatrix, orient towards and join pheromonal choruses. *Anim. Behav.* 75: 673–680. https://doi.org/10.1016/j.anbehav.2007.07.021

Linn, C. E., & Roelofs, W. L. 1983. Effect of varying proportions of the alcohol component on sex pheromone blend discrimination in male Oriental fruit moths. *Physiol. Entomol.* 8: 291–306. https://doi.org/10.1111/j.1365-3032.1983.tb00361.x

Linn, C. E., & Roelofs, W. L. 1985. Response specificity of male pink bollworm moths to different blends and dosages of sex pheromone. *J. Chem. Ecol.* 11: 1583-1590.

Llaurens, V., Whibley, A., & Joron, M. 2017. Genetic architecture and balancing selection: the life and death of differentiated variants. *Mol. Ecol.* 26: 2430–2448. https://doi.org/10.1111/mec.14051

Massey, J. H., Chung, D., Siwanowicz, I., Stern, D. L., & Wittkopp, P. J. 2019. The yellow gene influences drosophila male mating success through sex comb melanization. *ELife* 8: e49388. https://doi.org/10.7554/ELIFE.49388

McKinnon, J. S., & Pierotti, M. E. R. 2010. Colour polymorphism and correlated characters: Genetic mechanisms and evolution. *Mol. Ecol.* 19: 5101–5125. https://doi.org/10.1111/j.1365-294X.2010.04846.x

Mérot, C., Llaurens, V., Normandeau, E., Bernatchez, L., & Wellenreuther, M. 2020. Balancing selection via life-history trade-offs maintains an inversion polymorphism in a seaweed fly. *Nat. Commun.* 11: 1-11. https://doi.org/10.1038/s41467-020-14479-7

Millar, J. G. 2000. Polyene Hydrocarbons and Epoxides: A Second Major Class of Lepidopteran Sex Attractant Pheromones. *Annu. Rev. Entomol.* 45: 575-604.

Mochizuki, F., Fukumoto, T., Noguchi, H., Sugie, H., Morimoto, T., & Ohtani, K. 2002. Resistance to a mating disruptant composed of (Z)-11-tetradecenyl acetate in the smaller tea tortrix, Adoxophyes honmai (Yasuda) (Lepidoptera: Tortricidae). *Appl. Entomol. Zool.* 37: 299–304.

Nokelainen, O., Galarza, J. A., Kirvesoja, J., Suisto, K., & Mappes, J. 2022. Genetic colour variation visible for predators and conspecifics is concealed from humans in a polymorphic moth. *J. Evol. Biol.* 35: 467–478. https://doi.org/10.1111/jeb.13994

Nokelainen, O., Hegna, R. H., Reudler, J. H., Lindstedt, C., & Mappes, J. 2012. Trade-off between warning signal efficacy and mating success in the wood tiger moth. *Proc. Royal Soc. B.* 279: 257–265. https://doi.org/10.1098/rspb.2011.0880

Nokelainen, O., Lindstedt, C., & Mappes, J. 2013. Environment-mediated morph-linked immune and life-history responses in the aposematic wood tiger moth. *J. Anim. Ecol.* 82: 653–662. https://doi.org/10.1111/1365-2656.12037

Nokelainen, O., Valkonen, J., Lindstedt, C., & Mappes, J. 2014. Changes in predator community structure shifts the efficacy of two warning signals in Arctiid moths. *J. Anim. Ecol.* 83: 598-605. https://doi.org/10.1111/1365-2656.12169

Ojala, K., Lindström, L., & Mappes, J. 2007. Life-history constraints and warning signal expression in an arctiid moth. *Funct. Ecol.* 21: 1162–1167. https://doi.org/10.1111/j.1365-2435.2007.01322.x

Papke, R. S., Kemp, D. J., & Rutowski, R. L. 2007. Multimodal signalling: structural ultraviolet reflectance predicts male mating success better than pheromones in the butterfly Colias eurytheme L. (Pieridae). *Anim. Behav.* 73: 47-54. https://doi.org/10.1016/j.anbehav.2006.07.004

Pashley, D. P., Hammond, A. M., & Hardy, T. N. 1992. Reproductive Isolating Mechanisms in Fall Armyworm Host Strains (Lepidoptera: Noctuidae). *Ann. Entomol. Soc. Am*, 85: 400–405. https://academic.oup.com/aesa/article/85/4/400/24551

Pham, H. T., Elgar, M. A., van Lieshout, E., & McNamara, K. B. 2022. Experimental immune challenges reduce the quality of male antennae and female pheromone output. *Sci. Rep.* 12: 1–9. https://doi.org/10.1038/s41598-022-07100-y

Pham, H. T., McNamara, K. B., & Elgar, M. A. 2020. Socially cued anticipatory adjustment of female signalling effort in a moth: Female density-dependent signalling. *Biol. Lett.* 16: 20200614. https://doi.org/10.1098/rsbl.2020.0614

Pham, H. T., McNamara, K. B., & Elgar, M. A. 2021. Age-dependent chemical signalling and its consequences for mate attraction in the gumleaf skeletonizer moth, *Uraba lugens. Anim. Behav.* 173: 207–213. https://doi.org/10.1016/j.anbehav.2020.12.010

Regnier, F. E., & Law, J. H. 1968. Insect pheromones. *J. Lipid Res.* 9: 541-551 https://doi.org/10.1016/S0022-2275(20)42699-9

Roelofs, W. L., & Comeau, A. 1969. Sex Pheromone Specificity: Taxonomic and Evolutionary Aspects in Lepidoptera. *Science* 165: 398-400.

Rojas, B., Burdfield-Steel, E., Pakkanen, H., Suisto, K., Maczka, M., Schulz, S., & Mappes, J. 2017. How to fight multiple enemies: Target-specific chemical defences in an aposematic moth. *Proc. Royal Soc. B* 284: 20171424. https://doi.org/10.1098/rspb.2017.1424

Rojas, B., Mappes, J., & Burdfield-Steel, E. 2019. Multiple modalities in insect warning displays have additive effects against wild avian predators. *Behav. Ecol. Sociobiol.* 73: 1-12. https://doi.org/10.1007/s00265-019-2643-6

Rojas, B., Profile, S., Gordon, S. P., & Mappes, J. 2015. Frequency-dependent flight activity in the polymorphic wood tiger moth, Parasemia plantaginis. *Curr. Zool.* 61: 765-772. https://doi.org/10.1093/czoolo/61.4.765

Rönkä, K., De Pasqual, C., Mappes, J., Gordon, S., & Rojas, B. 2018. Colour alone matters: no predator generalization among morphs of an aposematic moth. *Anim. Behav.* 135: 153–163. https://doi.org/10.1016/j.anbehav.2017.11.015

Rönkä, K., Valkonen, J. K., Nokelainen, O., Rojas, B., Gordon, S., Burdfield-Steel, E., & Mappes, J. 2020. Geographic mosaic of selection by avian predators on hindwing warning colour in a polymorphic aposematic moth. *Ecol. Lett.* 23: 1654–1663. https://doi.org/10.1111/ele.13597

Ruther, J., Matschke, M., Garbe, L. A., & Steiner, S. 2009. Quantity matters: Male sex pheromone signals mate quality in the parasitic wasp Nasonia vitripennis. *Proc. Royal Soc. B.* 276: 3303–3310. https://doi.org/10.1098/rspb.2009.0738

Schöfl, G., Heckel, D. G., & Groot, A. T. 2009. Time-shifted reproductive behaviours among fall armyworm (Noctuidae: Spodoptera frugiperda) host strains: evidence for differing modes of inheritance. *J. Evol. Biol.* 22: 1447–1459. https://doi.org/10.1111/j.1420-9101.2009.01759.x

Shuker, D. M., & Kvarnemo, C. 2021. The definition of sexual selection. *Behav. Ecol.* 32: 781–794. https://doi.org/10.1093/beheco/arab055

Sinervo, B., & Lively, C. M. 1996. The rock-paper-scissors game and the evolution of alternative male strategies. *Nature* 380: 240–243. https://doi.org/10.1038/380240a0

Sinervo, B., & Svensson, E. 2002. Correlational selection and the evolution of genomic architecture. *Heredity* 89: 329–338. https://doi.org/10.1038/SJ.HDY.6800148

Sinervo, B., Svensson, E., & Comendant, T. 2000. Density cycles and an offspring quantity and quality game driven by natural selection. *Nature* 406: 985–988.

Sollai, G., Solari, P., & Crnjar, R. 2018. Olfactory sensitivity to major, intermediate and trace components of sex pheromone in Ceratitis capitata is related to mating and circadian rhythm. *J. Insect Physiol.* 110: 23–33. https://doi.org/10.1016/j.jinsphys.2018.08.007

Steiger, S., & Stökl, J. 2014. The Role of Sexual Selection in the Evolution of Chemical Signals in Insects. *Insects* 5: 423–438. https://doi.org/10.3390/insects5020423

Suomalainen, V. E. (1938). Die erblichkeitverhältnisse des männlichen dimorphismus bei parasemia plantaginis. *Hereditas* 24: 386-390.

Svensson, E., Sinervo, B., & Comendant, T. 2001. Density-dependent competition and selection on immune function in genetic lizard morphs. *PNAS.* 98: 12561–12565. https://doi.org/10.1073/pnas.211071298

Svensson, M. 1996. Sexual selection in moths: the role of chemical communication. *Biol. Rev.* 71: 113-135.

Symonds, M. R. E., & Elgar, M. A. 2008. The evolution of pheromone diversity. *Trends Ecol. Evol.* 23: 220–228. https://doi.org/10.1016/j.tree.2007.11.009

Symonds, M. R. E., Johnson, T. L., & Elgar, M. A. 2012. Pheromone production, male abundance, body size, and the evolution of elaborate antennae in moths. *Ecol. Evol.* 2: 227–246. https://doi.org/10.1002/ECE3.81

Tabata, J., Noguchi, H., Kainoh, Y., Mochizuki, F., & Sugie, H. 2007. Behavioral response to sex pheromone-component blends in the mating disruption-resistant strain of the smaller tea tortrix, Adoxophyes honmai Yasuda (Lepidoptera: Tortricidae), and its mode of inheritance. *Appl. Entomol. Zool.* 42: 675–683. https://doi.org/10.1303/aez.2007.675

Tillman, J. A., Seybold, S. J., Jurenka, R. A., & Blomquist, G. J. 1999. Insect pheromones-an overview of biosynthesis and endocrine regulation. *Insect Biochem. Mol. Biol.* 29: 481-514.

Umbers, K. D. L., Symonds, M. R. E., & Kokko, H. 2015. The mothematics of female pheromone signaling: Strategies for aging virgins. *Am. Nat.* 185: 417–432. https://doi.org/10.1086/679614

van Geffen, K. G., Groot, A. T., van Grunsven, R. H. A., Donners, M., Berendse, F., & Veenendaal, E. M. 2015. Artificial night lighting disrupts sex pheromone in a noctuid moth. *Ecol. Entomol.* 40: 401–408. https://doi.org/10.1111/een.12202

van Schooten, B., Meléndez-Rosa, J., van Belleghem, S. M., Jiggins, C. D., Tan, J. D., Owen McMillan, W., Papa, R., Piedras, R., Juan, S., & Rico, P. 2020. Divergence of chemosensing during the early stages of speciation. *PNAS.* 117: 16438-16447 https://doi.org/10.1073/pnas.1921318117

van Wijk, M., Heath, J., Lievers, R., Schal, C., & Groot, A. T. 2017. Proximity of signallers can maintain sexual signal variation under stabilizing selection. *Sci. Rep.* 7. https://doi.org/10.1038/s41598-017-17327-9

Wellenreuther, M., Svensson, E. I., & Hansson, B. 2014. Sexual selection and genetic colour polymorphisms in animals. *Mol. Ecol.* 23: 5398–5414. https://doi.org/10.1111/mec.12935

Wilson, R., Burnet, B., Eastwood, L., & Connolly, K. 1976. Behavioural pleiotropy of the yellow gene in Drosophila melanogaster. *Genet. Res.* 28: 75–88. https://doi.org/10.1017/S0016672300016748

Winters, A. E., Lommi, J., Kirvesoja, J., Nokelainen, O., & Mappes, J. 2021. Multimodal Aposematic Defenses Through the Predation Sequence. *Front. Ecol. Evol.* 9: 657740. https://doi.org/10.3389/fevo.2021.657740

Wong, B. B. M., & Candolin, U. 2005. How is female mate choice affected by male competition? *Biol. Rev.* 80: 559–571. https://doi.org/10.1017/S1464793105006809

Wright, S. 1982. The shifting balance theory and macroevolution. *Annu. Rev. Genet.* 16: 1-20.

Wyatt, T. D. 2014. Pheromones and animal behavior: chemical signals and signatures. Cambridge University Press.

Xu, H., Zhou, G., Dötterl, S., Schäffler, I., von Arx, M., Röder, G., Degen, T., Chen, L., & Turlings, T. C. J. 2019. The Combined Use of an Attractive and a Repellent Sex Pheromonal Component by a Gregarious Parasitoid. *J. Chem. Ecol.* 45: 559–569. https://doi.org/10.1007/s10886-019-01066-4

Xue, H. J., Wei, J. N., Magalhães, S., Zhang, B., Song, K. Q., Liu, J., Li, W. Z., & Yang, X. K. 2016. Contact pheromones of 2 sympatric beetles are modified by the host plant and affect mate choice. *Behav. Ecol.* 27: 895–902. https://doi.org/10.1093/beheco/arv238

Zakir, A., Khallaf, M. A., Hansson, B. S., Witzgall, P., & Anderson, P. 2017. Herbivore-induced changes in cotton modulates reproductive behavior in the moth Spodoptera littoralis. *Front. Ecol. Evol.* 5: 49. https://doi.org/10.3389/fevo.2017.00049

ORIGINAL PAPERS

I

EVOLUTIONARY IMPORTANCE OF INTRASPECIFIC VARIATION IN SEX PHEROMONES

by

Chiara De Pasqual, Astrid T. Groot, Johanna Mappes & Emily Burdfield-Steel 2021

Trends in Ecology and Evolution 36: 848-859.

https://doi.org/10.1016/j.tree.2021.05.005

Trends in
Ecology & Evolution

CellPress

Evolutionary importance of intraspecific variation in sex pheromones

Chiara De Pasqual,[1,*] Astrid T. Groot,[2] Johanna Mappes,[1,3] and Emily Burdfield-Steel[2]

Sex pheromones in many insect species are important species-recognition signals that attract conspecifics and inhibit attraction between heterospecifics; therefore, sex pheromones have predominantly been considered to evolve due to interactions between species. Recent research, however, is uncovering roles for these signals in mate choice, and that variation within and between populations can be drivers of species evolution. Variation in pheromone communication channels arises from a combination of context-dependent, condition-dependent, or genetic mechanisms in both signalers and receivers. Variation can affect mate choice and thus gene flow between individuals and populations, affecting species' evolution. The complex interactions between intraspecific and interspecific selection forces calls for more integrative studies to understand the evolution of sex pheromone communication.

Sex pheromones and their functions

Pheromones are semiochemicals involved in intraspecific communication, where species-specific signals are released by a sender that modify the behavior of a receiver [1]. Sex pheromones signal attraction and selection of potential mates. Sex pheromones are used by a variety of animal species, but the focus of this Review is on insect sex pheromones, as most pheromone research has been conducted on this class of animals. The traditional view is that sex pheromones are important species recognition signals to distinguish between species and thus under **stabilizing selection** (see Glossary) [2]. As closely related species can have similar sex pheromone signals that may only differ in the ratio of the different chemical constituents, the signaler and the receiver need to be finely tuned to recognize each other [3]. Small changes in pheromone release rate or ratio of the chemical constituents can affect attraction of the receiver [4].

Since sex pheromones are also mate-recognition signals acting within species, their roles in reproductive isolation and speciation processes are important. Reproductive isolation can be shaped by **reproductive character displacement** [5]. In sex pheromone communication channels, reproductive character displacement has been found in closely related, sympatrically occurring taxa [6,7], which lessens communication interference between closely related and **sympatric species**, but can generate variation between populations that lead to divergence [5]. Intraspecific variation between geographically distant populations has been described in many species [8–10], suggesting that such communication interference and other environmental factors affect variation in pheromone communication.

Even though intraspecific variation between geographically isolated populations is generally accepted, variation within populations is still thought to be less common. Johansson and Jones [11], however, reviewed the role of sex pheromones in mate choice, showing the possibility of variation in these signals even within populations. We now know that sex pheromones can even be plastic, and this **plasticity** can be translated into quantitative [12,13] or qualitative [14]

Highlights

Sex pheromones have traditionally been viewed as species recognition signals and thus are expected to show little within-species variation in composition and amount. Current research, however, reveals a high degree of intraspecific variation in sex pheromone communication channels, suggesting they are molded by multiple selection forces.

Sex pheromone signaling can be costly and plastic, and signals as well as responses may be affected not only by between-species interactions (e.g., communication interference, host plants, and geographic variation), but also by within-species interactions (mate choice) in addition to the genetic architecture, physiological state, and previous experience of individuals.

As variation in sex pheromone communication can be induced by intrinsic factors, its evolution may not only follow ecological speciation, but also be the driver of divergence.

[1]Department of Biological and Environmental Science, University of Jyväskylä, P.O. Box 35, FI-40014, Jyväskylä, Finland
[2]Institute of Biodiversity and Ecosystem Dynamics (IBED), University of Amsterdam, P.O. Box 94240, Amsterdam 1098 XH, The Netherlands
[3]Organismal and Evolutionary Biology Research Program, University of Helsinki, P.O. Box 4, FI-00014 Helsinki, Finland

*Correspondence:
chiara.depasqual@jyu.fi (C. De Pasqual).

intrapopulational differences. This can take place over an individual's lifespan [12,15], as a consequence of seasonal development [13], or due to the vicinity of heterospecifics during development [14]. Since sex pheromones can provide information on the general condition of the signaler, and the receiver can select based on this variation, intraspecific variation in sex pheromones must be more common than previously assumed.

Why has intraspecific variation been overlooked?

Although chemical communication is considered to be the oldest form of communication [16], humans are mostly visually oriented and thus much of the research focused on sexual signaling has been on variation in visual signaling. Also, it has proven challenging to analyze individual-level variation in chemical extracts. For example, identification of the first moth sex pheromone required thousands of individuals pooled together [17], naturally obscuring any intrapopulation variation. In addition, research on sexual selection and sexual conflict theory is affected by biases of sex roles and assignment to different taxa in sexual selection research [18]. In moths, females are typically thought to be the signalers and release the sex pheromone, while males are the responding sex, which could explain the higher volume of literature on female sex pheromone variation in moths [19–22]. Males, however, also produce and release sex pheromone [23] to which females may respond, but this aspect has been little explored (Box 1). Finally, female-signaling insect systems are well-represented amongst pest species, making them economically important. Sex pheromones are commonly used to monitor the presence and abundance of these pests and to disrupt mating. These applied aspects of insect chemical ecology likely increased the focus on sex pheromones at the species level. Thus, it is possible that, to date, our understanding of sex pheromone evolution, and in particular how intraspecific variation can affect a single species, has been held back by our research focus.

Mechanisms underlying intraspecific sex pheromone variation

Both sender and receiver are involved in the process of mate attraction, and intraspecific variation in pheromone communication can either arise as a consequence of physiological variation in the signaler (sender-specific driver of variation) or the receiver (receiver-specific driver of variation). To

Box 1. Visual versus chemical signals

In butterflies, mate attraction is typically determined by visual cues, while sex pheromones are usually involved in short-range attraction. Current research is uncovering many aspects of sex pheromone roles in butterflies, suggesting that chemical signals are much more involved than previously expected in diurnal species that commonly rely on visual cues. *Heliconius* butterflies (see Figure 1C,D in main text) are well known for exhibiting Müllerian mimicry, in which unrelated and chemically defended species mimic each other warning signal to more efficiently advertise their unpalatability to predators. When two species share identical wing pattern, such as *Heliconius timareta* and *Heliconius melpomene* in Peru, chemical signals are important to discriminate against heterospecifics [103]. Sex pheromones have a great importance in conspecific recognition, act as premating barriers and affect mate choice [104]. As male pheromones and female preference have been found to be heritable at least in some *Heliconius* butterfly species [105], pheromone communication may play a role in their speciation process. Thus, even in the visually-orientated butterflies, pheromones and other chemical signals, are used as premating barriers in addition to wing patterns, making these species multimodal signalers [106].

In *Bicyclus anynana*, the male sex pheromone can provide a wealth of information on the signaler, from the male's age to individual identity [100]. This pheromone is also a plastic trait as a consequence of a more or less climatic favorable season for their reproductive success [38], which has implications in their mating success [13]. Their sex pheromone can be more variable within populations than between populations and possibly acts as precursors of an ongoing speciation process [8].

In both *Heliconius* and *Bicyclus* study systems, short-range chemical signals are thus involved in species recognition and therefore under natural selection, but have also been shown to act in intraspecific mate assessment and thus under sexual selection. These examples show that in a male-signaling, female responding system, the progress of our understanding of the roles of pheromone signals seems to run counter to that in the more commonly studied females-signaling systems.

Glossary

Assortative attraction or assortative mating: individuals with similar phenotypes are attracted and mate with one another more frequently than expected under random mating.

Cuticular hydrocarbons (CHCs): molecules on the surface of adult insects, generally serving as antidesiccation compounds. In a number of species, CHCs have also be found to play important roles in insect communication. One of these roles is attraction and selection of potential mates.

Ecological speciation: a form of speciation that arises as a consequence of reproductive isolation due to a change in ecological factors (e.g., change in the host plant, presence of predators or parasites and, in general, any environmental factor).

Genetic correlation: two or more sets of genetic loci expected to covary as a consequence of, for example, linkage disequilibrium or pleiotropy.

Mating disruption: a pest management technique which prevent individuals to successfully locate potential mates through the release of a synthetic sex pheromone that mimics the species sex pheromone.

Plasticity: the phenomenon for which organisms with the same genotype manifest alternative phenotypes in response to environmental conditions, experienced usually during developmental time.

Preference function: along a continuous range of a signaling trait values, most receivers express a preference for intermediate values, while few show preference towards extreme values.

Reproductive character displacement: selection to avoid hybridization between species. When two species have overlapping habitats and (signaling) traits, divergence in one or more traits evolves in response to unfavorable interactions, such as cross-species attraction. When the divergence is due to selection to avoid hybridization between races/strains within species, the term reinforcement is used.

Runaway selection: due to genetic correlation between the sender and receiver's trait, changes in the sender's signal are inherited together with changes for the preference for that signal. This leads to a positive feedback loop, whereby both signal and

add another layer of complexity, both the sender and the receiver experience physiological changes within their lifetime, adding within-individual variation to the between-individual variation at the population level. In addition, senders may become receivers and vice versa.

Sender's perspective

Diet, age, mating status, and vicinity to conspecifics are among the mechanisms that can produce physiological changes and contribute to the maintenance of intraspecific variation in pheromone production. These changes can lead to both within- and between-individual variation. When considering intraspecific sex pheromone variation, it is important to first consider how they are produced. In some species, pheromone components and precursors are sequestered from the diet, while in others they are produced *de novo* within the animal [24]. Although both forms of pheromone production rely on the resources available, pheromones derived from the diet will be more vulnerable to environmental and host-related effects, and thus to external conditions. In contrast, species that produce their pheromones *de novo* are probably less susceptible to externally induced variation, but may exhibit variation due to internal processes. This is especially likely if there are costs to pheromone production, as this could result in trade-offs with other processes. Sex pheromones can indeed be costly: nutritional state influences the amount of pheromone produced in cockroaches [12], smaller female moths have lower fitness when they are stimulated into pheromone signaling [25] and pheromone composition predicts the fitness of female moths [26]. The **signaling sex** may communicate its nutritional state to the receiver [27]. There is also ample evidence that sex pheromones are affected by suboptimal diet at the larval stage [15,28], in adults [12], or at both life stages [15]. Pheromone production can be recouped if a higher nutritional diet is obtained [12], resulting in a dynamic environment–individual interaction. Regardless of how sex pheromones are produced, their variation can also be a consequence of the internal physiological state and its changes, such as mating status [29,30] or aging [31,32]. Generally, a greater investment in pheromone signaling is expected in older unmated females [33]. Older females may also call earlier in the night to avoid competition with more attractive young females [34].

Other mechanisms that can affect pheromone production and release are pathogens [19], volatiles from host plants [31], conspecifics [35,36], or prey [37]. Verheggen *et al.* [37] recently found that pheromone production in the Asian lady beetles (*Harmonia axyridis*) (Figure 1A) is conditioned by the presence of prey, as exposure to volatiles of the prey initiated pheromone production. Also, the female calling behavior of the gregarious beet webworm moth (*Loxostege sticticalis*) is affected by the vicinity to male conspecifics [38]. Pheromone release can also be increased by crowding and sexual competition; for example, in the American grasshopper (*Schistocerca americana*) [35]. It remains unclear if any of these changes represent adaptive strategies, that is, changes in resource allocation depending on individual status, or are simply nonadaptive responses to environmental triggers. Examples that do suggest a level of adaptive response to the environment are developmental plasticity due to the chemical environment, when individuals have been exposed to the chemical signals of heterospecifics [14], as a consequence of environmental temperature [39], seasonal variation [13], or nocturnal light pollution [40].

Finally, genetic and behavioral mechanisms may contribute to within-population pheromone variation. For example, a point mutation identified in the tobacco budworm (*Heliothis virescens*) (Figure 1B) translates to a sex pheromone signal with a higher or lower ratio of two pheromone components [41]. The different ratios of the two components affect the signal's attractiveness to males, and females releasing a signal with a higher ratio of the two components are less attractive than females releasing pheromone signal with a lower ratio [36]. Unattractive females can obtain matings while in close proximity to attractive females and, therefore, unattractive

preference for the signal become exaggerated over time.

Saltational event: evolutionary event which generates greatly or completely different phenotypes in a few generations, due to a mutation with major phenotypic effect. For example, a mutation in an enzyme that changes the stereochemistry of a pheromone component.

Signaling sex: the sex releasing a sexual communication signal to attract a potential mate.

Stabilizing selection: a form of selection in which the population mean trait converges to intermediate values, for example, when the mean pheromone signal is preferred over signals deviating from the mean.

Sympatric species: species with overlapping geographic ranges that are close enough to regularly interact.
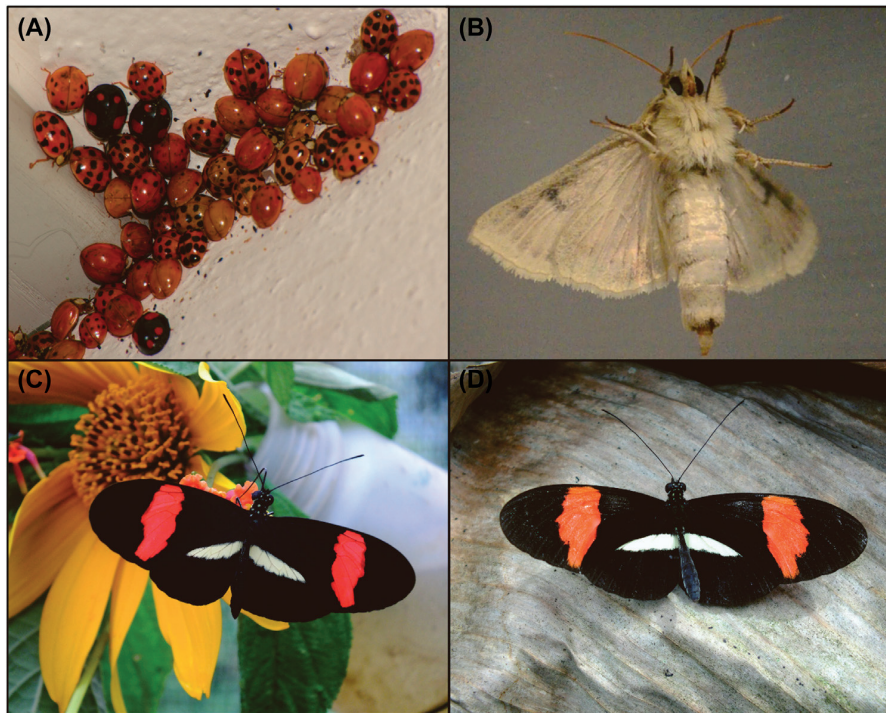
Trends in Ecology & Evolution

Figure 1. Examples of some species for which intraspecific variation in sex pheromone has been described in the literature. (A) Asian lady beetle (*Harmonia axyridis*) (Photo: Chiara De Pasqual). (B) Tobacco budworm (*Heliothis virescens*) (Photo: Jan van Arkel/IBED/UvA). (C) *Heliconius erato* and (D) *Heliconius melpomene* (Photo: Melanie Brien).

females are maintained in the population [36]. Similarly, in the European corn borer (*Ostrinia nubilalis*), two genetically identified sex pheromone strains [42] are maintained because males assortatively mate with females of their own pheromone strain [43].

Receiver's perspective

The traditional view holds that signalers and receivers are finely tuned, where the predicted shape of the receiver's **preference function** corresponds to the distribution of the female pheromone signal in the population. Experimental evidence from the 1970s and 1980s suggested that female pheromone signals experience stabilizing selection [3] and there is a lack of variation in male responses, because of optimal tuning to specific component ratios [2,4]. Variation in either the signaler or receiver, however, can introduce novelty in the communication channel even in established sender–receiver systems. For example, the rare receivers in the population that track changes in the pheromone signal [44], which might be possible through a **saltational event** [45]. New compounds in the signal may remain unperceived initially and only later do receivers evolve the preference for the new signal [46]. This scenario is known as the asymmetric tracking hypothesis [47]. An alternative and new conceptual model proposes that the preference trait evolves first in the receiver as a veiled preference, before the preferred trait is evolved in the signaler, and the receiver starts to select individuals with the newly preferred trait as soon as the trait arises [48].

Variation in receivers can take place at different levels, such as between and within individuals, and at different life stages, as a consequence of plasticity in the olfactory system [49]. In some

species, population density can contribute to variation in the morphological structures of the receiver as a consequence of developmental plasticity. For example, gum-leaf skeletonizer male moths (*Uraba lugens*) develop longer and more elaborate antennae to locate females in sparse populations [50]. This plasticity depends on the population density during the developmental stage and suggests that individuals can be more or less sensitive to pheromone signals.

Within the lifespan of an individual, plasticity in behavioral responses is well demonstrated in relation to circadian rhythms, as males of many moth species actively respond to pheromone sources at restricted times in the photoperiod [51]. A difference in gene expression in odor receptors seems to be at the base of this behavioral plasticity, linked to both the physiological state [52] and circadian rhythms [53] with, for example, effects on antennal sensitivity [54]. Responses to sex pheromones can also be modulated by experience, where pre-exposure to sex pheromones increases long-term sensibility and responsiveness [55,56]. The olfactory system dynamically adjusts to optimally perceive the surroundings [57] and differential receptor expression characterizes, for example, mated and unmated individuals [58]. After matings, some insects become less sensitive to sexual signals and cues for mating sites [59], either through a reduction in antenna sensitivity [54] or differences in pheromone processing in the central nervous system in the brain [60]. In other instances, environmental stress can have an effect on the receiver olfactory system. Sublethal pesticide doses was shown to increase the peripheral sensitivity of cotton leafworm (*Spodoptera littoralis*) males and increase their mating success [61]. A recent review [49] reports that the plasticity of the olfactory system occurs at all levels of the olfactory pathway. When studying the insect sensory system, it is challenging to integrate information from the periphery to the brain, therefore, we have just begun to understand these interactions. As the olfactory system can plastically respond to, for example, changes in the environment, physiological state, social interactions and experience, variation in the olfactory system among individuals is likely to lead to differences in sensitivity and responses.

Ecological consequences of intraspecific variation: effect on attractiveness and mating success

Intraspecific variation in sex pheromones can have important consequences in attractiveness, mating success, and mating behavior, even when the source of the variation is entirely environmental. Furthermore, these consequences can be sex specific.

Volatiles produced by host plants can affect the production and response to sex pheromones. This can in turn affect mate location by increasing the calling behavior of signalers, for example by augmenting the frequency and calling duration [62], or increasing pheromone release/production [63]. Alternatively, host plant volatiles can enhance receivers' attraction to pheromone signals [62,64], by attracting a higher number of individuals [62,64] or by reducing the time to locate the pheromone source [64]. Finally, host plant volatiles can alter the response to ratios of pheromone components [65]. Thus, in general, host plants can influence species reproductive success during different stages of the insect lifespan, through both resource acquisition at the larval stage, and the interaction of plant volatiles with adult signals and behavior. Oxidative stress and pathogen infections can also affect sex-pheromone based mate location [66,67]. Curiously, challenged immune systems and pathogen infections have been found to increase rather than decrease attraction in some insects, suggesting either terminal investment [67] or a strategy of pathogens to spread through populations via host manipulation [19]. Immune system challenges can also lead to sex-specific differences; for example, only female mating success might be affected [68]. As populations vary in parasite load [69], variation in immune challenge could thus impact the dynamics of both pheromone signaling and sexual selection. In addition to host plants and pathogens, sex pheromone production and mating success can be affected by seasonal

fluctuations. A good example is the squinting bush brown butterflies (*Bicyclus anynana*), in which males that emerge in the wet season produce more pheromone and have higher mating success than those that emerge in the dry season [13].

Mate assessment is based on intraspecific variation in sexually selected traits. Sex pheromones can be under sexual selection when used as indicators of mate quality. Variation in sex phero-mone composition can be used to avoid mating with siblings [70], which increases genetic variability and decreases the impact of deleterious alleles. For example, in *B. anynana*, females avoid mating with inbred males, which is assessed solely on the male-produced sex pheromone [71]. Sex pheromones are also used as signals to avoid matings that would lead to a lowered fitness and thus reduce the cost of matings. For example, sexually immature females of the cotton bollworm (*Helicoverpa armigera*) inhibit male attraction by temporarily releasing a repellent component in their pheromone signal [72]. Another example is the haplodiploid parasitic wasp, *Nasonia vitripennis*, in which females are more attracted to males with more pheromone, which correlates to a full sperm load [73]. As unfertilized eggs develop into males, this selection thus affects the population sex-ratio.

Sexual conflicts often arise over mating frequency. For example, in the parasitoid wasp families Pteromalidae and Braconidae, females tend to mate only once [30,74], while males mate multiple times. After mating, *Spalangia endius* (Pteromalidae) females actively resist males by increasing the release of a specific pheromone component, which prevents or terminates male courtship behavior [74]. Mated *Cotesia glomerata* (Braconidae) females stop releasing their attractive pheromone component in favor of the repellent only. These changes likely help mated females to avoid male harassment by reducing the attraction of males, and are also thought to help males locate virgin females, as their pheromone is distinct from mated females [30].

To conclude, intraspecific sex pheromone variation can have important consequences for the attractiveness and mating success of individuals, regardless of the source of this variation. If variation in signals and responses is heritable, there is the potential for different investment or responsive strategies to evolve.

Evolutionary consequences of intraspecific sex pheromone variation

Evolution in sexual communication systems can occur when heritable differences in signals and/or responses increase the survival and reproductive output of the individual (Figure 2 and Table 1). Below, we explore whether and how such evolution can occur in response to **ecological speciation** or as a driver of population divergence.

Ecological speciation

Many herbivore species use chemicals from their host plants as precursors for compounds that make up the pheromone signal. Thus, host plant shifts can directly affect pheromone composition. If this is followed by changes in responses, leading to **assortative mating** and reproductive isolation, ecological speciation can occur [75]. Changes in mating signals following a host plant shift has been experimentally shown in the mustard beetle (*Phaedon cochleariae*) [76], where **cuticular hydrocarbons** used as mate recognition cues differ depending on the host plant on which they feed. Similar changes are hypothesized to have contributed to the speciation process between two parental species of flea beetles (*Altica fragaria* and *Altica viridicyanea*) [77]. Pheromone divergence between populations linked to differences in host plants has also been hypothesized in the pine and larch strain of larch budmoth (*Zeiraphera diniana*) [78] and the chest-nut tortrix (*Cydia splendana*) [79], and may represent the initial step towards reproductive isolation in these species. Changes in host plants, however, do not automatically lead to differences in sex
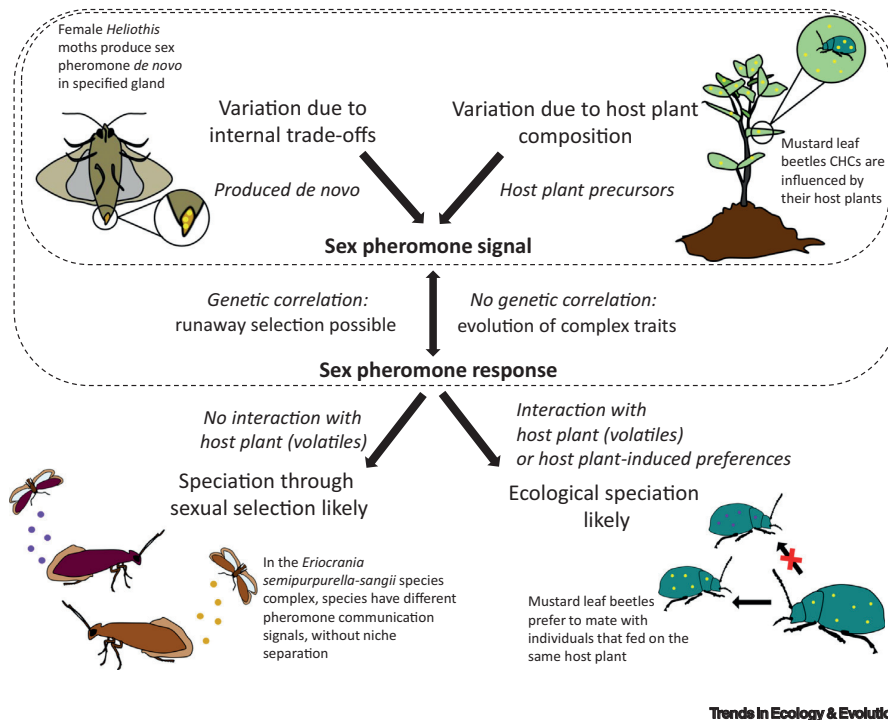
Figure 2. Some possible routes to speciation through variation in pheromone production and perception in herbivorous insects. Arrows indicate two of the possible alternative routes in the evolution of sex pheromone signals and responses. Pictures indicate examples discussed in this paper, clockwise from top left: *Heliothis* spp. [24]; mustard leaf beetle (*Phaedon cochleariae*) [76]; and *Eriocrania semipurpurella–sangii* [84].

pheromones [80] or to host shift [81], suggesting that other processes are also involved in speciation events.

Plants damaged by conspecific herbivores can negatively affect the premating and mating behavior in the moth species *S. littoralis* [82]. Host plant volatiles released as a result of herbivory can decrease the calling behavior of the signaler and negatively impact the mating success. This interaction may result in an avoidance of egg laying on a suboptimal plant that has high competition for resources or is attractive to the enemies of the herbivore [82]. Thus, interactions between host plants and pheromone signals and/or responses are complex and not straightforward, and how these interactions are involved in ecological speciation likely depends on the plants and insects involved.

Pheromone differentiation as driver of speciation

Reproductive isolation can also take place without ecological differentiation, through divergence in mate preference [83]. For example, in the primitive moth *Eriocrania semipurpurella–sangii* species complex, the ratios of components in the female pheromone do not only reflect geographic differences between populations, but also identify different species [84]. Since all the species share the same host plant, this is a case in which reproductive isolation likely has evolved as a result of divergence in sexual communication [84]. Such divergence could happen if some individuals of the choosing sex have a preference for a specific pheromone signal and

Table 1. Examples of intraspecific sex pheromone variation for which causes and/or consequences of the variation has been described and genetic basis or heritability is known

Species name	Level of variation[a]		Cause of variation	Consequences of the variation	Genetic basis known or heritability calculated	Refs
	Quantitative[b]	Qualitative[c]				
Tobacco budworm (*Heliothis virescens*)	—[d]	Yes	Single point mutation	Female attractiveness and mating success affected	Genetic basis known	[21,36,41]
	Yes	Yes	Pathogen infection boosted immune system	Sex specific: females mating success affected		[68]
Subflexus straw (*Heliothis subflexa*)	—	Yes	Phenotypic plasticity	Increase in **assortative attraction**	Genetic basis known	[14,100]
Squinting bush brown (*Bicyclus anynana*)	Yes	—	Seasonal polyphenism or age	Mating success affected	Heritability known	[13,101]
Parasitic wasp (*Nasonia vitripennis*)	Yes	—	Sperm limitation (highly correlated with pheromone titer)	Attractiveness and mating success affected	Genetic basis known	[73]
Burying beetle (*Nicrophorus vespilloides*)	Yes	Yes	Diet or age, or parasite load	Male attractiveness affected	Unknown	[102,103]

[a]Variation in sex pheromones can take two forms: quantitative and qualitative.
[b]Quantitative when the variation is referred to the total amount of the components.
[c]Qualitative when the variation is referred to the relative amounts and/or ratio of the components.
[d]Refers to not tested or not reported.

choose those signalers. A good example comes from *B. anynana*, in which females developed a mating bias towards the pheromone signal they were exposed to, even when this was a new signal. As this mating bias can be transmitted to the next generation without the offspring being exposed to the new pheromone signal [85], such heritable learning can drive the evolution of assortative mating and speciation. The potential of sex pheromones as drivers of speciation has been hypothesized for some *B. anynana* populations in which sex pheromone differentiation was found to be higher than genetic differentiation, suggesting that pheromone divergence precedes genetic divergence [8] (Box 2).

Potential mechanisms that can introduce variation and novelty in sex pheromones lie in the sequence variability of the genes involved in determining component ratios in pheromone signals [86], such as gene duplication and amino acid substitution in genes coding for specific enzymes in the pheromone pathways [87]. Variation in response and preference for pheromone signals may be due to divergence and evolution in olfactory receptors [88]. For example, chemosensory divergence in odorant receptors in the peripheral nervous system associated with reproductive isolation, have been found in two rarely hybridizing *Heliconius* species [58]. In *O. nubilalis*, however, genetic differences in the central nervous system (i.e., neurogenesis), instead of the

Box 2. Ecological and evolutionary consequences in the burying beetle (*Nicrophorus vespilloides*)

A comprehensive example of intraspecific sex pheromone variation that merges ecological and evolutionary consequences of its variation comes from burying beetles (*Nicrophorus vespilloides*). Intraspecific differences in sex pheromone have been shown to reflect males' condition, with males being more attractive when they are in better nutritional condition, of older age, have a larger body size and bear less parasites [101]. Burying beetles start to attract females once they have found a carcass suitable for reproduction, by releasing a sex pheromone. Both parents are known to feed on the carcass, so it is beneficial to both the parents and the offspring to mate only after a carcass has been found. Interestingly, males that have already performed brood care produce and release a higher amount of sex pheromone, and attract more females. Because of this positive loop, in which individuals that perform parental care produce more sex pheromone and become more attractive to females, the interplay between food source and heightened expression of secondary sexual trait (sex pheromone) has been suggested to have contributed to the evolution of parental care [102].

odorant receptors, were recently found to be primarily responsible for the differential male response [89]. The discovery of the genetic architecture underlying variation in sexual signals and responses has just recently begun, and so far mostly focused on Lepidoptera [90]. Recent studies suggest that phenotypic variation in sex pheromones can be the result of single gene [42] or locus [91] variation or due to a number of loci [92]. In the moths species studied so far, the genomic regions involved in signaling and response are unlinked. This makes **runaway selection** unlikely and the lack of **genetic correlation** favors the evolution of complex traits [90]. Thus, how phenotypic variation in pheromone communication may lead to reproductive isolation and, ultimately, to speciation is still an evolutionary mystery.

How easily can pheromone communication channels evolve?

As insect pheromones are used for pest management tactics, we have some knowledge on the evolution of sex pheromones in response to anthropogenically induced selection, which shows that pheromone communication channels can evolve relatively rapidly. Techniques such as pheromone trapping and **mating disruption** are attractive alternatives to pesticides, but the continuous use of artificial sex pheromones as disruptants may exert selective pressure on pest species to change their sexual signals and evolve resistance to it [93], meaning that males are no longer disrupted by the artificial pheromone composition. Cases of such emerging resistance can be viewed as natural field experiments showing that sexual communication systems can sometimes evolve quickly. The first field case of male resistance to pheromone-based traps was reported in Japan, where males of the smaller tea tortrix (*Adoxophyes honmai*) stopped responding to synthetic lures after about 10 years of treatment, causing the efficacy of mating disruption to drop from >96% to <50% [94]. The strong selective pressure exerted on this species resulted in the evolution of a resistant population, in which females changed their pheromone composition and males broadened their pheromone response [95,96]. This is unlikely to be an isolated case, as intraspecific variation observed in the cosmopolitan pest, codling moth, *Cydia pomonella*, shows the same potential for a shift in female sexual signal in response to mating disruption [20].

The continuous use of artificial pheromones can have an effect on both signalers and receivers. The signaling sex may evolve a different pheromone signal [95,97] and/or modify their behavior, by releasing pheromone for a longer time [98]. Pre-exposure to sex pheromones can affect gene expression of chemosensory genes and olfactory sensitivity [53] and result either in enhanced [55,56] or reduced [99] receiver sensitivity. All these factors can contribute to the evolution of resistance to artificial pheromones, and suggest that signaler and receiver can evolve and change in short time periods. Such quick evolutionary responses to selection indicate that there is a high level of standing genetic variation in populations on which selection can act.

Concluding remarks and future perspective

There is an increasing awareness that intraspecific variation in sex pheromone communication channels, both in the signaler and the receiver, is more common than previously assumed. There is also an increasing number of studies that point to the existence of within-population variation, as these signals are used for mate assessment and choice. Thus, sex pheromone signals and responses can be under multiple selection forces (see Outstanding questions) and seem able to evolve relatively quickly. This is particularly well represented by the fact that wild populations have been found to evolve resistance to synthetic sex pheromone composition, which suggests a high level of standing genetic variation within species on which selection can act. Finally, it is important to realize that individuals in nature can be affected simultaneously by several external factors (e.g., pathogens, vicinity to conspecifics, and predators), which can trade off with the physiology of the individual, so that populations always show some degree of

Outstanding questions

How do interspecific and intraspecific selection forces interact and cause population divergence? Individuals often are under multiple selective pressures resulting from abiotic and biotic sources, which may act in opposite, similar or complex ways.

Is only one sex the signaler and the other sex the receiver, or is there mutual mate choice? As signalers may become receivers in different phases of mate assessment, it is important to determine which signals are being used by which sex at the different phases, and how these signals may interact or evolve separately.

How much of the intraspecific variation is determined by genetic factors and how much plasticity occurs in pheromone signals and responses? Since both sex pheromone signals and responses have been found to be plastic, and plasticity can accelerate as well as impede evolution, we need to gain more knowledge on the fitness consequences and heritability of this plasticity.

When using artificial pheromone blends for mating disruption, could resistance development be delayed by using variable pheromone lures? Knowledge on the level of intraspecific variation in the pheromone signal may be used to develop lures with different pheromone blends to reduce evolution of resistance to pheromone artificial traps.

variation. We therefore need to deepen our understanding on how variation in pheromone communication channels affects and is affected by the interaction between the internal state of signalers and receivers, their mutual mate choice and their environment. We also need a better understanding of the mechanisms behind this variation, whether these are genetic or plastic, and which molecular pathways are involved. With all this information it will be possible to move the field forward and better understand how the evolution of chemical communication between the sexes affects speciation.

Declaration of interests

The authors declare no conflict of interests.

References

1. Karlson, P. and Lüscher, M. (1959) 'Pheromones': a new term for a class of biologically active substances. *Nature* 183, 55–56
2. Linn, C.E. and Roelofs, W.L. (1985) Response specificity of male pink bollworm moths to different blends and dosages of sex pheromone. *J. Chem. Ecol.* 11, 1583–1590
3. Li, C.E., Jr and , C.E., Jr and Roelofs, W.L. (1983) Effect of varying proportions of the alcohol component on sex phero-mone blend discrimination in male oriental fruit moths. *Physiol. Entomol.* 8, 291–306
4. Baker, T.C. *et al.* (1981) Sex pheromone dosage and blend specificity of response by oriental fruit moth males. *Entomol. Exp. Appl.* 30, 269–279
5. Pfennig, K. and Pfennig, D. (2009) Character displacement: ecological and reproductive responses to a common evolu-tionary problem. *Q. Rev. Biol.* 84, 253–276
6. Yang, C.Y. *et al.* (2015) Sex pheromones and reproductive isolation in five mirid species. *PLoS One* 10, e0127051
7. Meier, L.R. *et al.* (2016) Synergism between enantiomers creates species-specific pheromone blends and minimizes cross-attraction for two species of cerambycid beetles. *J. Chem. Ecol.* 42, 1181–1192
8. Bacquet, P.M. *et al.* (2016) Differentiation in putative male sex pheromone components across and within populations of the African butterfly *Bicyclus anynana* as a potential driver of repro-ductive isolation. *Ecol. Evol.* 6, 6064–6084
9. Darragh, K. *et al.* (2020) Species specificity and intraspecific variation in the chemical profiles of *Heliconius* butterflies across a large geographic range. *Ecol. Evol.* 10, 3895–3918
10. Gao, K. *et al.* (2020) Geographic variation in sexual communi-cation in the cotton bollworm, *Helicoverpa armigera*. *Pest Manag. Sci.* 76, 3596–3605
11. Johansson, B.G. and Jones, T.M. (2007) The role of chemical communication in mate choice. *Biol. Rev.* 82, 265–289
12. Jensen, K. *et al.* (2017) Change in sex pheromone expression by nutritional shift in male cockroaches. *Behav. Ecol.* 28, 1393–1401
13. Balmer, A.J. *et al.* (2018) Developmental plasticity for male sec-ondary sexual traits in a group of polyphenic tropical butterflies. *Oikos* 127, 1812–1821
14. Groot, A.T. *et al.* (2010) Phenotypic plasticity in sexual commu-nication signal of a noctuid moth. *J. Evol. Biol.* 23, 2731–2738
15. Schultzhaus, J.N. *et al.* (2018) High fat diet alters *Drosophila melanogaster* sexual behavior and traits: decreased attractiveness and changes in pheromone profiles. *Sci. Rep.* 8, 1–13
16. Wyatt (2014) Animals in a chemical world. In *Pheromones and Animal Behavior: Chemical Signals and Signatures* (2nd edn), pp. 1–48, Cambridge University Press
17. Butenandt, V.A. (1959) Uber den sexsual-lockstoff des seidenspinners *Bombyx mori*. *Reindarstellung und konstitution*. *Z. Naturforschg, b* 14, 283
18. Green, K.K. and Madjidian, J.A. (2011) Active males, reactive females: stereotypic sex roles in sexual conflict research? *Anim. Behav.* 81, 901–907
19. Burand, J.P. *et al.* (2005) Infection with the insect virus Hz-2v alters mating behavior and pheromone production in female *Helicoverpa zea* moths. *J. Insect Sci.* 5, 6
20. Duménil, C. *et al.* (2014) Intraspecific variation in female sex pheromone of the codling moth *Cydia pomonella*. *Insects* 5, 705–721
21. Groot, A.T. *et al.* (2014) Within-population variability in a moth sex pheromone blend: genetic basis and behavioural consequences. *Proc. R. Soc. Lond. B* 281, 20133054
22. Cruz-Esteban, S. *et al.* (2018) Geographic variation in phero-mone component ratio and antennal responses, but not in attraction, to sex pheromones among fall armyworm populations infesting corn in Mexico. *J. Pest. Sci.* 91, 973–983
23. Birch, M.C. *et al.* (1990) Scents and eversible scent structures of male moths. *Annu. Rev. Entomol.* 35, 25–54
24. Foster, S.P. and Anderson, K.G. (2015) Sex pheromones in mate assessment: analysis of nutrient cost of sex pheromone production by females of the moth *Heliothis virescens*. *J. Exp. Biol.* 218, 1252–1258
25. Harari, A.R. *et al.* (2011) Fitness cost of pheromone production in signaling female moths. *Evolution* 65, 1572–1582
26. Blankers, T. *et al.* (2021) Sex pheromone signal and stability covary with fitness. *R. Soc. Open Sci.* In press
27. Foster, S.P. and Johnson, C.P. (2011) Signal honesty through differential quantity in the female-produced sex pheromone of the moth *Heliothis virescens*. *J. Chem. Ecol.* 37, 717–723
28. Darragh, K. *et al.* (2019) Male pheromone composition de-pends on larval but not adult diet in *Heliconius melpomene*. *Ecol. Entomol.* 44, 397–405
29. Lu, Q. *et al.* (2017) Sex pheromone titre in the glands of *Spodoptera litura* females: circadian rhythm and the effects of age and mating. *Physiol. Entomol.* 42, 156–162
30. Xu, H. *et al.* (2019) The combined use of an attractive and a repellent sex pheromonal component by a gregarious parasitoid. *J. Chem. Ecol.* 45, 559–569
31. Domínguez, A. *et al.* (2019) Influence of age, host plant and mating status in pheromone production and new insights on perception plasticity in *Tuta Absoluta*. *Insects* 10, 256
32. Kartika, T. *et al.* (2021) Influence of age and mating status on pheromone production in a powderpost beetle *Lyctus africanus* (Coleoptera: Lyctinae). *Insects* 12, 8
33. Umbers, K.D. *et al.* (2015) The mothematics of female phero-mone signaling: strategies for aging virgins. *Am. Nat.* 185, 417–432
34. Pham, H.T. *et al.* (2021) Age-dependent chemical signalling and its consequences for mate attraction in the gumleaf skele-tonizer moth, *Uraba lugens*. *Anim. Behav.* 173, 207–213

35. Stahr, C. *et al.* (2013) Chemical identification, emission pattern and function of male-specific pheromones released by a rarely swarming locust, *Schistocerca americana*. *J. Chem. Ecol.* 39, 15–27

36. van Wijk, M. *et al.* (2017) Proximity of signallers can maintain sexual signal variation under stabilizing selection. *Sci. Rep.* 7, 1–10

37. Verheggen, F. *et al.* (2020) The production of sex pheromone in lady beetles is conditioned by presence of aphids and not by mating status. *J. Chem. Ecol.* 46, 590–596

38. Xiao, Y. *et al.* (2020) Male ventroposterior brush display increases the sexual receptivity of females in the gregarious beet webworm *Loxostege sticticalis* (Lepidoptera: Crambidae). *J. Insect Behav.* 33, 184–192

39. Dion, E. *et al.* (2016) Phenotypic plasticity in sex pheromone production in *Bicyclus anynana* butterflies. *Sci. Rep.* 6, 1–13

40. Van Geffen, K.G. *et al.* (2015) Artificial night lighting disrupts sex pheromone in a noctuid moth. *Ecol. Entomol.* 40, 401–408

41. Groot, A.T. *et al.* (2019) Within-population variability in a moth sex pheromone blend, part 2: selection towards fixation. *R. Soc. Open Sci.* 6, 182050

42. Lassance, J.M. *et al.* (2010) Allelic variation in a fatty-acyl reductase gene causes divergence in moth sex pheromones. *Nature* 466, 486–489

43. Malausa, T. *et al.* (2005) Assortative mating in sympatric host races of the European corn borer. *Science* 308, 258–260

44. Baker, T.C. (2002) Mechanism for saltational shifts in pheromone communication systems. *Proc. Natl. Acad. Sci. U. S. A.* 99, 13368–13370

45. Shimomura, K. *et al.* (2016) Saltational evolution of contact sex pheromone compounds of *Callosobruchus rhodesianus* (Pic). *Chemoecology* 26, 15–23

46. Niehuis, O. *et al.* (2013) Behavioural and genetic analyses of *Nasonia* shed light on the evolution of sex pheromones. *Nature* 494, 345–348

47. Phelan, P.L. (1992) Evolution of sex pheromones and the role of asymmetric tracking. In *Insect Chemical Ecology: An Evolutionary Approach* (Roitberg, B.D. and Isman, M.B., eds), pp. 265–314, Springer Science & Business Media

48. Moehring, A.J. and Boughman, J.W. (2019) Veiled preferences and cryptic female choice could underlie the origin of novel sexual traits. *Biol. Lett.* 15, 20180878

49. Anton, S. and Rössler, W. (2020) Plasticity and modulation of olfactory circuits in insects. *Cell Tissue Res.* 1–16

50. Johnson, T.L. *et al.* (2017) Anticipatory flexibility: larval population density in moths determines male investment in antennae, wings and testes. *Proc. R. Soc. Lond. B* 284, 20172087

51. Groot, A.T. (2014) Circadian rhythms of sexual activities in moths: a review. *Front. Ecol. Evol.* 2, 43

52. Jin, S. *et al.* (2017) Olfactory plasticity: variation in the expression of chemosensory receptors in *Bactrocera dorsalis* in different physiological states. *Front. Physiol.* 8, 672

53. Wan, X. *et al.* (2015) Synthetic pheromones and plant volatiles alter the expression of chemosensory genes in *Spodoptera exigua*. *Sci. Rep.* 5, 1–11

54. Sollai, G. *et al.* (2018) Olfactory sensitivity to major, intermediate and trace components of sex pheromone in *Ceratitis capitata* is related to mating and circadian rhythm. *J. Insect Physiol.* 110, 23–33

55. Anderson, P. *et al.* (2003) Pre-exposure modulates attraction to sex pheromone in a moth. *Chem. Senses* 28, 285–291

56. Anderson, P. *et al.* (2007) Increased behavioral and neuronal sensitivity to sex pheromone after brief odor experience in a moth. *Chem. Senses* 32, 483–491

57. Levakova, M. *et al.* (2018) Moth olfactory receptor neurons adjust their encoding efficiency to temporal statistics of pheromone fluctuations. *PLoS Comput. Biol.* 14, e1006586

58. van Schooten, B. *et al.* (2020) Divergence of chemosensing during the early stages of speciation. *Proc. Natl. Acad. Sci. U. S. A.* 117, 16438–16447

59. Kromann, S.H. *et al.* (2015) Concurrent modulation of neuronal and behavioural olfactory responses to sex and host plant cues in a male moth. *Proc. R. Soc. Lond. B* 282, 20141884

60. Gadenne, C. *et al.* (2001) Transient post-mating inhibition of behavioural and central nervous responses to sex pheromone in an insect. *Proc. R. Soc. Lond. B* 268, 1631–1635

61. Lalouette, L. *et al.* (2016) Unexpected effects of sublethal doses of insecticide on the peripheral olfactory response and sexual behavior in a pest insect. *Environ. Sci. Pollut. Res.* 23, 3073–3085

62. Gharaei, A.M. *et al.* (2020) Modulation of reproductive behavior of *Diaphania indica* (Lepidoptera: Pyralidae) by preferred and non-preferred host plants. *J. Insect Behav.* 33, 105–115

63. Xu, H. and Turlings, T.C. (2018) Plant volatiles as mate-finding cues for insects. *Trends Plant Sci.* 23, 100–111

64. McCormick, A.C. *et al.* (2017) Exploring the effects of plant odors, from tree species of differing host quality, on the response of *Lymantria dispar* males to female sex pheromones. *J. Chem. Ecol.* 43, 243–253

65. Hoffmann, A. *et al.* (2020) A plant volatile alters the perception of sex pheromone blend ratios in a moth. *J. Comp. Physiol.* 206, 553–570

66. Ruiz-Guzmán, G. *et al.* (2021) Interactions between oxidative stress and attractiveness to mates and individual mate choice in the beetle *Tenebrio molitor*. *Ethology* 127, 109–116

67. Reyes-Ramírez, A. *et al.* (2019) Female choice for sick males over healthy males: consequences for offspring. *Ethology* 125, 241–249

68. Barthel, A. *et al.* (2015) Sex-specific consequences of an induced immune response on reproduction in a moth. *BMC Evol. Biol.* 15, 1–12

69. Altizer, S.M. *et al.* (2000) Associations between host migration and the prevalence of a protozoan parasite in natural populations of adult monarch butterflies. *Ecol. Entomol.* 25, 125–139

70. Derstine, N.T. *et al.* (2017) Evidence for sex pheromones and inbreeding avoidance in select North American yellowjacket species. *Entomol. Exp. Appl.* 164, 35–44

71. van Bergen, E. *et al.* (2013) The scent of inbreeding: a male sex pheromone betrays inbred males. *Proc. R. Soc. Lond. B* 280, 20130102

72. Chang, H. *et al.* (2017) A pheromone antagonist regulates optimal mating time in the moth *Helicoverpa armigera*. *Curr. Biol.* 27, 1610–1615

73. Ruther, J. *et al.* (2009) Quantity matters: male sex pheromone signals mate quality in the parasitic wasp *Nasonia vitripennis*. *Proc. R. Soc. Lond. B* 276, 3303–3310

74. Mowles, S.L. *et al.* (2013) A female-emitted pheromone component is associated with reduced male courtship in the parasitoid wasp *Spalangia endius*. *PLoS One* 8, e82010

75. Fitzpatrick, B.M. (2012) Underappreciated consequences of phenotypic plasticity for ecological speciation. *Int. J. Ecol.* 2012, 256017

76. Geiselhardt, S. *et al.* (2012) Looking for a similar partner: host plants shape mating preferences of herbivorous insects by altering their contact pheromones. *Ecol. Lett.* 15, 971–977

77. Xue, H.J. *et al.* (2016) Contact pheromones of 2 sympatric beetles are modified by the host plant and affect mate choice. *Behav. Ecol.* 27, 895–902

78. Emelianov, I. *et al.* (2001) Host-induced assortative mating in host races of the larch budmoth. *Evolution* 55, 2002–2010

79. Bengtsson, M. *et al.* (2014) Pheromone races of *Cydia splendana* (Lepidoptera, Tortricidae) overlap in host plant association and geographic distribution. *Front. Ecol. Evol.* 2, 46

80. Frago, E. *et al.* (2019) Common pheromone use among host-associated populations of the browntail moth, *Euproctis chrysorrhoea*, displaying different adult phenologies. *Entomol. Gen.* 39, 295–306

81. Vuts, J. *et al.* (2018) Host shift induces changes in mate choice of the seed predator *Acanthoscelides obtectus* via altered chemical signalling. *PLoS One* 13, e0206144

82. Zakir, A. *et al.* (2017) Herbivore-induced changes in cotton modulates reproductive behavior in the moth *Spodoptera littoralis*. *Front. Ecol. Evol.* 5, 49

83. Svensson, E.I. (2012) Non-ecological speciation, niche conservatism and thermal adaptation: how they are connected? *Org. Divers. Evol.* 12, 229–240

84. Lassance, J.M. *et al.* (2019) Pheromones and barcoding delimit boundaries between cryptic species in the primitive moth genus *Eriocrania* (Lepidoptera: Eriocraniidae). *J. Chem. Ecol.* 45, 429–439

85. Dion, E. *et al.* (2020) Early-exposure to new sex pheromone blends alters mate preference in female butterflies and in their offspring. *Nat. Commun.* 11, 1–8

86. Lassance, J.M. *et al.* (2013) Functional consequences of sequence variation in the pheromone biosynthetic gene pgFAR for *Ostrinia* moths. *Proc. Natl. Acad. Sci. U. S. A.* 110, 3967–3972

87. Buček, A. *et al.* (2015) Evolution of moth sex pheromone composition by a single amino acid substitution in a fatty acid desaturase. *Proc. Natl. Acad. Sci. U. S. A.* 112, 12586–12591

88. de Fouchier, A. *et al.* (2017) Functional evolution of Lepidoptera olfactory receptors revealed by deorphanization of a moth repertoire. *Nat. Commun.* 8, 1–11

89. Koutroumpa, F.A. *et al.* (2016) Genetic mapping of male pheromone response in the European corn borer identifies candidate genes regulating neurogenesis. *Proc. Natl. Acad. Sci. U. S. A.* 113, E6401–E6408

90. Groot, A.T. *et al.* (2016) The genetic basis of pheromone evolution in moths. *Annu. Rev. Entomol.* 61, 99–117

91. Byers, K.J. *et al.* (2020) A major locus controls a biologically active pheromone component in *Heliconius* melpomene. *Evolution* 74, 349–364

92. Byers, K.J. *et al.* (2021) Clustering of loci controlling species differences in male chemical bouquets of sympatric *Heliconius* butterflies. *Ecol. Evol.* 11, 89–107

93. Evenden, M.L. (2016) Mating disruption of moth pests in integrated pest management: a mechanistic approach. In *Pheromone Communication in Moths: Evolution, Behavior and Application* (Allison, J. and Cardé, R.T., eds), pp. 365–393, University of California Press

94. Mochizuki, F. *et al.* (2002) Resistance to a mating disruptant composed of (Z)-11-tetradecenyl acetate in the smaller tea tortrix, *Adoxophyes honmai* (Yasuda) (Lepidoptera: Tortricidae). *Appl. Entomol. Zool.* 37, 299–304

95. Mochizuki, F. *et al.* (2008) Sex pheromone communication from a population resistant to mating disruptant of the smaller tea tortrix,

Adoxophyes honmai Yasuda (Lepidoptera: Tortricidae). *Appl. Entomol. Zool.* 43, 293–298

96. Tabata, J. *et al.* (2007) Sex pheromone production and perception in the mating disruption-resistant strain of the smaller tea leafroller moth, *Adoxophyes honmai*. *Entomol. Exp. Appl.* 122, 145–153

97. Evenden, M.L. and Haynes, K.F. (2001) Potential for the evolution of resistance to pheromone-based mating disruption tested using two pheromone strains of the cabbage looper, *Trichoplusia ni*. *Entomol. Exp. Appl.* 100, 131–134

98. Hodgdon, E.A. *et al.* (2020) Synthetic pheromone exposure increases calling and reduces subsequent mating in female *Contarinia nasturtii* (Diptera: Cecidomyiidae). *Pest Manag. Sci.* 77, 548–556

99. Judd, G.J. *et al.* (2005) Reduced antennal sensitivity, behavioural response, and attraction of male codling moths, *Cydia pomonella*, to their pheromone (E, E)-8, 10-dodecadien-1-ol following various pre-exposure regimes. *Entomol. Exp. Appl.* 114, 65–78

100. Groot, A.T. *et al.* (2013) One quantitative trait locus for intra-and interspecific variation in a sex pheromone. *Mol. Ecol.* 22, 1065–1080

101. Nieberding, C.M. *et al.* (2012) Cracking the olfactory code of a butterfly: the scent of ageing. *Ecol. Lett.* 15, 415–424

102. Chemnitz, J. *et al.* (2015) Beyond species recognition: somatic state affects long-distance sex pheromone communication. *Proc. R. Soc. B* 282, 20150832

103. Chemnitz, J. *et al.* (2017) Staying with the young enhances the fathers' attractiveness in burying beetles. *Evolution* 71, 985–994

104. Mérot, C. *et al.* (2015) Beyond magic traits: Multimodal mating cues in *Heliconius* butterflies. *Evolution* 69, 2891–2904

105. Darragh, K. *et al.* (2017) Male sex pheromone components in *Heliconius* butterflies released by the androconia affect female choice. *PeerJ* 5, e3953

106. González-Rojas, M.F. *et al.* (2020) Chemical signals act as the main reproductive barrier between sister and mimetic *Heliconius* butterflies. *Proc. R. Soc. Lond. B* 287, 20200587

107. Merot, C. *et al.* (2017) What shapes the continuum of reproductive isolation? Lessons from *Heliconius* butterflies. *Proc. R. Soc. Lond. B* 284, 20170335

II

MORPH-LINKED PHEROMONE SIGNALING AND MALE RECRUITMENT IN A POLYMORPHIC MOTH

by

Chiara De Pasqual, Eetu Selenius, Emily Burdfield-Steel & Johanna Mappes 2022

Manuscript

Request a copy from author.

III

THE ROLE OF MORPH-SPECIFIC CALLING BEHAVIOR AND PHEROMONE BLEND ON FEMALE ATTRACTIVENESS IN A POLYMORPHIC MOTH SPECIES

by

Chiara De Pasqual, Astrid T. Groot, Eetu Selenius, Cristina Ottocento, Juan A. Galarza, Melanie N. Brien, Emily Burdfield-Steel & Johanna Mappes 2022

Manuscript

Request a copy from author.

IV

HETEROZYGOTE ADVANTAGE AND PLEIOTROPY CONTRIBUTE TO INTRASPECIFIC COLOR TRAIT VARIABILITY

by

Chiara De Pasqual, Kaisa Suisto, Jimi Kirvesoja, Swanne Gordon, Tarmo Ketola & Johanna Mappes 2022

Evolution 76: 2389-2403.

https://doi.org/10.1111/evo.14597

Heterozygote advantage and pleiotropy contribute to intraspecific color trait variability

Chiara De Pasqual,[1,2,3] **Kaisa Suisto,**[1] **Jimi Kirvesoja,**[1] **Swanne Gordon,**[4] **Tarmo Ketola,**[1] **and Johanna Mappes**[1,2]

[1]*Department of Biological and Environmental Science, University of Jyväskylä, Jyväskylä 40014, Finland*

[2]*Organismal and Evolutionary Biology Research Program, University of Helsinki, Helsinki 00014, Finland*

   [3]*E-mail: chiara.depasqual@jyu.fi*

[4]*Department of Ecology and Evolutionary Biology, Cornell University, Ithaca, New York 14853*

The persistence of intrapopulation phenotypic variation typically requires some form of balancing selection because drift and directional selection eventually erode genetic variation. Heterozygote advantage remains a classic explanation for the maintenance of genetic variation in the face of selection. However, examples of heterozygote advantage, other than those associated with disease resistance, are rather uncommon. Across most of its distribution, males of the aposematic moth *Arctia plantaginis* have two hindwing phenotypes determined by a heritable one locus-two allele polymorphism (genotypes: WW/Wy = white morph, yy = yellow morph). Using genotyped moths, we show that the presence of one or two copies of the *yellow* allele affects several life-history traits. Reproductive output of both males and females and female mating success are negatively affected by two copies of the *yellow* allele. Females carrying one *yellow* allele (i.e., Wy) have higher fertility, hatching success, and offspring survival than either homozygote, thus leading to strong heterozygote advantage. Our results indicate strong female contribution especially at the postcopulatory stage in maintaining the color polymorphism. The interplay between heterozygote advantage, *yellow* allele pleiotropic effect, and morph-specific predation pressure may exert balancing selection on the color locus, suggesting that color polymorphism may be maintained through complex interactions between natural and sexual selection.

KEY WORDS: Color locus, heterozygote advantage, intraspecific trait variation, life-history traits, pleiotropy, wood tiger moth.

The origin and maintenance of polymorphism—the co-occurrence of more than two distinct morphs—within natural populations constitute a long-standing conundrum in evolutionary biology (Ford 1945; Huxley 1955; White 2017). Drift alone can erode phenotypic variation from populations in a few hundred generations (Nevo et al. 1997). If traits are under selection, polymorphism is even more puzzling. Theory predicts that traits contributing to the fitness of individuals should be under strong natural and stabilizing selection and drive the more fit morph to fixation (Endler, 1988; Cardé and Baker, 1984). Still, color polymorphic populations are widespread in nature (e.g., Sinervo and Lively 1996; Pryke and Griffith, 2007; Maan and Cummings, 2008; Hegna et al., 2015). Traits (i.e., coloration) may be shaped by complex evolutionary processes through multiple and nonmutually exclusive selective pressures (Gray and McKinnon, 2007), which drive and maintain phenotypic variation and genetic diversity in nature (Fisher 1930; Ford 1945).

Coloration, for example, plays an important role in a variety of ecological and physiological processes (Endler and Mappes, 2017; Cuthill et al., 2017), from camouflage (Duarte et al., 2017), to warning coloration (Mappes et al., 2005) and sexual selection (Maan and Cummings, 2008). Thus, color polymorphism may be the result of natural selection (Gray and McKinnon 2007), sexual selection (Wellenreuther et al., 2014), their combination (Maan and Cummings, 2008), and/or pleiotropic effects (i.e., when a single locus affects two or more phenotypic traits) because color

morphs are often genetically correlated with other traits (McKinnon and Pierotti 2010). Alternative color morphs often differ in features other than color (McKinnon and Pierotti 2010). For example, variable morph-specific behavioral strategies, such as territoriality (Sinervo and Lively 1996), aggressiveness and dominance (Pryke and Griffith 2007), or alternative reproductive strategies, may exist (Sinervo and Lively 1996; Tuttle 2003).

Complex phenotypes can be controlled by simple genetic mechanisms (i.e., one or few genes). In *Drosophila melanogaster*, a gene responsible for cuticle pigmentation, *yellow*, has pleiotropic effects on other traits in males. The lack of function of the *yellow* gene disrupts body pigmentation expression, male courtship behavior, and mating success (Bastock 1956; Wilson et al., 1976; Massey et al., 2019) caused by a morphological and structural change on the leg section used by the male to grasp the female (i.e., sex comb) (Massey et al., 2019). In the case of the common wall lizard (*Podarcis muralis*), the simple genetic basis of the color polymorphism leads to pleiotropic effects in numerous traits (Andrade et al., 2019), including morphology (Sacchi et al., 2007), behavior (Abalos et al., 2016), physiology (Galeotti et al., 2010), immunology (Calsbeek et al., 2010), and reproduction (Galeotti et al., 2013).

Intraspecific color polymorphism maintenance typically requires some form of balancing selection, achieved through color morph fluctuations resulting from negative frequency-dependent selection (FDS) (Wellenreuther et al., 2014) or independent of the relative abundance of a morph (Pryke and Griffith, 2007; Hedrick et al., 2016). Negative FDS mediated by sexual selection can maintain multiple color morphs in natural populations, for example, through alternative male reproductive strategies in the side-blotched lizards (*Uta stansburiana*) (Sinervo and Lively 1996), through rare morph advantage in guppies (*Poecilia reticulata*) (Hughes et al., 2013), or through FD sexually antagonistic selection in blue-tailed damselflies (*Ischnura elegans*) (Svensson and Abbott, 2005; Svensson et al., 2005). In populations with stable morph frequencies, nonrandom mating, in concert with other selective forces, can prevent the loss of color morphs through within-morph mating (i.e., assortative mating) (Pryke and Griffith 2007) or can promote morph maintenance through disassortative mating that maintains high heterozygosity and genetic variation within a population (Hedrick et al., 2016; Maisonneuve et al., 2021).

The presence of two different alleles at a locus (i.e., heterozygosity) provides a basis for phenotypic variation within populations, for example, by expressing alternative color morphs. If heterozygote individuals have a fitness advantage over the homozygote ones, the persistence of phenotypic polymorphism and genetic variability can be aided through heterozygote advantage (Fisher 1922, 1930; Hedrick 2012). Despite decades of research, the majority of studies have focused on heterozygote

advantage as a phenomenon of disease resistance, especially in humans (e.g., the sickle cell anemia, Allison 1954; AIDS, Carrington et al. 1999), in the environment (e.g., pesticide resistance, Greaves et al. 1977; infection resistance, Frelinger, 1972), or to maximize fecundity in livestock (Gemmell and Slate 2006). Recently, due to the advantages of the modern molecular biological methods, there is an increasing number of studies focusing on the role of heterozygote advantage in color polymorphic wild populations (Krüger et al., 2001; Coulson et al., 2011; Hedrick et al., 2014; Llaurens et al., 2017; Strickland et al., 2021). Heterozygote advantage is not an easy task to study in wild populations. The challenges lie in gathering life-history traits of the different genotypes and, sometimes, the lack of knowledge of the genetic basis of the polymorphic trait.

Compelling examples of the fitness advantage of heterozygote individuals are phenotypic variability of sexually selected traits (Coulson et al., 2011; Krüger et al., 2001; Johnston et al., 2013; Hedrick et al., 2014; Maisonneuve et al., 2021), concurrently with other selective forces. In the common buzzard (*Buteo buteo*), the plumage color polymorphism is maintained through heterozygote advantage, which counterbalances maladaptive assortative mate choice due to maternal sexual imprinting (Krüger et al., 2001). The color coat of wolves in Yellowstone National Park represents another well-known example, whose stable color polymorphism maintenance is due to heterozygote advantage (Coulson et al., 2011; Hedrick et al., 2014) coupled with weak selection (Hedrick et al., 2014) and a strong contribution of disassortative mating (Hedrick et al., 2016). Complex polymorphisms can thus be maintained by the interplay of multiple selective pressures, of which heterozygote advantage is one vastly understudied mechanism, and which altogether may determine phenotype-specific advantages culminating in the coexistence of multiple phenotypes.

The wood tiger moth (*Arctia plantaginis*) represents a compelling study species to investigate how different selective pressures can act on a single color locus and maintain within-population trait variation. In this system, male hindwing coloration is determined by a simple genetic basis (Suomalainen 1938; Nokelainen et al., 2022b; Brien et al., 2022): a one locus-two allele polymorphism (dominant W allele and recessive y allele), which translates into white (genotype: WW, Wy) and yellow (genotype: yy) males. Because this is an aposematic moth species, the color trait is not only used for intraspecific communication (i.e., sexual selection) but also to advertise their unpalatability to predators (i.e., interspecific communication). Previous studies have indeed shown that multiple selective pressures act on the male coloration. The two male morphs are differently protected against predators (Nokelainen et al., 2014; Rojas et al., 2017; Winters et al., 2021), with yellow males generally having higher survival (Nokelainen et al., 2012; Rojas et al.,

Figure 1. Examples of wood tiger moth hindwing coloration in Finland. Female's (top row) hindwing coloration varies from light to dark red, whereas male's (bottom row) coloration is either white (left and middle photo) or yellow (right photo). White males can either be WW or Wy for the dominant W color allele, whereas yellow males are homozygote for the y recessive allele. Photos: Chiara De Pasqual.

2017). In addition, male morph mating advantage is dependent on the morph frequency (Gordon et al., 2015) and males that origin from "mixed-morph lines" have higher mating success compared to the moths that originated from more monomorphic lines (Gordon et al., 2018), which suggests that heterozygote advantage may also contribute to the color polymorphism in this species. Here, we test the hypothesis that heterozygote advantage is contributing to male hindwing color polymorphism in the wood tiger moth. By using genotyped lines of moths reared in a greenhouse and life-history traits collected across 19 generations (i.e., 7 years), we subjected the three color genotypes (WW, Wy, and yy) to multiple tests. We test whether heterozygote individuals have (1) higher mating success, either through higher probability of copulating (copulation observations) or lower probability of unsuccessful matings; (2) higher reproductive output by testing fecundity, fertility, and hatching success; and (3) higher longevity by testing the adults' life span.

Material and Methods
STUDY SPECIES

The wood tiger moth (*Arctia plantaginis*) (formerly *Parasemia plantaginis*; Rönkä et al., 2016) is a polymorphic and aposematic moth species. The male hindwing coloration is determined by a simple genetic mechanism where a one locus-two allele (W and y allele) polymorphism translates into white (WW or Wy genotype) or yellow (yy genotype) male morphs (Suomalainen 1938; Nokelainen et al., 2022b) (Fig. 1). Females do not phenotypically express the male color alleles as their hindwing coloration

varies continuously from yellow to red but pass the color alleles to their offspring (Nokelainen et al., 2022b) (Fig. 1). The wood tiger moth is a capital breeder; it does not feed at the adult stage, making the larval diet very important for both their development and the adult stage (e.g., sperm quality, egg numbers) (Tammaru and Haukioja 1996). Adults only live for 1 or 2 weeks after their emergence and spend their adulthood looking for suitable mates. Females lay on average 250 eggs within a few days from the copulation event. Larvae hatch after about 7 days (Chargé et al., 2016), and start feeding on a variety of weedy plants (e.g., *Plantago* sp., *Taraxacum* sp., and *Rumex* sp.).

In Finland, the wood tiger moth has one generation per year and the flight season happens between mid-June and mid-July, depending on the latitude. It is both a diurnal and crepuscular species as it flies during daytime hours (Rojas et al., 2015), but shows mate searching flying activity between ∼5:00 p.m. and 10:00 p.m. with mating activity that can extend into the night (Nokelainen et al., 2012; Gordon et al. 2015) and a mating peak in laboratory around sunset (∼10:00 p.m. to 11:00 p.m.) (pers. obs.). Under laboratory conditions, it can produce up to three generations per year.

MOTHS REARING AND STOCK MAINTENANCE

The laboratory stock was established in 2013 at the Department of Biological and Environmental Science, University of Jyväskylä (Finland) and new individuals were introduced yearly to the stock to maintain the genetic variability. During the stock maintenance, individual females were offered one randomly selected male to ensure offspring's paternity. Greenhouse

temperature roughly followed the outdoor temperature (20–25°C) and natural light. Individuals were paired in 13 × 7 × 9 cm ($h \times w \times l$) transparent plastic boxes with mesh on the lid. Each box was provided with a small piece of moistened paper, where the moths could drink, and to offer a substrate for later oviposition. Three genotype lines have been established in the stock for experimental purposes, each one composed by numerous families. To avoid high inbreeding coefficient that could affect the moths' survival and the experimental results, controlled matings are performed in each generation to ensure the most variable genotype-family combination. The life-history traits (fecundity, fertility, hatching success, offspring survival, and mating success) analyzed in this article come from 19 generations (i.e., 7 years) of data collection. Because mating pairs for the stock maintenance are not individually observed for successful copulation events, we followed a subset of these matings to determine whether heterozygote individuals have higher probability of copulating. These same individuals were then used to test for the individual's longevity. We introduce here the terminology used in the following sections; at the precopulatory stage, we use "copulation probability" to define the likelihood of the paired individuals to copulate; at the postcopulatory stage, we use "reproductive output" when referring to fecundity, fertility, and hatching success, and "mating success" to refer to the likelihood of reproductive failure. Finally, throughout this work, when referring to "genotype," we refer to the sire or dam's genotype.

PRECOPULATORY STAGE: COPULATION PROBABILITY AND MATING DELAY

We followed a total of 292 pairs, of which 180 were white (87 WW and 61 Wy genotypes) and 112 were yellow (yy) males. Among females, 73 were WW, 53 Wy, and 89 yy (see the Supporting Information for the complete crossing scheme). Each male was paired with a single female. Pairs were set at 4:00 p.m. and observed until midnight, approximately 1 hour after sunset when moths were not active anymore. All moths were 1–7 days old. We considered a mating to be successful if the mating pair was successfully formed within the 8 hours of observation. Otherwise, we considered it as not successful. We recorded the copulation success of each pair and the time it took to start mating (henceforth "mating delay").

POSTCOPULATORY STAGE: REPRODUCTIVE OUTPUT

To test the reproductive output of the different genotypes, we compared the fecundity (number of eggs), fertility (number of hatched larvae), and hatching success over 19 generations (i.e., 7 years) of life-history trait data collected during routine maintenance of the common garden stock population. Because individuals had been reared in the greenhouse for several generations, we controlled for the effect of inbreeding coefficient by adding it

as fixed effect and tested its potential interactive effect with the genotype in the analyses of reproductive traits (see the Supporting Information for inbreeding coefficient calculation and Table S1). For each mating pair, the number of laid eggs was counted 4 days after the female had laid her first egg, and larvae were counted 14 days after the first one had hatched. The hatching success was calculated as the total number of larvae that hatched divided by the total number of eggs the female had laid. Larvae were divided to groups of 30, 14 days after hatching. This counting gives us an indication of the genotype's survival. We also tested for genotype differences in oviposition day and hatching day (i.e., the number of days it took for each individual to, respectively, lay the first egg or for the first larvae to hatch). A total of 2714 genotyped individuals were used for these analyses, of which 1566 were sires (111 WW, 522 Wy, and 933 yy) and 1148 were dams (150 WW, 351 Wy, and 647 yy).

POSTCOPULATORY STAGE: MATING SUCCESS

Because the life-history trait data collected during stock maintenance mainly take into account successful matings and thus represent fitness after selection, it is important to separately analyze those who failed either to mate or produce viable offspring. Because the lack of offspring also translates in the lack of full known genotype, we classified individuals either as having a W (either WW or Wy genotype) or a y (i.e., yy genotype) allele. We identified three stages of failure: no eggs laid (i.e., no eggs), eggs were laid but no larvae hatched (i.e., egg hatching), and larvae hatched but none reached adulthood (i.e., adult eclosion). A total of 1059 matings (out of 2357 set) were considered unsuccessful (44.9%) with 1568 individuals and 561 pairs included in the analyses.

LONGEVITY OF GENOTYPES

To follow individual longevity but avoid multiple matings, we removed the male from the mating box at about 1:00 p.m. the day after the mating and kept them in separated jars to follow their longevity.

STATISTICAL ANALYSES

All analyses were performed in Rstudio (version 1.4.1717) (R Core Team 2013). The effect of individual full-allele combinations (i.e., genotype) was tested both at the pre- and postcopulatory stage. Because several traits showed a general disadvantage of the yy genotype at the postcopulatory stage, we tested the effect of the y allele at the pair level. We classified the pairs either based on the number of y alleles in the pair (henceforth "number of y allele," from 0 when both individuals are WW, to 4 when both are yy) or based on individuals that either had one W allele or both yy alleles (henceforth "pair type"). This allowed to test, respectively, for the effect of the y allele regardless of, or considering, the sex of the moth (see Table S2 for the sample size and

Figure 2. Summary of the effect of the genotype and alleles on the traits included in the analyzes. "No difference" refers to no effect of the genotype/alleles on the denoted trait. Full-colored moths/dots indicate that the corresponding genotype/alleles plays a role on the denoted trait, and the size of the moth indicates the higher or lower trait output in genotype comparison. Grayscale moths/dots indicate no significant effect of such genotype/allele; however, the size hints at the higher or lower trend on such trait.

spelled-out pair classification, and Fig. 2 for a summary of the results).

Moth weight and age

Because mate choice and mating success can be affected by size and age, we tested whether genotype differences existed among the moths used at the precopulatory (weight and age) and postcopulatory stage (weight only) by fitting linear models with either "weight" or "age" as response variables and male or female genotype as fixed effects using the "lm" function ("stats" package version 4.1.1). We compared the mean weight and age between genotypes with F-tests implemented with the "aov" function ("stats" package) and performed pairwise post hoc comparisons by estimated least-square means using the "lsmeans" function (Tukey HSD adjustment; "lsmeans" package version 2.30-0).

Precopulatory stage: Copulation probability and mating delay

Copulation event was recorded as a binary variable: 1 if the pair formed, 0 otherwise. At the individual level, we first tested for differences in the copulation probability by setting two Generalized Linear Models (GLMs) (one for males and one for females) with "copulation probability" as response variable, modeled with binomial distribution, and genotype, weight, their interaction, and age as fixed factors. We included the interaction between genotype and weight because of significant differences in weight be-

tween genotypes (reported later). We tested the overall effects of the variables with Chi-square test implemented with the "anova" function.

We then analyzed the mating delay. Across years, the trials were performed by using moths reared in the three different generations, thus carried out in slightly different seasonal time. Because they mate preferentially 1–2 hours before the sunset (pers. obs.) and the sunset time is ∼9:30 p.m. in the first and third generations, and ∼11:00 p.m. during the second generation, we first tested whether the mating delay (response variable) was significantly affected by the generation time (fixed factor) and controlled for the effect of the year (random effect) with a Cox Proportional Hazard Model (henceforth "Cox model") (function "coxph," "survival" package, version 3.2-11). Because the mating delay was significantly affected by generation ($\chi^2 = 143.14$, df = 2, $P \leq 2.2 \times 10^{-16}$) with the second generation (mean $\pm$ SE = 334 ± 20 min) leading to higher mating delay compared to the first (mean $\pm$ SE = 262 ± 11 min; estimated marginal means = -0.726 ± 0.111, $z = -6.552$, $P \leq 0.0001$) and the third (mean $\pm$ SE = 246 ± 11 min) generations (estimated marginal means = -0.905 ± 0.216, z-ratio = -3.67, P-value = 0.0001), we standardized the mating delay to make it comparable for later analyses by centering the mean (mean = 0 and SD = 1). We tested the effect of genotype, weight, age, and generation (fixed effects) on mating delay (response variable) with two Cox models: one for males and one for females. The

male model included the interactions "genotype x generation," "genotype x weight," and age as fixed effects, whereas the female model included the interaction genotype by weight, generation, weight, and age. We did not fit "genotype × generation" interactions because we did not test WW females in the second generation. We then tested for the effect of "number of y allele" and "pair type" on copulation probability (response variable 1) and mating delay (response variable 2) by fitting two GLMs with binomial responses (for response variable 1) and two Cox models (for response variable 2).

Postcopulatory stage: Reproductive output

To test for differences in the number of eggs, larvae, and hatching success (response variables), we fit four Generalized Linear Mixed Models (GLMMs) per response variable: two with Poisson distribution and two with negative binomial distribution, of which two accounted for zero inflated distribution ("glmmTMB" function from "glmmTMB" package version 1.1.3). We included genotype, weight, inbreeding coefficient, and two interactions ("genotype × weight" and "genotype × inbreeding coefficient") as fixed effects and family as random effect to control for the effect of relatedness. We standardized both the weight and the inbreeding coefficient variables (by centering the mean and SD = 1) to include them in the interaction with a discrete variable (the genotype). The model with the lowest AIC value was selected as the best (Table S8, Panel a). For all three response variables, we used type III analyses of variance to test for the effect of the interactions on the response variable, and if the effects were not significant ($P > 0.05$), we removed the interactions from the final model. Finally, we performed genotype pairwise comparisons based on estimated marginal means ("emmeans" function of the "emmeans" package, version 1.7.2).

In addition, by considering pairs with only one WW and one yy individual (which ensures Wy offspring), we tested whether the heterozygote advantage could come from the dam or sire's side. We thus tested whether fecundity, fertility, and hatching success (three response variables) differed between pairs (fixed factor) by fitting two GLMs per response variable, one with a Poisson and the other with a negative binomial distribution. We chose the models with negative binomial distribution due to their lower AIC (Table S3).

Finally, we tested for the effect of genotype, "number of y allele," and "pair type" (fixed factors) on the number of days both to lay eggs (response variable "oviposition day") and for the eggs to hatch (response variable "hatching day") by setting two GLMMs per response variable, one with Poisson and one with negative binomial distribution, genotype as fixed factor and generation as random factor, and four GLMs with the same response variables and distribution, but either "number y allele" or

"pair type" as fixed effects. GLMs with Poisson distribution were chosen because of their lower AICs (Table S4).

Postcopulatory stage: Mating success

To test for differences in mating success between the W and y allele (fixed factor) and "pair type" (fixed factor), we fit four GLMMs, one to test for the allele effect regardless of sex, two models considering moth sex (one for males and one for females), and the final one for the pair effect. In all the four models, we determined the probability of successfully mating (response variable) by the count of successful over the unsuccessful matings through the "cbind" function and modeled with binomial distribution. We set generation as a random effect and used the function "weights" to specify the total number of matings that were set per generation. We tested the effect of the "pair type" using pairwise comparisons based on estimated marginal means (Tukey HSD adjustment).

Longevity of genotypes

To test whether longevity differed between genotypes, we fit two Cox models, one for males and one for females, with individuals' life span (days) as response variable, and genotype as fixed factor.

Results

MOTHS WEIGHT AND AGE

Genotype did not affect male ($F_{(2;1199)} = 2.567$, $P = 0.077$) or female ($F_{(2;938)} = 0.246$, $P = 0.782$) weight of the individuals used in the postcopulatory analyses, but did for those used at the precopulatory stage. In both sexes, WW individuals were significantly heavier than yy individuals (estimated marginal means; males = 14.10 ± 3.39, $t = 4.162$, $P = 0.0001$; females = 16.40 ± 6.18, $t = 2.652$, $P = 0.0233$), and WW females were also heavier compared to Wy females (estimated marginal means = 23.69 ± 7.03, $t = 3.371$, $P = 0.0026$). Age did not differ between male ($F_{(2,257)} = 0.898$, $P = 0.409$) or female genotypes ($F_{(2,212)} = 1.357$, $P = 0.26$).

PRECOPULATORY STAGE: COPULATION PROBABILITY AND MATING DELAY

Although we found genotype-specific differences in weight, the copulation probability in either sex was not affected by their interaction (males: genotype × weight = $\chi^2_{(2,250)} = 5.5438$, $P = 0.0625$; females: genotype × weight = $\chi^2_{(2,205)} = 2.4382$, $P = 0.2955$). Copulation probability was not affected by male or female genotype, male weight, or male and female age (Table S5, Panel a). Interestingly, the heavier the female, the lower the copulation probability (GLM; Estimate = -0.4776 ± 0.1538, $z = -3.106$, $P = 0.0019$). The mating delay was significantly affected by the generation, suggesting that environmental cues

(e.g., the sunset/light) may influence the mating behavior (Table S5, Panel b). Males took significantly longer in the second generation compared to the first and third (coxph; second vs. first; $\exp(\text{coef}) = 2.1212 \pm 0.2739$, $z = 2.745$, $P = 0.0060$; second vs. third; $\exp(\text{coef}) = 2.7969 \pm 0.2884$, $z = 3.567$, $P = 0.0004$), whereas females took significantly longer only compared to the third generation (coxph; second vs. third; $\exp(\text{coef}) = 3.0448 \pm 0.3285$, $z = 3.389$, $P = 0.0007$). Besides the effect of the environmental cues, no other traits played a significant effect on the mating delay. These include the lack of interaction between male genotype and generation (LR test; $\chi^2 = 0.8738$, df $= 2$, $P = 0.6460$), the lack of genotype-specific effect of weight (LR test; male genotype $\times$ weight: $\chi^2 = 3.1702$, df $= 2$, $P = 0.2049$; female genotype $\times$ weight: $\chi^2 = 1.5839$, df $= 2$, $P = 0.4530$), and lack of significant effect of genotype, weight or age, both in males and females (Table S5, Panel b).

Although there was no precopulatory selection at the individual level, a closer look at the copulation probability and mating delay suggests that the allele combination may play an indirect role in these traits, at least for some genotypes. The number of y alleles in the mating pair significantly affected the copulation probability ($\chi^2 = 12.996$, df $= 4$, $P = 0.0113$), where pairs with zero y alleles had a higher copulation probability in general, and significantly higher than pairs with one, two, and three y alleles (Table S6). We found, however, no significant effect of the pair type ($\chi^2 = 3.6337$, df $= 3$, $P = 0.3038$) on the copulation probability, suggesting a general effect of the allele combinations on the mating success rather than sex-specific contribution. For the mating delay, we found somewhat the opposite pattern, as it did not differ according to the number of y alleles (LR test; $\chi^2 = 2.26$, df $= 4$, $P = 0.6872$) but the "femyy + maleW-allele" pair type mated significantly faster than all the other pair types (Table S7). Genotype-specific advantages might be relative to the mating partner and thus can arise at the pair level.

POSTCOPULATORY STAGE: REPRODUCTIVE OUTPUT

For the six final models selected, the lowest AICs were given by the zero inflated with negative binomial models (Table S8, Panel a). Neither interactions ("genotype $\times$ weight" and "genotype $\times$ inbreeding coefficient") were significant and were excluded from the final models (Table S8, Panels b–d). This suggests that weight and inbreeding coefficient did not affect the reproductive output in a genotype-specific manner, despite, for example, genotype-specific differences in the inbreeding coefficient. Although the genotype did not explain the mean differences in fecundity, fertility, and hatching success (Table S9 [Panels b and d], Table S10 [Panel b], Table S11 [Panel b], and Table 1 [Panels a and c]), it had a strong effect on the probability of reproductive failure. This suggests that genotypes differ in their

likelihood of reproductive failure rather than the number of eggs, larvae, or proportion of eggs hatched.

Genotype, female weight, and inbreeding coefficient had a significant effect on the fecundity trait (Table S9, Panel a). yy males had significantly fewer eggs (mean $\pm$ SE $= 149.2 \pm 3.7$) compared to WW (mean $\pm$ SE $= 167.9 \pm 8.3$) and Wy (mean $\pm$ SE $= 171.6 \pm 4.9$) males (Table S9, Panel c; Fig. 3a). yy females laid a significantly lower number of eggs (mean $\pm$ SE $= 161 \pm 4.2$) compared to Wy females (mean $\pm$ SE $= 202.3 \pm 5$) but not compared to WW females (mean $\pm$ SE $= 166.2 \pm 7.8$) (Table S9, Panels d and e; Fig. 2d). The yy genotype disadvantage was due to both a lower egg count and a higher probability of failing to have eggs at all, both in males and females (Table S9, Panels c and e). Weight had a significant effect in females (Table S9, Panel a) with the heavier the female, the higher the number of eggs laid (Table S9, Panel e), whereas no significant effect of the weight was detected for males (Table S9, Panel a). Weight had a significant effect on the count (number) of eggs laid but did not affect the probability of zero count (Table S9, Panel e). No interaction between inbreeding coefficient and genotype was detected but the inbreeding coefficient had a significant effect on the number of eggs laid (Table S9, Panel a), with the higher its value, the lower the egg count (Table S9, Panels c and e). Interestingly, this did not affect the probability of having zero eggs (Table S9, Panels c and e).

Wy females had a significantly lower probability of egg hatching (i.e., having larvae) failure (Table 1, Panel b; Fig. 3e). This was not repeated in males, as yy males had lower probability of having larvae than WW and Wy males (Table S10, Panel b; Fig. 3b). The significant differences were in the probabilities of failure (zeroes) and not in the number (count) of larvae. Therefore, the female Wy advantage is due to the significantly lower probability in failing to have larvae at all compared to the other two genotypes (Table 1, Panel b). The effect of female weight on fertility was significant (Table S10, Panel a) with the heavier the female, the higher the number of larvae that hatched (Table 1, Panel b). This was not seen in males (Table S10, Panel c). The inbreeding coefficient had a significant effect on the fertility trait (Table S10 [Panel c] and Table 1 [Panel b]), where the higher the inbreeding coefficient, the lower the number (count) of larvae in males only (Table S10, Panel c) but not in females (Table 1, Panel b). In addition, the higher the inbreeding coefficient, the higher the probability of zero larva both in males and in females (Table S10 [Panel c] and Table 1 [Panel b]).

The hatching success was significantly affected by the individual genotype (Table S11 [Panel c] and Table 1 [Panel d]), with Wy females having a higher likelihood of hatching success compared to the other two genotypes (WW mean $\pm$ SE $= 0.46 \pm 0.03$, Wy mean $\pm$ SE $= 0.67 \pm 0.02$, yy
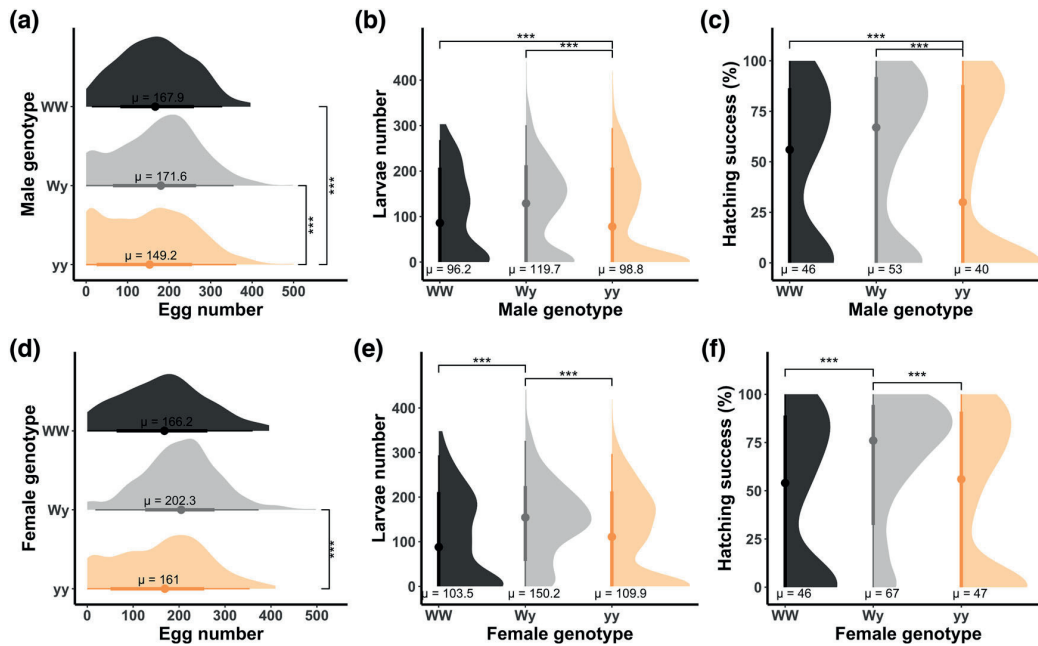
Figure 3. The graph illustrates differences in the fecundity, fertility, and hatching success between genotypes, in males (top row) and females (bottom row). Statistically significant differences are marked with asterisks.

mean $\pm$ SE $= 0.47 \pm 0.02$; Table 1, Panel d; Fig. 3f). In males, the yy genotype had a lower likelihood of hatching success than the other two genotypes (WW mean $\pm$ SE $= 0.46 \pm 0.04$, Wy mean $\pm$ SE $= 0.53 \pm 0.02$, yy mean $\pm$ SE $= 0.40 \pm 0.01$; Table S11, Panel c, Fig. 3c). We found, therefore, strong female heterozygote advantage in fertility and hatching success expressed in their higher likelihood of having larvae and higher likelihood of hatching success. Weight had a significant effect in males but not in females (Table S11, Panel a). Interestingly, the heavier the male, the lower the probability of hatching success (Table S11, Panel c). The inbreeding coefficient significantly affected males and females (Table S11 [Panel c] and Table 1 [Panel d]) with lower probability of hatching success as its value increases.

Finally, the Wy advantage does not seem to be due to either maternal or paternal effect. The number of eggs (glm.nb; estimate $= 0.031 \pm 0.127$, $z = 0.24$, $P = 0.81$), larvae (glm.nb; estimate $= -0.066 \pm 0.175$, $z = -0.377$, $P = 0.706$), or the hatching success (glm.nb; estimate $= 0.164 \pm 0.288$, $z = 0.589$, $P = 0.556$) did not differ between pairs where either the dam or the sire was WW and the other yy. This suggests that the higher Wy fitness is due to the allele combination (W and y) per se, rather than being determined by the dam or sire's side. We found no differences based on the individual genotype or due to the effect of the pair for the oviposition day and hatching day (Table S4) suggesting no particular effect of the color locus on these traits.

POSTCOPULATORY STAGE: MATING SUCCESS

With 78% of the unsuccessful matings having eggs and larvae, the mating failure is more likely to take place at the postcopulatory rather than precopulatory stage. The most sensitive stage seems to be the egg-hatching stage (62%), which was significantly higher than matings that had no eggs (23%; $\chi^2 = 17.89$, df $= 1$, $P = 2.335 \times 10^{-5}$) and than matings that had no adult eclosing (15%; $\chi^2 = 28.69$, df $= 1$, $P = 8.502 \times 10^{-8}$). No differences were found between the no-egg and adult-eclosing stage ($\chi^2 = 1.68$, df $= 1$, $P = 0.19$). There was no effect of either the sire or the dam at the different stage levels (no-eggs stage, $\chi^2 = 0.56$, df $= 1$, $P = 0.46$; egg-hatching stage, $\chi^2 = 0.072$, df $= 1$, $P = 0.79$; adult-eclosing stage, $\chi^2 = 0.13$, df $= 1$, $P = 0.72$), suggesting no sex-specific cause of failure. Y-allele individuals (i.e., yy genotype) had a significantly higher probability of failing to have offspring than W allele individuals (W vs. y; estimate $= -0.075 \pm 0.007$, $z = -10.475$, $P \leq 2 \times 10^{-16}$). These results were likely influenced by females, as y allele females failed significantly more than W allele females (W vs. y females; estimate $= -0.272 \pm 0.020$, $z = -13.38$, $P \leq 2 \times 10^{-16}$), whereas y allele males had significantly higher probability of succeeding in having offspring compared to W allele males (W vs. y males; 0.044 ± 0.011, $z = 4.014$, $P = 5.97 \times 10^{-5}$). The generation effect accounted in average for 15% of the variation in the probability of failing (16% in females and 13% in males). At the pair level, "female yy + male yy" and "female yy + male W-allele" pair types had the lowest probability of having

Table 1. Panels (a) and (c) report female genotype pairwise comparisons for the fertility and hatching success traits. Panels (b) and (d) report the GLMM output for, respectively, the fertility and hatching success trait in females.

Fertility:

(a) Pairwise comparisons based on estimated marginal means; Tukey HSD adjustment

Contrast	Estimate	SE	df	t	P
Wy-WW	0.074	0.082	925	0.894	0.644
Wy-yy	0.035	0.052	925	0.674	0.779
WW-yy	−0.039	0.078	925	−0.495	0.874

(b) Zero inflated; Intercept = Wy genotype

	Estimate	SE	z	P	
Count model					
Intercept	5.054	0.040	125.31	**<2 × 10⁻¹⁶**	
WW genotype	−0.073	0.082	−0.89	0.371	
yy genotype	−0.035	0.052	−0.67	0.500	
Weight	0.217	0.026	8.47	**<2 × 10⁻¹⁶**	
Inbreeding coefficient	−0.010	0.027	−0.39	0.699	
Zero inflated model					
Intercept	−2.456	0.229	−10.721	**<2 × 10⁻¹⁶**	
WW genotype	1.225	0.310	3.946	**7.95 × 10⁻⁵**	
yy genotype	1.566	0.249	6.293	**3.12 × 10⁻¹⁰**	
Weight	−0.026	0.087	−0.300	0.764	
Inbreeding coefficient	0.432	0.092	4.682	**2.84 × 10⁻⁶**	

Hatching success:

(c) Pairwise comparisons based on estimated marginal means; Tukey HSD adjustment

Contrast	Estimate	SE	df	t	P
Wy-WW	0.089	0.068	834	1.324	0.382
Wy-yy	0.041	0.044	834	0.933	0.619
WW-yy	−0.048	0.064	834	−0.756	0.730

(d) Zero inflated; Intercept = Wy genotype

	Estimate	Std. Error	z	P	
Count model					
Intercept	4.284	0.034	126.06	**<2 × 10⁻¹⁶**	
WW genotype	−0.089	0.068	−1.32	0.185	
yy genotype	−0.041	0.044	−0.93	0.351	
Weight	0.0004	0.021	0.02	0.986	
Inbreeding coefficient	0.005	0.023	0.23	0.817	
Zero inflated model					
Intercept	−2.350	0.230	−10.215	**<2 × 10⁻¹⁶**	
WW genotype	1.210	0.312	3.876	**0.0001**	
yy genotype	1.559	0.251	6.216	**5.12 × 10⁻¹⁰**	
Weight	−0.005	0.089	−0.060	0.952	
Inbreeding coefficient	0.382	0.095	4.003	**6.25 × 10⁻⁵**	

offspring (estimated marginal means = 0.0527 ± 0.0426, $z = 1.239$, $P = 0.6023$), whereas the probability of failing significantly differed between all the other pair comparisons (P-values < 0.05) (Table S12; Fig. 4).

LONGEVITY OF GENOTYPES

All males' genotypes had similar life spans after mating once (LR test; males; $\chi^2 = 0.297$, df = 2, $P = 0.862$), whereas WW females lived significantly longer than the other two female genotypes

(coxph; WW vs. Wy; exp(coeff) = 2.2614 ± 0.3653, $z = 2.234$, $P = 0.0255$; WW vs. yy; exp(coeff) = 1.99 ± 0.3192, $z = 2.156$, $P = 0.0311$). For a summary of these results, see Figure 2.

Discussion

We investigated the effect of color alleles and genotypes from pre- to postcopulatory stage in maintaining warning color polymorphism within wood tiger moth populations. Carrying one or
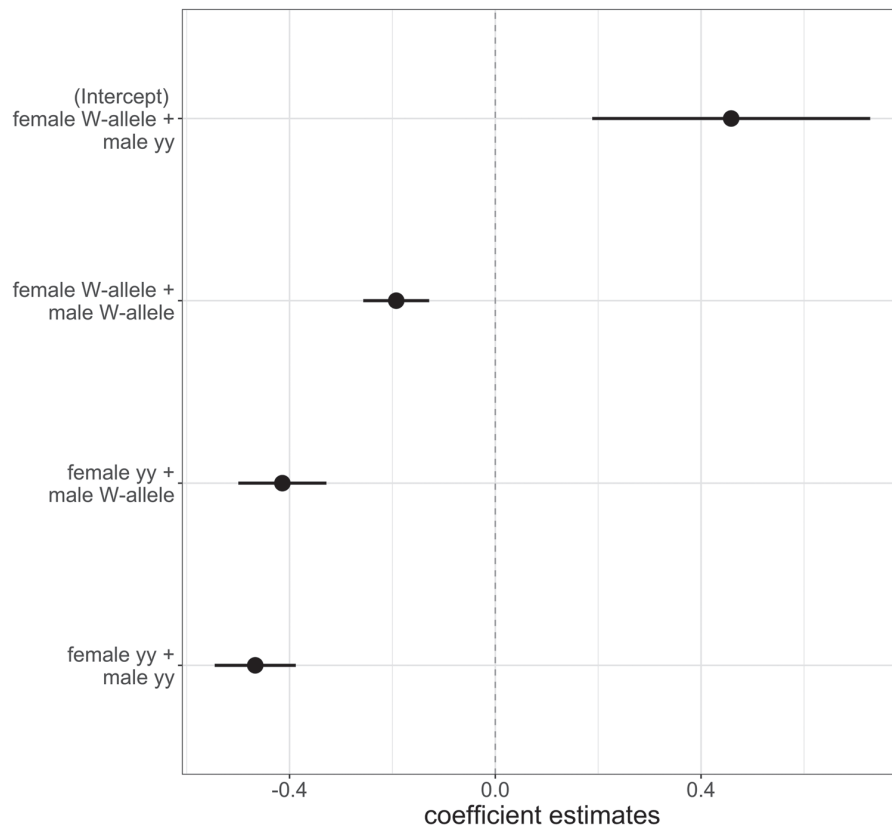
Figure 4. Coefficient estimates of the probability of pair type's mating success. Except for the nonsignificant difference between "female yy + male yy" and "female yy + male W-allele," all the other pairwise comparisons were significantly different (see Table S12 for a reference).

two copies of the yellow allele affected the reproductive fitness in a stage-specific way, from higher likelihood of reproductive output when females carry one copy of the allele (i.e., heterozygote advantage), to lower likelihood of reproductive output success and lower mating success when individuals carry two copies. Thus, the *yellow* allele might have a pleiotropic effect on several life-history traits that can contribute to the maintenance of polymorphism in male coloration. Although we found little contribution of male genotype across the reproductive sequence, female genotype had a significant effect, especially for reproductive success, and likely therefore contributes to the persistence of polymorphism in male coloration. Although all the genotypes, regardless of the sex, had an equal copulation probability and mating delay, Wy females had higher reproductive output (fertility and hatching success) and thus higher offspring survival. Pairs with yy females had shorter mating delay and were more likely to fail in having any offspring. The presence of the *yellow* allele affected the fitness both at the individual and pair level, such as a lower reproductive output in males, and across different steps of the reproductive process for females. Our results thus show the role of genotype-dependent female reproductive success in maintaining male hindwing coloration. Overall, these results suggest that the color locus is pleiotropic with a number of life-history traits, allowing for the maintenance of within-species phenotypic diversity.

WEAK EFFECT OF PRECOPULATORY SELECTION

At the precopulatory stage, 43% of paired individuals did not copulate suggesting some form of female or male rejection. The lack of copulation probability and mating delay differences between genotypes suggests that precopulatory selection may be a weak selective force on the genotypes and, at this stage, neither males nor females can avoid mating with partners with lower fitness prospects. These results are in accordance with a previous study with a similar mating experiment setup that showed equal mating probability between white and yellow phenotypes (Chargé et al., 2016). The hypothesis that sexual selection is more likely to take place after the copulation event, rather than resulting from precopulatory selection, may be further supported by the low (23%) percentage of failed matings that did not have

eggs, a proxy for the lack of copulation event. However, we cannot exclude that the lack of differences in the copulation probability may have been masked by a trade-off between securing at least one mating (and therefore some offspring) and exerting mate choice (see Kokko and Mappes 2005).

Females of different species have been shown to exert stronger sexual selection when presented with a choice (Dougherty and Shuker, 2015). Virgin females, due to the uncertainty of finding a second mate and the risk of dying unmated, are expected to be less choosy and may accept to mating randomly if they fear no further male will be encountered (Kokko and Mappes, 2005; Dougherty and Shuker, 2015). In addition, individuals may get choosier in later matings (Kokko and Mappes 2005; Gao et al., 2020), which might explain the lack of differences in copulation probability. This explanation may also be supported by the lack of differences in the mating delay; if any choice were to be made based on some trait, it might have been translated into a different mating delay. Instead, the mating delay was higher in the second generation because this species is mostly sexually active around sunset (pers. obs.), which is about 2 hours later than the first and third generations (~11:30 p.m. vs. ~9:30 p.m.). Other studies on the species have shown that differences in male copulation probability, and particularly the white male advantage, may be condition dependent (stress-induced condition; Nokelainen et al., 2012), due to the effect of white mixed-lineage advantage (more heterozygous individuals; Gordon et al., 2018), or context dependent, in which the most common morph has higher mating success (Gordon et al., 2015). Mating differences, or lack thereof, in the wood tiger moth may be, therefore, determined by the ecological context or be based on a different trait (e.g., the sex pheromone).

HETEROZYGOTE ADVANTAGE FOR THE MAINTENANCE OF COLOR POLYMORPHISM

At the postcopulatory stage, we found a significant effect of the genotype on fecundity, fertility, and hatching success. In particular, heterozygote (Wy) females had higher likelihood of fertility, offspring survival, and hatching success than the other two genotypes, suggesting that male hindwing coloration is maintained by a rather strong heterozygote advantage effect. The Wy advantage does not seem to be due to either dam or sire's effect (i.e., Ww × yy pairs do not show differences in their reproductive output) or due to differences in oviposition or hatching strategies, suggesting that the heterozygote advantage is a consequence of the W and y allele combination. Wy females had, indeed, a significantly lower probability of zero fertility, which translated into higher hatching success than both the homozygotes. Our results add to a few other known cases of heterozygote advantage (*Buteo buteo*, Krüger et al., 2001; *wolves*, Hedrick et al., 2014; *Heliconius numata*, Jay et al., 2021). The advantage of the dominant (W)

allele in our species does not appear to change for fitness-related measures supported by the general advantage of Wy (and WW genotype) and over the general disadvantage of the yy genotype throughout the reproductive output, a pattern somewhat opposite to the wolf of the Yellowstone National Park (Coulson et al., 2011; Hedrick et al., 2014). In contrast, the heterozygosity advantage in the wood tiger moth may be context dependent: in mating probability either due to female choice or intrasexual competition (Gordon et al., 2018), in the reproductive output (this study), or as defense against predators (Winters et al., 2021), which suggests the importance of considering both natural and sexual selective processes.

PLEIOTROPIC EFFECT OF THE YELLOW ALLELE

The presence of one or two copies of the *yellow* allele affected several steps of the reproductive sequence, from copulatory probability, to mating delay, reproductive output, and mating success, especially in females. Females carrying one *yellow* allele (i.e., Wy) had higher reproductive output than the other two genotypes (i.e., heterozygote advantage). The W and y allele combination might therefore lead to a genetic compatibility advantage that give rise to increased offspring survival, and higher likelihood of hatched eggs (i.e., hatching success). Bearing two copies of the *yellow* allele affected other traits of the reproductive sequence, such as copulation probability, mating delay, and mating success of pairs with yy females. About 55% of the pairs with yy females copulated (against, e.g., 80% of WW × WW pairs), whereas pairs with yy females and white (i.e., WW or Wy) males copulated faster than the other pair types. WW and Wy males have higher reproductive output than yy males, which might be a reason why yy females are more willing to accept white males compared to the yy males. Females carrying two copies of the *yellow* allele had a higher likelihood of reproductive failure regardless of the male they mated with. Mating with a yy female may thus be particularly costly to males.

We thus suggest that genotypic differences in life-history traits are likely due to pleiotropic effects of the *yellow* allele, especially because these effects are expressed in females without the yellow phenotype. The pleiotropic effect of the *yellow* allele extends to males as well. The male yellow coloration confers better protection against predators (Nokelainen et al., 2012, 2014; Rojas et al., 2017), but there are trade-offs with the mating probability (Nokelainen et al., 2012), the reproductive output (Gordon et al., 2018; this study), and their ability to disperse (Gordon et al., unpublished). Recent examples of the pleiotropic effect of color loci on life-history traits have been found in the warningly colored seed bug (*Lygaeus simulans*) (Balfour et al., 2018) and *Heliconius numata* (Jay et al., 2021).

The male coloration in the wood tiger moth is likely regulated by a *yellow*-family gene (Brien et al., 2022), which is

conserved across insects (Ferguson et al., 2011) and has well-known functions in the melanin production pathway (Wittkopp et al., 2002). *Yellow* genes have also been shown to have pleiotropic effects on life-history and behavioral traits (Bastock, 1956; Massey et al., 2019; Connahs et al., 2021). Loss of the *yellow* gene function in *D. melanogaster* results in reduced mating success due to changes in the courtship behavior (Bastock, 1956) and to structural changes in the sex combs used to grasp the female (Massey et al., 2019). The *yellow* gene has the opposite effect in *Bicyclus anynana* where its expression needs to be suppressed for the males to properly express courtship behavior (Connahs et al., 2021). Thus, we suspect that the *yellow* locus is influencing life-history traits as well as wing coloration also in the wood tiger moth, although the exact genetic mechanism is yet unknown.

GENERAL IMPLICATIONS FOR THE MAINTENANCE OF THE COLOR POLYMORPHISM

Across its distribution, the wood tiger moth shows a striking level of phenotypic diversity, both across and within populations (Hegna et al., 2015). From our results, the overall advantage of individuals bearing at least one dominant W allele, and the pleiotropic effect of the *yellow* allele, could theoretically explain populations that are naturally W male biased, such as the Finnish population. However, the 2:1 (white:yellow) ratio expected by the dominant W allele advantage is hardly found in natural populations, even in the light of the higher likelihood of y-bearing individuals to show disadvantage along the reproductive sequence. This suggests that other mechanisms and selective forces are at play. The extensive literature on this study system shows indeed that male morphs experience a multitude of morph-specific selective pressures, from predation (Nokelainen et al., 2012, 2014; Rojas et al., 2017, Winters et al., 2021) linked also to light environment (Nokelainen et al., 2022a), to immune response (Nokelainen et al., 2013), and density-dependent effects (Gordon et al., 2015). This likely affects the expected ratio of white and yellow morphs in natural populations. Future quantifications of the genotype frequencies of natural populations will shed more light on the mechanisms maintaining both alleles.

PRE- AND POSTCOPULATORY EFFECT OF TRAITS BEYOND COLOR GENOTYPE

Mate choice and mating probability may also be based on size or age. However, no effect of age in either sex or male weight was found to affect the copulation probability and mating delay despite white males being heavier than yellow males (and in general, WW individuals being heavier than the other two genotypes). The lack of age and weight effect could be due to the lack of mate choice or adaptation to lab conditions. Although female weight did not play a role in mating delay, it was interesting to

notice that the heavier the female, the lower her copulation probability. A similar result was found in azure damselflies males (*Coenagrion puella*) in which lighter males have higher mating success (Banks and Thompson, 1985). Banks and Thompson (1985) put forward the hypothesis that heavier males may be less active due to their bigger size, thus less likely to find a female. As in our experiment, mating trials were carried out in a confined space and females were more easily spotted by males than in a natural scenario, the lower copulation probability of heavier females may be due to between-females behavioral differences. For instance, heavier wood tiger moth females may be more prone to actively reject males than lighter females. Although this hypothesis should be properly tested, it has been already shown that in Lepidoptera male harassment can be costly to females (Merrill et al., 2018) and females actively reject males to the point they can override male preference (Chouteau et al., 2017). This behavioral hypothesis is also in line with the lowest yy female mate acceptance toward males carrying the W allele that lacks the deleterious elements associated with the y allele when expressed in homozygote yy males (at least for the reproductive success).

At the reproductive output stage, female, and not male, weight played a significant role in fecundity and fertility, despite larger males produce bigger spermatophores (Chargé et al., 2016). Not surprisingly, heavier females laid more eggs. This is in accordance with Santostefano et al. (2018) and it is expected because this species is a capital breeder and females are born with all the eggs that can be potentially fertilized (Tammaru and Haukioja, 1996). Heavier females also had higher fertility. Therefore, female weight may be a trait that males could select for. It is interesting to notice that heavy males had lower hatching success. A previous study on the wood tiger moth (Santostefano et al., 2018) showed that mating with heavier males led to a lower number of eggs laid. Because heavier males produce bigger spermatophores (Chargé et al., 2016), a negative correlation between male weight and hatching success may be the result of trade-offs; being heavy and therefore having invested more resources into mass development may trade-off with the quality of the spermatophore, or heavier males may spend more energy than lighter males in finding and/or courting a female, therefore lowering the resources available for spermatophore production.

Conclusion

Altogether these results suggest that wood tiger moth male coloration is maintained through stage-specific color allele and genotype advantages across the reproductive sequence, from copulation probability to offspring survival. Although individuals do not seem to avoid mating with partners with lower fitness prospects, the strong female heterozygote advantage in fertility,

hatching success, and offspring survival offers a powerful mechanism for both alleles to be maintained within the population. Male hindwing coloration seems also to be maintained through the pleiotropic effect of the *yellow* allele, which affects specific traits of the reproductive sequence, from shortening the mating delay, to being correlated with higher reproductive failure and in general, with the reproductive output. In nature, populations are typically exposed to complex ecological interactions, multiple mechanisms, and selective forces. Such multiple mechanisms concurrently interact and allow for life-history trait variability maintenance through pleiotropy (this study, Mérot et al., 2020) and thus maintain complex color polymorphisms even in the situation when selection is positively frequency dependent (Gordon et al., 2015; Chouteau et al., 2016).

AUTHOR CONTRIBUTIONS

ACKNOWLEDGMENTS

CONFLICT OF INTEREST

The authors declare no conflict of interest.

DATA ARCHIVING

The data used in this study are accessible from the following repository: https://doi.org/10.5061/dryad.g1jwstqth. The data will be released after 6 months.

REFERENCES

Abalos, J., Pérez i de Lanuza, G., Carazo, P. & Font, E. (2016) The role of male coloration in the outcome of staged contests in the European common wall lizard (*Podarcis muralis*). *Behaviour*, 153, 607–631.

Allison A.C. (1954) Protection afforded by sickle-cell trait against subtertian malarial infection. *Br. Med. J.*, 1, 290–294.

Andrade, P., Pinho, C., Pérez i de Lanuza, G., Afonso, S., Brejcha, J., Rubin, C.J., Wallerman, O., Pereira, P., Sabatino, S.J. & Bellati, A. (2019) Regulatory changes in pterin and carotenoid genes underlie balanced color polymorphisms in the wall lizard. *Proc. Natl. Acad. Sci. USA*, 116, 5633–5642.

Balfour, V.L., Aumont, C., Dougherty, L.R. & Shuker, D.M. (2018) The fitness effects of a pale mutant in the aposematic seed bug *Lygaeus sim-*

ulans indicate pleiotropy between warning coloration and life history. *Ecol. Evol.*, 8, 12855–12866.

Banks, M.J. & Thompson, D.J. (1985) Lifetime mating success in the damselfly *Coenagrion puella. Anim. Behav.*, 33, 1175–1183.

Bastock & M. (1956) A gene mutation which changes a behavior pattern. *Evolution*, 10, 421–439.

Brien, M.N., Orteu, A., Yen, E.C., Galarza, J.A., Kirvesoja, J., Pakkanen, H., Wakamatsu, K., Jiggins, C.D. & Mappes, J. (2022) Colour polymorphism associated with a gene duplication in male wood tiger moths. *bioRxiv*, https://doi.org/10.1101/2022.04.29.490025.

Cacoyianni, Z., Kovacs, I.V. & Hoffmann, A.A. (1995) Laboratory adaptation and inbreeding in *Helicoverpa punctigera* (Lepidoptera, Noctuidae). *Australian journal of zoology*, 43(1), 83–90.

Calsbeek, B., Hasselquist, D. & Clobert, J. (2010) Multivariate phenotypes and the potential for alternative phenotypic optima in wall lizard (*Podarcis muralis*) ventral colour morphs. *Journal of evolutionary biology*, 23(6), 1138–1147.

Cardé, R.T. & Baker, T.C. (1984) Sexual communication with pheromones. In Chemical ecology of insects (ed. W. J. Bell & R. T. Cardé), pp. 355–383. Chapman & Hall, Lond.

Carrington, M., Nelson, G.W., Martin, M.P., Kissner, T., Vlahov, D., Goedert, J.J., Kaslow, R., Bushbinder, S., Hoots, K. & O'Brien, S.J. (1999) HLA and HIV-1: heterozygote advantage and B* 35-Cw* 04 disadvantage. *Science*, 283(5408), 1748–1752.

Chargé, R., Wedell, N., Lindstedt, C., Hämäläinen, L., Övermark, E. & Mappes, J. (2016) Variation in male fertility in a polymorphic moth, *Parasemia plantaginis. Animal Behaviour*, 111, 33–40.

Chouteau, M., Arias, M. & Joron, M. (2016) Warning signals are under positive frequency-dependent selection in nature. *Proceedings of the national academy of Sciences*, 113(8), 2164–2169.

Chouteau, M., Llaurens, V., Piron-Prunier, F. & Joron, M. (2017) Polymorphism at a mimicry supergene maintained by opposing frequency-dependent selection pressures. *Proceedings of the National Academy of Sciences*, 114(31), 8325–8329.

Connahs, H., Tan, E., Dion, E., Ter, Y., Matsuoka, Y., Bear, A. & Monteiro, A. (2021) Developmental plasticity in male courtship in *Bicyclus anynana* butterflies is driven by hormone regulation of the yellow gene. *bioRxiv*, https://doi.org/10.1101/2021.06.13.448264.

Coulson, T., MacNulty, D.R., Stahler, D.R., VonHoldt, B., Wayne, R.K. & Smith, D.W. (2011) Modeling effects of environmental change on wolf population dynamics, trait evolution, and life history. *Science*, 334(6060), 1275–1278.

Cuthill, I.C., Allen, W.L., Arbuckle, K., Caspers, B., Chaplin, G., Hauber, M.E., Hill, G.E., Jablonski, N.G., Jiggins, C.D., Kelber, A., Mappes, J., Marshall, J., Merrill, R., Osorio, D., Prum, R., Roberts, N.W., Roulin, A., Rowland, H.M., Sherratt, T.N., Skelhorn, J., Speed, M.P., Stevens, M., Stoddard, M.C., Staurt-Fox, D., Talas, L., Tibbetts, E. & Caro, T. (2017) The biology of color. *Science*, 357(6350), eaan0221.

Dougherty, L.R. & Shuker, D.M. (2015) The effect of experimental design on the measurement of mate choice: a meta-analysis. *Behavioral Ecology*, 26(2), 311–319.

Duarte, R.C., Flores, A.A. & Stevens, M. (2017) Camouflage through colour change: mechanisms, adaptive value and ecological significance. *Philosophical Transactions of the Royal Society B: Biological Sciences*, 372(1724), 20160342.

Endler, J.A. (1988) Frequency-dependent predation, crypsis and aposematic coloration. *Philosophical Transactions of the Royal Society of London. B, Biological Sciences*, 319(1196), 505–523.

Endler, J.A. & Mappes, J. (2017) The current and future state of animal coloration research. *Philosophical Transactions of the Royal Society B: Biological Sciences*, 372(1724), 20160352.

Ferguson, L.C., Green, J., Surridge, A. & Jiggins, C.D. (2011) Evolution of the insect yellow gene family. *Molecular biology and evolution*, 28(1), 257–272.

Fisher, R.A. (1922) XXI.— On the dominance ratio. *Proceedings of the royal society of Edinburgh*, 42, 321–341.

———. (1930) The evolution of dominance in certain polymorphic species. *The American Naturalist*, 64(694), 385–406.

Ford, E.B. (1945) Polymorphism. *Biological Reviews*, 20(2), 73–88.

Frelinger, J.A. (1972) The maintenance of transferrin polymorphism in pigeons. *Proceedings of the National Academy of Sciences*, 69(2), 326–329.

Galeotti, P., Pellitteri-Rosa, D., Sacchi, R., Gentilli, A., Pupin, F., Rubolini, D. & Fasola, M. (2010) Sex-, morph-and size-specific susceptibility to stress measured by haematological variables in captive common wall lizard *Podarcis muralis. Comparative Biochemistry and Physiology part A: Molecular & Integrative Physiology*, 157(4), 354–363.

Galeotti, P., Sacchi, R., Pellitteri-Rosa, D., Bellati, A., Cocca, W., Gentilli, A., Scali, S. & Fasola, M. (2013) Colour polymorphism and alternative breeding strategies: effects of parent's colour morph on fitness traits in the common wall lizard. *Evolutionary Biology*, 40(3), 385–394.

Gao, K., van Wijk, M., Clement, Z., Egas, M. & Groot, A.T. (2020) A life-history perspective on sexual selection in a polygamous species. *BMC evolutionary biology*, 20, 1–10.

Gemmell, N.J. & Slate, J. (2006) Heterozygote advantage for fecundity. *PLoS One*, 1(1), e125.

Gordon, S.P., Kokko, H., Rojas, B., Nokelainen, O. & Mappes, J. (2015) Colour polymorphism torn apart by opposing positive frequency-dependent selection, yet maintained in space. *Journal of Animal Ecology*, 84(6), 1555–1564.

Gordon, S.P., Burdillat, S. & Mappes, J. (2018) Phenotype-dependent mate choice and the influence of mixed-morph lineage on the reproductive success of a polymorphic and aposematic moth. *Evolutionary Ecology*, 32(4), 427–441.

Greaves, J.H., Redfern, R., Ayres, P.B. & Gill, J.E. (1977) Warfarin resistance: a balanced polymorphism in the Norway rat. *Genetics Research*, 30(3), 257–263.

Gray, S.M. & McKinnon, J.S. (2007) Linking color polymorphism maintenance and speciation. *Trends in ecology & evolution*, 22(2), 71–79.

Hedrick, P.W. (2012) What is the evidence for heterozygote advantage selection? *Trends in ecology & evolution*, 27(12), 698–704.

Hedrick, P.W., Stahler, D.R. & Dekker, D. (2014) Heterozygote advantage in a finite population: black color in wolves. *Journal of Heredity*, 105(4), 457–465.

Hedrick, P.W., Smith, D.W. & Stahler, D.R. (2016) Negative-assortative mating for color in wolves. *Evolution*, 70(4), 757–766.

Hegna, R.H., Galarza, J.A. & Mappes, J. (2015) Global phylogeography and geographical variation in warning coloration of the wood tiger moth (*Parasemia plantaginis*). *Journal of Biogeography*, 42(8), 1469–1481.

Hughes, K.A., Houde, A.E., Price, A.C. & Rodd, F.H. (2013) Mating advantage for rare males in wild guppy populations. *Nature*, 503(7474), 108–110.

Huxley, J. (1955) Morphism and evolution. *Heredity*, 9(1), 1–52.

Jay, P., Chouteau, M., Whibley, A., Bastide, H., Parrinello, H., Llaurens, V. & Joron, M. (2021) Mutation load at a mimicry supergene sheds new light on the evolution of inversion polymorphisms. *Nature genetics*, 53(3), 288–293.

Johnston, S.E., Gratten, J., Berenos, C., Pilkington, J.G., Clutton-Brock, T.H., Pemberton, J.M. & Slate, J. (2013) Life history trade-offs at a single locus maintain sexually selected genetic variation. *Nature*, 502(7469), 93–95.

Kokko, H. & Mappes, J. (2005) Sexual selection when fertilization is not guaranteed. *Evolution*, 59(9), 1876–1885.

Krüger, O., Lindström, J. & Amos, W. (2001) Maladaptive mate choice maintained by heterozygote advantage. *Evolution*, 55(6), 1207–1214.

Llaurens, V., Whibley, A. & Joron, M. (2017) Genetic architecture and balancing selection: the life and death of differentiated variants. *Molecular ecology*, 26(9), 2430–2448.

Maan, M.E. & Cummings, M.E. (2008) Female preferences for aposematic signal components in a polymorphic poison frog. *Evolution*, 62(9), 2334–2345.

Maisonneuve, L., Beneteau, T., Joron, M., Smadi, C. & Llaurens, V. (2021) When do opposites attract? A model uncovering the evolution of disassortative mating. *The American Naturalist*, 198(5), 625–641.

Mappes, J., Marples, N. & Endler, J.A. (2005) The complex business of survival by aposematism. *Trends in ecology & evolution*, 20(11), 598–603.

Massey, J.H., Chung, D., Siwanowicz, I., Stern, D.L. & Wittkopp, P.J. (2019) The yellow gene influences *Drosophila* male mating success through sex comb melanization. *Elife*, 8, e49388.

Mérot, C., Llaurens, V., Normandeau, E., Bernatchez, L. & Wellenreuther, M. (2020) Balancing selection via life-history trade-offs maintains an inversion polymorphism in a seaweed fly. *Nature communications*, 11(1), 1–11.

Merrill, R.M., Neggazi, S., Morrison, C.R., Crisp, R. & McMillan, W.O. (2018) Experimental manipulation of Heliconius warning patterns reduces harassment of previously mated females. *bioRxiv*, https://doi.org/10.1101/437525.

McKinnon, J.S. & Pierotti, M.E. (2010) Colour polymorphism and correlated characters: genetic mechanisms and evolution. *Molecular ecology*, 19(23), 5101–5125.

Nevo, E., Kirzhner, V., Beiles, A. & Korol, A. (1997) Selection versus random drift: long–term polymorphism persistence in small populations (evidence and modelling). *Philosophical Transactions of the Royal Society of London. Series B: Biological Sciences*, 352(1351), 381–389.

Nokelainen, O., Hegna, R.H., Reudler, J.H., Lindstedt, C. & Mappes, J. (2012) Trade-off between warning signal efficacy and mating success in the wood tiger moth. *Proceedings of the Royal Society B: Biological Sciences*, 279(1727), 257–265.

Nokelainen, O., Lindstedt, C. & Mappes, J. (2013) Environment-mediated morph-linked immune and life-history responses in the aposematic wood tiger moth. *Journal of Animal Ecology*, 82(3), 653–662.

Nokelainen, O., Valkonen, J., Lindstedt, C. & Mappes, J. (2014) Changes in predator community structure shifts the efficacy of two warning signals in Arctiid moths. *Journal of Animal Ecology*, 83(3), 598–605.

Nokelainen, O., de Moraes Rezende, F., Valkonen, J.K. & Mappes, J. (2022a) Context-dependent coloration of prey and predator decision making in contrasting light environments. *Behav. Ecol.*, 33, 77–86.

Nokelainen, O., Galarza, J.A., Suisto, K., Kirvesoja, J. & Mappes, J. (2022b) Genetic colour variation visible for predators and conspecifics is concealed from humans in a polymorphic moth. *Journal of Evolutionary Biology*, 35, 467–478

Pryke, S.R. & Griffith, S.C. (2007) The relative role of male vs. female mate choice in maintaining assortative pairing among discrete colour morphs. *Journal of evolutionary biology*, 20(4), 1512–1521.

R Core Team. (2013) R: a language and environment for statistical computing. R Foundation for statistical computing, Vienna.

Rojas, B., Gordon, S.P. & Mappes, J. (2015) Frequency-dependent flight activity in the colour polymorphic wood tiger moth. *Current Zoology*, 61(4), 762–772.

Rojas, B., Burdfield-Steel, E., Pakkanen, H., Suisto, K., Maczka, M., Schulz, S. & Mappes, J. (2017) How to fight multiple enemies: target-specific

chemical defences in an aposematic moth. *Proceedings of the Royal Society B: Biological Sciences*, 284(1863), 20171424.

Rönkä, K., Mappes, J., Kaila, L. & Wahlberg, N. (2016) Putting *Parasemia* in its phylogenetic place: a molecular analysis of the subtribe Arctiina (Lepidoptera). *Systematic Entomology*, 41(4), 844–853.

Roush, R.T. (1986) Inbreeding depression and laboratory adaptation in *Heliothis virescens* (Lepidoptera: Noctuidae). *Annals of the Entomological Society of America*, 79(4), 583–587.

Sacchi, R., Scali, S., Pupin, F., Gentilli, A., Galeotti, P. & Fasola, M. (2007) Microgeographic variation of colour morph frequency and biometry of common wall lizards. *Journal of Zoology*, 273(4), 389–396.

Santostefano, F., Galarza, J.A. & Mappes, J. (2018) Testing the direct and genetic benefit hypotheses of polyandry in the wood tiger moth. *Behavioral Ecology and Sociobiology*, 72(7), 1–10.

Sinervo, B. & Lively, C.M. (1996) The rock–paper–scissors game and the evolution of alternative male strategies. *Nature*, 380(6571), 240–243.

Strickland, L.R., Fuller, R.C., Windsor, D. & Cáceres, C.E. (2021) A potential role for overdominance in the maintenance of colour variation in the Neotropical tortoise beetle, *Chelymorpha alternans*. *Journal of Evolutionary Biology*, 34(5), 779–791.

Suomalainen, E. (1938) Die Erblichkeitsverhältnisse des männlichen Dimorphismus bei Parasemia plantaginis. *Hereditas*, 24(3), 386–390.

Svensson, E.I. & Abbott, J. (2005) Evolutionary dynamics and population biology of a polymorphic insect. *Journal of evolutionary biology*, 18(6), 1503–1514.

Svensson, E.I., Abbott, J. & Härdling, R. (2005) Female polymorphism, frequency dependence, and rapid evolutionary dynamics in natural populations. *The American Naturalist*, 165(5), 567–576.

Tammaru, T. & Haukioja, E. (1996) Capital breeders and income breeders among Lepidoptera: consequences to population dynamics. *Oikos*, 561–564.

Tuttle, E.M. (2003) Alternative reproductive strategies in the white-throated sparrow: behavioral and genetic evidence. *Behavioral Ecology*, 14(3), 425–432.

Wellenreuther, M., Svensson, E.I. & Hansson, B. (2014) Sexual selection and genetic colour polymorphisms in animals. *Molecular ecology*, 23(22), 5398–5414.

White, T.E. (2017) Digest: strengthening the link between sexual selection and color polymorphism. *Evolution*, 71, 1913-1914.

Wilson, R., Burnet, B., Eastwood, L. & Connolly, K. (1976) Behavioural pleiotropy of the yellow gene in *Drosophila melanogaster*. *Genetics Research*, 28(1), 75–88.

Winters, A.E., Lommi, J., Kirvesoja, J., Nokelainen, O. & Mappes, J. (2021) Multimodal aposematic defenses through the predation sequence. *Frontiers in Ecology and Evolution*, 9, 657740.

Wittkopp, P.J., Vaccaro, K. & Carroll, S.B. (2002) Evolution of yellow gene regulation and pigmentation in *Drosophila*. *Current Biology*, 12(18), pp. 1547–1556.

Associate Editor: M. Kronforst
Handling Editor: T. Chapman

Supporting Information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Table Supp. info 1. Table a) reports the model selection for testing if inbreeding coefficients differ at the genotype level, and genotype pairwise comparisons.

Table Supp. Info 2. Sample size of the individuals tested and all possible pair combinations used in the experiments.

Table Supp. Info 3. The table below reports the model selection for the analyzes that tested whether the heterozygote advantage was linked to the sire or dam's side.

Table Supp. Info 4 The upper table below reports the model selection for the oviposition and hatching day considering both models at the individual level (males and females) and at the pair level (number of yellow alleles in the pair and the pair type).

Table Supp. Info 5. Table a) reports the effect of the genotype, weight and age on the mating probability, for males (upper part) and females (lower part) separately through Chi-square test.

Table Supp. Info 6. The upper part of the table reports the effect of the number of *yellow* allele and the pair type on the copulation probability through LR-test.

Table Supp. Info 7. The upper table reports the effect of the number of yellow allele and the pair type on the mating delay through LR-test.

Table Supp. Info 8. Table a) reports the model selection for the fecundity, fertility and hatching success traits for males and females.

Table Supp. Info 9. Table a) reports the effect of genotype, weight and inbreeding coefficient on the fecundity of males and females.

Table Supp. Info 10. Table a) reports the effect of genotype, weight and inbreeding coefficient on the fertility of males and females.

Table Supp. Info 11. Table a) reports the effect of genotype, weight and inbreeding coefficient on the hatching success of males and females.

Table Supp. Info 12. The table reports the pairwise comparisons between pair type for the mating success.