The << specific resistance >> model: how the epidemiological context can drive the direction of sexual selection

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The « specific resistance » model: how the epidemiological context can drive the direction of sexual selection

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Summary:

In many species, males have evolved exaggerated secondary sexual traits, for which females have evolved a strong preference. It is however unclear what makes attractive males beneficial to females, and why is female choice, despite being costly, so ubiquitous. The "good genes" model stipulates that secondary sexual traits, that make males more attractive, indicate that these males carry genetic variants that increase their fitness, and thus that will increase the fitness of their offspring, representing an indirect benefit to female. This implies an additive genetic correlation between male attractiveness and offspring fitness. Also, female choice should reduce genetic variation in males, as the chosen males are always the more attractive ones. How is genetic variation maintained in males is a question known as the "lek paradox". Two scenarios, both implying pathogens and resistance to pathogens, could explain how genetic variation is maintained under the "good genes" hypothesis: the "specific resistance" model, and the "general immunocompetence" model. A key difference between the two models is that under the "specific resistance" model, the epidemiological context in which female choice occurs could have an important impact on the outcome of sexual selection, whereas in the "general immunocompetence" model, chose males should always be the same ones, regardless of the currently present pathogens. In this thesis, we experimentally tested this context-dependence by measuring the impact of the presence of pathogens on the identity of the more sexually successful males in *Drosophila melanogaster*. We also tested how the correlation between male sexual success and offspring resistance would differ depending on the epidemiological context in which mating choice was done. Last, we used Pool-sequencing to look for single nucleotide polymorphisms (SNPs) associated with male sexual success, and to investigate if the level of genetic differentiation between sexually

successful versus unsuccessful males would depend on their pathogen exposure. We found results consistent with the "specific resistance" model, as we found evidence in support with the idea that the epidemiological context in which sexual selection takes place has a crucial role on its outcome, and on the sign of the genetic correlation between male sexual success and offspring resistance.

Résumé :

Chez de nombreuses espèces, les mâles ont développé des traits sexuels secondaires pour lesquels les femelles ont acquis une forte préférence. La raison pour laquelle choisir ces mâles est bénéfique pour les femelles reste incertaine, ainsi que, en dépit de son cout, le choix de la femelle reste omniprésent. La théorie des « bons gènes » stipule que ces traits qui rendent les mâles attractifs indiquent qu'ils portent des variants génétiques augmentant leur fitness, ainsi que celle de leur descendance, ce qui représente un bénéfice indirect pour les femelles. Ceci implique une corrélation génétique additive entre l'attractivité des mâles et la fitness des descendants. De plus, le choix des femelles devrait réduire la variance générique chez les mâles, puisque ce sont toujours les plus attractifs qui sont choisi, or elle est maintenue, ce qui représente un paradoxe. Deux scénarios impliquant l'influence des pathogènes et de la résistance à ces derniers peuvent expliquer comment, dans le cadre de la théorie des « bons gènes », cette variance est maintenue : le model de la « résistance spécifique », et celui de « l'immunité générale ». La principale différence entre les deux réside dans le fait que, dans le premier model, le contexte épidémiologique peut influer sur le sens de la sélection sexuelle, alors que dans le second, les mâles choisis seront toujours les même, peu importe le contexte. Dans cette thèse, nous avons expérimentalement testé cette dépendance au contexte épidémiologique en mesurant l'impact de la présence de pathogène sur l'identité des mâles ayant le plus de succès auprès de femelles chez la mouche *Drosophila melanogaster*. Nous avons également testé si le sens de la corrélation entre l'attractivité des mâles et la résistance des descendants dépend du contexte épidémiologique dans lequel le choix du partenaire est fait. Finalement, nous avons séquencé des groupes d'individus afin de rechercher des polymorphismes à nucléotide simple pouvant être associés à l'attractivité des mâles, et

également afin d'étudier si le niveau de différenciation génétique entre mâles plus ou moins à succès dépend du leur exposition aux pathogènes. Nous avons obtenu des résultats en accord avec le model de la « résistance spécifique », à savoir que le contexte épidémiologique à un rôle crucial sur les conséquences de la sélection sexuelle.

General Introduction:

Darwin's theory of evolution has become a universally accepted paradigm explaining the tremendous diversity among living species, with natural selection being its main driver. In order to survive and to reproduce, individuals need to be adapted to their environment, thus the more adapted an individual is, the higher his chances of reproducing and transmitting his genes are (Darwin 1859). However, another type of selection has been suggested by Darwin himself. During his journey, he observed several individuals exposing incredibly exaggerated traits as ornaments. Because of their bright colours or extravagant shape, these traits did not seems to be adaptation due to natural selection. It even seemed to Darwin that such traits would be a handicap, for example by making its owner more detectable by predators. Interestingly, those traits were usually observed in male individuals, and females seemed to have a preference for these ornamented males. With this, the principle of sexual selection was proposed. Where natural selection is the consequence of competition among individuals for access to resources and for survival, sexual selection comes from competition for access to a mating partner, or more precisely, its gametes (Andersson 1994; Shuker 2010). The identity of the male that will gain access to the female is based on several criteria, and thus some males will be less likely to reproduce and transmit their genes to the next generation, even though their ability to survive is as good as the one of more attractive males. It has been shown that female preference can be a very powerful agent in the evolution of males' phenotype (Kirkpatrick 1982), resulting in the evolution of these ornaments that can be either morphological or behavioural. Classic examples of such traits are the peacock male, and its impressive colourful tail, or the deer males, fighting each other to gain access to a female.

The identity of the male that will access to a female is generally determined through the interaction of two mechanisms: female choice and male-male interactions (Hunt et al. 2009; McGhee, Fuller, and Travis 2007; Candolin 1999), which can also be referred to as intra-sexual and inter-sexual selection. Often, males not only compete with each other for the attention of the female by being more attractive, but also by physically interacting. Thus, secondary sexual traits can be selected for being beneficial in regards with both mechanisms. But sexual selection is not only happening when a female choses a male, or when males compete with each other to gain access to their female. In cases where female has a chance to mate with multiple partners, then selection becomes also post-copulatory, based on traits that will increase the paternity likelihood of one male over others (Birkhead and Pizzari 2002). This selection is mediated by both sperm competition and cryptic female choice. Sperm competition can be defined as the competition between sperms from different males for access to fertilization (Parker 1970; Møller and Ninni 1998; Birkhead 1998). Cryptic female choice refers to a form of female choice, which consists in the female controlling fertilization through physical or chemical mechanisms, and thus having the ability to select which sperm will or will not fertilize her eggs (Birkhead 1998; Eberhard 1996; Firman et al. 2017).

1. How do females benefit from mating with the "best" males?

Even though sexual selection is a well-accepted theory, it is yet not clear what are, if there are any, the direct/indirect benefits and costs for females to choose a partner based on a particular trait (i.e on the male attractiveness) or on the outcome of male-male interactions. Female choice is based on secondary sexual traits that can be considered as signals reflecting the male's value as a sexual partner, and the honesty of a signal is crucial for females to benefit

from their choice. But for males, it would probably be beneficial to "cheat", and to produce a misleading signal that would indicate an exaggerated value of the individual. However, this would lead to the selection of low-quality cheating males, using fake signals. There are two principal models that explain why natural selection does not favour cheating, and thus why honesty is maintained (Biernaskie, Grafen, and Perry 2014). The first one concerns the cost of dishonesty, and stipulates that producing a fake signal is simply too costly (Számadó 2011). The second one, the "indicator" hypothesis, says that individual fitness is tightly linked to the signal quality, so that these signals cannot be faked (Smith and Harper 1995; Hill 2011). In both cases, females should benefit from choosing males with more developed secondary sexual traits, as these should always be honest signals. The nature of the benefits a female would earn from her choice can vary quite a lot. Direct benefits can be for example based on territory, resources (e.g nutritional gift), or parental care (Andersson 1994; W. D. Hamilton 1990; Hoelzer 1989; Evans and Moller 1996). Indirect benefits are generally pointing to alleles transmitted from the preferred males, which will increase offspring fitness (and thus the female indirect fitness) (W. Hamilton and Zuk 1982; Heywood 1989; Fisher 1930). The exact phenotypic effects of these alleles, as their importance for female choice, is still debated. Here, I will describe three different explanations for potential indirect genetic benefits that females will gain from their choice: the Fisherian runaway process, disassortative mating, and the "good genes" model, the latter being the one I focused on in my thesis.

1.1 The Fisherian runaway process

In 1915, Fisher suggested a model explaining the existence of male secondary sexual traits and female preference for them (Fisher 1915). This model, known as the “sexy sons” model or as the Fisherian runaway selection process, raises the idea that by choosing attractive males, females will have attractive sons that will have a higher chance to reproduce. The model stipulates that male secondary sexual traits were originally non-sexual traits, selected by natural selection, for which female developed a preference through, for example, a sensory bias (Fuller, Houle, and Travis 2005; Dawkins and Guilford 1996). This preference gave males with developed traits an advantage over others and lead to the evolution of even more developed traits and of a stronger female preference. At some point, the trait might have become negatively selected by natural selection, no longer being an honest index of quality anymore. But females still indirectly benefited from using it as a criteria to choose their partner, as their sons would inherit the genes responsible for the trait, making them more attractive to other females. With this, Fisher suggested that genetic components for female preference and male sexual traits could explain the maintenance of both: the offspring of a female showing a strong preference for exaggerated sexual traits will carry alleles for the same preference and traits, thus leading to a coevolution between female preference and male sexual traits (Fisher 1930). Several models of this runaway selection have been described since. Kirkpatrick (1982) used a two-locus model of a hypothetical haploid population, with one loci determining female preference for a male particular trait, and the other loci determining that male trait (which reduces viability). He confirmed that female preference is a force strong enough to maintain such a male trait, despite being counter-selected by natural selection. However, this model assumes no cost of female preference. In another model,

Pomiankowski (1987) took these costs (i.e predation risk, energy spent, risks of pathogen transmissions) into account, and showed that a coevolution between male exaggerated traits and female choice can happen, but only if females obtain some other indirect benefits (i.e genetic benefits to the offspring) in addition to the Fisherian advantages. Still, the Fisherian runaway selection process, or "sexy sons" model, is supported by several studies, as shown in a meta-analysis from Prokop et al. (2012). As mentioned before, sperm competition can also be a source of variance in male mating success, thus the "sexy sons" model has also been extrapolated to post-copulatory selection as the sexy-sperm theory (McNamara, Van Lieshout, and Simmons 2014).

1.2 Disassortative mating

Disassortative mating refers to individuals exhibiting a preference for sexual partners that are genetically dissimilar. In particular, one example of dissimilarities is based on the major histocompatibility complex (MHC). MHC genes play an important role in disease resistance, and heterozygosity in MHC genes is advantageous, especially when facing multiple species or strains of pathogens (A. L. Hughes and Nei 1992). Choosing a partner with dissimilar MHC genes is believed to play a role in limiting inbreeding and improving offspring immunity (Milinski 2006a; Penn and Potts 1999; Huchard et al. 2013). It has been often raised that sexual selection plays a crucial role in the maintenance of MHC diversity, which mediates the extent of resistance (Edwards and Hedrick 1998; Apanius et al. 2017), and that female choice can be driven by males' MHC genes. For example, in sticklebacks females preference have been shown to be positively correlated with the diversity of males' MHC profile (Reusch et al. 2001; Eizaguirre et al. 2009). Also, humans and mice seem to prefer MHC-dissimilar partners (Chaix,

Cao, and Donnelly 2008; Potts, Manning, and Wakeland 1991; Penn and Potts 1999). Evidence found in several other systems shows that mating with dissimilar partners brings benefits to the offspring (Butlin et al. 1984; Day and Butlin 1987; Hori 1993; Schilthuizen 2007; Horton et al. 2013), or simply aims to avoid inbreeding (Szulkin et al. 2009; Walker et al. 2017; Leedale et al. 2020)

1.3 The "good genes" model

Finally, one of the most mentioned models of sexual selection, and the one I focused on in my thesis, is called the "good genes" model. This model suggests that male secondary sexual traits are honest indicators of males' quality, which can be defined as an unmeasured trait that is positively linked to fitness (Wilson and Nussey 2010), and that by choosing males with more developed traits, females will ensure that their offspring will receive genes that will increase non-sexual aspect of their fitness. The idea that secondary sexual traits reflect genetic quality (i.e the fact that the male carries alleles that increase non-sexual aspect of fitness) is based on the assumption that male attractiveness is positively correlated with other fitness related traits through some pleiotropic effects (Zahavi 1975, 1977). In other words, the good-genes model implies that there is a positive genetic correlation between male attractiveness (secondary sexual traits) and non-sexual fitness. Here, secondary sexual traits are believed to be condition-dependent. Condition can be tricky to define, and here I will use the definitions suggested by Hill (2010), with condition being define as "the relative capacity to maintain optimal functionality of essential cellular processes", or also as "the capacity to withstand environmental challenges". These definitions differs from the one suggested by Rowe and Houle (1996), where condition is defined as the amount of resources an individual is able to

allocate to the maintenance and production of traits increasing fitness. According to Hill (2010), condition is determined by the individual's genotype and its somatic and epigenetic state. Thus, secondary sexual traits condition-dependence may implies that males carrying genetic variants increasing their condition will exhibit more developed secondary sexual traits. By selecting those individuals, females will transmit these good genes to their offspring. As mentioned earlier, these good genes are believed to be linked to male sexual success due to an additive genetic correlation, but the nature of the benefits brought by these good genes is still debated. These different models (the Fisherian runaway process, disassortative mating, and the "good-genes" model) explaining how females can indirectly benefit from their choice are not necessarily mutually exclusive. A meta-analysis done by Prokop et al. (2012) compared results from studies testing the relationship between male sexually selected traits and offspring traits related to fitness through either attractiveness, so corresponding to the Fisher model, or other fitness components, such as life history traits, so corresponding to the "good genes" model. As they found more evidence supporting the Fisherian process, they also found a positive correlation between male attractiveness and offspring condition and immunocompetence, which is in support of the "good genes" model. The functions of the traits mediated by these "good genes", and thus the benefits they represent, can be diverse. And according to Iwasa and Pomiankowski (1991), they need to be linked to the individual's overall condition.

2. The maintenance of genetic variation, a problem called the Lek paradox

A point that has raised many questions is the fact that directional female choice (i.e a choice that is always favouring the same trait quality, which is the case in both the Fisherian runaway

process and the “good genes” model) should intuitively lead to a decrease in males’ genetic variance and to the decline of female choice, as without variance within males females should have no reason to choose some males over others (Kotiaho, Simmons, and Tomkins 2001; Tomkins et al. 2004; Kotiaho 2002; Rowe and Houle 1996; Pomiankowski and Moller 1995). But female choice has been maintained, and so has been male genetic variance. A solution to this paradox, called the “Lek paradox”, has been suggested by Rowe and Houle (1996), based on the dependence of secondary sexual traits to condition. But the mechanisms leading to the maintenance of variation in condition, and thus in male sexual traits, are still debated.

What maintains the variation relevant for sexual selection is a specific case of a broader discussion on what maintains additive genetic variation in general. One explanation is mutation-selection balance, which implies that in a population the rate at which deleterious alleles appears by mutation is at least equal to the rate at which selection eliminates these deleterious alleles (Crow and Kimura 1971; Lynch 2010). Another explanation is that selection varies with time and/or space, which is called fluctuating selection (Taylor 2008; Bell 2010). Both explanations can be related to sexual selection and to the question of the maintenance of additive genetic variance in sexual traits. How is variation in secondary sexual traits maintained, and what is the nature of the non-sexual aspects of fitness that represent indirect benefits of mating choice under the “good genes” hypothesis, are questions often raised in the field of sexual selection.

For mating choice to evolve in a population there must be enough fitness variance among individuals. Under the “good genes” hypothesis, secondary sexual traits are condition dependent. General condition is based on numerous traits, and thus on many genes, representing a large mutational target (Rowe and Houle 1996; Dugand, Tomkins, and

Kennington 2019). Thus, the maintenance of variation in condition has been suggested to be based on the mutation-selection balance, as the appearance of deleterious mutation is a bias strong enough to maintain fitness variation in the population, allowing not only the evolution of mate choice but its maintenance, a key point of the previously mentioned lek paradox. But another force might explain the maintenance of fitness variation, which implies host-pathogen co-evolution, as suggested by Hamilton and Zuk (1982). Pathogens are an important factor of selection as they impact condition and viability, and numerous studies have thus invoked the idea that, in sexual selection, there is a role for pathogens.

3. A role for pathogens

As explained earlier, the "good genes" hypothesis stipulates that secondary sexual traits, that increase male mating success, are signaling the fact that the male is also carrying alleles that improve non-sexual aspects of fitness. Resistance to pathogens (in a broad sense, including immune defense, tolerance, and behavioral avoidance) is often invoked as one of these non-sexual fitness components, meaning that female preference for developed secondary sexual traits will select for higher resistance, and thus generate indirect benefit through the transmission of resistance alleles to the offspring (Hamilton and Zuk 1982; Adamo and Spiteri 2005). A very recent study on birds has put in light similarities in terms of selective pressure between genes related to immunity and genes related to feather coloration, which brings new support to the idea that resistance to pathogen could be involved (Jaiswal et al. 2021). Different models aim to explain the role of pathogens in sexual selection and in the maintenance of males' genetic variation. The first one, the "specific resistance" model, implies fluctuating selection due to host-pathogen coevolution. The second one, the "general-

297 immunocompetence" model, consider pathogens in the context of mutation-selection
298 balance.

299

300 *3.1 The "specific resistance" model*

301 Hamilton and Zuk have suggested in 1982 a potential solution to the "lek paradox" implying
302 pathogens under the "good genes" hypothesis (Hamilton and Zuk 1982). They proposed that
303 male sexual traits capture variation in condition, which depends on the host's resistance to
304 currently present pathogens. Female will prefer males that are in better condition, meaning
305 males specifically resistant to the currently present pathogen pool (here, resistance can be
306 specific to either pathogen species, strain or genotype). Co-evolution between host and
307 parasite, also known as the "Red queen dynamic" (Lively and Morran 2014; Brockhurst et al.
308 2014), is believed to maintain additive genetic variation in host resistance and parasite
309 virulence, by creating a co-evolution cycle implying host's resistance alleles and pathogen's
310 virulence alleles (Balenger and Zuk 2014). Moreover, this idea is consistent with the fact that
311 genotype-by-environment interactions are believed to have a role in sexual selection and
312 could facilitate the maintenance of variation in secondary sexual traits (Hanna Kokko and
313 Heubel 2008; Ingleby, Hunt, and Hosken 2010). Here, male resistance will result on the specific
314 interaction between the host genotype and the currently present pathogens. Thus, as male
315 mating success (mediated by sexual traits) is condition dependent, females will favour males
316 that are specifically more resistant to currently prevalent pathogens. This implies that females
317 will benefit from their choice only if their offspring encounters a similar epidemiological
318 context (i.e a similar pathogen pool). Also, male additive genetic variation for pathogen
319 resistance can in that case only be detected by females when males are exposed to infection,

and thus we can expect absence of pathogens to eliminate any positive additive genetic correlation between male attractiveness and resistance. In this hypothesis, that we called the "specific resistance" hypothesis, the epidemiological context in which mating choice is done is crucial, as it will impact the identity of the more sexually successful males.

A particular situation that should also be considered is a case where resistance is not specific, but comes with a strong cost. In absence of pathogen, that cost would come with no benefits, and would thus be a handicap. So here, a positive genetic correlation between resistance and secondary sexual trait would only appear in presence of pathogens. This would make the context important, but only in terms of presence/absence of pathogens, without male and offspring having to be exposed to the same pathogen pool.

3.2 The "general-immunocompetence" hypothesis

The "general-immuncompetence" hypothesis also posits that secondary sexual traits are signals for the general condition of the individual, which is here directly determined by the individual's genotype. Condition will here determine the individual general level of immunocompetence, on which will depend resistance to a broad range of pathogens. This implies that males of a better condition will be more resistant in general, and will exhibit more developed secondary sexual traits regardless of the epidemiological context. This means that the identity of the most attractive males is not expected to change in the presence of different pathogen pools, and that females will always benefit from their choice independently of the fact that their offspring and the father do or do not face a similar epidemiological context. Also, as here condition is determined by numerous physiological traits, and is thus mediated

342 by a high number of genes, it represent a large mutational target (Rowe and Houle 1996;
343 Dugand, Tomkins, and Kennington 2019). Secondary sexual traits should thus capture, through
344 condition, an important part of the genetic variation for fitness that is in this case maintained
345 through mutation-selection balance. And here, variation cannot be maintained through co-
346 evolution, as general-immunocompetence does not lead to any co-evolution cycle with
347 pathogens.

348 The relative importance of the "specific resistance" and the "general-immunocompetence"
349 hypotheses in sexual selection is unknown (Zuk and Wedell 2014). The key distinction between
350 these hypotheses is the importance of the epidemiological context on the genetic correlation
351 between male attractiveness and resistance to pathogen. Under the "specific resistance"
352 hypothesis, variation in male attractiveness should capture genetic variation in resistance
353 specific to currently present pathogens (i.e to which males have been exposed). In absence of
354 pathogens, there should be no positive correlation between male attractiveness and either
355 male resistance, offspring resistance or both. Thus, females should only benefit from their
356 choice through offspring resistance when both the father and the offspring are exposed to
357 similar pathogens. However, under the "general-immunocompetence" hypothesis, male
358 attractiveness is expected to always be positively correlated with his resistance and the one
359 of his offspring.

360

361 *4. How to test the importance of these two hypotheses*

362 Variance in male resistance on which female choice is based might not necessarily be additive
363 genetic variance. It could come from the fact that, for example, some males may be either

364 homozygous or heterozygous for some loci that are linked to resistance. In that case, there
365 can be no direct transmission of resistance to the offspring, or at least not only depending on
366 the male's resistance status. For this reason, it is essential to investigate not only the effect of
367 male resistance on female mating choice, but also the impact these factors have on offspring
368 resistance. And it is also crucial, in order to test the importance of both the "specific
369 resistance" and the "general-immunocompetence" hypotheses, to control the
370 epidemiological context. While under the "general-immunocompetence" hypothesis the
371 epidemiological context has no importance, under the "specific resistance" hypothesis,
372 weather males are or are not exposed to pathogens during mating choice might lead to
373 different conclusions on not only the existence but the sign of the genetic correlation between
374 male attractiveness and offspring resistance. The specificity of the resistance might imply that
375 in absence of infection with the targeted pathogen, there might be no more reliability of
376 secondary sexual traits as a signal for resistance.

377 Investigating the potential role of resistance to pathogens in mating choice and the relative
378 importance of both the "general-immunocopetence" and the "specific resistance" hypotheses
379 is relevant for understanding to consequences of sexual selection. It also addresses the
380 questions of the maintenance of mating choice and male genetic variation, and of selection
381 for resistance. Under the "general-immunocompetence" hypothesis, sexual selection will
382 select for general condition, which is mediated by many traits and depends on mutation-
383 selection balance. Thus, sexual selection will help purging deleterious mutations. Here, both
384 resistance and attractiveness (i.e, secondary sexual traits) will be positively selected, as a
385 consequence of being directly dependent on condition. Also, female choice will always be
386 adaptive, regardless on fluctuations in the currently present pathogen pool. However, under

387 the “specific resistance” hypothesis, female choice could be maladaptive, in cases where
388 males and offspring do not encounter the same pathogen pool. But in a more stable
389 environment, sexual selection could select directly for resistance, and thus increase the
390 response to the appearance of new pathogens. Also, under this hypothesis, maintenance of
391 the genetic variation will not be based on mutation-selection balance, but on changes in the
392 pathogen pool. Getting a better understanding on the relative influence of both hypotheses
393 should bring relevant insights on the mechanisms leading to selection and evolution of
394 resistance to pathogens, mating choice, and the maintenance of genetic variation in males’
395 secondary sexual traits.

396 Testing for the differences between the two hypotheses requires looking at the genetic
397 correlation between attractiveness and resistance in both presence and absence of
398 pathogens. However, so far most studies investigating the role of pathogens and pathogen
399 resistance in sexual selection have been based on the “general-immunocompetence”
400 hypothesis, as the alternative implying “specific-resistance” has not been experimentally
401 addressed. The relationship between male attractiveness, pathogens and offspring resistance
402 have been experimentally studied in several ways and models, with various results.

403 As mentioned before, for offspring resistance to be predicted by male attractiveness, there
404 must be a genetic correlation between secondary sexual traits and resistance. There are quite
405 a few studies that have investigate the idea that male attractiveness can predict offspring
406 resistance, in the sense that more attractive male should sire offspring more resistant to
407 pathogens. Some only looked at the relationship between male attractiveness and their own
408 resistance to pathogen or parasites, without investigating offspring resistance, so the additive
409 genetic correlation between attractiveness and resistance was not investigated (Kennedy et

410 al. 1987; Martin and Johnsen 2007). In *Drosophila melanogaster*, experimental evolution
411 studies have again shown a relationship between resistance and mating success, but with
412 opposed results. Rolff and Kraaijeveld (2003) found a positive correlation between *D.*
413 *melanogaster* males' parasitoid-resistance and their mating success. In a study involving
414 experimental evolution, McKean and Nunney (2008) found that males from lines with an
415 increased sexual selection, which were more competitive than control males, had reduced
416 immune functions. Still, there has been no investigation on offspring resistance and its
417 relationship with sire attractiveness. Other studies have however tackled this question.
418 Offspring resistance and growth was shown to be linked to father's ornaments in sticklebacks,
419 as offspring from brighter fathers were more resistant but grew more slowly (Barber et al.
420 2001). Yet, fathers' pathogenic status was not investigated. Raveh et al. (2014) showed in mice
421 that offspring sired by preferred males exhibit a higher tolerance to pathogen injection, but
422 female preference was only measured in the absence of pathogens, and in this case
423 preference seems to be linked to MHC compatibility. No relationship was found between
424 offspring immunocompetence and father attractiveness (here nuptial gift quality) in the
425 scorpion fly *Panorpa vulgaris* (Joachim Kurtz 2007). Similarly, no relationship between male
426 mating success and offspring resistance after infection with *Pseudomonas aeruginosa* has
427 been found in *Drosophila melanogaster* (Guncay et al. 2017). A positive genetic correlation
428 between a secondary sexual trait (beak color) and immunity was found in the Zebra fish using
429 a large breeding design (Birkhead et al. 2006). In lizard, male throat coloration have also been
430 shown to be genetically positively correlated with immunity. However, a negative genetic
431 correlation between some aspects of the courtship song and immunity was shown in crickets
432 (Simmons, Tinghitella, and Zuk 2010). Thus, results are so far equivocal. Most of these studies
433 indicate a link between pathogens and mating choice, but the importance of the pathogenic

context in which mating choice is done was not tested. And among all these studies, there are none in which exposure to pathogens or parasites has been experimentally controlled.

Therefore, the relationship between male attractiveness and offspring resistance to a pathogen needs to be studied in situations where males have been either exposed or not exposed to an infection with the same pathogen. An important point to raise is also that even if I have mostly mentioned female choice as the main mechanism determining the identity of the mating male, male-male interactions can also be considered in this study. Traits determining the outcome of these interactions are also likely to be condition-dependent, and thus to be linked to pathogen resistance. As for attractiveness, male success when interacting with competitors may also be genetically linked to resistance. But here again, this relationship could depend on the epidemiological context, and the identity of males outcompeting others might change in regards with currently present pathogens. Testing the importance of epidemiological context in which sexual selection takes place, and thus distinguishing the relative importance of the "specific resistance" and the "general-immunocompetence" hypotheses is a novel approach, and is the main aim of my thesis.

5. Drosophila melanogaster as a model to study sexual selection and pathogen resistance

In this project we used, as model, *Drosophila melanogaster.* This model has been often used in biology studies to investigate evolution and sexual selection (e.g., Promislow et al. 1998; McKean and Nunney 2008; Guncay et al. 2017). In this species, males do not only have developed morphological secondary sexual traits, but they do have evolved a behavioural secondary sexual trait consisting in a complex courtship behaviour that includes several steps

(Greenspan and Ferveur 2000; Immonen and Ritchie 2012). It is known that this particular courtship behaviour has an influence on whether or not a female will accept a male as reproductive partner. Females are, in this species, the deciding sex. This means that ultimately, even if intra-sexual male competition has an importance in sexual selection (Saltz and Foley 2011), it is females that are in control of the identity of their partner (Billeter et al. 2012; Baxter et al. 2018). Besides males' courtship behaviour, females also base their decision on olfactory and visual signals emitted by males (Billeter and Wolfner 2018).

Drosophila melanogaster has also been broadly used as a host model to study host-pathogen, parasite, and parasitoid relationships (Martins et al. 2013; McGonigle et al. 2017; Ye, Chenoweth, and McGraw 2009; Kraaijeveld and Godfray 2008; Vijendravarma, Kraaijeveld, and Godfray 2009; Vijendravarma et al. 2015; Wölfle, Trienens, and Rohlfs 2009). The pathogen we used in this project is *Pseudomonas entomophila*, a gram-negative bacteria, and a natural pathogen of *D. melanogaster* in the wild (Buchon et al. 2009; Vodovar et al. 2005; Liehl et al. 2006). This bacteria is known to be highly virulent for *D. melanogaster*, infecting the gut, and can induce, at high dose, death within 72 hours. Flies get infected with this pathogen by ingestion. We chose to work with this pathogen model as it is convenient to grow and maintain, and can be orally transmitted, which mean that a high number of flies can be infected at the same time. Oral infections with this or other pathogens have already been used in several studies, and are ecologically more relevant than systemic infections (Nehme et al. 2007; Basset et al. 2000; Vodovar et al. 2005; Buchon et al. 2009). Also, even if *P. entomophila* is quite virulent, infected flies can still be manipulated within a few days after infection. Another important point is that there has been evidence for genetic variation in *D. melanogaster* for resistance to *P. entomophila* (Martins et al. 2013; Sleiman et al. 2015).

6. Thesis overview

In this project we studied the importance of the epidemiological context on the genetic correlation between male attractiveness and resistance with three different approaches.

In the first chapter, we investigated the additive genetic correlation between sexual success and resistance by testing the within population relationship between male sexual success and offspring resistance and if this relationship would depend on the epidemiological context. To do this, we performed mating trials in which two males, both either infected or sham treated, were competing for a female. In parallel, offspring from each males was generated and infected, and its resistance was measured. Next, we looked for a correlation between male attractiveness (i.e the outcome of mating trials) and offspring resistance. We found a significant context dependence, as we observed that more attractive males (i.e males that were winners in the mating trials) sire more resistant offspring, but only when mating choice was done in presence in pathogens.

In the second chapter, we also investigate the importance of the epidemiological context in sexual selection, but this time using populations that have been selected for resistance to *P. entomophila*, and their corresponding control populations. We performed mating trials in which two males (one resistant and one control) competed for a female, in situations where both were first either exposed to the pathogen or sham treated. Again, we found evidence for an importance of the pathogenic environment on the direction of mating choice. When males were first exposed to the pathogen, resistant males were more likely to win the competition. But in absence of pathogens, both control and resistant males were as likely to win.

In the third chapter, we used genomic data to investigate the potential genetic differentiation between males whose attractiveness was assessed in different epidemiological context. We also looked at genetic differentiation between post-infection survivors and sham treated flies, and if the level of this genetic differentiation with regard to attractiveness is influenced by the pathogenic environment. To do so, we sampled males from a single population after performing mating trials in both presence and absence of exposure to the pathogen. We pooled together males considered as either “winners” or “losers”, for both situations (with and without pathogen). Then we performed pool-sequencing on each sample, and we aimed to compare allele frequencies of SNPs in each sample. Unfortunately, we were not able to detect SNPs that showed significant differences in allele frequency in regards to the different samples.

The “specific-resistance” model predicts that the genetic correlation between father sexual success and offspring resistance will only be positive when fathers and offspring have been exposed to the same pathogens. However, in the 3 chapters we only used one pathogen, and thus we did not address the question of whether or not our results would have been similar using other pathogens, and even other types of stress. Environmental stress may have an impact on attractiveness in vertebrates (Moore et al. 2016), it is thus reasonable to imagine that our conclusions could be extended to other stress than pathogenic infection. Also, we did not investigate situations where fathers and offspring would have been exposed to different pathogens, so there is a possibility that our results are due to presence versus absence of pathogens, but not to anything linked to specificity. This means that up to that point, the “specific resistance” model is a label, as in our experiments we did not test for resistance specificity. The specificity of resistance is a central point of our theory, and so we

developed a master project in which David Simonin, a master student that I supervised, tested if the additive genetic correlation between male sexual success and offspring resistance would change when males and offspring are not exposed to the same pathogen, but to different ones. Also, he tested if the "specific resistance" model could be extended other environmental stress than pathogens, such as heat shock and starvation. His master report can be found in the appendix 1 of this thesis.

Finally, in a second project that I also supervised, Louaï Maarachli, another master student, investigated the impact of infection on males' courtship behaviour. More precisely, he measured the intensity of courtship as the time spent courting within a precise time frame, and looked if this intensity would change when males are infected with *Pseudomonas entomophila*. As for the previous project, his report has been added as appendix 2 in this thesis.

Chapter 1:

Sexual selection favours good or bad genes for pathogen resistance depending on males' pathogen exposure

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Abstract

Resistance to pathogens is often invoked as an indirect benefit of female choice, but experimental evidence for links between father's sexual success and offspring resistance is scarce and equivocal. Two proposed mechanisms might generate such links. Under the first, heritable resistance to diverse pathogens depends on general immunocompetence; owing to shared condition-dependence, male sexual traits indicate immunocompetence independently of the male's pathogen exposure. In contrast, the Hamilton-Zuk and similar hypotheses posit that sexual traits only reveal heritable resistance if the males have been exposed to the pathogen. The distinction between the two scenarios has been neglected by experimental studies. We show that *Drosophila melanogaster* males that are successful in mating contests (one female with two males) sire sons that are substantially more resistant to the intestinal pathogen *Pseudomonas entomophila* – but only if the males have themselves been exposed to the pathogen before the mating contest. In contrast, sons of males sexually successful in the absence of pathogen exposure are less resistant than sons of unsuccessful males. We

detected no differences in daughters' resistance. Thus, while sexual selection may have considerable consequences for offspring resistance, these consequences may be sex-specific. Furthermore, contrary to the "general immunocompetence" hypothesis, these consequences can be positive or negative depending on the epidemiological context under which sexual selection operates.

Introduction

The "good genes" hypothesis for sexual selection posits that traits enhancing male mating success are indicators that the male carries genetic variants improving non-sexual components of offspring fitness (relative to alternative alleles, i.e., "bad genes")(Hanna Kokko et al. 2003a). In genetic terms, this means a positive correlation between a male's sexual traits and his breeding value for non-sexual fitness components (Hunt et al. 2004; Prokop et al. 2012). One fitness component often invoked in this context is resistance to pathogens and parasites: female preference for costly male display traits is hypothesized to bring indirect genetic benefits in terms of offspring resistance (Adamo and Spiteri 2005; Hamilton and Zuk 1982; Roberts, Buchanan, and Evans 2004; Koch, Josefson, and Hill 2017), and sexual selection is proposed to act in synergy with natural selection for improved resistance (Tomkins et al. 2004; Birkhead et al. 2006) (Here we use resistance in a broad sense of reducing the impact of pathogen exposure on host fitness, including behavioural avoidance, barriers to infection, immune defence and physiological tolerance of infection.) Despite its intellectual appeal and the research effort devoted to it, this idea remains controversial (Prokop et al. 2012; Balenger and Zuk 2014; Zuk and Wedell 2014; Hughes 2015). In particular, very few studies experimentally tested the prediction that more sexually attractive or successful males actually

587 do sire offspring more resistant to pathogens; their results are equivocal. In the three-spined
588 stickleback, offspring of fathers with a stronger ornament (redder belly) became less heavily
589 infected upon experimental exposure to a cestode parasite (Barber et al. 2001). In contrast, in
590 *Drosophila*, survival after a bacterial infection did not differ between offspring of sexually
591 successful versus unsuccessful males (Guncay et al. 2017). Female mice mated to their
592 preferred males did produce offspring more resistant to *Salmonella* than females mated to
593 non-preferred males (Raveh et al. 2014), but this appears mediated by MHC heterozygote
594 advantage (Ilmonen et al. 2007), and thus supports the "compatible genes" hypothesis
595 (Tregenza and Wedell 2000) rather than the "good genes". In trout, offspring survival under
596 conditions favouring opportunistic pathogens was positively correlated with father's melanin
597 ornamentation, but negatively with carotene ornamentation; it is not clear which plays a
598 greater role in female choice (Jacob et al. 2010). No relationship between father's
599 attractiveness and measures of offspring immune response was found in scorpion flies (Kurtz
600 2007; Kurtz and Sauer 1999), whereas in ostrich one of several measures of plumage positively
601 correlated with one of three measures of immune response (Bonato et al. 2013). Similarly
602 mixed results about additive genetic correlations between sexually selected traits and
603 resistance have emerged from quantitative genetic estimates (Birkhead et al. 2006; Simmons,
604 Tinghitella, and Zuk 2010; Svensson, McAdam, and Sinervo 2009; Milinski 2006b; Rantala et
605 al. 2012; Lawniczak et al. 2007) and experimental evolution (Rolff and Kraaijeveld 2003;
606 McKean and Nunney 2008; Hangartner et al. 2015, 2013).

607 The study we report here suggests that those mixed results can be at least in part explained
608 by a distinction between two ways in which a positive correlation between a male's sexual
609 traits and his breeding value for pathogen resistance could be generated. The currently

prevailing view is that variation in pathogen resistance relevant for sexual selection is largely due to general immunocompetence that determines resistance to a broad range of pathogens, and which depends on (or is an aspect of) the individual's physiological condition (Folstad and Karter 1992; Roberts, Buchanan, and Evans 2004; Birkhead et al. 2006). The condition is thought to be heritable because it captures a significant part of genetic variance for fitness maintained by mutation-selection balance and other mechanisms; sexual display traits evolve to be honest signals of condition (Rowe and Houle 1996; Tomkins et al. 2004), and thus of immunocompetence (Hill 2011; Birkhead et al. 2006; Koch, Josefson, and Hill 2017).

An alternative scenario, first proposed by Hamilton and Zuk (Hamilton and Zuk 1982), assumes that variation in resistance is specific to pathogen species or genotypes, which undergo constant turnover; male sexual traits reveal heritable resistance to currently prevalent parasites and pathogens (rather than general immunocompetence). This correlation is generated by differential consequences of pathogen exposure for the health of males with different degrees of resistance, and these health consequences are revealed by sexual display traits (Hamilton and Zuk 1982; Adamo and Spiteri 2005; Eshel and Hamilton 1984; Charlesworth 1988; Howard and Lively 2004; Adamo and Spiteri 2009; Westneat and Birkhead 1998). Thus, male sexual traits only "capture" variation in resistance to pathogens to which the males have been exposed (Westneat and Birkhead 1998). In the absence of any pathogen, resistant males are not expected to be healthier and thus not more sexually attractive or successful (Westneat and Birkhead 1998); they may be less successful if resistance carries a physiological cost (Adamo and Spiteri 2005). Thus, under this "specific resistance" scenario the identity of "good genes" depends on the environmental context; offspring resistance is an

633 indirect benefit of mating choice only if both fathers and offspring are exposed to the same
634 pathogen (Hamilton and Zuk 1982; Adamo and Spiteri 2005).

635 Both these scenarios have been originally invoked in the context of display traits targeted by
636 mate choice, but may apply as well to traits involved in intra-sexual competition for mates, as
637 these traits are also costly and likely condition-dependent, and often are the same traits as
638 those involved in mate choice (Hunt et al. 2009). The relative and absolute importance of
639 these two hypothetical scenarios linking pathogen resistance and sexual selection remains
640 unresolved (Zuk and Wedell 2014). Yet, the predictions about consequences of sexual
641 selection differ between these scenarios in a crucial way. Under the "general
642 immunocompetence" scenario, fathers' sexual success predicts offspring resistance to diverse
643 pathogens irrespective of whether or not the fathers have been exposed to any pathogens
644 (Westneat and Birkhead 1998). In contrast, under the "specific resistance" scenario, sexually
645 successful males sire offspring with higher resistance to a pathogen only if the males have
646 themselves been exposed to the pathogen while they were developing their sexual traits;
647 sexual success in the absence of pathogens does not predict offspring resistance (Westneat
648 and Birkhead 1998).

649 The aim of the present study was to test these distinct predictions. To our knowledge, the
650 distinction has not been experimentally addressed; in none of the experimental studies
651 summarized above were the fathers experimentally exposed to pathogens, although in some
652 (Barber et al. 2001; Jacob et al. 2010; Svensson, McAdam, and Sinervo 2009) they might have
653 been naturally exposed. We tested if sexually successful *Drosophila melanogaster* males sire
654 offspring more resistant to an intestinal pathogen (*Pseudomonas entomophila*) than
655 unsuccessful males, and, crucially, if this depends on whether the males' success is determined

after they have been exposed to the pathogen. This pathogen causes substantial mortality in *Drosophila*, and fly populations harbour natural genetic variation in resistance to this pathogen. This variation has been found associated with differences in ROS production, tendency to lose gut wall integrity and activity of gut repair (Sleiman et al. 2015; Vijendravarma et al. 2015). In contrast, genetically higher resistance to *P. entomophila* does not seem to be mediated by greater expression of antimicrobial peptides or reduced ingestion of the bacteria (Sleiman et al. 2015; Vijendravarma et al. 2015), in spite of flies being able to learn to avoid this pathogen (Babin et al. 2014).

We staged mating contests in which two males (sires) from a single outbred population competed for a female, where either both sires were previously exposed to the pathogen or both were sham-treated. *Drosophila* females have full control over mating, and although the outcome of such contests is affected by male-male agonistic interactions, it contains a large component of female choice (Baxter et al. 2018). Subsequently, we quantified pathogen resistance of offspring sired by these winner and loser males before the infection treatment and the mating contest. This excluded potential non-genetic effects of father's infection or contest outcome on offspring resistance, and prevented potential transmission of the pathogen from infected fathers to offspring. Mean resistance of the offspring was thus an unbiased estimate of the sire's breeding value (his "genetic quality") for that trait (Hunt et al. 2004; Falconer and Mackay 1996), allowing us to test its relationship with attractiveness.

678 **Material and Methods**

679 *(a) Fly maintenance*

680 We used flies from a population collected in 2007 in the canton of Valais, Switzerland, and
681 maintained in the laboratory since at a population size of >1000 adults. Flies used in the
682 experiments were raised at 25°C, relative humidity 55% and 12:12 photoperiod on standard
683 yeast-cornmeal-sugar medium under density of about 250 larvae per bottle with 30 ml of food
684 (controlled by egg counting). Virgin flies of both sexes were collected within 12 h of
685 emergence. Virgin females were maintained in groups in food vials until used in the
686 experiment; their virginity was verified by the absence of larvae. All fly transfers were done
687 under light CO_2 anaesthesia.

688

689 *(b) Father's sexual success and offspring resistance*

690 The design of our main experiment is summarized in Fig. 1. Immediately after being collected,
691 sires were dusted with red or blue powder (Sennelier), then maintained for 72 hours in groups
692 of about 50 in vials with food. Subsequently, each sire was placed with two virgin females in a
693 vial containing 10 ml of food and given 48 hours to mate before being removed for the next
694 step of the experiment. Females were given another 24 hours to lay eggs before being
695 removed from the vials; the vials were then kept until offspring collection.

696 After removal from the mating vials we haphazardly paired a red-dusted and a blue-dusted
697 sire; each sire duo was then subject to either the infection or the sham treatment (described
698 below) for 20 h. After the infection or sham treatment, each sire duo was transferred to a new

699 food vial divided by a removable longitudinal partition (Supplementary Figure 1). The sires
700 were placed on one side of the partition and a virgin female on the other side; they were
701 maintained so overnight to let them habituate and the CO_2 effect wear off. The next morning
702 (40 h after the beginning of the infection or sham treatment), we removed the partition,
703 bringing the two sexes together. We observed the flies until the first mating occurred; the
704 male that mated was defined as the "winner" and its less successful counterpart the "loser".
705 Replicates in which no mating occurred within 2 h or in which one or both males were dead
706 before the mating contest were discarded. Where mating occurred, flies were retained in the
707 vial and the survival of "winner" and "loser" males until 72 h post-infection was recorded.

708 To assess resistance of the offspring, 17 days after initial mating (4-6 days after adult eclosion)
709 we collected 10 female and 10 male offspring per sire. The offspring were orally infected (in
710 single sex groups) as described below and subsequently transferred to food vials; the number
711 of dead and alive flies was scored at 24, 48 and 72 hours from beginning of the infection
712 treatment.

713 This entire experiment was performed in three blocks spread over several months. Per block
714 and infection/sham treatment we assessed the resistance of offspring of five winner-loser
715 duos (3 blocks × 2 treatments × 5 duos × winner and loser × 2 sexes × 10 offspring = 1200
716 offspring in total). The design was paired in that we compared offspring of winner and loser
717 from the same duo, i.e., two sires that directly competed with each other (see section 2e). If
718 either sire of a duo failed to produce enough offspring, the entire duo was discarded to avoid
719 a sampling bias. To obtain this number of replicates, many more mating contests were set to
720 allow for sire mortality prior to contest, unresolved contests (i.e., no mating) and insufficient
721 number of offspring (i.e., fewer than 10 offspring of each sex for either sire of a winner/loser

pair). Thus, the number of replicate duos whose offspring's resistance was assessed was smaller than the total number of mating contests.

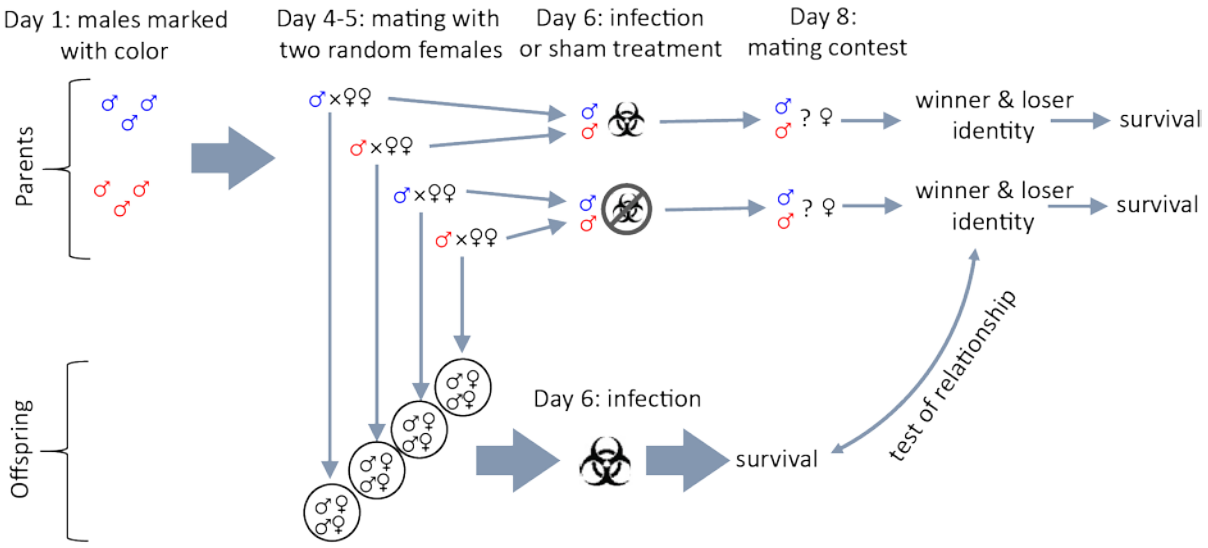


Figure 1. The design of the experiment to study the relationship between a sire's sexual success and his breeding value for resistance to *P. entomophila*. For explanations see Methods.

(c) Bacterial culture and infection protocol

As the experimental pathogen we used *Pseudomonas entomophila*, a gram-negative bacterium originally isolated from *D. melanogaster,* which is virulent upon intestinal infection at sufficiently high doses (Vijendravarma et al. 2015; Vodovar et al. 2005). The *Pseudomonas entomophila* strain was originally provided by Bruno Lemaitre (Vodovar et al. 2005) and maintained at –80°C. Cultures were first initiated on solid medium (triptone, yeast, NaCL, agar and 5% milk). Milk was added to screen colonies for protease activity, which is a marker of virulence and which will form a pale halo around the colony. A single colony from the plate was used to initiate culture in 50 ml of liquid medium (with the same composition as the solid

737 media but without agar and milk) for 24 hours at 28.5°C on a shaker at 190 rpm. The 50 ml of
738 culture were then transferred into 200 ml of fresh medium and kept in the same conditions
739 for another 24 hours. The content was subsequently centrifuged for 20 minutes at 4°C and
740 3000 rpm. The pellet was resuspended in 0.9% NaCl solution to the optical density (OD) of 200
741 at 600 nm. For infection of the sires and their male offspring, the final bacterial suspension
742 was obtained by adding the same volume of a 5% sucrose solution, reducing the final OD to
743 100. The same bacterial concentration was used to infect the female offspring in the first
744 experimental block; however, it resulted in over 90 % mortality for daughters of all sire
745 categories. Aiming to reduce mortality and thus to increase the resolution of potential
746 differences in daughter resistance, for the remaining two experimental blocks we halved the
747 final concentration used to infect female offspring to OD 50. The infectious suspension was
748 always prepared on the day when the flies were to be infected.

749 Prior to infection flies were first starved for 2 hours in empty vials to increase their
750 consumption of bacteria. For the infection treatment, the flies were transferred to vials with
751 a filter paper disc soaked with 100 µl of bacterial mix placed on top of agarose and left there
752 for 20 hours. Subsequently, they were transferred to vials with food and monitored for
753 survival until 72 h from the onset of infection. Based on previous studies (Bou Sleiman et al.
754 2015; Vijendravarma et al. 2015; Vodovar et al. 2005), comparing survival at 72 h post-
755 infection offers good resolution of differences between treatments in resistance to *P.*
756 *entomophila*. For the sham treatment, sires were manipulated in the same way as sires in the
757 infection treatment except that the paper disk was infused with 100 µl of 50:50 mixture of 0.9
758 % NaCl and 5 % sucrose.

759

(d) Infection and the ability to mate

In order to verify if our infection treatment impaired males' ability to mate in the absence of male-male competition or mate choice, in a separate experiment we performed mating trials that excluded these factors. Virgin males (raised and handled as in the main experiment) were either infected with *P. entomophila* or sham-treated as described above. Thereafter a single male and a virgin female were placed on opposite sides of a vial divided by a partition, as in mating contests described above and left to habituate overnight. The next day, the partition was removed and the mating trial started and we scored whether mating occurred within the 2 h period. Replicates in which the male was dead or immobile before the trial were discarded, leaving 29 males in the infection treatment and 50 in the sham treatment.

(e) Statistical analysis

All statistical analyses were performed using R (version 3.5.1) and the RStudio plugin (version 1.1.463). Colour of the powder used to mark males had no detectable effect on their probability of winning (p = 0.37, binomial test), in agreement with our previous unpublished results. We focused on offspring resistance in terms of the likelihood of surviving 72 h post-infection. Using survival until 48 h post-infection led to the same conclusions; statistics for both time points are reported in Supplementary Table S1. With offspring survival as the binary response variable, we used the glmer function of R package *lme4* to fit generalized mixed models with logit link and binomial error distribution. Mating outcome (winner or loser), treatment (infection or sham) and offspring sex (where both sexes were analysed together) were the fixed effects. The main unit of replication – winner-loser duo – was included as a

782 random explanatory variable; block was also treated as a random variable (an alternative
783 analysis with block treated as a fixed factor resulted in the same conclusions). To test directly
784 if survival odds ratios differed between sons and daughters of sires of the two treatments, we
785 also fitted generalized mixed models separately for infected and sham-treated sires and
786 tested for the interaction between contest outcome and offspring sex with the likelihood ratio
787 test. Marginal means were estimated with *emmeans*; pairwise contrasts were performed with
788 pairs function of the *emmeans* package. A further analysis was performed with father's
789 success in the mating contest and father survival (as a binary variable: the fathers were either
790 dead or alive after 72 hours) as fixed factors, only including data from the infected treatment.
791 Because the infectious dose used for female offspring in blocks 2 and 3 was reduced compared
792 to block 1 (see above), we repeated all analyses involving female offspring with data from
793 blocks 2 and 3 only. None of the conclusions were affected; thus, we only report the analysis
794 including all the blocks.

795

796 **Results**

797 *(a) Father's sexual success predicts sons' resistance*

798 The relationship between a sire's winning versus losing the mating contest and *P. entomophila*
799 resistance of his offspring depended on offspring sex (contest outcome × sire infection
800 treatment × offspring sex interaction: $\chi^2_1 = 7.4$, $p = 0.0067$, likelihood ratio test, GLMM on
801 probability of surviving 72 h post-infection; for detailed analysis see Supplementary Table
802 S1a). This justified splitting the analysis by offspring sex.

803 The relationship between father's success and pathogen resistance of his male offspring had
804 opposite signs depending on whether or not the contest took place after pathogen exposure
805 (contest outcome × sire infection treatment interaction: $\chi^2_1 = 38.6$, $p < 0.0001$, Supplementary
806 Table S1b). When the fathers were infected prior to the contest, the odds of surviving 72 h
807 post-infection were five times greater for sons of winners than for sons of losers (Fig. 2a,c;
808 odds ratio 5.1, $z = 5.83$, $p < 0.0001$). The opposite was the case for sham-treated sires – here
809 the winners' sons were half as likely to survive infection than losers' sons (Fig. 2a,c; odds ratio
810 0.49, $z = 2.6$, $p = 0.007$). These differences were consistent among three experimental blocks
811 performed weeks apart, despite considerable variation among blocks in overall mortality
812 (Supplementary Figure S2a).

813 In contrast to sons, we did not detect any relationship between the father's winning versus
814 losing the mating contest and his daughters' survival upon infection (contest outcome $\chi^2_1 =$
815 0.02, $p = 0.89$; contest outcome × sire infection $\chi^2_1 = 3.0$, $p = 0.083$). The pattern of survivorship
816 differences did resemble that for sons (Fig 2b), but was not consistent among blocks
817 (Supplementary Figure S2b); odds ratio for daughters of winners versus losers was 1.44 for
818 infected sires ($z = 1.14$, $p = 0.25$) and 0.65 for sham-treated sires ($z = 1.35$, $p = 0.18$). Daughters
819 suffered higher mortality than sons ($\chi^2_1 = 303.5$, $p < 0.0001$), and this was consistent across
820 the three experimental blocks (Supplementary Figure 2), despite daughters in blocks 2 and 3
821 being infected with a reduced dose of the pathogen (see Methods).

822 To compare these survival odds ratios for daughters with those for sons, we tested for an
823 interaction between mating outcome and offspring sex separately for infected and sham
824 treated sires. Although this test was not significant for sham-treated sires ($\chi^2_1 = 0.5$, $p = 0.48$),
825 it was for infected sires ($\chi^2_1 = 9.8$, $p = 0.0017$). Thus, even if daughters of infected winners

might have been somewhat more resistant than daughters of infected losers, father's success made less difference to their odds of surviving the infection than it did to that of the sons.

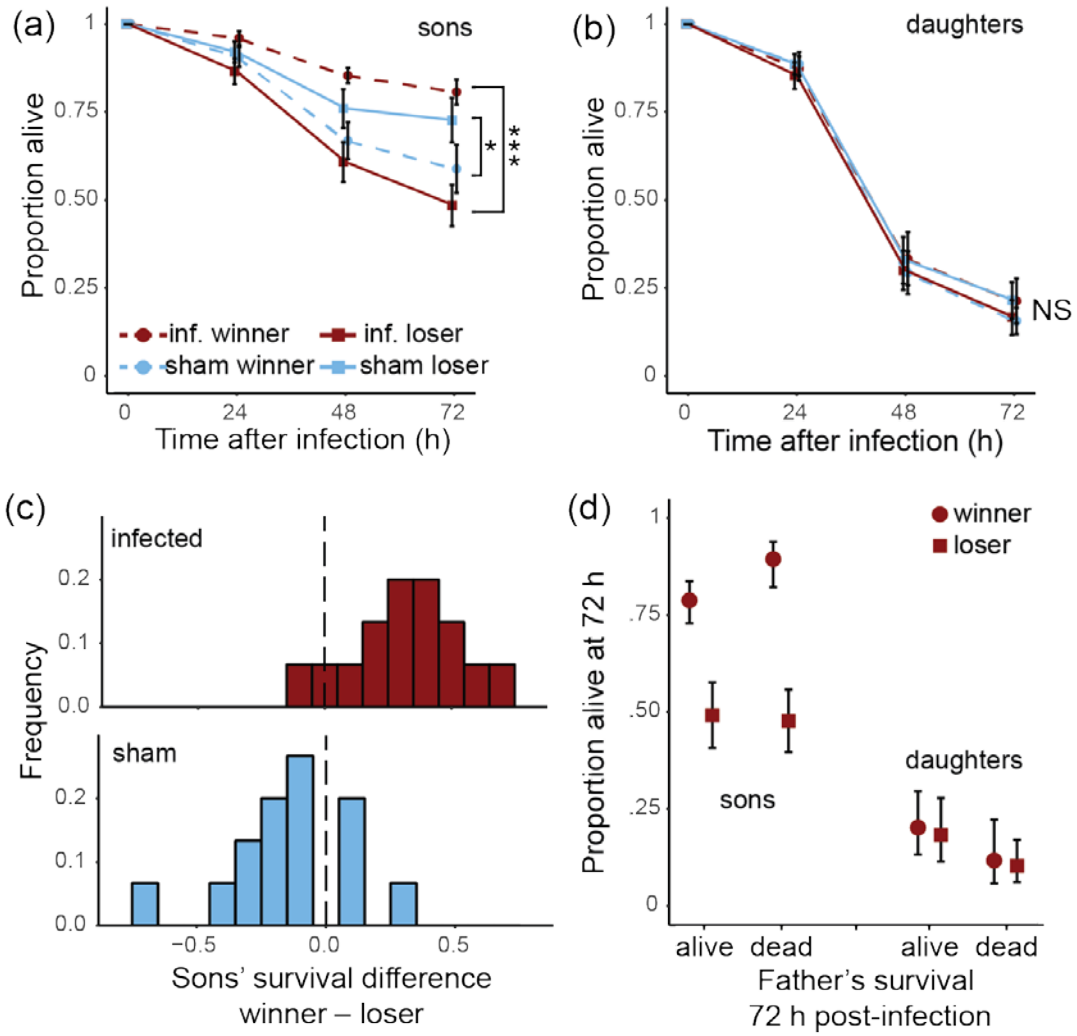
We monitored the survival of sires after the mating contest. Only four out of 50 sham-treated sires died within 72 h. As expected, mortality was higher among infected sires. Infected winners had a higher likelihood than losers of surviving until 30 h after the end of the contest (i.e., 72 h post-infection). Among all replicates in which contest between infected sires was resolved, 26 out of 32 winners and 11 out of 32 losers survived ($p = 0.0003$, Fisher's exact test); for the subset of sires whose offspring resistance was assayed, 13 out of 15 winners and 7 out of 15 losers survived ($p = 0.05$). This demonstrates that, unsurprisingly, fathers that were phenotypically more resistant in terms of mortality were more likely to win the mating contest. However, when father's survival 72 h post-infection was added to the statistical model as a binary explanatory variable, it was not associated with differences in sons' survival upon infection ($\chi^2_1 = 1.2$, $p = 0.26$; Supplementary Table 2). In other words, both among winners and among losers, sires that died had sons as susceptible as the sons of sires that survived the infection (Fig. 2d). This shows that sons' survival upon infection was better predicted by the father's success in the mating contest than by the father's own survival.

(b) Infection does not impair the ability to mate

While the above results are consistent with the "specific resistance" hypothesis, how confident can one be that they are mediated by sexual selection, in particular in the case of infected males? The mating contests took place 40 h after the onset of infection (Fig. 1), when mortality had already started to occur; about 40% of replicates set up for the mating contests

were discarded because at least one of the two males was dead or inactive. One could thus question whether the winner/loser outcome for infected males reflects male-male competition or female choice rather than the losers simply being too morbid to court and mate. Based on qualitative observations, all males involved in the mating contests were active and courted at least some of the time. Furthermore, if a substantial number of infected males had indeed been unable to mate, we should have seen more cases of mating failure during the contest between infected than between sham-treated males. This was not the case; in both treatments about 25% of contests did not produce mating within the 2 h of contest duration (11/43 between infected versus 17/71 between sham-treated, $p = 1.0$, Fisher's exact test).

As a further test of the infected males' ability to mate, we performed a separate experiment in which a single infected or sham-treated male was allowed to interact with two virgin females for 2 h, in the same time frame as in the mating contests. In this setting, the proportion of males that failed to mate was not significantly different between treatments (6/29 = 21% for infected, 16/50 = 32% for sham treated; $p = 0.31$, Fisher's exact test). These results show that, in spite of pathogen virulence, our infection treatment did not impair the males' ability to mate within the time frame of the mating contests. Thus, the outcome of the mating contests can be attributed to the relative sexual competitiveness/attractiveness of the males.



868

869 Figure 2. The relationship between the father's sexual success and offspring resistance to *P.*
870 *entomophila*. (a) Post-infection survival curves of sons and (b) of daughters of sires that won versus
871 lost a mating contest, depending on whether the sires were themselves exposed to the pathogen prior
872 to the mating contest (inf.) or not (sham). (c) The distribution of pairwise differences in the proportion
873 of sons' surving 72 h post infection for each winner/loser sire duo, depending on the sire's treatment.
874 (d) The proportion of offspring of each sex surviving 72 h post infection broken down by sire's
875 winner/loser status and his own survival 72 h post-infection (only for sires subject to the infection
876 treatment prior to mating contest). Symbols in (a), (b) and (d) are means ± SE.

877

878

Discussion

We found that fathers that are more successful in a mating contest sire sons that are more resistant to *P. entomophila* – if the contest takes place after the fathers have been exposed to the pathogen. In contrast, males that win the contest in the absence of *P. entomophila* exposure sire sons that are less resistant to the pathogen. These differences in resistance are manifested, respectively, as five- and two-fold differences in odds of surviving 72 h post-infection. The experimental design allowed us to exclude non-genetic paternal effects of winning versus losing or of pathogen exposure (such as transgenerational immune priming (Roth et al. 2010)) on offspring resistance. Thus, our results are most parsimoniously interpreted as mediated by additive effects of genes passed on by the sires, as postulated under the "good genes" hypothesis.

These results demonstrate that the relationship between male traits under sexual selection and the males' breeding value ("genetic quality") for resistance to a pathogen can depend strongly on the epidemiological context under which competition for mates and mate choice take place. They support the scenario envisioned by Hamilton and Zuk (1982) and Adamo and Spiteri (2009), under which male sexual traits reflect health as determined by their interactions with the pathogen, and thus can only reveal the male's breeding value for resistance if the male has been exposed to the pathogen. They do not support the "general immunocompetence" scenarios, which postulate a positive genetic correlation between sexual success and resistance to pathogens irrespective of pathogen exposure, mediated by shared condition-dependence of sexual traits and immunocompetence (Roberts, Buchanan, and Evans 2004; Birkhead et al. 2006).

This conclusion is consistent with findings in ecological genetics of pathogen resistance in *Drosophila*. If variation in pathogen resistance were mainly mediated by a condition-dependent general immunocompetence, resistance to different pathogens should be highly positively correlated. Yet, in *Drosophila* natural genetic variation in resistance seems largely uncorrelated across different pathogens (Lazzaro, Sackton, and Clark 2006; Martins et al. 2013). Even variation in resistance to the same pathogen may have different genetic bases depending on the route of infection: experimental populations that evolved high resistance to oral infection with *P. entomophila* showed no changes in resistance to systemic infection and vice versa (Martins et al. 2013). Furthermore, flies raised on a nutrient-poor larval diet show similar resistance to *P. entomophila* as flies raised on standard diet, despite being only half the normal body weight (Vijendravarma et al. 2015), suggesting that resistance to this pathogen is largely condition-independent.

Without prior exposure to the pathogen, males that sired more resistant sons were less successful in the mating contests, although the magnitude of the difference was smaller than between the offspring of infected winner and loser males. This is interesting because two independent experimental evolution studies failed to detect any costs of improved *P. entomophila* resistance in terms of larval fitness traits, larval competitive ability, stress resistance or reproductive output (Gupta, Ali, and Prasad 2013; Faria et al. 2015). This suggests that traits under sexual selection are more sensitive to subtle trade-offs of resistance than life history traits under natural selection. Interestingly, the success of an infected father in the mating contest predicted his sons' resistance better than the father's own post-infection survival. Both of these findings are consistent with the notion that sexually selected traits are particularly sensitive to heritable differences in the physiological condition of the organism

924 (Tomkins et al. 2004; Rowe and Houle 1996; Hill 2011) – with the twist that in the absence of
925 pathogens the resistant individuals may actually be in lower condition because of physiological
926 trade-offs of resistance.

927 An unexpected aspect of our results is the apparent sex-specificity of the relationship between
928 father's sexual success and offspring resistance. Although the effects on daughters tended in
929 the same direction as those on sons, they were not significant; the mating outcome × offspring
930 sex interaction indicates that they were significantly smaller (in terms of odds ratio) than on
931 sons. Although not generally the case for *P. entomophila* infections in *D. melanogaster* (Siva-
932 Jothy et al. 2018), in our study females showed a substantially lower post-infection survival
933 than males. Halving the bacterial concentration used to infect daughters (in the last two
934 experimental blocks, see Methods) did little to change this. Possibly, the effect of genes
935 passed on by winner versus loser fathers on offspring resistance vanishes as the overall
936 virulence of the infection increases, which could explain the absence of detectable effects on
937 daughters' survival. Alternatively, alleles that differentiate winners from losers may have truly
938 sex-specific effects on offspring resistance. This possibility is supported by increasing evidence
939 that natural genetic variation may affect pathogen resistance in sex-specific or even sexually
940 antagonistic way (Vincent and Sharp 2014; Roved, Westerdahl, and Hasselquist 2017). Under
941 this interpretation, the indirect genetic benefits of sexual selection in terms of pathogen
942 resistance could be largely limited to male offspring.

943 This study demonstrates that consequences of sexual selection for offspring pathogen
944 resistance can be large and strongly context-dependent. It implies that sexual selection will
945 promote the evolution of pathogen resistance when the pathogen is prevalent in the
946 population, but will oppose it when the pathogen is absent. Females that mate with successful

947 males will benefit in terms of offspring fitness if both generations are exposed to the pathogen
948 (because their offspring will be more resistant) or if both experience no pathogen pressure
949 (because the offspring will be genetically less resistant and thus avoid paying the pleiotropic
950 costs of resistance). However, "good genes" may become "bad genes" if the epidemiological
951 situation changes radically between the generations, as inherent in the Hamilton-Zuk
952 (Hamilton and Zuk 1982; Eshel and Hamilton 1984) and Adamo-Spiteri (Adamo and Spiteri
953 2005, 2009) models. It remains to be tested to what degree sexual selection in the presence
954 of *P. entomophila* affects offspring resistance to other pathogens and vice versa. Nonetheless,
955 it is clear that in this system and under the type of mating competition implemented here,
956 male sexual success is not an unconditional predictor of offspring resistance. The hypothesis
957 that sexually selected traits reveal the breeding value for general immunocompetence
958 independently of pathogen exposure may well still apply to other species and other
959 pathogens. However, our results support the call for a greater experimental effort to test
960 hypotheses assuming that the link between heritable pathogen resistance and sexual traits is
961 generated by interactions of males with specific pathogens (Balenger and Zuk 2014; Zuk and
962 Wedell 2014).

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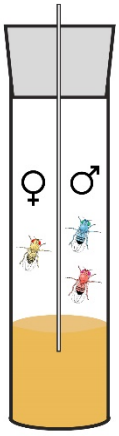
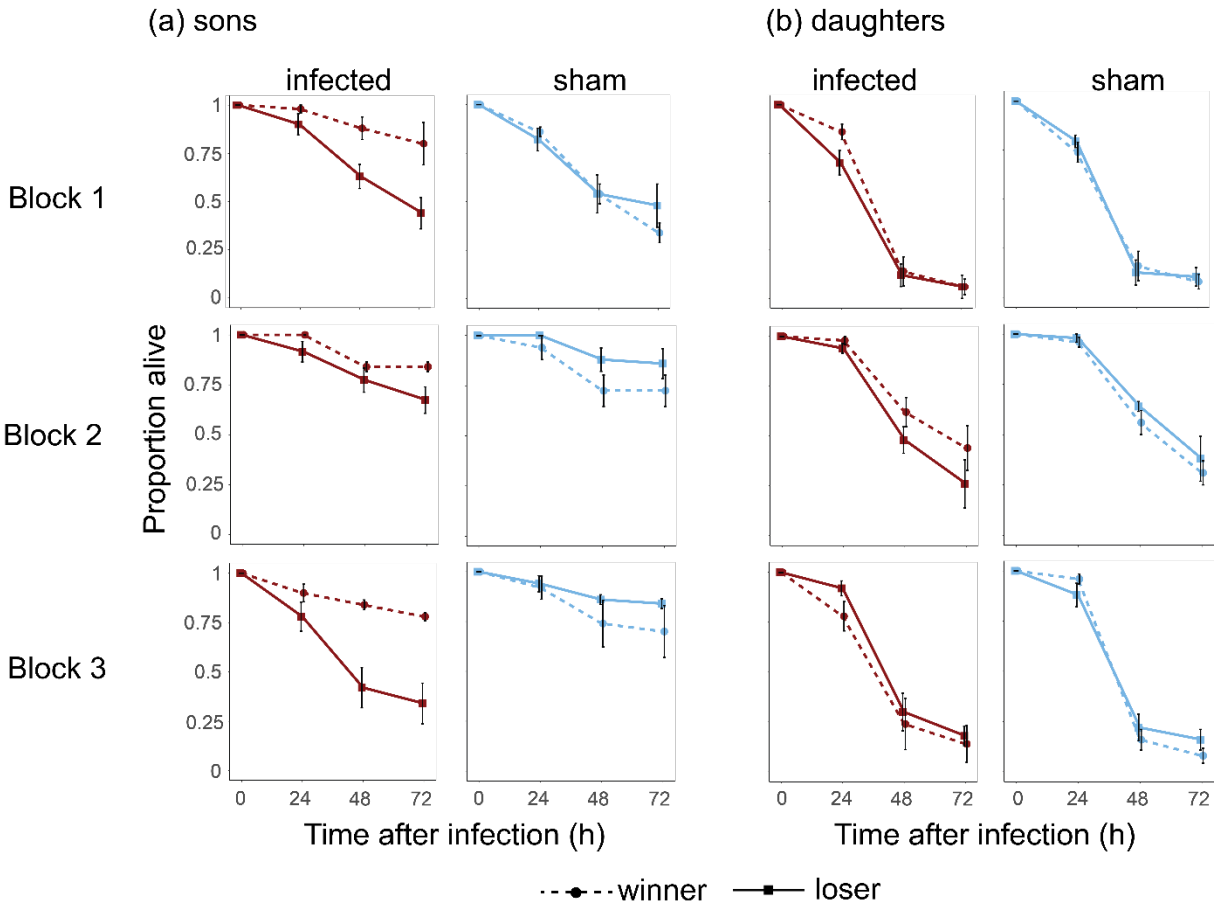
Supplementary material:

Supplementary Figures S1 and S2.

Supplementary Tables S1 and S2.



Supplementary Figure 1. A scheme of the vial used for mating contests. Flies of the two sexes are placed on opposite sides of a cardboard divider and let acclimatize. The assay is initiated when the separator is removed through the slit in the plug.



977

978 Supplementary Figure S2. Post-infection survival curves of offspring of winner and loser fathers (the

979 same data as in figure 2a,b) split by experimental block. (a) sons and (b) daughters. Symbols are

980 means ± SE.

981

Supplementary Table 1. The results of likelihood ratio tests of factors included in the generalized mixed model fitted to offspring survival until 72 h from the beginning of infection.

(a) Joint analysis of offspring of both sexes

Factor	χ^2_1	p
Mating outcome (winner/loser)	2.1	0.14
Treatment (infection/sham)	0.02	0.89
Mating outcome × treatment	28.2	< 0.0001
Offspring sex	303.5	< 0.0001
Mating outcome × offspring sex	3.0	0.081
Treatment × offspring sex	0.1	0.81
Mating outcome × treatment × offspring sex	7.4	0.0067
Block (random)	15.7	< 0.0001

(b) Separate analysis for offspring of each sex

	Sons		Daughters	
Factor	χ^2_1	p	χ^2_1	p
Mating outcome (winner/loser)	5.57	0.018	0.02	0.89

Treatment (infection/sham)	0.01	0.92	0.01	0.96
Mating outcome × treatment	38.61	< 0.0001	3.01	0.083
Block (random)	4.17	0.041	10.19	0.0014

Supplementary Table 2. Likelihood ratio test of father's success versus father's post-infection survival as predictors of offspring survival 72 h post-infection (GLMM with binomial error distribution and logit link). Only sires subject to the infection treatment are included.

	Sons		Daughters	
Factor	χ^2_1	*p*	χ^2_1	*p*
Mating outcome (winner/loser)	33.9	<0.0001	0.1	0.75
Father's survival (dead/alive)	1.2	0.26	2.6	0.10
Mating outcome × father's survival	1.8	0.18	0.0	0.98
Block (random)	4.5	0.033	15.3	< 0.0001

Chapter 2:

Sexual selection and pathogens: context-dependent link between "good genes" for resistance and mating success in *Drosophila*

Patrick Joye, Sakshi Sarda and Tadeusz J. Kawecki

Abstract

A popular version of the “good genes” hypothesis stipulates a positive genetic correlation between traits that enhance male mating success and resistance to pathogens and parasites. Empirical support for this idea is unconvincing. Using *Drosophila melanogaster* and its pathogen *Pseudomonas entomophila*, we demonstrate such a positive genetic correlation and show that it is contingent on prior exposure of males to pathogens. We used *Drosophila melanogaster* males from four populations that were highly resistant to *P. entomophila* as a result of selection and from four populations with typical levels of susceptibility. We let these genetically resistant and susceptible males compete for females, whereby both males had been either exposed to the pathogen or sham-treated. With prior pathogen exposure the resistant males were more likely to mate first than susceptible males, revealing a positive genetic correlation between mating success and resistance. In contrast, the competitive mating success of resistant and susceptible males did not differ in the absence of pathogen exposure. Nearly all pathogen-exposed males mated rapidly in a non-competitive setting; thus, the results competitive trials reflect sexual selection. Our results contradict the notion

1016 that genetically more resistant males should be more sexually successful irrespective of
1017 pathogen exposure. Rather, they support an alternative scenario where males' sexual traits
1018 reflect their resistance and tolerance to currently present pathogens; implying that positive
1019 genetic correlation between sexual traits and pathogen resistance is generated by pathogen
1020 exposure. Thus, whether sexual selection promotes pathogen resistance or not may depend
1021 on the epidemiological context.

1022

1023 **Introduction**

1024 The “good genes“ hypothesis for sexual selection stipulates that males with more pronounced
1025 secondary sexual traits also carry alleles that on average improve non-sexual aspects of fitness
1026 (Kokko et al. 2003; Prokop et al. 2012). An aspect of fitness hypothesized to play a particularly
1027 important role in sexual selection is resistance to pathogens and parasites (Hamilton and Zuk
1028 1982; Roberts et al. 2004; Adamo and Spiteri 2005). Thus, applied to pathogen resistance the
1029 "good genes" hypothesis predicts a positive additive genetic correlation between pathogen
1030 resistance and secondary sexual traits that enhance male mating success (Hunt et al. 2004).
1031 Evidence in support of this prediction is at best mixed (Barber et al. 2001; Birkhead et al. 2006;
1032 Kurtz 2007; Svensson et al. 2009; Simmons et al. 2010; Bonato et al. 2013; Raveh et al. 2014;
1033 Guncay et al. 2017). Here we report an experiment that indicates that genetic correlation
1034 between male mating success and resistance to pathogens may be contingent on
1035 epidemiological context in which sexual selection operates, a factor largely overlooked in
1036 previous experimental tests of the above prediction.

1037 The theory underlying the "good genes" hypothesis generally assumes that variation in male
1038 secondary sexual traits reflects variation in male "condition", which is broadly (if vaguely)
1039 defined as the general health and vigour, physiological robustness and the amount of
1040 metabolic reserves (Pomiankowski 1987; Iwasa and Pomiankowski 1994; Rowe and Houle
1041 1996; Westneat and Birkhead 1998; Hill 2011). However, different versions of the theory
1042 postulate two broadly different mechanisms to generate the positive genetic correlation
1043 between secondary sexual traits and pathogen resistance (Westneat and Birkhead 1998).

1044 Under the first mechanism, resistance to a large variety of pathogens is mediated by general
1045 immunocompetence, which in turn directly depends on condition. Condition in turn depends
1046 on many underlying traits and thus presents a large mutational target, capturing much of
1047 genetic variation segregating in the population. Thus, under this "general
1048 immunocompetence" scenario, the positive genetic correlation between secondary sexual
1049 traits and pathogen resistance results from their mutual dependence on genetically variable
1050 condition (Rowe and Houle 1996; Tomkins et al. 2004).

1051 Under the second mechanism, pathogen resistance is determined by the interaction between
1052 the host's genotype and the specific pathogen, with different genetic variants conferring
1053 resistance to different pathogen species and strains. In this view, resistance is not mediated
1054 by condition. Rather, male condition, and thus the expression of secondary sexual traits, is
1055 strongly affected by the outcome of the interaction between the currently or recently
1056 prevalent pathogens and the genetic resistance profile of the male, i.e., whether the male has
1057 been infected and how sick he has become (Hamilton and Zuk 1982; Read 1988; Westneat and
1058 Birkhead 1998; Adamo and Spiteri 2005). This "resistance-dependent condition" model thus

emphasizes genotype-environment interactions and posits that variation in condition, and thus in sexual traits, captures the variation in resistance to the current pathogen pressure.

These two mechanisms are not mutually exclusive and both could contribute to genetic correlation between pathogen resistance and male secondary sexual traits. However, a key difference between those two models is the role of the epidemiological context in the expression of this genetic correlation, leading to contrasting predictions. Under the "general immunocompetence" scenario male sexual success is predicted to be positively genetically correlated with resistance regardless of whether or not the males have been exposed to pathogens. On the contrary, under the “specific resistance" model a positive genetic correlation between male mating success and resistance to a pathogen only becomes expressed if the males are exposed to the pathogens as they are developing the traits that determine their mating success. In the absence of pathogen exposure no such positive correlation is predicted (because the resistance variants bring no benefit to condition); it can even be negative if the resistance is costly (Westneat and Birkhead 1998; Zuk and Wedell 2014; Joye and Kawecki 2019).

A recent study has provided experimental support for this second scenario in the fruit fly *Drosophila melanogaster* in the context of infection with the intestinal bacterial pathogen *Pseudomonas entomophila* (Joye and Kawecki 2019). Using a breeding design, it has shown that males with a higher breeding value for resistance to the pathogen are more likely to "win" a mating contest (i.e., mate first in a 2 males + 1 female setting), but only when both males have been exposed to the pathogen prior to the mating contest (Joye and Kawecki 2019). When males have not been exposed to the pathogen, those with a higher breeding value for resistance are more likely to "lose" the mating contest. Thus, the sign of the additive genetic

correlation between resistance to *P. entomophila* and mating success in that experiment was reversed depending on the epidemiological context in which the mating success was assessed.

In the present study we use a complementary approach, based on correlated responses to selection (Falconer and Mackay 1996), to test if there is a genetic correlation between male mating success and pathogen resistance, and if this correlation depends on the epidemiological context under which mating success is evaluated. We used four *Drosophila melanogaster* populations that evolved high resistance to *P. entomophila* as a result of laboratory selection and four corresponding unselected control populations that show a typical degree of susceptibility to the pathogen (Martins et al. 2013); hereafter we refer to these populations as "resistant" and "susceptible", respectively. No trade-offs of the improved resistance in life history traits, larval competitive ability, and stress resistance have been detected, and the resistant population have retained a high level resistance over several years after the selection had ceased (Faria et al. 2015; Kawecki 2019).

We first confirmed that males from the "susceptible" and "resistant" populations still differ in resistance to infection, and verified that the infection treatment does not render males incapable of mating. Then, in the main experiment, we performed mating contests where males from the resistant and susceptible populations competed for females, with both males either previously exposed to the pathogen or subject to a sham treatment. The first male to mate was scored as the "winner". Under the “general immunocompetence” scenario, the resistant males were predicted to have a higher success in the mating contests than susceptible males regardless of prior pathogen exposure. By contrast, the “specific resistance” scenario predicted a higher mating success of resistant than susceptible males only in the context of exposure to *P. entomophila*; in the absence of exposure susceptible males should

be as successful, or even more successful than resistant males. Finally, if neither version of the "good genes" hypothesis applies in this system, resistant males should have had no mating advantage irrespective of pathogen exposure.

Material and methods

Fly origin and maintenance

Males used in this experiment were collected from populations originally described by Martins et al. (2013; see there for detailed information). All were derived from a population collected in the wild in Portugal. Four populations were subject to experimental selection for resistance against intestinal infection by *Pseudomonas entomophila* (labelled BactOral populations in Martins et al. 2013 and referred to here as "resistant"). Briefly, each generation adult flies of both sexes were exposed to food containing *P. entomophila* at a dose causing substantial mortality, and the next generation was bred from the survivors. In parallel, four control ("susceptible") populations were subject to a similar manipulation, but with a sham infection treatment. The resistant and susceptible populations have been maintained for many generations in our lab without any particular selection regime.

Females used in this study were from a population collected in 2007 in the canton of Valais, Switzerland, and maintained in the laboratory since then, with a population size of more than 1000 adults. We used females from a totally different population, unrelated to the resistant and susceptible populations, because females from the resistant or susceptible populations might show a biased preference for males of their own population.

All flies used in this study were kept in controlled conditions (25°C, 55% of humidity, 12:12 LD cycle), in bottles filled with 30 ml of standard fly food (cornmeal, sugar, yeast and agar). Density was maintained at approximatively 250 larvae per bottle. Flies were all collected as virgins less than 12 hours after emergence, and kept in bottles of about 50 individuals of the same sex until used in the experiment. All fly manipulations were performed under CO_2 anaesthesia.

Bacterial culture

Pseudomonas entomophila is a gram-negative bacteria and, at high doses, a virulent natural intestinal pathogen of *D. melanogaster*. The pathogen induces a strong immune response in the gut and is mostly cleared within 24 h; most mortality occurs between 24 and 72 h post-infection, largely as a consequence of the loss of gut integrity (Vodovar et al. 2005; Chakrabarti et al. 2012; Vijendravarma et al. 2015). The *P.entomophila* strain was provided by Bruno Lemaître (Vodovar et al. 2005), who also provided the strain used to impose selection (Martins et al. 2013), and kept at -80°C. *P. entomophila* was first cultured on solid LB medium, supplemented with 5% of skim milk in order to screen colonies for protease activity, a marker of virulence (Rondon et al. 2000). A single colony was selected from the agar plate and transferred to 50ml liquid LB media, incubated for 24 h at 28.5°C and shaken at 190 rpm, then mixed with 200ml fresh LB media and incubated for another 24 h under the same conditions.

To collect the bacteria for infection, we centrifuged the culture for 20 minutes at 4°C and 3000 rpm. The pellet was diluted using 0.9% NaCl solution until reaching an OD_{600} (optical density at 600 nm) of 200. To increase the nutrition value of the pellet suspension and to induce the

bacterial consumption by the flies, 5% sucrose solution was added to the pellet suspension and the final concentration of the bacteria was OD_{600} 100.

Oral infection protocol

Oral infections were always performed the same day as bacterial suspension was collected. Flies were first starved for 2 hours in empty vials, to increase their consumption of bacteria and thus the infection efficiency. Flies were then placed in vials with agarose gel, on top of which we placed a filter paper disc soaked with 100 µl of bacterial mix (or 0.9% NaCl solution for sham treatments). After 20 hours, flies were transferred to fresh food vials.

Survival after infection

To test the effectiveness of the infection and to verify that the resistant population were indeed still resistant even though selection had been discontinued several years earlier, we compared resistance of males from the two sets of populations to *P. entomophila*. 2-day old virgin males were subject to the infection protocol described above in groups of 10 in a single vial (two replicates for each of the four resistant and four susceptible populations). Their survival was monitored over 72 hours from the onset of the infection treatment.

Mating ability of infected males

The interpretation of the mating contests described below rests on the assumption that they are determined by sexual selection, i.e., by female choice and/or male-male interference, also in the case where the males are subject to the infection treatment. This interpretation would be undermined if the infection rendered a substantial proportion of males too sick to be able to mate. To verify that this is not the case, we tested the ability of infected males to mate when presented by ample opportunity to mate (three virgin females) in the absence of competing males.

Five to six days old virgin males from the resistant and susceptible populations were subject to the infection treatment as described above. Twenty hours from the onset of the infection treatment the males were transferred singly to vials with regular food, partitioned in half by a cardboard separator. Three 5 days old virgin females were introduced on the other side of the partition and the flies were allowed to acclimatize overnight. Next morning around 9 a.m. the partitions were removed and the vials were monitored for mating for 2 hours; the time from the partition removal to the first mating in each vial was recorded to the nearest minute. The experiment was performed in two blocks on two consecutive days.

Mating contests

To test the predictions formulated in the introduction we performed mating contests by placing one virgin female with one resistant and one susceptible male, where males were either both previously exposed to the pathogen or both sham treated. The populations were paired (resistant population 1 with susceptible population 1, etc.), i.e., all assays were

performed between one resistant a one susceptible male of the same population pair (1, 2, 3 or 4). After being collected, males were coloured with red or blue powder (Sennelier) to enable identification, and then kept in food vials for 48 hours. Males were then randomly placed in duos (one resistant male and one susceptible male marked with different colours were then haphazardly paired) in vials containing regular food. In half of the pairs, the resistant male was blue, in the other half red. 48 hours later in half of the replicates, both males where subject to the oral infection protocol with *P.entomophila* described above, and the other half was sham-treated. In each case, colour combinations were equally distributed, same for all 4 population pairs. After infection, each pair was transferred to a vial with 10 ml of food. A random female from the Valais population was also transferred to each of those vials, and was kept separated from the males with a piece of cardboard separating the vial in half. Flies were kept in these conditions overnight to acclimate to this new environment and wear off potential effects of CO_2 anaesthesia. The separator was removed the next morning, around 9 a.m., and the vials were monitored for mating. As soon as mating occurred, the identity of the mating male (i.e., resistant versus susceptible, based on colour) was recorded as a “winner”, and the other as a “loser”. If a male died before or during the mating contest or showed abnormally low activity, the replicate was removed from the experiment. The replicate was also removed if no mating was observed within the two hour period. In total, we performed 121 successful mating contests, 71 for the sham treatment, and 50 for the infected treatment. Following the successful trials, the females were discarded, and the males were maintained together in the vial and their survival was checked at 72 h counted from the onset of the infection or sham treatment.

Statistical analysis

All statistical analysis and figures were performed using the software R (version 3.2.2) and the RStudio plugin (version 0.99.489). Survival differences between susceptible and resistant populations 72 h post-infection was analysed with a generalised linear mixed model, with the counts of alive and dead files as a binomial response variable, population resistance status (i.e., resistant or susceptible) as a fixed factor and replicate population as a random factor.

The main interest in the non-competitive mating trials of infected males was to verify if most of them are capable of mating. However, we also used Cox regression to test for differences in time to the first mating (mating latency), with population resistance status as the fixed factor and replicate population and block as random factors. Replicates where no mating occurred within 2 h were treated as censored.

To test if the identity of winner males (i.e., resistant versus susceptible) in the mating contests was influenced by the treatment (i.e., whether or not males have been exposed to pathogens), we did a likelihood ratio test comparing two generalised linear mixed models, fitted using the glmer function of the "lme4" R package, in which the response binary variable was winner males identity. One of the two models also included treatment as a fixed factor, and both models included the colour scheme as a fixed factor. In the model with treatment, the colour scheme × treatment interaction was also included. Both models contained population pair (1-4) as a random factor. We used the "emmeans" R package to perform a contrast analysis to test if resistant winner proportion was different, in each treatment, from 50 %, which is what we would expect if both susceptible and resistant males would have the same success. We also tested the survival of infected males used in mating contests, in two different generalized linear mixed models, one with the population resistant status (i.e., resistant or susceptible) as

1236 the sole fixed factor, and one additionally including mating contest outcome (i.e, winner or
1237 loser), and its interaction with population resistance status as fixed factors.

1238

1239 **Results**

1240 *Survival and mating ability of infected males*

1241 Post-infection survival assays showed that resistant populations were indeed still resistant, as
1242 73.8 % (SE: 0.049) of resistant flies were still alive 72 h after infection, versus 18.8 % (SE: 0.043)
1243 for susceptible flies (Fig 1A; GLMM, $\chi^2_1 = 27.8$, $p < 0.001$).

1244 A substantial proportion of infected males, in particular those from the susceptible
1245 populations, died prior to the non-competitive mating assay (48% in susceptible populations,
1246 and 22% in control populations), but most of those that survived mated within an hour.
1247 Resistant males mated sooner on average than susceptible males (Fig. 1B; median mating
1248 latency 15 versus 22 min, Cox regression $\chi^2_1 = 5.9$, $p = 0.015$). Nonetheless, only three males
1249 (1 out of 74 resistant and 2 out of 48 susceptible) failed to mate within 2 h. Thus, nearly all
1250 males subject to the infection treatment that remained alive retained the competence and
1251 motivation to mate.

1252

1253 *Mating contests*

1254 We found that the likelihood of resistant versus susceptible males winning a mating contest
1255 (i.e., mating first) depended on the pathogen context in which the contests took place (Fig 2;
1256 $\chi^2_1 = 10.4$, p = 0.005, LRT). When the males were exposed to *P.entomophila* prior to the trials,

more than 3/4 of contests (38 versus 12) were won by males from the resistant populations, a proportion significantly different from 0.5 (z = 3.2, p = 0,0013, estimated marginal mean 0.79, 95% confidence interval CI = [0.63,0.89]). By contrast, in absence of exposure to pathogens (after the sham treatment), the proportions of resistant and susceptible winners were almost identical, as we observed respectively 37 and 34 winners from the resistant and susceptible populations (z = 0.3, p = 0.80, estimated marginal mean 0.52, 95% CI = [0.38, 0.65]). We found no effect of the colour of the powder used to mark males (χ^2_1 = 0.79, p = 0.37), and no significant interaction between treatment and powder colour (χ^2_1 = 3.25, p = 0.071).

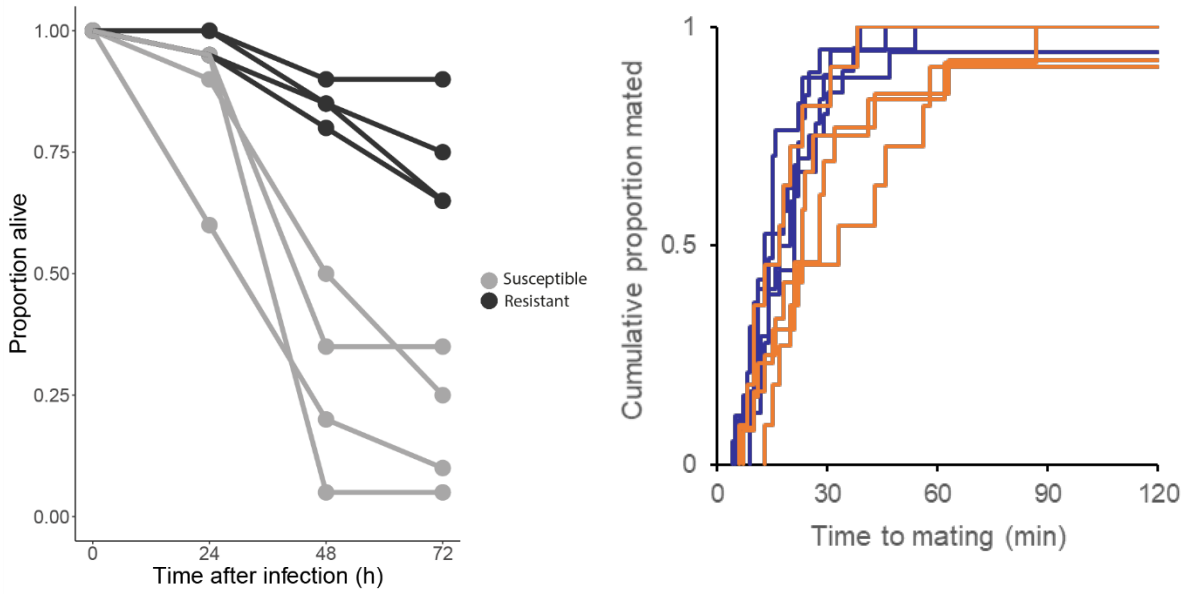


Figure 1: (A) Survival of male flies from the four resistant (dark) and four susceptible (light) populations after oral infection with *Pseudomonas entomophila*. The starting time point (0) corresponds to the onset of the infection treatment. (B) Time to first mating by males subject to oral infection with *P. entomophila* when presented with three virgin females and no competing males. Time = 0 corresponds

to removal of partition separating the sexes; this is 42h after the onset of the infection treatment. *N* = 17-20 per resistant population, 11-13 per susceptible population.

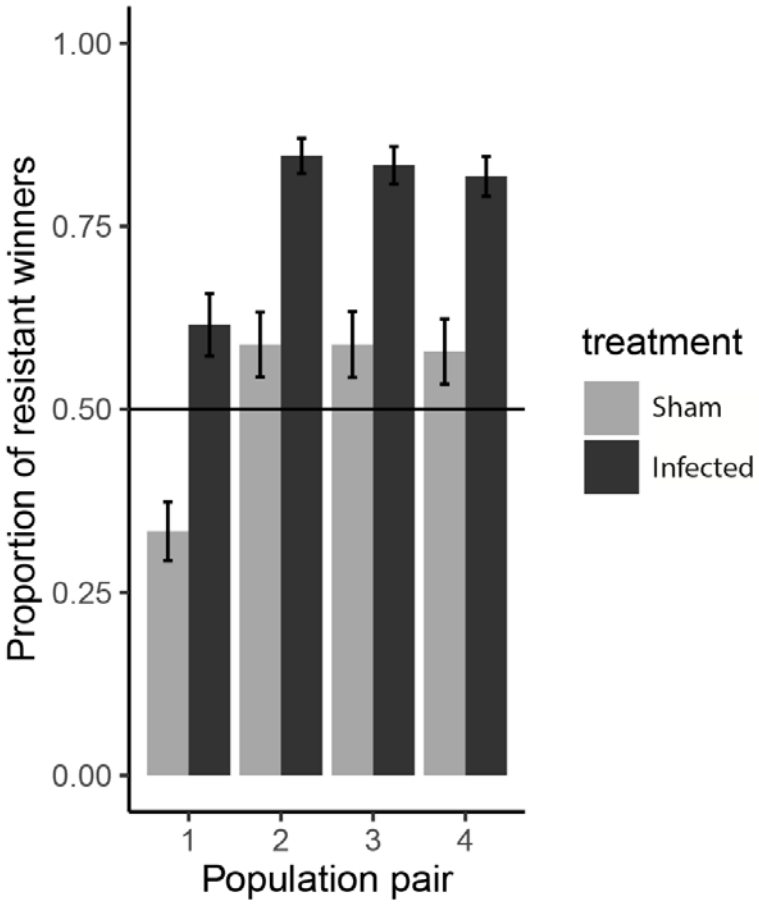


Figure 2: Proportion of resistant males among winners of the mating contests for each population pair and infection treatment. The horizontal line corresponds to equal likelihood of winning for resistant and susceptible males. Error bars represent the standard error.

As expected, we found that resistant males used in mating contests were more likely to be alive 72 hours after infection than susceptible males ($\chi^2_1 = 14.4$, $p < 0.001$, LRT). However, in the statistical model that included both mating contest outcome (i.e., winner or loser) and population resistance status (i.e., resistant or susceptible) as factors, we found that survival after 72h was only explained by mating contest outcome (Fig 3; $\chi^2_1 = 13.2$, $p < 0.001$, LRT), and not anymore by the population type ($\chi^2_1 = 1.3$, $p = 0.26$, LRT). We found no interaction between the two factors ($\chi^2_1 = 0.25$, $p = 0.62$, LRT). Thus, even though pathogen-exposed resistant males were less likely to die and more likely to win the mating contest than pathogen-exposed susceptible males, resistant losers were as likely to die as susceptible losers, and resistant winners as likely to die as susceptible winners.

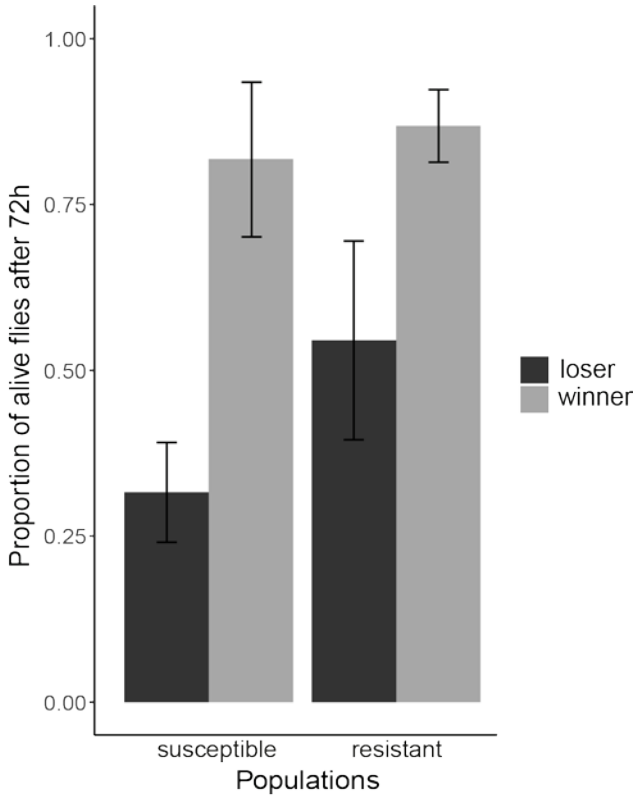


Figure 3: Survival of the infected males used in mating trials at 72 hours after the onset of the infection treatment. Error bars represent standard errors.

Discussion

We performed mating contests between males from *D. melanogaster* populations that differed genetically in terms of resistance to oral infection with *Pseudomonas entomophila* as a result of laboratory selection (Martins et al. 2013). We found that males from the four resistant populations had a higher mating success than males from the susceptible control populations, but only when the males were exposed to the pathogen before mating contests. Without prior pathogen exposure, the resistant and susceptible males were equally likely to win the mating contests (i.e., to be the first to mate with the female). Thus, the relationship between genetically-based difference in resistance and male mating success was contingent upon the pathogen exposure context in which the mating contests took place.

Rather than resulting from female choice and male-male competition, the outcome of mating contests might have been explained by the loser males being morbid to the degree that prevented mating. This was not the case: when presented with ample mating opportunities in the absence of other males, nearly all males form both sets of populations mated within an hour despite being infected. Although the susceptible males were slower in achieving mating, only two (out of 48) infected susceptible males failed to mate within 2 hours, compared to one (out of 74). The poor performance of the susceptible populations in the competitive mating contests between infected males must therefore have been driven by female choice and/or male-male interference. Hence, the dependence of the outcome of the mating contests on the infection treatment can be attributed to context-dependent sexual selection.

This context-dependence of the link between resistance and mating success contradicts the predictions of the "general immunocompetence" version of "good genes" hypothesis, which posits that the link should be expressed irrespective of the males' pathogen exposure. Rather,

1318 the fact that the identity of sexually favoured males depends on the pathogenic context
1319 provides support for the "specific resistance" version of the hypothesis, originally proposed
1320 by Hamilton and Zuk (Hamilton and Zuk 1982; Eshel and Hamilton 1984; Adamo and Spiteri
1321 2005). This hypothesis posits that the interaction between male genotype and the pathogenic
1322 environment generates variation in resistance, which in turn impacts condition and thus male
1323 traits that promote mating success. This implies that positive genetic correlation between
1324 resistance and mating success will only be detectable when pathogens are present, as we have
1325 found.

1326 The detailed mechanisms underlying these results remain unclear. The mating contests we
1327 employed cannot separate female choice from male-male interference. However, females
1328 have a higher degree of control over mating in *Drosophila* (Billeter et al. 2012), and while the
1329 outcome of such trials is affected by male-male interference, it appears largely determined by
1330 female choice based on male sexual traits including the quality and intensity of male courtship
1331 (Baxter et al. 2018). Males have evolved an energetically costly courtship behaviour, which
1332 consist of several complex steps (Greenspan and Ferveur 2000; Immonen and Ritchie 2012),
1333 and is a key secondary sexual trait. Males that are more adversely affected by infection may
1334 have been less able or less motivated to court; other pathogens have been shown to cause
1335 reduction in locomotor activity or time spent asleep in *Drosophila* (Vale and Jardine 2015).
1336 However, the difference between resistant and susceptible males that we observed after
1337 infection may be mediated by other traits, as *Drosophila* females also use other olfactory,
1338 visual and tactile signals in mate choice (Billeter and Wolfner 2018). Irrespective of the
1339 mechanism, the strong relationship we found between the outcome of the mating contest

and the subsequent survival of the male supports the link between mating success and post-infection condition.

This study complements the findings of a previous study that used a breeding design to study additive genetic correlation between resistance to *P. entomophila* and male mating success in within a single population of *D. melanogaster* (Joye and Kawecki 2019). Both studies demonstrate that this correlation is positive if the males have been exposed the pathogen when their mating success is assessed. However, the two studies differ in the outcome in the absence of pathogen: while the present study finds no difference in mating success between genetically resistant and susceptible males, Joye and Kawecki (2019) found that males siring more resistant offspring had a lower mating success. This difference could reflect different experimental approaches (correlated response to selection versus sire-offspring correlation) or different gene pools (originating, respectively, from Portugal and Switzerland). However, there is evidence that, in the absence of pathogen exposure, the resistant populations used in the present study show a reduction in competitive paternity, a measure of male sexual success that combines both pre-copulatory and post-copulatory aspects over several days (Kawecki 2020). Thus, even though we find no evidence for it in the present study, the two previous studies (Joye and Kawecki 2019; Kawecki 2020) suggest that genetically-based resistance to *P. entomophila* has a mild cost in terms of sexual success in the absence of pathogen.

Obviously, the conclusion that a positive genetic correlation between resistance to a pathogens and sexually selected traits is contingent on the exposure to the pathogen can at this stage only be drawn for this one host-pathogen system. However, if the kind of genotype-environment interactions that underlie it are widespread in other systems, that might explain the mixed results of the studies that attempted to test for this genetic correlation. None of

these studies deliberately exposed the males to pathogens or parasites prior to assessment of their secondary sexual traits or mating success. It is suggestive that the strongest evidence for the predicted positive correlation came from studies that were carried out in the field (Svensson et al. 2009), in a large captive breeding colony (Birkhead et al. 2006), or using males directly sourced from nature (Barber et al. 2001). Conversely, studies performed on lab-bred populations found a negative or no correlation (Kurtz and Sauer 1999; Kurtz 2007; Simmons et al. 2010; Guncay et al. 2017). It is tempting to speculate that the latter were effective at excluding pathogens whereas in the former the males were exposed to some pathogens or parasites that had differential impact on condition – and thus on the expression of secondary sexual traits – of males with different degree of resistance.

The distinction between these two mechanisms generating a link between resistance and sexual success is not only important for finding evidence for "good genes", but also affects several aspects of sexual selection. Under the “general immunocompetence” mechanism "good genes" are universal; thus, females should benefit from mating with healthy / high condition males regardless on the epidemiological environment condition their offspring encounter. In contrast, under the “specific resistance” mechanism "good genes" show strong genotype-environment interactions; thus, female preference for healthy males is only beneficial if offspring are exposed to a similar community of pathogens as the males (Hamilton and Zuk 1982). This also means that sexual selection would not favour resistance in absence of pathogens, and might even select against it if resistance is costly (Adamo and Spiteri 2005). In contrast, under "general immunocompetence" sexual selection always favours resistance, even where there are no pathogens around. Finally, the "general immunocompetence" scenario envisions no selective mechanism for the maintenance of genetic variation for

resistance and sexually selected traits; rather, it is assumed to be maintained by mutation pressure (Rowe and Houle 1996; Dugand et al. 2019). In contrast, with the genotype-environment interactions inherent to the "specific resistance", spatial and temporal variation in the pathogen abundance community composition, as well as host-pathogen coevolutionary dynamics, would help maintain genetic variation for resistance and thus for secondary sexual traits, as first proposed by Hamilton and Zuk (1982). Thus, finding out which of the two mechanisms is more important in generating additive genetic correlation between pathogen resistance and secondary sexual traits is highly relevant for understanding of the consequences of sexual selection.

Chapter 3:

The impact of pathogen presence on the genomic differences between individuals of different sexual success levels

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Abstract:

The “good gene” theory in sexual selection stipulates that secondary sexual traits are additively genetically correlated with non-sexual fitness-related traits. Resistance to pathogens is often invoked as one of these traits. Secondary sexual traits are thus believed to capture genetic variation for resistance. Two different scenarios can explain the existence of this genetic correlation, the “general immunocompetence” model and the “specific resistance”. The key distinction between the two models is that in the “specific resistance” one, sexual traits will only capture variation for resistance when sexual selection occurred in presence of the pathogens against which individuals are resistant. In this study, we used pool-sequencing to investigate the level of genomic differentiation (SNPs) between successful and unsuccessful males in different epidemiological contexts, and between resistant and control individuals. Under the “specific resistance” model, we would predict that any genomic differentiation would vary with respect to the epidemiological context, but would be constant under the “general-immunocompetence” model. However, we detected almost no genetic differentiation between the different treatments. This could suggest that the differences are small, but numerous, which could be in support of the “general-immunocompetence” model, in which resistance depends on individual condition, and is thus based on many different

region of the genome. However, the absence of detectable genetic variation prevents us from making any reliable conclusions.

Introduction

In sexual selection, male mating success is based on secondary sexual traits (Kokko et al. 2003). Under the "good gene" theory, these traits are additively genetically correlated with non-sexual aspects of fitness, so with traits that are not directly linked to sexual success. Thus, secondary sexual traits are believed to capture genetic variation in these non-sexual fitness related traits (Hunt et al. 2004; Prokop et al. 2012), meaning that more sexually successful males will sire offspring with higher quality fitness-related traits. One often invoked hypothesis on the nature of these non-sexual aspects is resistance to pathogens and parasites. Pathogens and parasites are an important factor of selection, first of all because they are ubiquitous in nature, and because host-pathogen co-evolution, as turnovers of the pathogen community, are believed to continuously generate additive genetic variation for fitness (Lively and Morran 2014; Brockhurst et al. 2014). Many studies have already investigated the phenotypic relationship between infection or pathogen load and sexual traits (e.g., Hamilton and Zuk 1982; Weatherhead et al. 1993; Liljedal et al. 1999; Brawner et al. 2000; Kortet et al. 2004; Costa and Macedo 2005; Ezenwa and Jolles 2008; Gilbert et al. 2016). However, they do not directly address the question of whether or not this correlation is genetic, which is a crucial point of the "good-genes" theory. If additive genetic variation in pathogen resistance would indeed be captured by secondary sexual traits, we could predict a positive genetic correlation between male attractiveness, thus sexual success, and resistance to pathogens. This prediction has already been experimentally studied, with mixed results (Barber et al. 2001;

Svensson, McAdam, and Sinervo 2009; Birkhead et al. 2006; Bonato et al. 2013; Kurtz 2007; Raveh et al. 2014; Guncay et al. 2017).

Theories about a genetic correlation between resistance and male secondary sexual traits are generally based on the condition-dependence of these sexual traits, condition being considered as the individual general health state (Pomiankowski 1987; Rowe and Houle 1996; Iwasa and Pomiankowski 1994; Westneat and Birkhead 1998; Hill 2011). Individual that exhibit more developed secondary sexual traits should thus be the ones in better condition. So the genetic correlation between sexual success and pathogen resistance should depend on how condition and pathogen resistance are linked, which can be explained with at least two different scenarios (Westneat and Birkhead 1998). These two scenarios also bring up different explanations about the maintenance of male genetic variation despite sexual selection always favouring the most attractive males.

In the first scenario, pathogen resistance is dependent of the individual's general immunocompetence, which is mediated by condition, as is sexual success. Condition is dependent on resource acquisition and allocation, on how well the physiology functions (Andersson 1986; Hill 2011), and thus on many different region of the genome (Houle 1992) which makes it a large mutational target. This implies that mutation pressure should be strong enough to balance selection pressure and to maintain genetic variation in condition, and so in both resistance and sexual success (Rowe and Houle 1996; Dugand, Tomkins, and Kennington 2019). In this genic-capture model, both sexual success and resistance will capture genetic variation in condition, and will thus be positively genetically correlated (Tomkins et al. 2004)

The second scenario assumes specific pathogen resistance. Resistance specificity has been shown in some studies to be related to the host genotype in a number of specific loci not

1473 necessarily involved in resource acquisition or general physiological performance (Marlene
1474 Zuk and Wedell 2014; Luijckx et al. 2013; Bento et al. 2017; Koskella, Vergara, and Lively 2011).
1475 Also, genetic correlation between resistance to different pathogens has been shown to be
1476 often low or negative (Milinski 2006; Lazzaro, Sackton, and Clark 2006; Routtu and Ebert 2015;
1477 Martins et al. 2013; Adamo 2004). These two statements are not consistent with the idea of
1478 the condition-dependent general immunocompetence described in the previous paragraph.
1479 So in this alternative scenario, the relationship between condition and genetic resistance may
1480 be different, as here resistance results from the interaction of the host genotype and the
1481 currently present pathogen pool (Hamilton and Zuk 1982; Adamo and Spiteri 2005). The
1482 outcome of this interaction will affect the host's condition, and thus its sexual traits. This
1483 means that sexual success, still depending on condition, may capture genetic variation for
1484 specific resistance to a particular pathogen, when this pathogen is present. But this also means
1485 that changes in the pool of currently present pathogens context may impact condition, and
1486 thus sexual success. This is a crucial difference between the two scenarios: under the first one,
1487 the "general immunocompetence" scenario, sexual success and resistance should always be
1488 positively correlated, as individual in higher condition will exhibit both higher resistance and
1489 more developed secondary sexual traits. But under the "specific resistance" scenario, the
1490 genetic correlation between resistance and sexual success will only be positive in presence of
1491 pathogens, and will thus capture variation in resistance to currently prevalent pathogens. In
1492 absence of pathogens, we should not expect any correlation, or even maybe a negative one,
1493 if there resistance comes with a cost (Westneat and Birkhead 1998; Joye and Kawecki 2019).
1494 Besides, under the "specific resistance" scenario, temporal and/or spatial fluctuation of the
1495 epidemiological environment will contribute to the maintenance of male genetic variation, as
1496 here variation in sexual success will capture variation in condition, which will depend on the

interaction between the host genotype and the currently present pathogens. This also means that in this case, females should only benefit from their mating choice if both males and offspring are exposed to a similar pathogen pool (Hamilton and Zuk 1982).

We previously found evidence that, in Drosophila melanogaster, there is a positive correlation between the father's sexual success and offspring resistance. However, we also found that this correlation is only positive when male have been exposed to pathogens. In the case of the relationship between sexual success and offspring resistance, we found that the correlation even became negative in absence of pathogens (see chapter 1 and 2). These findings support the idea that the epidemiological context in which mating choice occurs can change the identity of the more successful males, and thus the consequences of sexual selection.

In this study, we intended to look at genetic differences between successful vs unsuccessful *Drosophila melanogaster* males in different epidemiological contexts. As the indirect benefits brought to females through their choice are genetically determined, and depend on male genetic variation, we wanted to use genomics tools to investigate hypothesis derived from the "general immunocomptecence" and the "specific resistance" hypotheses. We aimed to look for a signature of the genic capture of variation for resistance and sexual success. In other words, the aim of our study was to investigate if there are any differences in terms of single nucleotide polymorphisms (SNPs) between successful and unsuccessful males ("winners" and "losers"), in different epidemiological contexts, and also between individuals that survive an infection versus sham treated individuals. The different epidemiological contexts corresponded to different two different level of infection (low and high) and a sham treatment. In our previous study (see chapter 1), we showed that the presence of the pathogen during mating trials could change its outcome, and here we also wanted to test if

1520 the magnitude of the infection is important, or if it is only a matter of presence or absence of
1521 the pathogen. So in this study we used pool-sequencing on pools of *Drosophila melanogaster*
1522 males that have been tested for their sexual success in mating trials consisting in two males
1523 competing for a female, the first male to mate being considered as the "winner". We did these
1524 trials with both males either infected with high or low doses of *Pseudomonas entomophila*, or
1525 sham treated. In parallel, we also collected samples of males and females that have survived
1526 an oral infection with the same bacteria, and samples of sham treated males and females.
1527 Individuals of each treatment combinations were pooled together and a whole genome pool-
1528 sequencing was performed. With this, we aimed to address several questions.

1529 First, we wanted to know if "winners" and "losers" exhibit different SNPs frequencies, as
1530 genetic variation in sexual success is a prerequisite for the "good genes" model. Second, we
1531 wanted to check that, if we find differences between "losers" and "winners", the pattern of
1532 differentiation would change according to the epidemiological context in which sexual success
1533 was assessed. We expected under the "specific resistance" hypothesis that sexual success
1534 should capture variation in resistance, but only if there males have been exposed to pathogens
1535 prior to the mating trials. In accordance, we have recently found evidence that support the
1536 idea that resistance comes with a cost (Kawecki 2020; Joye and Kawecki 2019). Thus, we could
1537 expect that in absence of pathogens we may still find differences between "winners" and
1538 "losers", but either based on the same SNPs but in opposite directions, or on different sets of
1539 SNPs, as "winners" in one situation would become "losers" in the other, and vice-versa. On
1540 the other hand, under the "general immunocompetence" hypothesis, differences between
1541 "winners" and "losers" should be consistent regardless of the pathogenic context, as here
1542 sexual success should capture variation in condition, which is only genetically determined and

should not be impacted by the environmental context. Here, this variation is believed to be maintained by mutation load, as to be based on many region of the genome, and thus on many SNPs (Rowe and Houle 1996; Dugand, Tomkins, and Kennington 2019). Therefore we should not expect any particular region to strongly differ between “winners” and “losers”, regardless of whether or not there has been an exposure to pathogens. However, under the “specific resistance” hypothesis, sexual success should capture variation in resistance to a specific pool of pathogens. Here, variation is thought to be maintained through fluctuation in the currently present pathogen pool (Hamilton and Zuk 1982; Adamo and Spiteri 2005; Westneat and Birkhead 1998). This means that we should expect the difference between “winners” and “losers” to be based on a smaller number of SNPs, but with larger differences in terms of allele frequencies, and, and this is a key point, that this should only be the case when males were exposed.

Third, we wanted to know if the genetic differentiation between “winners” and “losers” is similar to the one between flies that have survived an infection and sham treated flies, and if this is the case regardless of the epidemiological context. Again, under the “general immunocompetence” hypothesis, the epidemiological context in which mating success has been tested should not be relevant, we should thus always expect the difference in allele frequencies between “winners” and “losers” to be correlated with the difference between survivors of infection and sham treated controls. But under the “specific-resistance” hypothesis, this correlation should only be observed in the case where mating success was assessed after an exposure to the pathogens. Indeed, under this scenario, the mating success should only capture variation in resistance when males are exposed to pathogens, but not in a pathogen-free environment.

Material and methods

Fly maintenance

Flies used in this experiment came from a *Drosophila melanogaster* population collected in the canton of Valais, in 2007, and maintained since then in a population cage with overlapping generations. Population size was more than 1000 adults, and flies where raised at 25°C, in a relative humidity of 55% and in a 12:12 photoperiod. We used a standard medium composed of yeast, cornmeal and sugar, in bottles with 30ml of food in which larvae where grown at a density of about 250 individuals (controlled by egg counting). Both males and females where collected as virgin within 12 hours after emergence, as where them maintained separated in small groups until used in the experiments. Female virginity was controlled by ensuring the absence of larvae. All flies where manipulated under CO_2 anaesthesia.

Bacterial culture and infection protocol

The pathogen used in this experiment was *Pseudomonas entomophila* (Pe), a gram-negative bacteria species that was originally isolated from *Drosophila melanogaster*. Pe is a virulent pathogen, causing intestinal infections (Vijendravarma et al. 2015; Vodovar et al. 2005). The original Pe strain was provided by Bruno Lemaître (Vodovar et al. 2005), and was kept at -80°C. We started cultures in Petri Dishes, on a solid medium composed of triptone, yeast, NaCl and agar, to which we added 5% of milk. The addition of milk was done in order screen for colonies' protease activity, forming a pale halo around it, which is a sign of virulence (Rondon et al. 2000). Then we initiated each liquid culture from a single colony from a plate. Liquid media composition was the same as the solid medium, but without agar. The colony was

placed in 50 ml of liquid medium for 24 hours at 28.5 °C, in a shaker at 190 r.p.m for oxygenation. Then, the 50 ml were transferred in 200 ml of new medium, and incubated for another 24 hours, in the same conditions. Next, we centrifuged the culture at 3000 r.p.m and at 4°C for 20 minutes, and removed the supernatant. The pellet was then suspended in NaCL solution (0.9%) until reaching the desired optical density (OD) at 600nm, which corresponded to OD 200 for the high dose treatment, and OD 40 for the low dose treatment. The bacteria solution was finally mixed with the same volume of 5% sucrose solution, so the final bacteria concentrations were, respectively, 100 and 20.

To increase the amount of bacteria ingested and to reduce variation in feeding, flies were starved 2 hours before infection, simply by being placed in empty vials. Next, we transferred flies in vials containing agarose, on top of which we placed a filter paper disc and 100 ul of bacterial solution, and left them so for 20 hours. Then we transferred them to new vials with food. For the sham treatment, we followed the exact same protocol, except that instead of the bacteria solution, we used a 50:50 mixture of 5% sucrose and 0.9% NaCl solutions.

Sexual success assays and winners/losers collection

Our aim was to generate pools of males considered as either sexually successful or not (winners or losers), after assessing their sexual success in situations with or without pathogen infection. Males were coloured, using red or blue powder (Sennelier), and then placed in bottles in groups or approximatively 50 individuals of the same colour, for 48 hours, so that they could clean themselves from the excess of powder. Next, males were randomly grouped in pairs (with one male of each coloration), and infected or sham treated. Each pair was then

placed in a new vial, which was divided in two by a removable partition made of plasticised cardboard. Both males, either infected (with a high or a low dose, i.e OD 100 or OD 20) or sham treated, were placed on one side, and on the other side we placed a random virgin female. Flies we maintained so overnight, so that they have time to habituate to this environment, and to make sure any CO2 effects disappears. The next morning, we removed the separation in all vials, allowing the two males and the female to be in contact. Flies were observed until one male ended mating with the female. The male mating was then defined as a "winner", and the other on as a "loser". If no mating occurred within 2 hours, or if one or both male were dead before that step, the replicate was discarded. Next, for each treatment, we collected winners and losers and placed them in 5ml screw cap tubes (so 6 different tubes, one for each winner/loser and high dose/low dose/sham combination), and were flash-frozen in liquid nitrogen, and stocked at -80°C until DNA extraction. In total, 140 winners and 140 losers were collected for each infection treatment, divided in two pools of 70 males for sequencing.

Collection of resistant and control flies

To collect individuals that survived after exposure to *Pseudomonas entomophila*, first males and females were collected as virgins, and then placed in vials with 10ml of food, in groups of 20 individuals (separated by sex). Then half of the groups were infected, and the other half were sham treated, as described above. For males, we used a high dose of bacteria (OD 100). Female susceptibility to *Pe* had been observed to be higher in this population (Joye and Kawecki 2019), so for them we used half the dose used for male (OD 50). Survival was recorded, in each vial, every 24 hours following the beginning of the infection. Surviving flies

were then collected once mean mortality among vials reached approximatively 30% for males and 50% for females (we wanted the collection time to be the same for males and females from the same block), which happened after 48 and 72 hours after the start of infection (48 hours for one block, 72 for the other one). Close to no mortality was observed the Sham treated flies, and they were collected at the same time. Flies were all placed in 5ml screw cap tubes, and flash-frozen in liquid nitrogen, before being stocked at -80°C until DNA extraction. 140 males and 140 females from each treatment were collected, so 560 individuals in total. Each group of 140 individuals was divided in two pools of 70 individuals (35 from each block) for sequencing.

DNA extraction, library preparation and sequencing

Each pool of 70 flies was first homogenized with beads beating, using 0.1 µm beads, and a cryolisis homogenizer (4°C, 6500 rmp for twice 30 seconds), with 700 µl of solution A [0.1 M Tris-HCl (pH 9.0), 0.1 M EDTA, 1% SDS]. We then added 84 µl of Proteinase K, and incubated the sample for 30 minutes at 56°C, and another 30 minutes at 70°C. Next, 250 µl aliquots were collected from each sample, in which 28 µl of RNAse A were added, followed by a 30 minutes incubation at 37°C. 39 µl of potassium acetate solution was added in each sample, and samples were mixed by inverting, incubated on ice for 30 minutes, and then centrifuged at 13000 rpm for 15 minutes. The supernatant was transferred in a new tube with one volume (approximatively 200 µl) of PCI (Phenol-Chlorophorm-Isoamyl alcohol), and samples were mixed by inverting, and centrifuged at 13000 rpm for 5 minutes. This step was repeated but this time using, instead of the PCI, 150 µl of pure Chloroform. 100 µl of the supernatant was transferred in a new tube in which we added 300 µl of 95% ice cold EtOH, before centrifuging

samples at 10000 rpm for 5 minutes. Then we removed the supernatant in each tube, washed the pellet with 1ml of 70% EtOH, and centrifuged the samples at 13000 rpm for 5 minutes. After that the EtOH was completely removed, and samples were dried for 10 minutes before being resuspended in 50 µl TE buffer. DNA extractions were send to the Genomic Technologies Facility of the University of Lausanne, where libraries were prepared using Nextera DNA Flex kit according to manufacturer specifications and sequenced on Illumina HiSeq 4000 with paired end sequencing and read length of 150 bases.

Mapping pipeline

Raw FASTQ reads were first trimmed and filtered using *cutadapt* v. 2.5 (Martin 2011), to remove sequencing adaptors and low-quality bases, with a minimum sequence length set on 75 bp, and a minimum base PHRED score set on 18. Then the quality of the trimmed reads was checked using FastQC v. 0.11.7 (Andrews 2015). Next, we used BWA –MEM v. 0.7.17 (Li 2013) to map the reads. The reference genome used was a compound reference composed of the genomes of *Drosophila melanogaster* (v.6.12), but also genomes of *D. melanogaster*'s natural pathogens and commensal: *Saccharomyces cerevisiae* (GCF_000146045.2), *Wolbachia pipientis* (NC_002978.6), *Pseudomonas entomophila* (NC_008027.1), *Commensalibacter intestini* (NZ_AGFR00000000.1), *Acetobacter pomorum* (NZ_AEUP00000000.1), *Gluconobacter morbifer* (NZ_AGQV00000000.1), *Providencia burhodogranariea* (NZ_AKKL00000000.1), *Providencia alcalifaciens* (NZ_AKKM01000049.1), *Providencia rettgeri* (NZ_AJSB00000000.1), *Enterococcus faecalis* (NC_004668.1), *Lactobacillus brevis* (NC_008497.1), and *Lactobacillus plantarum* (NC_004567.2) (Kapun et al. 2018). SAM files were then convert to BAM files with Samtools v. 1.10 (Li et al. 2009). PCR duplicates were

marked and removed with Sambamba v. 0.7.1 (Tarasov et al. 2015), and then we used GATK v. 4.1.3.0 (McKenna et al. 2010) to re-align sequences around indels. Finally, mapping quality was assessed using Qualimap V. 2.2.1 (García-Alcalde et al. 2012; Okonechnikov, Conesa, and García-Alcalde 2016) and MultiQC v. 1.8 (Ewels et al. 2016). Bam files were finally converted into a single mpileup file using samtools v. 1.10 (Li et al. 2009).

SNPs calling and filtering

To call SNPs from the mpileup file we used a software written by Martin Kapun, Pool SNP, that is based on UNIX and Python scripts (Kapun et al. 2018). The SNP calling parameters used were the following: 1) the minimum coverage was set to 10, and the maximum coverage percentile to be computed was set at 0.95; 2) the minimum alternative allele count across all samples and frequency were set at respectively 10 and 0.0001; 3) the minimum base pair quality for each nucleotide was set at 15 (for more information on the different parameters, see https://github.com/capoony/PoolSNP). We obtained a VCF (Variant call format) file containing all allele counts and frequencies for every position containing a SNP in at least one of our sample. SNPs around InDels, in transposable elements and in low coverage areas were filtered, and we converted the VCF to a Sync file. At this step, we had 1536002 SNPs. We removed SNPs that had, in a least one sample, a coverage lower than 40 for autosomes, and 20 for the X chromosome. All mitochondrial SNPs were removed, and also SNPs with, across all samples, a mean minor allele frequency lower than 0.05. Also, when looking at the Sync file, we realised that many SNPs had apparently more than 2 alleles. For some of them, this could be due to sequencing errors. Others might originate from paralogs genes, and have been mapped on the wrong gene due to their similar sequence. We decided to remove SNPs for

which the number of reads attributed to alleles 3 and 4 (pooled across samples) was greater than 3. Finally, we realized that some paired samples (for each treatment combination, e.g. "winners" and "sham treated", collected flies were pooled in two technical replicates of 70 flies) showed different patterns in terms of allele frequencies, although they were expected to be identical (except for sampling error). To measure that, we calculated the overall measure of this difference between all sample pairs for each SNP position as sum[(sample1-sample2)^2]/10, as there are 10 pairs of samples. This measure, from now referred to as DiffMS, basically corresponds to variance around a true mean of zero, as we should not expect any difference between paired samples. Next, we ran simulations assuming random sampling of SNPs and paired samples in order to obtain the expected value of DiffMS under pure random sampling. This was done with mean allele frequencies of 0.05, 0.1, 0.2, 0.3, 0.4, and 0.5, and coverage values of 20, 30, 40, 60, 80, and 110. We did one million runs per parameter combination. Then we used this to calculate the DiffMS treshold corresponding to a p-value of 10^{-5}. We finally removed SNPs with a DiffMS larger than the threshold for their mean allele frequency and coverage. With this, we removed 6633 SNPs, and finally ended up with a total of 908122 SNPs.

SNP allele frequency analysis

All analyses were performed on R (version 4.0.2). We first performed several principal components analyses on allele frequencies data of all SNPs to see if we could observe any difference between samples, using the "factoextra" R package. Then, to test if there were any SNPs that significantly differed in terms of allele frequencies between treatments, we ran a likelihood ratio test on all SNPs, one by one. To do so, we used the "mixed" function of the

"afex" R package (the "lme4" package was also required) on a model with combined minor and major allele counts as a binomial response variable, infection treatment and status (winner or loser) as fixed factors, and SNP ID as random factor. The "method" parameter was set to "LRT" for likelihood ratio test. Once we obtained a p-value for each SNP, we controlled for false discovery rate using the Storey method (Storey and Tibshirani 2003) and the "qvalue" R package (Storey 2002, 2003; Storey, Taylor, and Siegmund 2004; Storey 2011). We also wanted to compare the proportion of true null p-values (π_0) when comparing winners and losers for each of the infection treatment (sham, low and high infection). The proportion of true null p-values gives an indication on the number of non-significantly differentiated SNPs that can indeed be considered as true negative, and thus it gives an indication on the number of SNPs that, even if unidentified (because of unsignificant p-values), might be false-negative (Langaas, Lindqvist, and Ferkingstad 2005). To do so we ran the same analysis, but on three different models (one for each infection treatment), and with only status (winner or loser) as fixed factor. To test SNP differences between samples of control and post-infection survivors, we ran the same analysis as before but on a model in which the binomial response variable was again a combination of major and minor allele counts, this time with treatment and sex (and their interaction) as fixed factors, and SNP ID as random factor. Here we used both males and females as the genes could be sex specific. Indeed, in our previous study, the correlation between offspring resistance and fathers' sexual success was only found with sons, not with daughters (Joye and Kawecki 2019). All analyses were also performed using, as a response variable, the major allele frequency on which we applied an ArcSin square-root transformation (thus assuming a normal distribution this time), to see if we would obtain different results. Here our model was constructed using the "lm" function of the "lme4" R package. Sex and treatment (and interaction) were used as fixed factor.

Genetic diversity

In all samples we measured the expected heterozygosity, π, which is a common way of quantifying a population's genetic diversity, and corresponds to the proportion heterozygous sites expected under Hardy-Weinberg equilibrium (Nei 1973). We calculated for each sample and each chromosome arm, as the following: ExpH = sum(2*p*(1-p)) / N, where p is the frequency of the major allele at each site, and N is total number of sites.

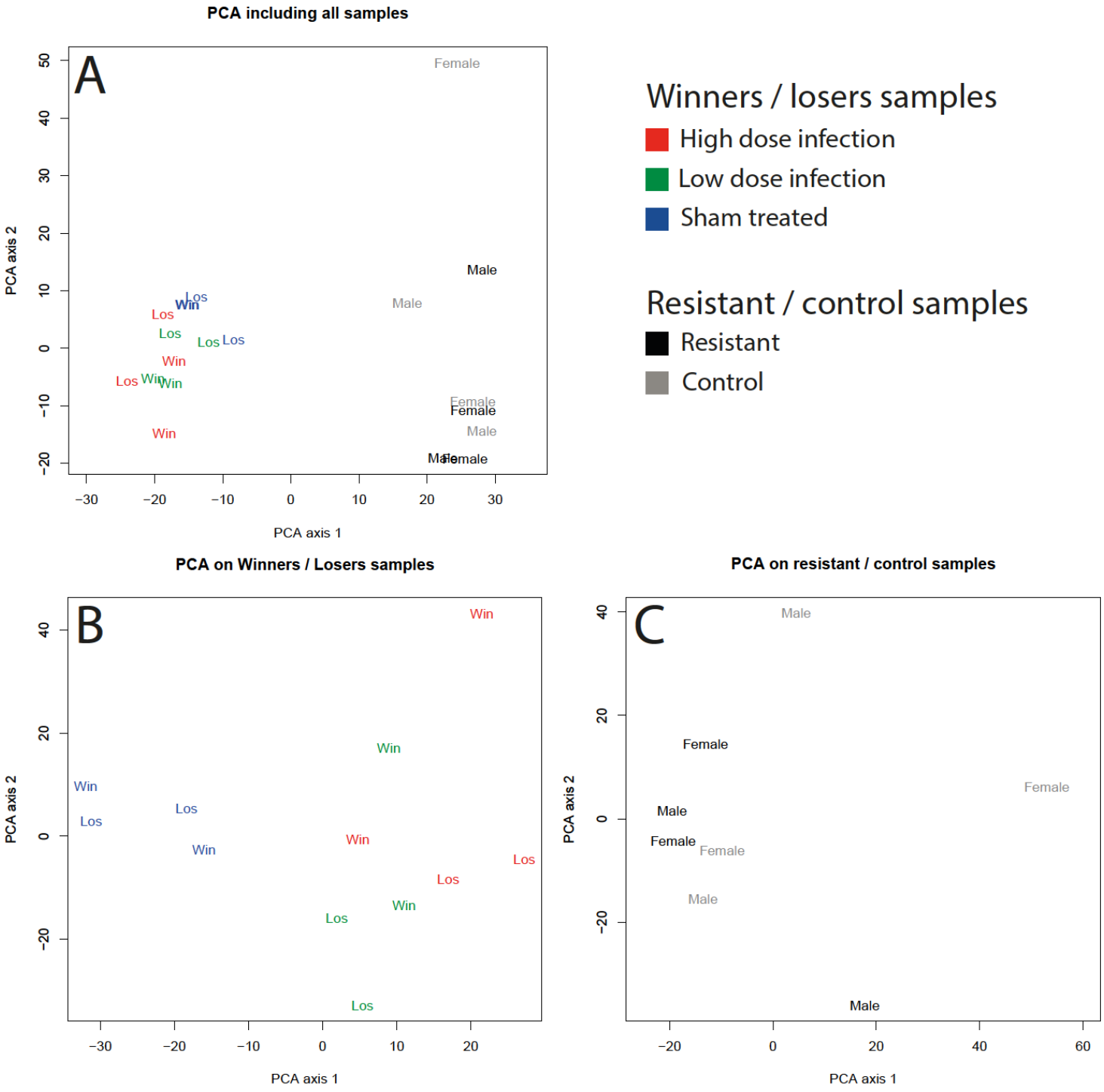
Results

The first principal component analysis we ran on the allele frequencies of all SNPs in all 20 samples explained about 19% of the variance with the two first axis (12.6 % axis 1, 6.6% for axis 2, Fig. 1A). Surprisingly, the first axis clearly separates samples from the two sets of experiments, with winner and loser samples on one side, and post-infection/sham survivors on the other side. Flies from both sets were collected from the same population, thus we did not expect the first axis to be determined by this factor. The second axis seems to separate one sample, sham treated females sample 2, from the rest. Therefore, after this first PCA, we decided to run new PCAs for both sets of experiments separately. In the second PCA we only included winner and loser male samples, in order to investigate if a genetic differentiation between losers and winners would be impacted by the context in which mating trials where done (with high, low or no exposure to the pathogen). Here, the two first axes explain 22% of the variance (12% for axis 1, 10 for axis 2, Fig. 1B). We can see that axis 1 separates sham treated samples from both low and high infection samples, but we did not find any separation based on the winner/loser status. The separation between sham treated and infected males

might reflect some resistance based differences, as during the sampling of “winners” and “losers”, flies that died between the beginning of the infection process and the end of the mating trial (i.e so potentially flies with a lower resistance) were discarded. This may have resulted in samples of infected “winners” and “losers” to be composed of flies with a higher resistance, on average, than flies of the sham treated samples. So in a third PCA we only included samples from post-infection survivors and sham treated males and females, to see if could find any evidence for a resistance-based genetic signature. This time, 34.3% of the variance is explained by the two first axis (19.2% for axis 1, 15.1 for axis 2, Fig 1C). However here this variance seems to come from a few particular samples that differ from others, even from the other sample they are paired to, which are technical replicates. This first axis clearly separates one of the sham treated females samples from others, which was already the case for the second axis of the PCA including all 20 samples.

When looking at SNP allele frequency differentiation between winners and losers from sham, low and high infected treatments, we found only one SNP which allele frequencies significantly differed according to infection, status and their interaction (Fig. 2A,B,C). We found that this SNP is fixed in both high infected losers samples. This SNP is located at position 7648524 on chromosome 2L, which corresponds to an intronic region of the gene CG43756, a regulator of the calcium-activated channel Slo (Schopperle et al. 1998; Zhou et al. 1999). A second SNP came out significant only according to the infection treatment, and is located at position 17604807 on the 3R chromosome, which does not correspond to any know gene. Running the same analysis on arcsine square root transformed allele frequencies did not revealed any significant SNPs. We separately compared winners and losers from each 3 infection treatments (sham, low and high infection) to look at the proportion of true null p-values (π_0,

not to be confuse with the expected heterozygosity π), which gives an indication about the amount of SNPs that may be differentiated, even if we did not have the power to detect them (Storey 2011). If this value was smaller in infected treatments, it could indicate that the genetic differentiation between winners and losers is larger even if we did not found SNPs with significantly different allele frequencies. However, we found almost identical π_0 values for each treatments (sham: $\pi_0 = 0.85$, low: $\pi_0 = 0.84$, high: $\pi_0 = 0.85$). We also calculated the true null p-value for the treatment*status (winner-loser) interaction.



1802

1803 Figure 1: Principal component analysis based on SNPs allele frequencies including A: All samples, B:

1804 Winner and Loser male samples, and C: Samples of resistant (flies that survived an infection) and

1805 control (sham treated) males and females. Colours refers to the infection treatment and levels of grey

1806 refers to the resistance status.

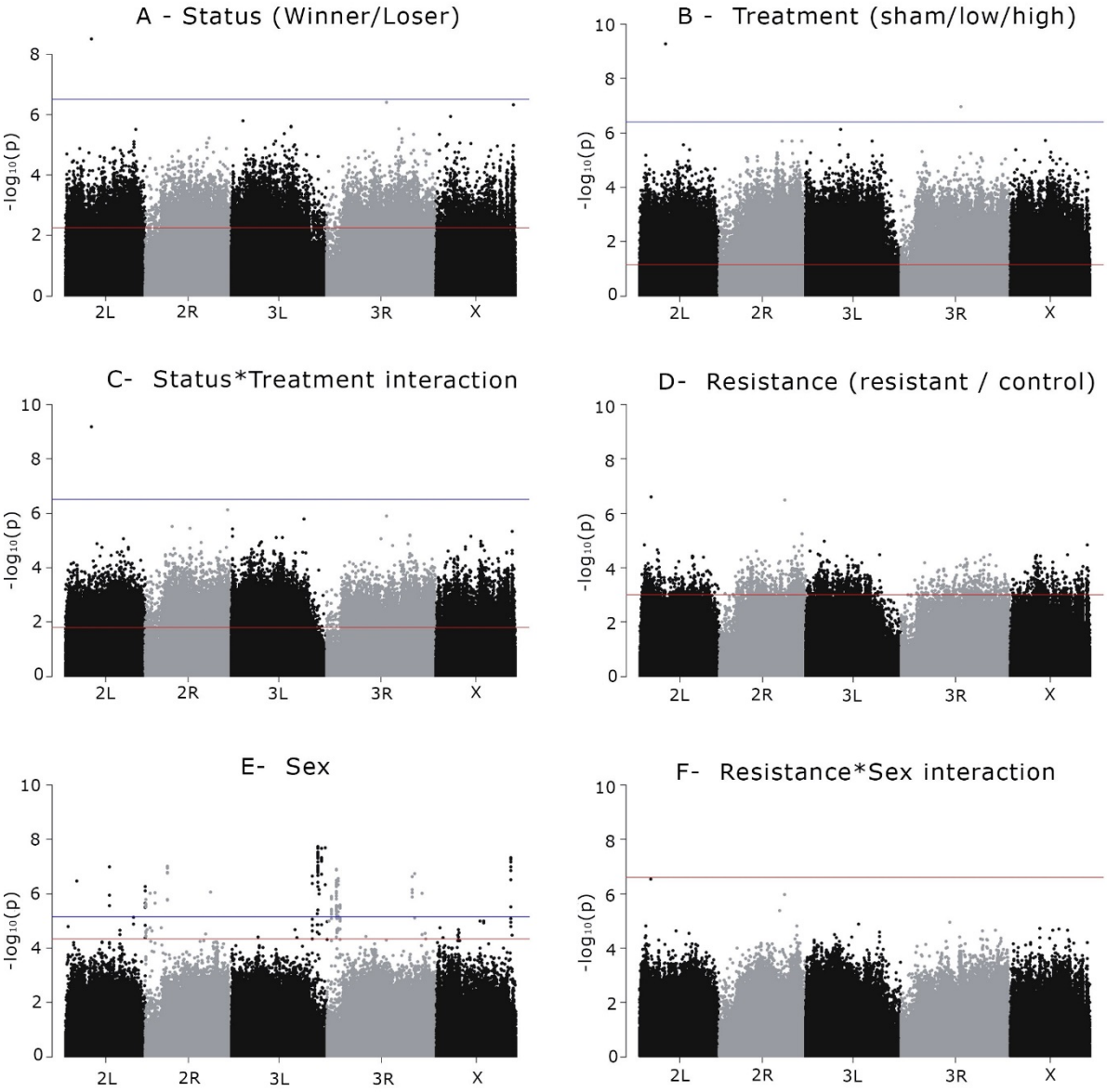


Figure 2: Manhattan plots for all tested SNPs among the 2L, 2R, 3L, 3R and X chromosomes. Plots A,B and C represent comparisons of samples of winners vs losers, and plots D,E and F represent samples of survivors vs sham treated flies. The red line represent the p-value threshold corresponding to a 0.2 adjusted p-value/q-value. When at least one SNP with an adjusted p-value lower than 0.05 was detected, a blue line was added to separate significant SNPs from others.

Comparing samples from male and female survivors after infection or sham treatment did not reveal any significant SNPs according to the treatment (Fig. 2D), and we found 115 significant SNPs according to sex (Fig. 2E). Among these 115, only 4 are situated in exonic regions and were identified as being part of 2 genes, vig2 and Mocs2. Those two genes are already known to have been duplicated from the 3R chromosome to the Y chromosome (Carvalho et al. 2015), and thus the SNPs we found are probably due to mapping on paralogous version of these genes on the Y chromosome. We believe that the other 111 SNPs, all situated in intronic regions, are also due to duplications. Also, we found no significant SNPs according the treatment * sex interaction.

The expected heterozygosity values measured for each chromosomes and each samples were very consistent between all samples (table 1). The mean expected heterozygosity values for each chromosome (X: $\pi = 0.002$, 2L: $\pi = 0.003$, 2R: $\pi = 0.004$, 3L: $\pi = 0.002$, 3R: $\pi = 0.002$) were lower that what we would expect in natural populations (e.g. mean expected heterozygosity values of 0.005, 0.007 were measured in two natural populations from Africa and North America, see Langley et al. 2012). This is not surprising, as it is know that laboratory populations have lower genetic diversity than wild populations (Lainhart et al. 2015; Gloria-Soria et al. 2019). The population used in our study has roughly half of the genetic diversity of natural populations, but its genetic diversity is very similar to the one of other laboratory populations that have been successfully used by our lab in another genomic study (Kawecki et al. 2020). We can thus assume that our results are not due to a lack of genetic variation.

Expected heterozygosity (π) in all samples

Chromosome	In this study	Natural populations (Langley et al. 2012)		Other laboratory populations (Kawecki et al. 2020)
X	0.0018	0.0038	0.0082	0.0009
2L	0.0031	0.0063	0.0084	0.0025
2R	0.0044	0.0058	0.0073	0.0024
3L	0.0025	0.0057	0.0078	0.0024
3R	0.0021	0.0048	0.0063	0.0020

Table 1: expected heterozygosity values obtained in all pool samples, for each chromosome. The values are about half lower than the ones from natural populations (Langley et al. 2012), but very similar to the ones of other laboratory populations used by our group (Kawecki et al. 2020).

Discussion

In this study we used pool sequencing on sexually successful and unsuccessful males, whose success has been determined in different pathogenic contexts and on individuals that have survived to an infection with *Pseudomonas entomophila* (Pe). We looked for evidence that a genomic differentiation between "winner" and "loser" males would depend on the presence of pathogens, and for a relationship with genomics bases of resistance. In our previous studies, we already found support for a genetic correlation between sexual success and resistance to pathogen, and also that the sign of this correlation depends on the pathogenic context in which sexual selection occurs (see chapters 1 and 2). To confirm the genetic basis of these results, it was necessary to find evidence based on genomic data. Evidence for a genetic basis of variation for resistance have already been found in *Drosophila melanogaster* (e.g. Lazzaro et al. 2004; Lazzaro et al. 2006; Wang et al. 2017). Also using *Pseudomonas entomophila* as pathogen, Sleiman and al. (2015) revealed genetic loci associated with susceptibility to infection, linked to the metabolism of reactive oxygen species.

By comparing pool-seq data from flies that survived an infection with data from control flies, we were expecting to find SNPs associated with resistance to Pe. However, we were not able to find any SNPs which had significantly different allele frequencies depending on the treatment (infected or not) after correction for multiple comparison. There is a possibility that the level of variation in the population we used was too low to be detected through our protocol. However, the population was the same used in chapter 1, in which variation for resistance was found, as we measured different levels of mortality in offspring from "winners" and "losers". It is also possible that our sampling method for resistant individuals was not

efficient enough to obtain pools of individual with levels of resistance that differs sufficiently to be detected.

In both the “general immunocompetence” and “specific-resistance” models, sexual success is believed to capture genetic variation for resistance. As we were unable to detect any genetic signature of variation for resistance, we might not expect to detect genetic differentiation based on sexual success, even if there could be SNPs affecting sexual success that are unrelated to pathogen resistance. The first question we assessed in our study was whether or not we could find SNPs associated with male sexual success, i.e males considered as “winners” and “losers” following mating trials. Analyses performed on pooled-sequencing data from winners and losers did not show any detectable difference in terms of SNP allele frequencies, regardless of the infection treatment (sham, low or high dose), except for one SNP, located in an intronic region of gene CG43756. And the analysis using ArcSin square-root transformed allele frequencies did not show any significant SNPs. A central point of our study was our second question, which was to know if any detectable differentiation between winners and loser would vary with respect of the pathogenic context in which mating trials were performed. Indeed, the key distinction between the “general immunocompetence” and the “specific resistance” models is that in the first one, resistance to pathogen and sexual success should always be positively genetically correlated, where under the “specific resistance” model, it should only be the case in a context with pathogens. In our previous study (see chapter 1), we found that “winner” males in a context with pathogen could become “losers” in absence of pathogens. Thus, we were aiming to see how the genetic differentiation between “winners” and “losers” would change depending on their infection level during mating trials. However, as we did not detect any differentiation at all, we could not answer

that question. Even when separating winners and losers, the proportion of true null p-values (π_0), that could somehow indicate the level of differentiation between samples of the different treatments even without significantly differentiated SNPs, did not differ at all. We expected to find differences that would either be observable in all treatments, which would have been in support of the "general immunocompetence" model, or only in samples that have been exposed to pathogens, consistently with the "specific-resistance" model. However, true null p-values of approximatively 0.85 actually could imply that around 15% of SNPs may have been somewhat differentiated between winners and losers, but that we did not have sufficient power to identify them. And the 0.66 π_0 calculated for the treatment*status (winner-loser) interaction could also represent a hint on how important this interaction may have been, even if undetected in our study.

Under the "general immunocompetence" model, variation in resistance should capture variation in condition. Condition can be defined as the individual general health, amount of metabolic reserves, and global physiological state (Westneat and Birkhead 1998; Hill 2011; Rowe and Houle 1996), and is believed to depend on many different traits, and thus to be mediated by numerous regions of the genome (Rowe and Houle 1996; Dugand, Tomkins, and Kennington 2019). We would have, in that case, expected the differentiation to be based on many different SNPs, regardless of the pathogenic context. But under the "specific resistance" model, it is condition that should capture variation in resistance, and the number of SNPs that are specific to a pathogen should be smaller, because specific resistance is likely to depend on genetic interactions between the host and the pathogen, as for example having specific receptors. So we would have expected any differentiation to be observed in a smaller number of SNPs, in more defined genomic regions. Though, not being able to detect any differences

could, in a way, be in support of the "general immunocompetence" model, as these differences would in that case be small, and not gathered in some particular regions. As mating success is believed to be condition-dependent, not being able to detect genetic variance for condition would explain the absence of difference between losers and winners. The "general immunocompetence" model stipulates that resistance to pathogen is also condition dependent, in contrast with the "specific resistance" model that says that condition depends on resistance. Again, if this is the case, then the fact that we found no SNPs with significantly different allele frequencies between flies that survived to an infection and sham treated flies could also be supportive of the "general immunocompetence" model. As for mating success, it is possible that variance for resistance depends directly on condition, and so is also mediated by numerous traits and thus numerous genomic regions. However, this would not be consistent with results found in chapters 1 and 2, which are supportive to the "specific resistance" model.

Several studies have found support for a genetic basis of different traits involved in sexual success, such as courtship behaviour, pheromones or sex combs, using a quantitative genetic approach (Gosden, Reddiex, and Chenoweth 2018) or by identifying candidate Loci/SNPs (Sisodia and Singh 2005; Singh and Singh 2016; Cloud-Richardson, Smith, and Macdonald 2016), and we could thus expect variation in sexual success to imply detectable genetic polymorphism. Using RNA-seq data, Höglund et al. (2017) were able to find SNPs associated with mating success in the bird species *Gallinago media*.But detecting associations between SNPs and some particular traits might be challenging. In 2015, Santure et al. aimed to investigate the architecture of eight quantitative traits (clutch size, egg mass, offspring weight, adult and fledgling weight, tarsus and wing length and exploratory behaviour) in two *Parus*

major populations, but they were not able to detect any signature, and they conclude that these traits were influenced by many genes of small effects (Santure et al. 2015). This conclusion could also be applied to our results. If the variation is based on small difference in many loci, it may have been too low to be detectable in our study. But also, if sexual success does indeed capture variation for resistance, which we could not detect, we might not expect to detect it either.

The genetic variation of the population used in this study, measured as the expected heterozygosity, is lower than what we would expected in wild populations. This is not surprising, as it is known that laboratory populations are known to show lower genetic variance. But this could also partially explain why we could not detect any differences. In both scenarios, mating success is believed to capture variation in condition, which should capture in turn an important part on the global variation. A low population's genetic variance is thus a reason to believe that the absence of measured differences may be due to a lack of detectability. . The fact that we were not able any differences does of course not necessarily mean that winners and losers are totally similar genetically speaking, but there is a possibility that there is no genetic variation affecting sexual success in the population used in this study. Variation might have been depleted due to a relatively small population size under strong selection in a constant environment. If that was the case, the "good-genes" model would not work in this population. However, we have reasons to believe that there is actually genetic variation for sexual success and resistance in this population, as we found, in chapter 1, different levels of resistance in sons of successful vs unsuccessful males.

Other unexpected results were found in this study. The principal component analyses that were performed on allele frequencies of all SNPs in each sample did not show what we would

expect under both scenarios. In the first PCA, including all winner/loser and resistant/control samples, we could observed a clustering of samples from both experiments, with winners and losers on one side, and resistant and control on the other side. The “winner-loser” experiment and the “resistance” experiment were not performed at the same time, which may have resulted in a change in the gene pool, due to some kind of selection within the generation during the process of obtaining the flies. In the PCA showing only winners and losers samples from the 3 infection treatments, the first axis separates sham treated samples from samples of the 2 infection treatments (i.e low and high doses). During the experiment, flies that died before or during the mating trials, or flies that did not mate within two hours, were discarded. In both low and high doses infection treatments, this may have resulted in selecting more resistant individuals. Thus, this first axis might reflect some genetic differences for resistance. However, in the third PCA with only survivors of infected and sham treated samples, this pattern was not observed. Selection for resistance with only 30% to 50% mortality may have been too small.

Samples from each sample pair (sample 1 and 2) are expected to be identical, as they are pure technical replicate. Knowing this, we were surprised to observe that in all PCAs, some samples from the same pair were not clustering together, or at least seem to differ more that samples from different treatment. This could be due to our choice of using Pool-sequencing instead of individual sequencing. Pool sequencing, despite its practical and financial benefits, has been criticized in regards to the precision of the SNP allele frequency data that is obtained with this method (Cutler and Jensen 2010; Anderson, Skaug, and Barshis 2014). But more recent studies have shown that allele frequencies data obtain with Pool-seq are reliable, and that Pool-seq is a valid method to obtain SNPs frequency data (Fracassetti, Griffin, and Willi 2015; Anand et

al. 2016). Of course, despite all our effort while running these experiments, including fly sampling, DNA extraction and sequencing, it is hardly possible to completely exclude the existence of potential mishandling that would cause these inconsistent results. Obviously, understanding what genetic differences are capture by sexual success and resistance, and how these are or are not impacted by the epidemiological context will need more investigation. Here, unfortunately the absence of detected difference does not allow us to make any reliable conclusions.

General discussion:

This project aimed to investigate the role of pathogens under the "good genes" model of sexual selection. Pathogens have been suggested to play an important role explaining both how mating choice might be indirectly beneficial to females, and how male genetic variation is maintained. But the mechanisms beneath that role remain unclear, as direct experimental evidence is rather scarce and equivocal, and at least two hypotheses can be made to explain how pathogens impact sexual selection: the "general-immunocompetence" and the "specific resistance" hypotheses. In both hypotheses, male attractiveness, based on secondary sexual traits, is condition dependent and is genetically correlated with resistance to pathogens. But in the "general-immunocompetence" model, it is the individual's condition that determines resistance, whereas in the "specific resistance" model, the degree of susceptibility is expected to result from the interaction between the host genotype and the currently present pathogens, and to have a large influence on condition. These two models are not necessarily mutually exclusive, but there are crucial differences between them that can impact the consequences of sexual selection. These differences concerns the mechanisms maintaining genetic variation in males, but also how sexual selection might improve the selection of resistance to pathogens. It is thus relevant to understand the relative importance of each of those two models in sexual selection.

The relationship between male attractiveness and both male and offspring resistance has already been studied, and results are equivocal (reviewed in the general introduction). In this project, we aimed to test how this relationship would be impacted by different epidemiological contexts. This is a key difference between the "general immunocompetence" and the "specific-resistance" models. Indeed, under the first one, male attractiveness is

2027 expected to always be positively genetically correlated with resistance to pathogens. But
2028 under the “specific resistance” model, this correlation could disappear in absence of
2029 pathogens, or even become negative. For this reason, we investigated the impact on the
2030 outcome of sexual selection of pathogen presence during mating trials (i.e., when male
2031 courtship behaviour / female choice happens) with different approaches. In all cases, we used
2032 “choice” designs, in which sexual success was assessed in situations with 2 males competing
2033 for a female, which is more ecologically relevant. The fact that we used “choice” designs
2034 instead of “no-choice” designs, in which each male would have been alone with a female, is
2035 not trivial and has been shown to have an importance in the measurement of sexual success
2036 (Dougherty and Shuker 2015).

2037 In the first chapter, we showed that more sexually successful males sire more resistant sons,
2038 but only when mating choice was done in presence of pathogens (i.e., when males where
2039 infected). Without pathogens, we found the opposite correlation. This result provides
2040 evidence for the importance of the epidemiological context in which sexual selection
2041 operates, and brings support to the “specific resistance” hypothesis. Results from in the
2042 second chapter are also in support of this hypothesis. Indeed, we showed that males from
2043 populations selected for resistance were more likely to mate when competing with males from
2044 control populations, but again only when both males were first exposed to pathogens. In
2045 absence of infection, resistant and control males showed similar mating success. These two
2046 studies represent strong support for the “specific resistance” hypothesis, as they bring to light
2047 the importance of the epidemiological context in sexual selection. Under the “general-
2048 immunocompetence”, we would have expect, in both studies, to obtain results that do not
2049 depend on the epidemiological context. Finally, we were not able to demonstrate that

differences in terms of SNPs between more or less attractive males are also context dependent. Indeed, in the 3rd chapter we pooled males either chosen or not by females in situations with or without pathogens, and used whole genome pool-sequencing to search for SNP based differences between the different pools. Our aim was to investigate how these differences depend on the epidemiological context, as a context-dependence would be in strong support with the "specific resistance" hypothesis. But we could not detect any difference, regardless of the infection treatment. This could be considered as consistent with the other model, the "general-immunocompetence" hypothesis. In this genic-capture model, condition-based variation is mediated by numerous small differences in multiple traits and thus multiple genomic regions (Rowe and Houle 1996; Dugand, Tomkins, and Kennington 2019), which makes differences possibly harder to detect. But it would obviously be a little bit far-fetched to conclude anything from this absence of results. Despite the absence of conclusive results from the pool-sequencing experiment, we were able in the two first chapter to highlight the importance of the context in which mating choice is done. With this, the role of pathogens in sexual selection might differ to what has been mainly thought so far, and may be highly relevant when it comes to the consequences of sexual selection.

The evolution and maintenance of mate choice, the maintenance of genetic variation in males, and the consequences of sexual selection in general have been largely discussed in literature. And if pathogens were often thought as being part of the story, in most of the cases it was under the "general-immunocompetence" hypothesis (Morehouse 2014; Rantala et al. 2012; Garratt and Brooks 2012; Boonekamp, Ros, and Verhulst 2008). But other recent studies have also brought some support to the alternative scenario, the "specific-resistance" hypothesis. The red queen dynamics on which is based the Hamilton and Zuk model (Hamilton and Zuk

1982) has found some support in studies using both experimental and natural populations (Bourgeois et al. 2017; Turko et al. 2018; Bérénos, Wegner, and Schmid-Hempel 2011; Decaestecker et al. 2007; Brockhurst et al. 2014), suggesting a context-dependence of sexual selection. Also, there has been evidence for a cost of genetically determined resistance (Kawecki 2020; Martins et al. 2013; Viney, Riley, and Buchanan 2005; Luong and Polak 2007), implying that resistance should be counter-selected in situations with low pathogen presence. Finally, the specificity of resistance has also received some support, as there are some evidence for a weak, sometimes negative genetic correlation for resistance to different species of pathogens (Martins et al. 2013; Adamo 2004; Lazzaro, Sackton, and Clark 2006; Milinski 2006b) or even different genotypes (Luijckx et al. 2013; Bento et al. 2017; Koskella and Lively 2009). All these findings bring support for the "specific resistance" hypothesis, or at least some aspects of it, as we found evidence for the importance of the epidemiological context on the genetic correlation between male attractiveness and offspring resistance. Indeed, the crucial point of this model is how, depending on the context, the positive genetic correlation between male attractiveness and offspring resistance can disappear or even become negative. We showed in two different experiment that this is the case, and that selected resistance can become disadvantageous in a situation where there are no pathogens.

These findings are relevant for the understanding of the role of pathogens in sexual selection, but also for the understanding of the general consequences of sexual selection. As we showed that there is a context-dependence of sexual selection, and found support for the "specific resistance" hypothesis, we brought new insights on different aspects of sexual selection. First, the maintenance of genetic variation in male sexual traits: in contrast to the "general-immunocompetence" hypothesis, under which genetic variation is maintained through

selection-mutation balance (Rowe and Houle 1996; Dugand, Tomkins, and Kennington 2019), the "specific resistance" hypothesis implies that variation is mediated through fluctuations in the currently present pathogen pool, as here resistance depends on a specific interaction between the host's genotype and some particular pathogen species or genotype (Zuk and Wedell 2014). This findings bring some support to an alternative solution to the Lek paradox, beside the already proposed genic-capture hypothesis (Rowe and Houle 1996), as here maintenance of variation is mediated by the context-dependence of sexual selection. Another important point is that, because of this context-dependence, female choice can only be beneficial to the female, and thus selected, if her offspring face to a similar pathogen pool than the one the male has been exposed to (Eshel and Hamilton 1984; Charlesworth 1988; Howard and Lively 2004). So far, the importance of fluctuations in the epidemiological context on the outcome of sexual selection has been neglected, but here we show that it is a crucial parameter that needs to be controlled when studying sexual selection and its general mechanisms.

In addition to this, one important consequence of the "specific resistance" hypothesis concerns the selection of resistance to pathogens. We have shown, in the first chapter, that resistance can be unfavourable in some circumstances. Indeed, we found that in an environment without pathogens, males siring more resistant offspring where in fact less likely to mate when competing with another male. And in the second chapter, even if we could not find evidence for a cost, we found that in absence of pathogens resistance and mating success were no longer correlated. In a recent study, Kawecki (2020) showed that resistant males were less sexually successful when the environment was pathogen-free. These findings are in support with the idea that resistance, but also mate choice, can be maladaptive if both parents

and offspring to not face similar pathogen pools. But if they do, then mate choice would potentially strengthen selection for resistance to, for example, a new pathogen. The environmental stability, at least in terms of pathogens, is thus a crucial point to take into account when studying sexual selection, and indirect benefits and costs of mating choice. With this we have shown how resistance to pathogens may play an important role in mediating the outcome of sexual selection.

A next step would be to keep investigating this at the genomic level. This would maybe allow to identify important regions for variation in resistance and attractiveness, but also to get a better understanding of how genetic differences between individuals considered as more or less attractive depend on the epidemiological context. The fact that we were not able to find any meaningful results should not mean that the investigation should stop here. Including genomic data to the general understanding of sexual selection is crucial, and so far there are too little experimental genomic data that are used in the field (Balenger and Zuk 2014; Wilkinson et al. 2015). This is why it would be important to put some more effort on detecting genomic differences based on attractiveness and resistance, and to measure how these differences vary in respect with pathogen exposure.

Beside the genomic aspects, evidence we found also bring new questions concerning the mechanisms that lead the epidemiological context-dependence of male sexual success. An interesting follow up to this project would be to understand which phenotypes link the differences in resistance with the differences in sexual success. Male sexual success is known to be based on several factors, such as courtship behaviour and olfactory compounds (Greenspan and Ferveur 2000a; Grillet, Dartevelle, and Ferveur 2006). When it comes to courtship, both the intensity and the quality should be considered. To analyse courtship

quality, measurements of male song characteristics could be used, such as song duration or the amount of pulses and sines, and inter-pulse intervals, that are known to have specific roles in attracting females and for which there are evidence of genetic variation (Schilcher 1976; Talyn and Dowse 2004; Arthur et al. 2013; Rybak et al. 2002). Several questions could be investigated to better understand how infection impacts attractiveness. The first step would be of course to measure how infection impacts these different aspects of male sexual success. This should help determining what are the phenotypical differences between resistant males and less resistant males that could impact their sexual success after exposure to pathogens. The effect of infection on courtship intensity has been investigated in a study ran by Louaï Maraachli, a master student, under my supervision (see appendix 2). In his project, Maraachli measured courtship intensity of males in situations with and without male-male competition, and with exposure to different doses of *Pseudomonas entomophila*. His results showed that courtship intensity, which was here measured as the proportion of a given time that a male spends courting, was lower when males where infected with a sufficient dose. These results bring new insights on the impact of infection on male investment in courtship, that is likely to mediate attractiveness, and thus on how more resistant males might be more attractive than less resistant males in presence of pathogens (assuming that the difference between resistant and susceptible males after infection would be similar to the one between infected males and sham treated males, which might not be the case).

As we showed in the first chapter that offspring resistance can predicted by male sexual success, the next step would be to try to predict resistance based on measures of courtship (intensity and song composition) or olfactory cues. And of course in the context of the "specific resistance" hypotheses, this should be done when males are exposed to either infection or to

a sham treatment, in order confirm that the resistance predictability is context-dependent, which would be consistent with our results. Finally, another approach would be to use the fly populations used in chapter 2 that have been selected for resistance to *Pseudomonas entomophila* (Martins et al. 2013), and the corresponding control populations, to measure the previously mentioned traits (olfactory and courtship component) after males have been exposed to the pathogen. We should expect, if the measured trait is implied in the context-dependence of attractiveness, to observe differences between resistant and control populations.

Finally, another direction that would be worth investigating is how specific our results are to 1) our model system and 2) to pathogenic, or even biotic, stresses. In this project we only used *Drosophila melanogaster* as a host, and *Pseudomonas entomophila* as pathogen. For this reason we are force to limit our conclusions to these species, and thus obtaining similar results using different host and/or pathogen species would represent strong support to our findings. Moreover, the "specific resistance" model assumes that the correlation between male sexual success and offspring resistance will only be positive if fathers and offspring have been both exposed to the same pathogen. Thus, it would be important to investigate how important the specificity of the resistance is by exposing fathers to a pathogen, and offspring to a different one. Also, so far we do not know if our conclusions are limited to pathogens. There is a possibility that the genetic correlation between male attractiveness and resistance, as its context-dependence, could also be found using not only other pathogen species, but maybe other kind of biotic or abiotic stresses. And so far we have shown that female choice is only beneficial if both parent and offspring face an infection with pathogens. But the "specific resistance" model implies that sexual success is mediated by a resistance that is believed to

be specific to the pathogen pool males and offspring are exposed to. To confirm this, exposing parents and offspring to different pathogens could to confirm the importance of that resistance specificity. Also, we should consider the possibility that the "specific resistance" model is not limited to pathogenic infections but can be extended to other types of stress, even abiotic stresses.

One of our master student, David Simonin, have done his project under my supervision on this precise topic. In his project (see appendix 1), Simonin investigated the link between male sexual success and offspring resistance, as we did in the first chapter, but using two different pathogens, *Pseudomonas entomophila* and *Metarhizium brunneum,* so that fathers and offspring were infected with a different pathogen. He found that when fathers were exposed to *M. brunneum*, offspring resistance to *P. entomophila* was negatively correlated with father sexual success, as it was the offspring from "losers" that survived better after being infected. However, when both fathers and offspring were exposed to *M. brunneum*, he did not detect any difference in resistance between offspring from "winners" and "losers".

He also tested if the results we found in chapter 1 could be reproducible using, instead of pathogens, abiotic stress sources (e.g., starvation and heat shock). Using a design very similar to ours, he aimed to measure if male sexual success could predict offspring resistance to heat shock and starvation, in situations where males where previously exposed or not to a heat shock. However, whether or not male sexual success was assessed after a heat shock or not, offspring resistance to both heat shock and starvation did not differ between offspring from "winners" or "losers".

To investigate the stress specificity of the relationship between sexual success and resistance, he also tested it in situations where males and offspring were exposed to different types of

stress (biotic vs abiotic). Little evidence was found in support of the "specific resistance" hypothesis, as at some point he found a negative correlation between male mating success and offspring resistance in cases where males where exposed to a heat shock and offspring were exposed to *M.brunneum*.

To conclude, sexual selection is a field where we have extensive theory. We have a lot of understanding on how it works at the phenotypic level. But the understanding of underlying genetic links and its consequences remains unsatisfactory. With this project, we have brought new insights on the role of pathogens in sexual selection, and, more important, how context-dependent sexual selection can be. Our results suggest that more attention should be paid to the context in further studies on sexual selection, and in particular studies investigating the relationship between male mating success and offspring viability should consider testing for any environmental effect, and including it in their experimental design.

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Appendix 1:

Master project

This appendix is a master project done by a master student, David Simonin, under the supervision of Patrick Joye and Tadeusz Kawecki.

Does male sexual success predict offspring resistance to stress?

David Simonin

Abstract

The relationship between male attractiveness, condition, and offspring resistance can be explained by two different. The “Specific resistance” model posits that males resistant to a currently prevalent pathogen will have a higher sexual success, but only when the pathogen is present, whereas the “general immunocompetence” model posits that males with good general resistance to all pathogens would be favoured, regardless of the context. In this project, we tested those two hypotheses. We used pathogen infections, but also extended the experiment to other biotic and abiotic stresses. We used *Drosophila melanogaster* to find a potential link between male’s sexual success and offspring resistance to different stresses (infection with the fungus *Metarhizium brunneum* or with the bacteria *Pseudomonas entomophila*, heat shock or starvation), and if this link depends on whether or not males have been stressed. We found some support in favour of the first model, as we found that sexually successful males exposed to a particular stress sire offspring less resistant to a different stress.

However, despite a positive link between sire resistance and offspring resistance to the same stress, we did not find any link between male's sexual success after exposure to a stress and offspring resistance to the same stress.

Introduction

It can sometimes be puzzling to understand how some traits that do not seem to be beneficial in terms of fitness are still maintained in some populations, notably for males. However, individual fitness is not only based on his ability to survive, but also to find a mating partner. That is why we can encounter these traits: they are useful in order to attract the opposite sex or compete with the other individuals of the same sex. There are many examples of these traits, called secondary sexual traits (Enquist and Arak 1993; Kodric-Brown and Brown 1984), and they can have different form, as for example a courtship dance for the peacock spider (*Maratus volans*) (Girard, Elias, and Kasumovic 2015), a gift for the bushcricket (*Tettigoniidae*)(Simmons 1999), a call song for the marsh frog (*Pelophylax ridibundus*) (Lukanov, Simenovska-Nikolova, and Tzankov 2014), a physical ornament-like weapon for the sand fiddler crab (*Uca pugilator*) (Allen and Levinton 2007) or a colourful body for the splendid fairy wren (*Malurus spendens*) (Brooker and Rowley 1995). But why should a female prefer males with the brightest colours or the one with the biggest display? In some cases, it is quite straight forward, as the female receives direct benefits by gaining food (Andersson 1994), territories (Reynolds and Gross 1990) or by avoiding potential infection (Price, Schulter, and

Heckman 1993) for example. But in many other cases, females do not seem to directly benefit from their choice, but through an increased viability of their offspring. These indirect benefits are nevertheless much less known, because hard to quantify. The “good genes” hypothesis posits that male’s traits determining their attractiveness are indicators of a good condition, which is determine by good genetic variants, i.e alleles that have the potential to increase offspring fitness (Andersson 1994).

The nature of these genes is still not well defined, but genes linked to resistance to pathogens are often invoked to play a role in sexual selection (Hamilton and Zuk 1982; Westneat and Birkhead 1998; Adamo and Spiteri 2005). In this hypothesis, the key link between attractiveness and genes is condition: if a male is attractive, it is because of its good condition that is due to its genes (Andersson 1986). We talk here about “condition-dependence”, because the condition is crucial in this mechanism. If a male has “bad genes”, it will result in a bad condition and thus in a handicap translated in a low attractiveness, which ensures the honesty of secondary sexual traits (Zahavi 1975; Grafen 1990; Sheldon and Verhulst 1996; Lochmiller and Deerenberg 2000). Therefore, females will benefit from developing a preference for these traits, even if the preference can be relatively costly (Dawkins and Guilford 1996). However, a problem known as the “lek paradox” may arise: if the females always prefer males with the same trait, it should result in a depletion of genetic variation. As the genetic diversity is depleted, the benefit brought from of the choice will disappear (Borgia 1979; Rowe and Houle 1996; Kokko and Lindstrom 1996; Ritchie 1996).

To explain this paradox, several hypothesis have been suggested. A first one, called the “general immunocompetence” hypothesis, argues that these condition-dependent traits become increasingly elaborated, and as condition, depend on the expression of many genes.

2314 The genetic variance is said to be “captured” by the condition and then affects sexual traits
2315 (Rowe and Houle 1996; Tomkins et al. 2004). The traits become a broader target to mutations
2316 and ensure honesty, because if one of these genes is deleteriously mutated, the trait will suffer
2317 from it. The erosion of genetic variation by selection is therefore balanced by this mechanism
2318 (Shuker and Simmons 2014). This hypothesis can also be approached in terms of resistance to
2319 pathogens. For a pool of pathogen present in a host population, the genetic variance in
2320 resistance will be “captured” by the condition dependent traits and the males that have a
2321 higher resistance against the broad diversity of pathogen, thus a good general
2322 immunocompetence, will exhibit the more attractive traits. Here, the term
2323 “immunocompetence” is not only restricted to immune reactions, but is extended to barriers,
2324 tolerance and avoidance behaviour that will reduce infection symptoms, and are heritable
2325 (Owens and Wilson 1999). In this hypothesis, the resistance of an individual is dependent of
2326 its condition, and this latter is independent of the environment. A male with a higher condition
2327 than other males will stay, relatively to other males, in a better condition regardless of which
2328 pathogens are present in the environment.

2329 Another hypothesis has focused essentially on the specificity of the resistance to pathogens
2330 (Hamilton and Zuk 1982). The term of specificity refers to the fact that resistance is mainly
2331 directed against one specific pathogen species or genotype. Here, the pathogen and the host
2332 are engaged in a non-ending arm race. Host condition, and thus host attractiveness, depend
2333 on resistance to the most prevalent pathogen. Here, the condition is dependent of the
2334 interaction between individuals’ genotype and the epidemiological context. This means that
2335 the condition will be good, and the individual attractive, if this individual has a good resistance
2336 to the currently prevalent pathogen. Once the resistance is spread, the pathogen may gain in

virulence, or another pathogen may become prevalent. Thus other host genetic variants will be advantageous, and the genetic diversity is maintained. We can assume here that in the absence of the pathogen, resistant males will not be favoured anymore, and could even counter selected if the resistance is costly. By choosing a male resistant to the currently prevalent pathogen in the population, a female will allow her offspring to have the best chance to resist to pathogens they are likely to encounter. But females will benefit from their choice only if both sires and offspring are exposed to the same pathogens.

These two hypotheses are not necessarily mutually exclusive and can both contribute to the genetic correlation between traits and fitness. Their relative and absolute importance remains unresolved (Zuk and Wedell 2014). However, a crucial point distinguishes them. Under the first hypothesis, males' relative condition and attractiveness is independent of the environmental epidemiological context. Whatever pathogens are present in the population, the males with the best condition will suffer less from them than the others and still be chosen by females. In contrast, under the second hypothesis, a male attractiveness, relatively to others, is strongly dependent of the environmental epidemiological context. A male's condition will depend on whether or not they can fight against the currently prevalent pathogen. Even if the first hypothesis is well supported by the scientific community (Birkhead et al. 2006; Roberts, Buchanan, and Evans 2004; Skarstein and Folstad 1996; Folstad and Karter 1992), Joye and Kawecki (2019) found interesting results in favour of the second one. In an experiment where a female had to choose between two males (both either infected or sham-treated), the relationship between the offspring resistance of each male and the status of their father (winner or loser, depending on the choice of the female) was assessed. They found that males that were more successful in mating contests sired sons that were

substantially more resistant to the pathogen *Pseudomonas entomophila* but only if the males have been themselves exposed to the pathogen before the mating contest. The tendency was inverted in the case of sham treatment.

In this study, we tested the importance of the context in which sexual selection occurs on the relationship between male attractiveness and offspring resistance, using a design similar to the one Joye and Kawecki (2019) used. As they already tested this with, as pathogens, a gram-negative bacteria, *Pseudomonas entomophila*, we used a different kind of pathogen, the fungus *Metarhizium brunneum*. Whereas the bacteria attacks the fly's intestines, the fungus, in the spore form, passes through the cuticle and then need several days for sporulation that lead to the death of the fly. We tested (I) if sexually successful *Drosophila melanogaster* males sire offspring more resistant to *M. brunneum* than unsuccessful males, and whether the male's success depends on a previous exposure to the pathogen. According to the first hypothesis, sexually successful males should sire offspring more resistant to *M. brunneum* than unsuccessful males, whether or not they have been exposed to the fungus. According to the second hypothesis, sexually successful males should sire offspring more resistant to *M. brunneum* than unsuccessful males, but only when males have been previously infected with *M. brunneum.*

A second question we wanted to raise was if the two hypothesis are only restricted to biotic stress (exposure to pathogens) or if they can be extended to abiotic stresses. In order to answer this question, we substituted the pathogen stress with a heat-shock stress. We tested (II) if sexually successful *Drosophila melanogaster* males sire offspring more resistant to heat shock than unsuccessful males, and whether the male's success depends on a previous heat shock.

The third question concerned the specificity of the stress. Indeed, to fully test the hypotheses of "specific resistance" and "general immunocompetence", we have to infect sires and offspring with different pathogens. In population of host that would encounter pathogens A and B, under the "general immunocompetence" hypothesis, sexually successful fathers exposed to pathogen A should sire offspring more resistant to both pathogen A and B, because fathers' heritable relative condition/attractiveness is independent of the pathogenic context, and chosen fathers will always be the ones with a higher general resistance. Under the "specific resistance" hypothesis, sexually successful fathers exposed to pathogen A should not sire offspring more resistant to pathogen B, and maybe even less resistant if specific resistance is costly. To test this, we looked if (IIIa) males that are sexually successful after exposure to *M. brunneum* sire offspring more resistant to *P. entomophila*. We also tested this using abiotic stresses. Then we tested (IIIb) if males that are sexually successful after heat shock sire offspring more resistant to *M. brunneum*, (IIIc) if males that are sexually successful after heat shock sire offspring more resistant to starvation, and (IIId) if males that are sexually successful after exposure to *M. brunneum* sire offspring more resistant to heat shock (fig. 1).

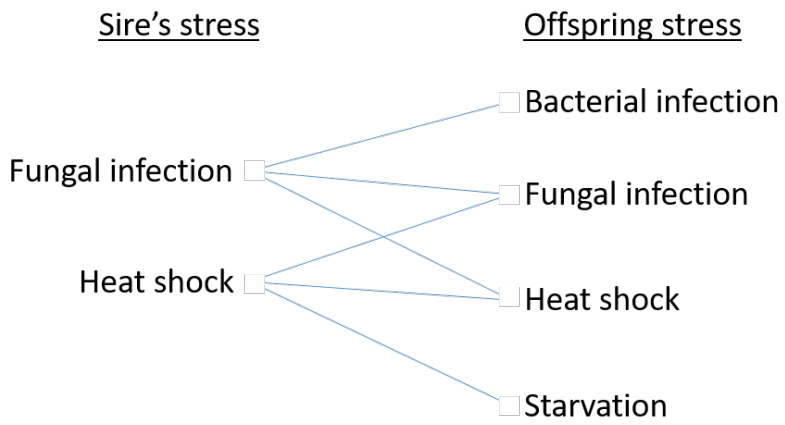


Figure 1. combinations of the different stresses used in the experiment

Additionally, to further analyse the relationship between male sexual success and offspring resistance, we looked at the direct relationship between father's resistance and offspring resistance. We also looked at the relationship between father's resistance and father's sexual success, in order to detect potential dishonest effort (see Copeland and Fedorka 2012).In this experiment, we used female mating choice between two males (both either stressed or sham-treated) determined the males' status, winner and loser. Male courtship is quite complex (Spieth 1974; Krstic, Boll, and Noll 2009) and is considered as a condition-dependent secondary sexual trait. Even if male-male competition can influence the courtship (Dow and Schilcher 1975; Partridge and Farquhar 1983), it is the female that has the final say by deciding or not to mate (Baxter et al. 2018). Thus, the mating outcome reflects the attractiveness of males.

In all experiments, offspring have been sired with other females than ones used for mating trials, and before infection/sham treatment, in order to avoid potential non-genetic transmission due to stress on fathers, but also any maternal investment effects. The

relationship between offspring resistance to stress and male status (winner or loser) was assessed.

We found little support in favour of “specific stress resistance”. This was translated by a general tendency for sexually successful males exposed to a stress A to sired offspring less resistant to a stress B.

Material and methods

Fly maintenance

The organism used for this experiment was *Drosophila melanogaster*. The flies came from a population collected in the canton of Valais (Switzerland) in 2007. The flies were maintained in lab conditions (photoperiod 12:12, 50% humidity, 26.5°C). The manipulations were made under anesthesia with CO2. The food used was a standard food based on agar, yeast, cornmeal, sucrose and Nipagin 20% (respectively 0.2g, 0.6g, 1.6g, 3.3g, 0.3ml) and water for a total volume of 10 ml per vial (or 30 ml per bottles). Larval density was controlled by egg counting (~250 eggs per bottle with food). The virgins were collected within the 12 hours following the emergence.

Male’s coloration

The design of the experiment was the following (fig.2): on day one, we collected the virgin flies and we coloured the males with powder (Sennelier). Half males were coloured in red, the other half in green. On day three, we placed each male in a vial with two virgin females of the

same age, in order to obtain offspring from each male. On day five, we separated males from females and we randomly grouped all males in pairs, with one male of each coloration, and we let the females laying eggs for two more days.

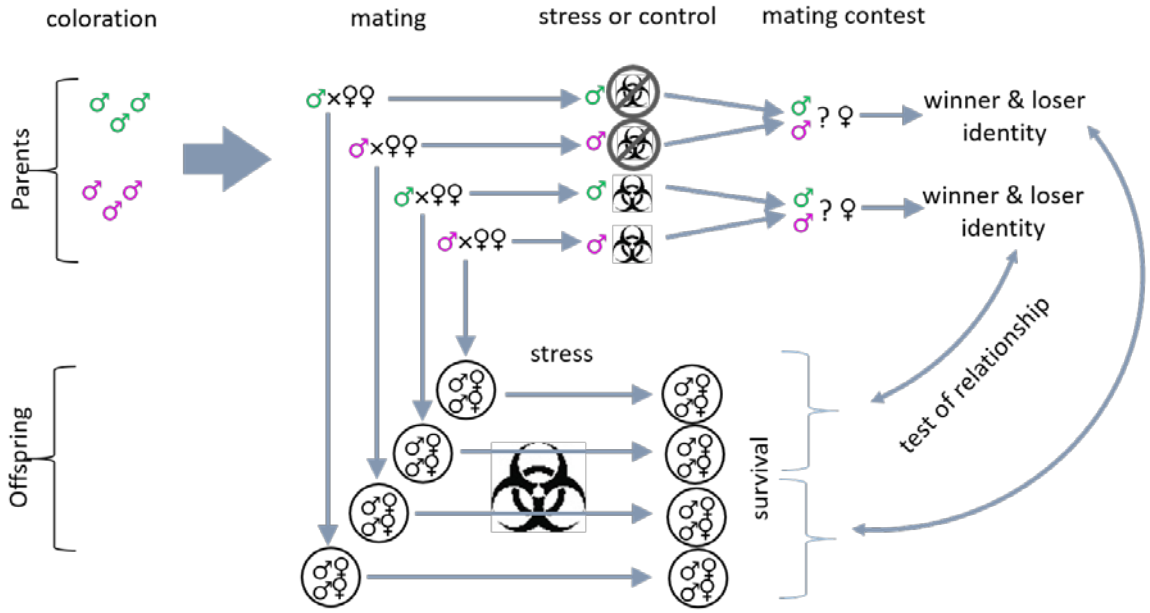


Figure 2. Design of the experiment to study the relationship between sire's sexual success and his breeding value for resistance different stresses (fungal infection, heat shock, bacterial infection or starvation).

Male status under fungal infection

On day six, we infected males by pairs in a solution of spores for half the males and we used a sham treatment for the other half (see "Fungal culture and infection" part). On day ten, we placed the males in new vials, but this time with a laminated paper inside, in order to separate the vial into two parts, and we put a virgin female on the other part. On day eleven, we removed the separation and we noted which male mated first (winner). The utility of the

separation was that after removing it, males and females were directly in presence of each other without having to use CO2 anaesthesia just before and to avoid a potential stress due to the manipulation. We observed all trials during 2 hours for potential mating. When mating occurred, the status (i.e winner or loser) of each male was recorded. In the infected treatment, we also noted which male from each pair survived longer than its competitor within a 7 days time frame. For the sham treatment, we infected both males of each pair after the mating trial and noted which of the two males died earlier than its competitor within a 11 days time frame. Replicates in which no mating occurred where discarded.

Male's status under heat shock

On day five, males were put by pairs on the same side of a vial separated into two parts by a laminated paper. On day six, they received a heat shock stress (for details see the "heat shock" part). They had then 30 minutes to recover, and a female was put in the other side of the vial. The separation was then removed, and we noted which male mated first (winner). We observed during 2 hours for potential mating. After the mating, we stressed all males with heat shock, and we noted which males survived when the other male of the pair died.

Offspring stress

On day 20, we collected offspring that were aged from 1 to 5 days and separated sexes and placed them into separated vials. The stress inflicted to offspring sired from infected father was either a heat shock, or an infection with either *M. brunneum* or *P. entomophila*. The stress

of offspring whose fathers were stressed by heat shock was either heat shock, starvation or an infection by *M. brunneum*. The number of individual in each vial varied between 7 and 10, depending of the number of offspring available. If possible, for each father, we set three vials (for the three stresses that were infection with *M. brunneum*, heat shock and starvation or infection with *P. entomophila*) of 10 sons, and three vials of 10 daughters. On day 21, offspring were stressed, and the survival was assessed during the following days (see below for the number of observations). We did four blocks for each sire stress (heat shock or *M. brunneum*). The sample size varied between 4'000 and 5'000 offspring for each combination of stress, for a total of 27'750 offspring, from 600 fathers and thus 300 pairs of males. We observed 361 mating, but we discarded those with no or too few offspring.

Fungal culture and infection

The stocks of *M. brunneum* were kept at -80°C. We spread out spores on a petri dish (9 cm ⌀) containing SDA (Sabouraud Dextrose Agar), Dodin (inhibitor of unwanted fungi growth), Chloramphenicol and Streptomycin sulphate (both inhibitors of bacteria). We let the petri dish at room temperature until germination. When the plate contained fully sporulated spores (when the plates was full of dark green spores), which correspond to about 107-108 spores, we poured 10 ml of 0.05% Triton X and scraped the plate. We washed twice by centrifugation, and then add 3 ml of 0.05% Triton X solution. Diluting this solution allowed us to count the concentration of spores with a Neubauer Chamber. The concentration of the undiluted solution was of $8x10^8$ spores/ml. The flies were dipped into a 2 ml Eppendorf containing a ten-fold dilution (~$8x10^7$ spores/ml). The sires were infected by pairs whereas the offspring were infected in group of ten. The sham treatment for sires consisted of dipping in a solution of

0.05% Triton X only. Once they were entirely dipped, we removed them with a brush and dried them on a filter paper for a few seconds, before putting them back in their vials. The offspring survival (proportion of living offspring) was noted at 120h, 144h, 168h, 192h, 216h and 240h post-infection.

Pilot for fungal infection

The dilution used for infection was chosen after pilot tests done with dilutions 1/10 and 1/100. It was important to choose a dose would impact male sucess. We ran mating trials between mating and sham treated males. When the infection was done using the 1/100 dilution, infected males were winners in 50% of the matings (39/78, odds ratio=1, p=1, fisher's exact test). With a 1/10 dilution, the proportion of infected winners decreased to 40% (37/95, odds ratio=0.41, p=0.004). We chose to use the 1/10 dilution, which was furthermore consistent with concentrations used in other studies (Keyser, Jensen, and Meyling 2016; Kohlmeier, Holländer, and Meunier 2016; Ríos-Moreno et al. 2017; Grizanova et al. 2019; Clifton et al. 2019).

Bacterial culture and infection

The protocols of culture and infection was the same as the ones used by Joye and Kawecki (2019). The Gram-negative bacteria *P. entomophila* stocks were stored in a freezer at -80°C. We laid out the bacteria on a petri dish containing triptone, yeast, NaCl and agar and 5% of milk. The point of adding milk was to allow us to screen colonies for protease activity (visible

from a pale halo around the colony), which is a marker of virulence (Rondon et al. 2000). We kept the petri dish for 3 days at a temperature of 26.5°C. Then, under the hood, we inoculated a single colony into 50 ml of liquid media. We took only one colony, in order to minimize as much as possible the genetic diversity (and thus virulence diversity). The composition of the liquid culture meda was the same as the solid media but without agar and milk. We incubated the solution for 24 hours on a shaker (190 rpm, 28.5°C). We then poured the 50 mL into 200 mL of fresh medium and incubated for another 24 hours. Then we collected bacteria by centrifugation (20 minutes at 4°C and 3000 rpm) and, after collecting the pellet, we adjusted its concentration to the optical density (OD) of 200 at 600 nm by resuspending it in 0.9% NaCl solution. We diluted then by half with a 5% sucrose solution, so the final OD was 100.

Oral infection was made by putting offspring in a vial filled with agar covered with a filter paper disc, on which we first placed 100 µL of the bacteria mix. Flies were kept so for 24 hours, and then placed again on standard food. Offspring survival was measured at 24h, 48h, 72h and 96h post-infection.

Heat shock

The heat shock stress was done at 40°C using a Percival incubator (I41-VL model). Temperature between 33 and 40 degrees are commonly chosen for experiments of heat shock, because they can correspond to natural stress the flies can encounter (Kilias and Alahiotis 1985). We chose to use 40 °C in order to minimize the age variation during the stress (when the temperature is higher, flies need less time to be stressed and each round of stress can be less spaced in time). In the incubator, the humidity was 50% and the light turned off.

The control flies were just kept in the dark, without temperature variation. To determine the lengths of the heath shock, that needs to reduce male attractiveness, we made mating contests between non-stressed males and males stressed during 30 minutes (followed by 30 minutes of recovery). Less than 40% of mating contests were won by stressed males (42/109, odds ratio=0.39, p=0.001). Concerning the offspring stress, we used a time of 70 minutes, to make sure to induce enough mortality. During the stress, 16 vials of 7 to 10 flies were disposed on a basket (30x20 cm), and each vial was separated from the others or the border by 3 cm. Per round of stress, two basket were incubated. Sire resistance to heat shock was assessed the day after for experiment (I), (II) and (IIId) (where sire stress was infection) and 2 days after for experiment (IIIa), (IIIb) and (IIIc) (where sire stress was heat shock).

Starvation

Concerning starvation stress, the offspring was simply put on vials containing water and agar. We assessed the survival each day for a total of seven days. As a measure of survival, we used the mean time of death for each vial.

Statistical analysis

We used the software R (v.3.6.1) for the statistical analysis. As the different offspring stresses act differently (heat shock acts directly, whereas an infection need hours or days to occur), we used different analysis. We used a Cox proportional hazards model to analyse the survival after fungal or bacterial infection. The model was fitted with offspring survival as the response

variable, sire stress treatment (infected or sham-treated for fungus; heated or control for heat shock), sire status (loser or winner), offspring sex (sons or daughters) as fixed effects, plus two random effects: the sire pair number and the experiment block (4 blocks where sire's stress was fungal infection/ sham treatment, 4 blocks where sire's stress was heat shock/ control). We used the function coxme available on the R package "coxme". We analysed heat shock survival with the glmer function of R package "lme4", based on the same model. The analysis of resistance to starvation was made with the lmer function of R package "lme4" with the mean time of death as response variable. We used the function anova from the R package "stats" for the analysis of interaction between fixed factors (sire's status × sire stress × offspring sex interaction, sire's status × sire stress interaction, sire's status × offspring sex interaction, sire stress × offspring sex interaction). When the interactions were far from significant (>0.1), we removed them and used the Anova function from the R package "car" for the analysis. When not, we analysed by contrasts with the emmeans function of "emmeans" package.

The relationship between offspring survival and sire survival was analysed through a second model with offspring survival as response variable, depending on sire treatment (early or late infection for fungus; once or twice for heat shock), sire survival (more/less resistant), and offspring sex (son or daughter) as fixed variable, and pair winner-loser and block as random variable. When sires stress was infection, we infected the sham-treated sires after the mating, and thus they were infected five days after the pre-mating infected males. This implied that some males were infected when they were six days old (early infection), and other when they were eleven years old (late infection). We kept this difference in the model because it is known that aging can have an effect on immune response (Zerofsky et al. 2005). When sires' stress

was heat shock, we stressed all males after the mating. Thus, it was the second stress for the males which were already heat shocked (heated twice), whereas it was the first stress for the sham-treated males (heated once). We kept this difference in the model because repeated exposure to heat shock can influence the resistance (Krebs and Loeschcke 1994). The same packages and function were used for this second analysis according to the stress.

Sire resistance to either heat shock or *M. brunneum* was analysed through, as before, through either a Cox proportional hazards model for infection and a GLMM for heat chock, with survival as response variable (more resistant or less resistant than its competitor), depending on sires treatment (infected or sham treated; heat shocked or control) and sires status (winner or loser) and pair winner-loser and block as random variable.

Results

a) Relationship between father success and offspring survival

(I) fungus (sires)/ fungus (offspring)

None of the different interactions were significant ($p>0.14$). The survival of the offspring did neither depend on sire's status (sire's status $\chi2 = 0.0052$, $p=0.94$; analysis of variance, cox regression on probability of surviving after fungal infection, Fig. 3a) nor on the sire infection (sire infection: $\chi2 =0.11$, $p=0.74$). Nevertheless, we found a strong effect of the offspring sex (offspring sex: $\chi2 =254$, $p<0.001$). The odds ratio for daughters versus sons was 0.56 at 144h and 0.45 at 192h.

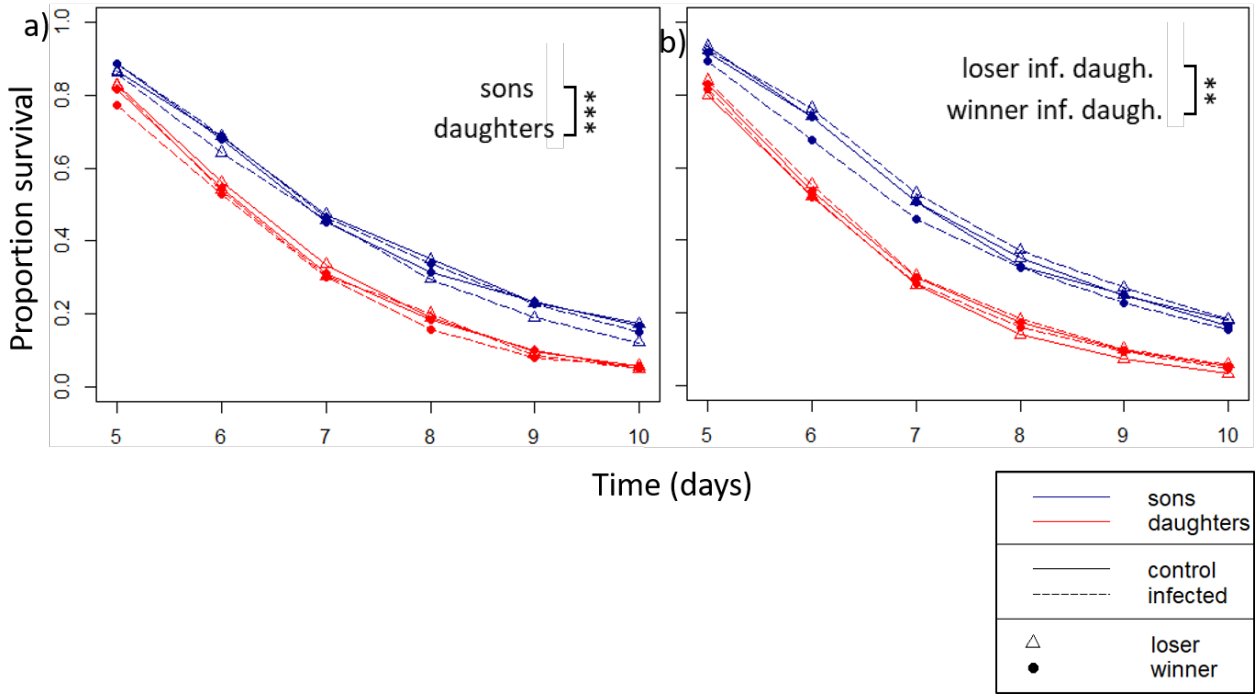


Figure 3. (a) The relationship between offspring resistance to *M. brunneum* and father's sexual success exposed to (a) *M. brunneum* or (b) heat shock.

(II) heat shock (sires) / heat shock (offspring)

Some interactions were not negligible, namely sire's status and offspring sex (sire's status × offspring sex interaction: $\chi 2$ = 3.12, p=0.077, Fig. 4a), sire's status and sire infection (sire's status × sire infection interaction: $\chi 2$ =5.67, p=0.017) and offspring sex and sire infection (offspring sex × sire infection interaction: $\chi 2$ =4.22, p=0.040). That is why we looked at the contrasts for each combination, but found only an effect for sham-treated males (loser-winner for sham/daughters: z. ratio= -1.54, p=0.12; loser-winner for stress/daughters: z=1.25, p=0.21; loser-winner for sham/sons: z=-3.51, p=0.0004; loser-winner for stress/sons: z=-0.80, p=0.42). Odds ratio for sons of sham-treated winner versus loser was 1.4, but this tendency is only present in one block, where sons of sham winner had a probability to survive more than two times (2.26) more than sons of sham looser, which skewed the overall result.

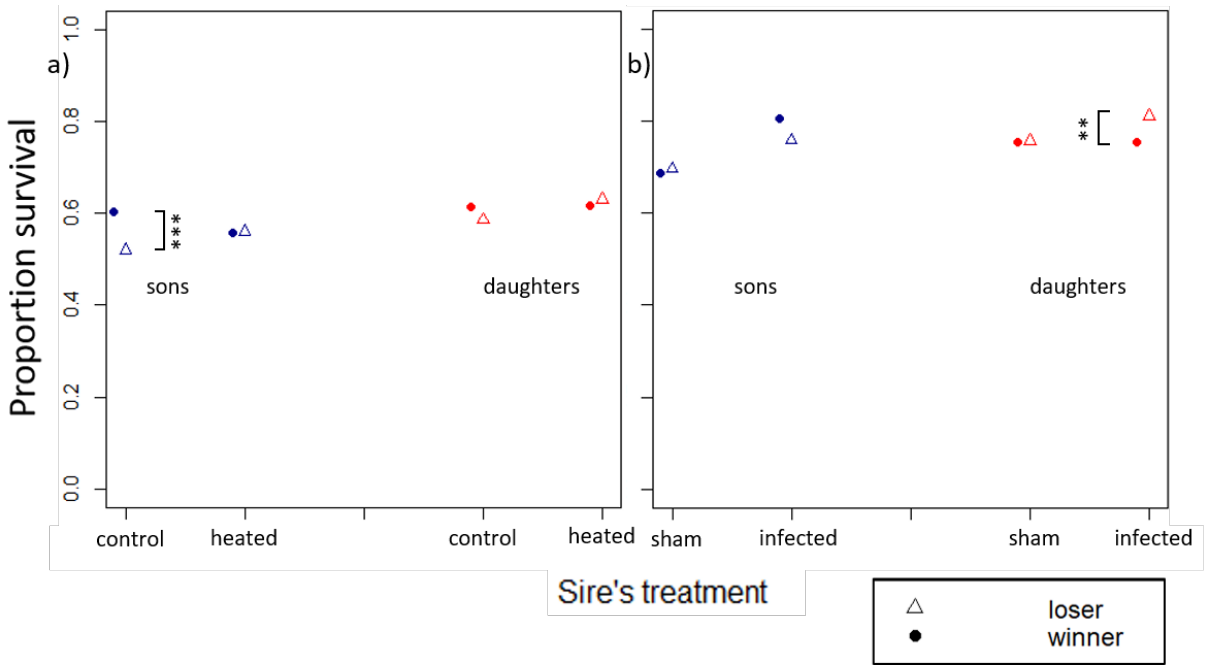


Figure 4. The relationship between offspring resistance to heat shock and fathers' sexual success exposed to (a) heat shock or (b) *M. brunneum*.

(IIIa) fungus (sires)/ bacteria (offspring)

None of the different interactions were significant ($p>0.44$). Offspring survival depended strongly on fathers' status (sire's status: $\chi2 = 16.05$, $p<0.001$, Fig. 5a) and the offspring sex (offspring sex: $\chi2 = 1064$, $p<0.001$) but not on sire infection ($\chi2 = 0.003$, $p=0.96$). Odds ratio for losers' offspring versus winner was 1.17 at 72h post-infection and 6.85 for sons versus daughters.

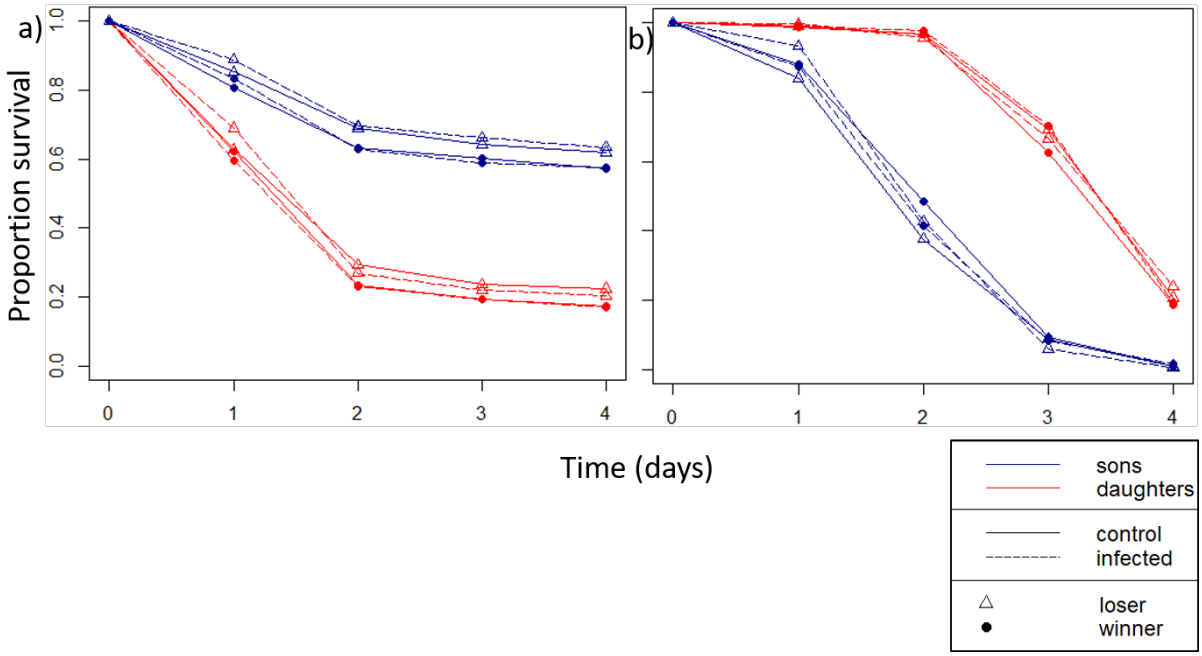


Figure 5. (a) The relationship between fathers' sexual success exposed to *M. brunneum* and offspring resistance to *P. entomophila*. (b) The relationship between fathers' sexual success after heat shock and offspring resistance to starvation

(IIIb) heat shock (sires) / fungus (offspring)

As the interactions between sires'1 status and offspring sex (sire status × offspring sex interaction: $\chi 2$ = 3.18, p=0.075, Fig. 3b) and between sire status and sire stress (sire status × sire stress interaction: $\chi 2$ = 6.04, p=0.014) were not negligible, we analysed by contrast. It indicated a significant effect only for the offspring of the stressed males (loser-winner for control/daughters: z. ratio= 1.27, p=0.20; loser-winner for stress/daughters: z=-1.67, p=0.094; loser-winner for control/sons: z=-0.82, p=0.41; loser-winner for stress/sons: z=-3.54, p=0.0004). Odds ratio for sons of heated losers versus heated winners was 1.53 at 144h and 1.28 at 196h. Even if the case of daughters of stressed sires was not significant, the tendency

that daughters of stressed loser survived more than stressed winner was consistent among the four blocks (odds ratio 1.15 at 144h and 1.09 at 196h).

(IIIc) heat shock (sires) / starvation (offspring)

As the relationship between sire status and sire stress seemed to be dependent of the sex (sire stress × sire status × offspring sex interaction: $\chi 2$ = 3.30, p=0.069, Fig. 5b), we separated the analysis for males and females. For sons, their survival did neither depend on sire status (sire status: $\chi 2$ = 0.03, p=0.86) nor on sire infection (sire infection: $\chi 2$ =0.52, p=0.47). For daughters, their survival did not depend on sire status (sire status: $\chi 2$ = 1.01, p=0.31) nor on sire infection (sire infection: $\chi 2$ = 0.20, p=0.66).

(IIId) fungus (sires) / heat shock (offspring)

For this experiment, the link between sire status and sire infection was influenced by offspring sex (sire infection × sire status × offspring sex interaction: $\chi 2$ = 5.07, p=0.024, Fig. 4a). We split offspring by sex for the analysis. The survival of sons did neither depend on sire status (sire status: $\chi 2$ = 2.21, p=0.14) nor on sire infection (sire infection: $\chi 2$ = 2.29, p=0.13). For daughters, we analysed by contrasts because of a significant effect of the interaction between sire status and sire infection (sire status × sire infection interaction: $\chi 2$ = 4.31, p=0.038). No effect was found for daughters whose father was sham treated (loser-winner sham-treated: Z=-0.076, p=0.94), but an effect was found for the daughters of the infected sires (loser-winner stressed: Z=-2.94, p=0.0033). Odds ratio for daughters of the infected losers versus infected winners

was 1.35. However, this tendency was only present on the half of the four blocks (one block indicated no tendency, one block indicated the opposite tendency).

b) Relationship between father survival and offspring survival

(I) fungus (sires)/ fungus (offspring)

We observed a marginally significant trend of offspring sex to influence the link between sire survival and sire infection (treatment × sire survival× offspring sex interaction: $\chi 2$ = 2.93, p=0.087), justifying the splitting into males and females.

For daughters, an interaction was observed between sire survival and treatment (treatment × sire survival interaction: $\chi 2$ = 12.49, p=0.00041). The analysis by contrast showed that for daughters whose father was infected after the mating contest, those whom father died before its competitor had higher survival than daughters whom father died after its competitor (z=3.08, p=0.0022). Odds ratio for daughters whom father died earlier VS after than its competitor was 1.20 at 144h and 1.63 at 196h. For daughters whose father was infected before the mating contest, they showed a tendency in the opposite direction, with daughters whom father died after its competitor which had a tendency to survive more than those whom father died before its competitor (z=1.91, p=0.056); the odds ratio for daughters whom father died after VS earlier than its competitor was 1.35 at 144h and 1.30 at 192h. For sons, survival was correlated with sire survival (sire survival: $\chi 2$ = 4.00, p=0.046) but not on the treatment (treatment: $\chi 2$ = 0.40, p=0.52). Sons whose father lived longer than its competitor were 1.24 times more likely to survive at 144h, and 1.29 at 196h.

(II) heat shock (sires) / heat shock (offspring)

The offspring survival depended strongly on sire survival (sire survival: $\chi 2$ =12.84, p=0.00034), but neither on treatment (treatment: $\chi 2$ =0.0095, p=0.92) nor on offspring sex (offspring sex: $\chi 2$ =0.94, p=0.33). Offspring whose sire survived longer were 1.5 times more likely to survive than offspring whose sire died earlier.

(IIIa) fungus (sires)/ bacteria (offspring)

The interaction between treatment and sire survival led to a separation by treatment for the analysis (treatment × sire survival interaction: $\chi 2$ =4.78, p=0.029). However, the offspring survival did neither depend on sire survival for lately infected sires (z=1.44, p=0.15) nor for early infected (z=-1.69, p=0.090).

(IIIb) heat shock (sires) / fungus (offspring)

The interaction between sire survival and offspring sex (offspring sex × sire survival interaction: $\chi 2$ =4.16, p=0.041) and between sire survival and treatment were not negligible (treatment × sire survival interaction: $\chi 2$ =2.93, p=0.087).The offspring survival of sons was not correlated with sire survival, neither for sons whose father was twice stressed (z=0.96, p=0.33) nor for once stressed (z=-0.95, p=0.34). The offspring survival of daughters depended on the sire survival but only for daughters whose father have been stressed twice (z=3.74, p=0.0002) and not for daughters whose father have been stressed once (z= 1.62, p=0.10).

Odds ratio for daughters whose twice-stressed father survived longer versus earlier than its competitor was 1.75 at 144h and 1.54 at 196h.

(IIIc) heat shock (sires) / starvation (offspring)

As explained earlier, we separated males from females for this analysis. For sons, their offspring depended on sire survival (sire survival: $\chi 2$ =11.75, p=0.00061) but not on the treatment (treatment: $\chi 2$ =2.29, p=0.13). Sons whose sire survived more had a mean of survival of 1.9 days, whereas sons whose sire survived less had a mean of survival of 1.6 days, giving that sons whose sire survived more versus sons whose sire survived less had odds of 1.19. For daughters, their survival did neither depend on sire survival (sire survival: $\chi 2$ =0.52, p=0.47) nor on treatment (treatment: $\chi 2$ =0.0005, p=0.98).

(IIId) fungus (sires) / heat shock (offspring)

As the interaction between offspring sex and treatment (offspring sex × treatment: $\chi 2$ =11.29, p=0.00078) and offspring sex and sire survival (offspring sex × sire survival interaction: $\chi 2$ =4.065, p=0.044) were important, we split into males and females. Offspring was only different for daughters (z. ratio= 2.16, p=0.031), and not for sons (z=-0.70, p=0.48). Daughters whose father died earlier versus after its competitor had odds ratio of 1.2.

c) Relationship between father's resistance and father sexual success

The survival of winner versus loser sires was dependent of their infection status (sire status × sires treatment interaction: F= 7.59, p=0.006, analysis of variance). The sires' success predicted their own survival to fungal infection when they have been infected before the mating contest (sire status under infection: F=33.34, p<0.001) but not when they have not been previously (sire status without infection: F=1.38, p=0.24). Odds for infected winner sires versus infected loser sires was 2.71, whereas for sham-treated winner sires versus sham-treated loser sires, odds was 0.79. The survival of winner versus loser sires was dependent of their stress status (sire status × sires treatment interaction: F= 13.06, p=0.0005). The sires' success predicted their own survival to heat shock when they have been heat shocked before the mating contest (sire status under infection: F=7.23, p=0.01) and also when they have not been shocked before the mating contest (sire status under infection: F=5.97, p<0.018). Odds for heat shocked winner fathers versus heat shocked loser fathers was 1.9, whereas for control winner fathers versus control loser fathers, odds was 0.43.

Discussion

We found that in some cases, fathers that were more successful in a mating contest when they were stressed sired sons less resistant to other stresses (fig. 6a and c). The experimental design was thought to avoid non-genetic paternal effect of winning versus losing the mating contest or stress exposure. These findings underline the importance of the environmental context under which competition for mates and mate choice takes place. They support partially the "specific resistance" model, which posits that females make their choice

depending on the currently prevalent stress in the population, because their offspring are likely to encounter this same stress. Nevertheless, if the offspring do not encounter the same stress, they should not be more resistant, and even less resistant in the case of a costly resistance. This hypothesis is partially supported, because we did not find any support that more sexually successful males under a stress sired offspring more resistant to this same stress (fig. 6a and c).

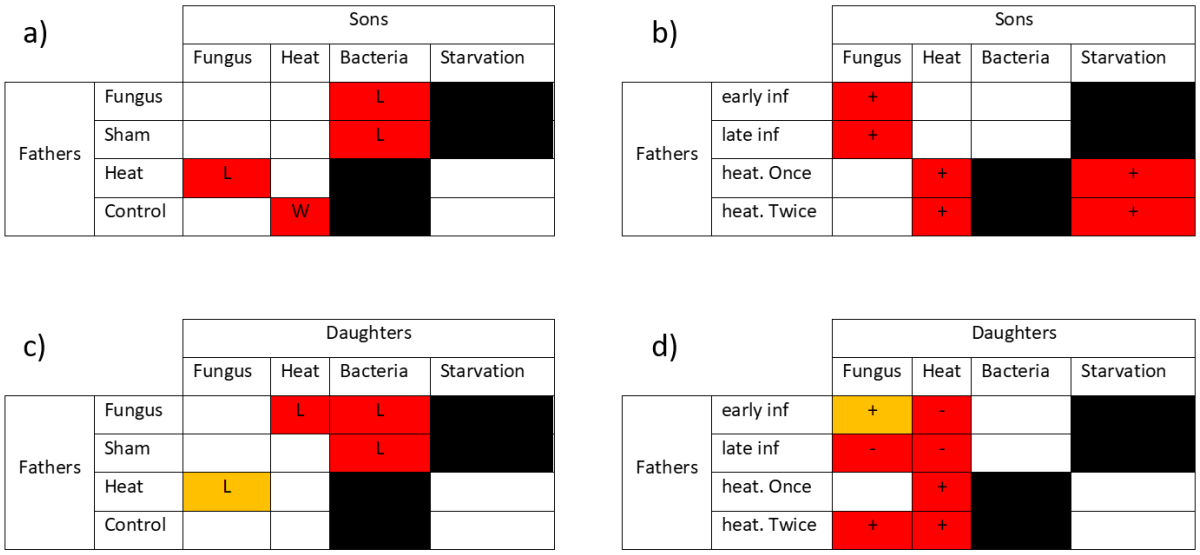


Figure 6. Summary of the different results. Relationship between father's sexual success and sons (a) or daughters (c) resistance to stress. The letter "L" indicates that offspring of the loser were more resistant, whereas the letter "W" indicates that offspring of the winner were more resistant. The color red correspond to a significant effect ($p<0.05$), orange to a tendency consistent among blocks ($0.1>p>0.05$), white to no tendency ($p>0.1$) and black to no test. Relationship between father survival and sons (b) or daughter (d) resistance to stress. The sign "+" indicates that fathers that survived more sired offspring more resistant, whereas the sign "-" indicates that fathers that survived less sired offspring more resistant.

We did not find any support to our first question, which was whether more sexually successful fathers sire offspring more resistant to a fungus than unsuccessful fathers, and whether the male success dependes on a previous exposure to the pathogen. The survival of the offspring seemed not to differ between winner and loser males. This result does not support the “good genes” hypothesis that says that males with best condition are chosen by females in order that her offspring will have good condition. An explanation could have been that the mating contest was too early after the infection, and the effect of infection was not yet physiologically important. If so, even non-resistant males did not suffer from this infection and could have kept their condition. But we measured that 10% of infected fathers died from the infection until the mating contest, indicating that the infection occurred well (28 death for 262 infected fathers, 3 death for 188 sham-treated fathers). However, as we found a positive link between male’s survival and offspring survival to infection (at least for sons), the mechanism of this resistance is probably not based on non-additive genetics components like individual factors or heterozygosity that would not have been inherited by offspring. The fact that females do not always show the same tendency of resistance was already found by Joye and Kawecki (2019). The fact that we found a positive link between sexual success and resistance of the males allows removing the question of a potential “dishonest” effort of the males (Copeland and Fedorka 2012). As the winner infected males survived longer than looser infected males, it would indicate that the less resistant males have a lower condition than the most resistant males. However, it is not so obvious to conclude in this way, because the mating could have an influence on the survival of the males. Another explanation could have been a low or non-heritability of the resistance, but the results showed a positive link between male’s survival and offspring survival to infection, at least for sons.

The second question, which was if the “good genes”, the “specific resistance” and the “general resistance” hypotheses are not only restricted to genes of resistance to pathogen but also to resistance to abiotic stresses, was also not answered. We did not find any link between offspring survival to heat shock and father’s status, and this regardless of if sires have been heat shocked or not. A potential explanation that low or non-heritability of the resistance to heat shock was eliminated, because we found a strong positive relation between sire survival to heat shock and offspring survival to heat shock. However, as the previous experiment, the effect of mating could have influenced the survival of the sires. Moreover, we found an interesting link between sire sexual success and sire resistance to heat shock, with an opposite sign according to the treatment before the mating. Thus, the stress seemed to influence the mating choice anyway, even if we could not detect it with the offspring survival.

For the third question, which was if the offspring survival to a stress A is influenced by the sexual success of their fathers under a stress B, we found some interesting supports in favour of the “specific resistance” model. This was translated by a tendency for offspring of winner males under a stress A to survive less under a stress B. In the case of “fungus (sires)-bacteria (offspring)”, we found that sexually successful fathers sired offspring less resistant to *P. entomophila*, regardless if the fathers have been infected by *M. brunneum* or sham-treated. However, the relationship between sire survival to fungus and offspring survival to bacteria did not show any tendency. This indicates that sires more resistant to fungus do neither sire offspring more resistant nor less resistant than sires less resistant to fungus.

For the case “heat shock (sires)-fungus (offspring)”, the “specific resistance” model was also supported. Offspring of heat shocked winner were less resistant to fungus than offspring of heat shocked losers. However, we did not find any relationship for offspring of control fathers.

These findings could indicate that these resistances are costly. To support this view, we have to compare with the “fungus (sires)-heat shock (offspring)” case. Here again, infected sexually successful fathers sired offspring less resistant to stress than infected sexually unsuccessful fathers, but this was only the case for daughters and not for sons.

In the last case, the “heat shock (sires) - starvation (offspring)”, we did not find any relationship between father sexual success under heat shock and offspring resistance to starvation. The case of starvation is a bit different from the three others stresses. It implies mainly the fat storage and the size of the fly (Chippindale, Chu, and Rose 1996; Harshman, Hoffmann, and Clark 1999). These results suggests that the mechanisms of resistance for heat shock and starvation are not implied in a trade-off between them. In fact, a surprising positive link was found between sire’s survival to heat shock and offspring resistance to starvation. The fact that we found, after death of flies under starvation, some mycelium on them, we can imagine that under starvation flies have to allocate energy for basic metabolism and neglect other functions. Thus, them that have the best immunology will survive longer.

The difference of survival between sons and daughters goes not in favour of some hypothesis saying that females should invest more in resistance than males (Zuk 1990; Rolff 2002). The main argument going in favour of a better resistance for females than males is that a male can mate several times within a not long interval, and thus can get a lot of offspring even if his lifespan is short, whereas a female need each time the duration of maturation of eggs and thus is more dependent of her lifespan. However, our results is in agreement with some experiments showing that drosophila females are more susceptible to infections than males (Shahrestani et al. 2018).

In summary, we found some supports in favour of “specific resistance” model. Sires with the highest condition after an exposition to a stress A are chosen by the females. Their offspring are less resistant to a stress B than offspring of unsuccessful males. This shows that the choice of the females is influenced by the stress context of the population. However, to prove fully the “specific stress resistance” we should have observe that sexually successful fathers exposed to a stress should sire offspring more resistant to this same stress. This claim should be observable as well under “general stress resistance” and “good genes” hypotheses, but we did not observe that for either fungal infection or heat shock, unlike Joye and Kawecki (2019) found with a bacteria. The fact that the heat shock and fungal resistance was heritable showed that the mechanism of resistance is probably due to heritable variation This study calls for a broader study with more stresses in order to analyse the “specific resistance” model.

Appendix 2

First step project

This appendix is a first step project done by a master student, Louaï Maarachli, under the supervision of Patrick Joye and Tadeusz Kawecki

How does infection impact male courtship behaviour?

Louaï Maarachli

Abstract

Sexual selection is the result of the competition for reproductive opportunities and leads to exaggerated sexual traits. The Hamilton-Zuk hypothesis states that secondary sexual traits are condition dependant and should therefore be affected by infection. Individuals in good condition should be able to display higher quality secondary sexual traits. By contrast, the terminal reproductive investment is an effect observed in a few different species where infected males display dishonest signals when under infection. In *Drosophila melanogaster,* one of those sexual trait is the courtship behaviour. This courtship behaviour is complex and an important component of the reproductive success of males. But it is not known yet how this courtship behaviour is impacted by infection. In this study, we use *Pseudomonas entomophila* to infect male flies and look at the effect of infection on courtship intensity as an indicator for courtship behaviour. Here we show that infection has an influence on courtship

intensity as infected males court less than non-infected ones. These results are a first peek at the effect of infection on courtship behaviour and are promising as they follow the Hamilton-Zuk hypothesis. However, further investigations are needed to assess the total impact of infection on courtship behaviour.

Introduction

Sexual selection can be summarized as a result of intersexual as well as intrasexual competition over reproductive opportunities. In most cases, the intrasexual aspect of sexual selection affects the males as they are the one competing for the access to females. However, the intersexual aspect of sexual selection is often carried out by the females expressing preferences to some male sexual traits. Those two aspects of sexual selection are closely related and do not exclude each other (Andersson 1994; Endler and Basolo 1998; Kotiaho 2002).

Some male sexual traits can be used for to competition such as armaments, while others can be used to please the preference of the female and influence them on their mate choice, such as coloration or odours. However, They are not exclusive and some of the armaments traits can be used as well by the female as a factor of choice and not only by the male as a competition tool (Berglund, Bisazza, and Pilastro 1996; Kotiaho 2002; Kotiaho, Simmons, and Tomkins 2001).

The secondary sexual traits of the males are therefore affected by sexual selection. Female preferences as well as competition select sexual traits and lead them to exaggeration. Indeed, displaying an exaggerated version of a trait preferred by females can lead to better

reproductive success. In one of the actual models of sexual selection, the “good gene hypothesis”, these traits are thought to be honest signals that indicate the individual’s genetic adaptation to its environment, representing “good genes”. For those assumptions to work, these traits must be costly and “condition-dependent”. The amount of energy allocated to the development of secondary sexual traits is dependent on the condition of the individual. This condition can be influenced by the environment, an individual well adapted to its environment will be able to allocate more energy to secondary sexual traits. However, genetics deleterious mutations can also affect the condition of an individual (David et al. 2000; Griffith, Owens, and Burke 1999).

The Hamilton-Zuk hypothesis states that infection of the males by parasites can have an influence on the sexual selection and the evolution of secondary sexual traits (Hamilton and Zuk 1982). The assumption would be that an infected male has less reproductive success than a non-infected one due to its “condition-dependent” traits. In an environment where the selective pressure by a parasite is high, resistant males can exhibit higher quality secondary sexual traits. Those males are preferred by females as they can transmit the resistant genes to their offspring by choosing them (Balenger and Zuk 2014). However, if the pathogen putting selective pressure should come to disappear, males having resistant genes for it might not be able to express higher quality secondary traits anymore. Indeed, having specific resistant genes can be costly and the effect of this cost in a pathogen-free environment could be lower quality secondary sexual traits. Therefore, the pathogenic diversity of the environment in which the female preference is done is highly influent on the direction of the selection. Some species display sexual traits that vary greatly between individuals, while other species have less variation. It is expected that this aspect of sexual selection would be more effective in a

species displaying great variation in sexual traits than in a more discreet one (Hamilton and Zuk 1982).

However, it is interesting to note that infection can have the opposite effect on sexual traits. In some cases, an individual can recognize that it has been infected. When reaching a certain threshold of infection, the individual can invest most of its energy to reproductive behaviours. This effect is called the terminal reproductive investment. This action lowers the long-term survival of the individual but will greatly increase its short-term reproductive success. This is known as a "dishonest signal". The individual will display sexual traits that are not representative of its actual state and therefore will misdirect the females to believe he is a good mating choice when he is not (Adamo 1999; Agnew et al. 1999; Bonneaud et al. 2004). This effect has been observed, for example, in the cricket species *Allonembius socius* (Copeland and Fedorka 2012).

Previous results showed that infection has an influence on sexual selection, resistant males were more chosen by female than susceptible ones in the presence of the pathogen. The question we are asking here is how does this impact courtship behaviour. The first hypothesis would be that a sick male displays less effective sexual signals while courting the female. While a second hypothesis would be that infected males display more effective sexual signals due to the terminal reproductive investment effect. In the context of courtship behaviour, looking at courtship intensity (i.e. the proportion of time spent courting in a set amount of time) can be a good indicator of the effect of infection on the overall courtship behaviour. Being less proficient in courtship than other males can be an indicator of bad condition. In the context of male-male competition, the males able to give more energy to courting and therefore able to court more should have more opportunities to mate. Whereas in the context of intersexual

competition, females should prefer males allocating more time to courting taking it as an honest signal of good condition.

In *D. melanogaster* the sexual signal mostly consists of a complex courtship ritual (Bastock and Manning 1955). The male fly will proceed to court the female following precise behaviours.

The objective of this project is to observe and measure the courtship intensity of males whether they are infected or not. It has already been observed that poor conditions can impact the courting intensity in horned dung beetles (Kotiaho 2002). Therefore, the concrete question is “How does infection impact male courtship intensity?”. We predicted an effect of the infection on the courtship intensity in *D. melanogaster* according to the Hamilton-Zuk hypothesis. This impact is expected to be a decrease in courtship intensity due to the infection. However, this impact might be the opposite in the context of terminal reproductive investment.

To test the changes in courtship intensity in *D. melanogaster* whether it is infected or not, we conducted a behavioural experiment. To infect the flies, we used *Pseudomonas entomophila* as it is a natural virulent pathogen of *D. melanogaster*. By putting an infected male fly with a female in a petri dish and filming them, we can measure their courting intensity. By then comparing this intensity with the one of control flies in the same conditions, we can measure the differences in their intensity and assess the impact of infection on courtship intensity on males. A second experiment putting the observed male in competition with another male in control conditions was done. As there is no competition in the first experiment, the infected males might allocate more resources to fighting the pathogen than they would in a competition setting. The competition setting is expected to stimulate the males to court at

their maximum. This stimulation could lead to different results between competition and non-competition settings.

Furthermore, to see if the decrease in intensity is not only due to the flies being incapacitated by the infection, we tested flies infected with a lower bacteria dosage. This lower bacteria dosage is expected to only activate the immune system of the flies without killing them.

In summary, we showed that infection has an impact on courtship intensity. Male flies infected with high dosage court significantly less than control flies in both competition and non-competition settings. These results support the Hamilton-Zuk hypothesis on the fact that male secondary sexual traits are condition-dependent, and those traits are impacted by the presence of the pathogen. This impact can have an influence on the mating choice.

Material and methods

Breeding

The population of flies used is called “Valais”, it is a wild type population of fruit fly that has been collected in Valais in 2007 and maintained in laboratory condition since. On the first day morning, the flies were left to reproduce on standard yeast-cornmeal food medium (cornmeal, agar, yeast, sucrose). On the evening of this same day, the flies were collected and put on an orange juice and yeast substrate for egg laying. The next day, the eggs were collected and counted. Groups of 200 were put back on standard yeast-cornmeal food medium for growth. Approximately twelve days later, the flies started to emerge. Around the start of emergence, when flies hatch from the pupa, virgin flies were collected. They are the

ones the experiments were conducted upon. The already hatched flies were eliminated in the morning and, on early afternoon, all the newly born flies were taken and sorted by sex. They were then left over five days on standard yeast-cornmeal food medium to mature.

After 6 days, and 24 hours before the observation, the females were put with males for 3 hours to mate. They were then separated again until the observation. This was done to make the female unreceptive to male courtship. Indeed, it has been observed that a female that has recently mated will be less likely to respond to the courtship efforts of the males while not stopping the males from courting them (Manning 1967). The unreceptivity of the females was essential to this experiment as we did not want any mating happening in the film time. Mating in *D. melanogaster* takes time and would make the film where it happened not usable.

Infection

To infect the flies with bacteria, we needed to prepare the bacteria so that they can orally infect the flies as well as having the right concentration for infection. To do that, we started by preparing the LB medium on which the bacteria would grow. The recipe for 500ml is 5g of Tryptone, 2.5g of yeast, 5g of NaCl, 7.5g of agar and 5ml of milk. All those ingredients except the milk were then put in water to reach 500ml and the solution was autoclaved. After autoclaving, the milk was added.

The milk was added to see if the bacteria were virulent. The bacteria used, Pseudomonas entomophilia have an enzyme that can denature lactose. Some mutation can alter enzyme

activity and with it, the virulence of the bacteria. When the enzyme is active, the medium gets clearer around the colony, indicating that it is virulent.

Pseudomonas entomophila were then plated on the petri dishes and left to grow for a few days. When the colonies appeared to have grown enough, petri dishes were put in the fridge for later uses. In the petri dishes, we could see if the colony was virulent or not thanks to the denaturation of lactose around it and could select only one of the said virulent colony. By using a single colony, we made sure that there was as little variation as possible in the genome of the bacteria used for the infection.

To grow bacteria and use them for oral infection, and in the meantime, obtain the right concentration, we prepared a liquid medium (without agar). The recipe for 250ml of this liquid lb is 250ml of water, 2.5g of tryptone, 2.5g of NaCl and 1.25g of yeast. The medium was put to autoclave, then put in an incubator for 24 hours. The medium was then centrifuged, and the pellet was collected. The concentration of bacteria in the precipitation is measured at 600nm and diluted with 0.9% NaCl solution to optical density (OD) 200 for the “High-infected” treatment and OD 20 for the “Low-infected” treatment. Each solution was then mixed at 50% with a 5% sucrose solution to finally obtain the wanted concentration, OD 100 for the “High-infected” treatment and OD 10 for the “Low-infected” treatment. Sucrose was added so that the flies had something to eat in the infection phase. The water and agar substrate does not have enough nutrients alone. Moreover, it increases the chances of the flies eating the liquid medium containing the bacteria to the concentration needed.

To infect the flies, they were first put to starve in a food-empty bottle for 3 hours. The aim of the starving process is to have higher chances for the flies to eat the liquid medium containing the bacteria when we try to infect them. They then were put on a water-agar substrate

covered by a filter paper soaked with 100ml of a solution of the concentration of bacteria wanted for each treatment. For the Control treatment, the bacteria were replaced with a 0.9% NaCl solution, also mixed with a 5% sucrose solution. The flies were left on this substrate for 24 hours.

Filming

Finally, when all the male's treatment and female's unreceptivity had been set, the preparation for the observation could start. The objective was to film and then measure the courtship intensity (i.e. the proportion of time spent courting in the time of the film) of the virgin male with a previously mated and therefore unreceptive female. The male was therefore put in a petri dish with a female. The petri dishes were filled with a substrate of apple juice and agar, this substrate had been chosen for its colour. The colour of the apple juice-agar medium was contrasting enough with the flies to be able to see them clearly on the cameras.

The petri dishes used had a slit on both side, permitting the placement of a plastic band. This plastic band was twice as long as the petri dish and had a half that was full and the other half that was pierced by a "window" allowing the flies to pass. The full half of the band was used to separate the male and the female for 24 hours before the filming. The flies were separated for 24 hours after placing them in the petri dishes to make them accustomed to the new environment.

The petri dishes were then put under the cameras (Logitech 905) and the band was moved to its "windowed" half to let the fly pass and meet each other (Fig. 1). The filming could then

start. The courtship was filmed for one hour and recorded using the ContaCam (ver. 3.0.0) program. We had 7 cameras and each camera could film 4 petri dishes at a time, making the total number of observed individual 28 per day of observation. As we had 3 blocs, the number of individual filmed totals at 84. However, the flies that mated or died during the films were eliminated as materials for observation leaving the total observed individual at 50.

Figure 1: Scheme of the petri dish setup with the plastic band. On the left, the petri dish is separated and there is no way from one side to the other. On the right, the "window" in the plastic band allows passage.

Single male assays

The first experiment was a single male assay, we put one male and one female in a petri dish and measured the courtship intensity of the male. For this experiment, we had three different treatment for the male flies: the "High-infected" flies that had been infected with a high dosage of bacteria of optical density (OD) 100, the "Low-infected" flies that had been infected with a lower dosage of bacteria (OD 10), and the "Control" flies that had not been infected but went through all the same manipulation as the other two treatment. The "High-infected" treatment is the flies infected with the standard dosage of bacteria that is our first subject of interest. By comparing the courtship intensity of this treatment to the control one, we were able to assess the impact of infection on the courtship intensity. However, we wanted to see if there was a difference in courtship intensity whether the flies were infected with a high

dosage of bacteria or with a lower dosage. Indeed, with a high dosage, the fly might simply be incapacitated by the infection and this could hinder its courtship intensity. To observe to which point this courtship intensity is affected by the dosage of bacteria used for infection, we used another treatment. This treatment, with a lower bacteria dosage should only stimulate the immune system without killing the flies. The flies were put in petri dishes and put under cameras, the 1-hour films that resulted from this were the subject of the observation and data collection (see “data collection” part).

Two males assays

In the second experiment performed in this study, we wanted to observe the effect of infection in a competition setting. To observe male-male competition between the two males, we need to have two males in the box competing for one female. To differentiate the two males, they were coloured with coloration powder. This coloration was done 3 days in advance to the observation to let the fly some time to clean themselves as it has been observed that they are mostly doing that in the first 48 hours after the infection. The coloration is done by putting coloration powder in a vial with the flies and mixing it gently so that the powder deposes itself on the flies. The flies are then put back in their bottle.

The observations were made on the male-male competition between the same treatments used in the first experiment and a control fly (i.e. “high-infected”-Control; “Low-infected”-Control; Control-Control). To avoid any bias from the different colorations, half of the flies from each treatment were coloured in red and the other half in green. That way, in half of the observation, the focal fly was green and on the other half it was red. Finally, for the

measurements, the treated male fly was selected as a focal fly (the “High- infected” fly for the “High-infected”-Control tests, the “Low-infected” fly for the “Low infected”-Control tests and one of the control flies for the Control-Control tests).

Data collection

Data were extracted from the video by a human observer. Courtship intensity was recorded as such: The observer recorded if the male is courting or not every 30 seconds of the video, making therefore 120 observations per individual. For the first experiment, we had 3 blocks of 7 films each capturing 4 individuals, adding up to 28 individuals per blocks and a total of 84 individuals. However, due to death, mating or escapes during the filming, the total number of individuals considered is 50, 15 for Control and Low-infected and 20 for High-infected. For the second experiment, the setup was the same (i.e. 7 films of 4 individuals) but we had 2 blocks, adding up to a total of 56 individuals. However, the same events occurred in this experiment and in the first one. Some of the flies either died, mated or escaped. Those were not considered, reducing the total of individuals considered to 32, 11 Control, 10 Low- infected and 14 High-infected. The observations were done blindly.

Statistical analysis

The statistical analysis was performed in R, using the “lme4”, “afex” and “emmeans” packages. A repeated measure type analysis was performed on the data using a generalized linear mixed model (GLMM). Taking the courtship intensity as a binomial response variable, the treatment as a fixed variable, the video time as a continuous variable and the individual as a random

variable. The interaction between the treatment and video time was tested as well. But as it was not significant, it was discarded for the analysis.

Pairwise comparisons of the least square means between the three different treatments were performed as post-hoc tests. The same analysis was performed for the data of both experiment. However, for the second experiment, the interaction between treatment and colour was tested as well. Yet it was not significant and was therefore discarded. For the two analyses, the 5 first minutes of observation were discarded as they were probably affected by the recent opening of the separation, disrupting the behaviour of the flies.

Results

Single male assays

According to the Hamilton-Zuk hypothesis, sexual traits should be condition-dependant. To test that we set up males of different condition (i.e. different levels of infection) to courtship a female. Each male was put alone with a female for one hour and the time spent courting in this time span was measured as an indicator of courtship intensity. The males were either control, highly infected or lowly infected. The proportion of individual courting every 30 seconds for each treatment (that has been averaged to every 5 minutes for visibility in figure 2) shows a trend of the courtship mean going up with time. This trend is supported by the p-value (Chisq 27.51, df = 1, $p < 0.0001$) of the effect of time on the courtship intensity variable given by the GLMM. In addition, we can see that the three different treatment seem to have a different overall mean of courtship intensity. But they seem to be affected the same way by

the time (Chisq = 1.07, *df* = 2, *p* = 0.59) given by the GLMM on the effect of the interaction Treatment * Video time.

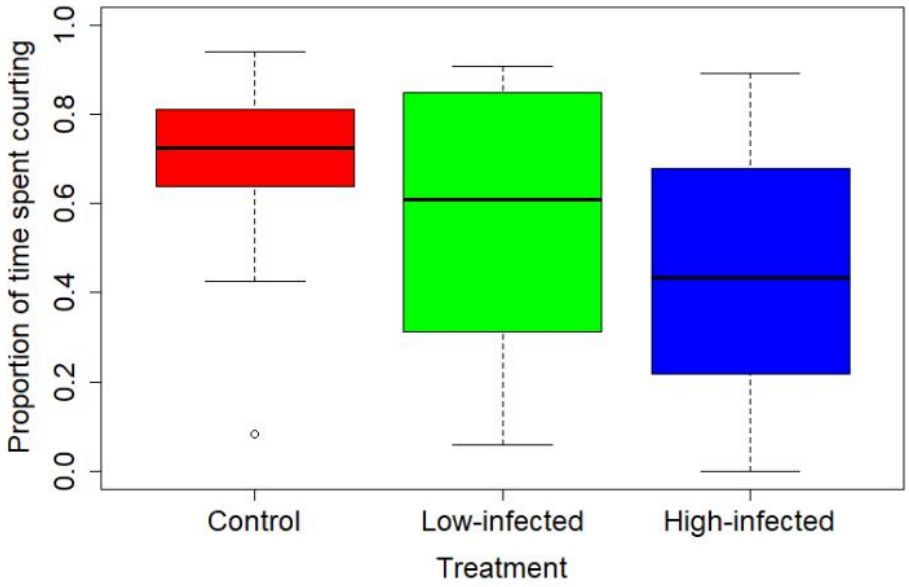


Figure 3: Boxplots of the proportion of time spent courting for each treatment in the single male assays. In Red Control, in Green Low-infected and in Blue High-infected.

The overall proportion of time spent courting, indicator of courting intensity, for each treatment (Fig. 3) Shows high intensity for Control (~0.7) to a medium intensity for low infected (~0.6) and finally to a low intensity for High-infected (~0.4). The GLMM gives a P-value (Chisq = 6.02, *df* = 2, *p* = 0.05) indicating an effect of the treatment on the courtship intensity. Post-Hoc tests for pairwise comparison of the least square means between each of the treatments showed significant differences between Control and High-infected *(z-ratio* = 2.471, *df* = inf, *p* = 0.0359). Indicating that the High-infected flies court significantly less than the

controls. The Low-infected to Control (*z-ratio* = 2.471, *df* = inf, *p* = 0.6190) and Low-infected to High- infected (*z-ratio* = -1.472, *df* = inf, *p* = 0.3042) comparison gave no significant differences.

The proportion of individual that court for a given proportion of time for each treatment (Fig. 4) gives details about the distribution among individual males of courtship intensity between each treatment. The Control treatment shows high proportion of individuals from 0.6 to 1 (25-50%). And very few individuals from 0.4 to 0 (~5%). The Low-infected treatment showed a different pattern with more stable proportion all over the spectrum. However, it still shows a higher proportion of individual between 0.8 and 1 (~35%). The High-infected treatment on the other hand, shows lower proportions of individuals between 0.4 and 0.6 (~5%) as well as between 0.8 and 1(~20%).

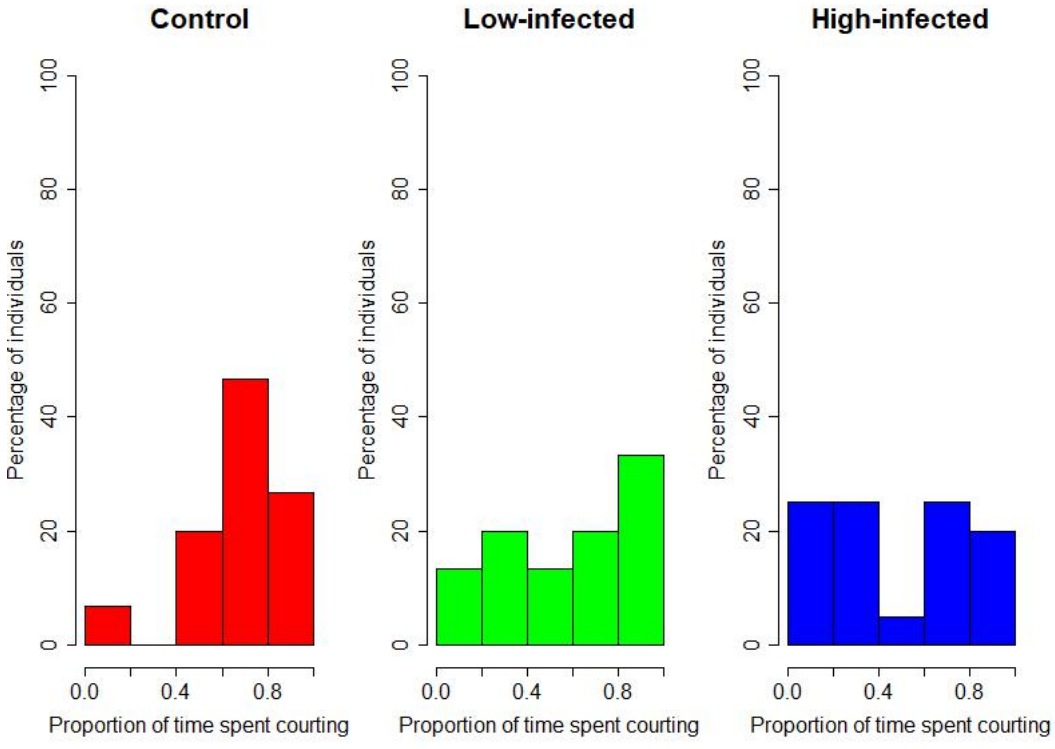
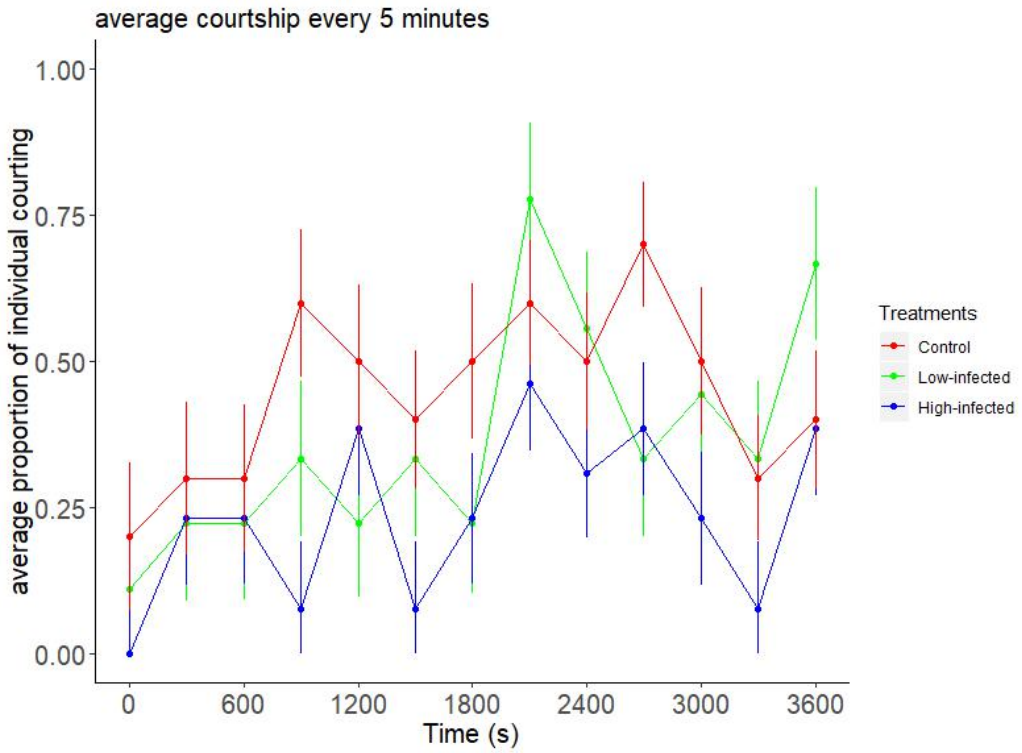


Figure 4: Histograms of the proportion of individual by the proportion of time spent courting in the single male assays. From left to right: Control, Low-infect, High-infected. In Red Control, in Green Low-infected and in Blue High-infected. On the x-axis, the percentage of individuals. On the y-axis the proportion of time spent courting

Two males assays

In the two male assays, we tested the effect of competition settings on the impact of infection on the courtship intensity (indicated here by the proportion of time spent courting). To do that we put two males in competition to court an unreceptive female and measured the proportion of time spent courting of the male of interest. As for the single male assay, the average of courtship means every 30 seconds for each treatment (Fig. 5) shows an overall higher courtship intensity with higher video time supported by the GLMM (*Chisq* = 14.74*, df* = 1 *p* = 0.0003). The three treatments seem to show the same overall different mean of courtship intensity trend than in the first experiment. In addition, the time seems to affect all three treatment the same way as well according to the GLMM (*Chisq* = 4.88, *df* = 2, *p* = 0.09).



3173

3174 Figure 5: Plot of the average courtship mean for each treatment in the two male assays. On this
3175 plot, the data was averaged every 5 minutes for visibility. In Red Control, in Green Low-infected and
3176 in Blue High-infected. On the x-axis, the time of the video in seconds. On the y-axis, The average
3177 proportion of individual courting.

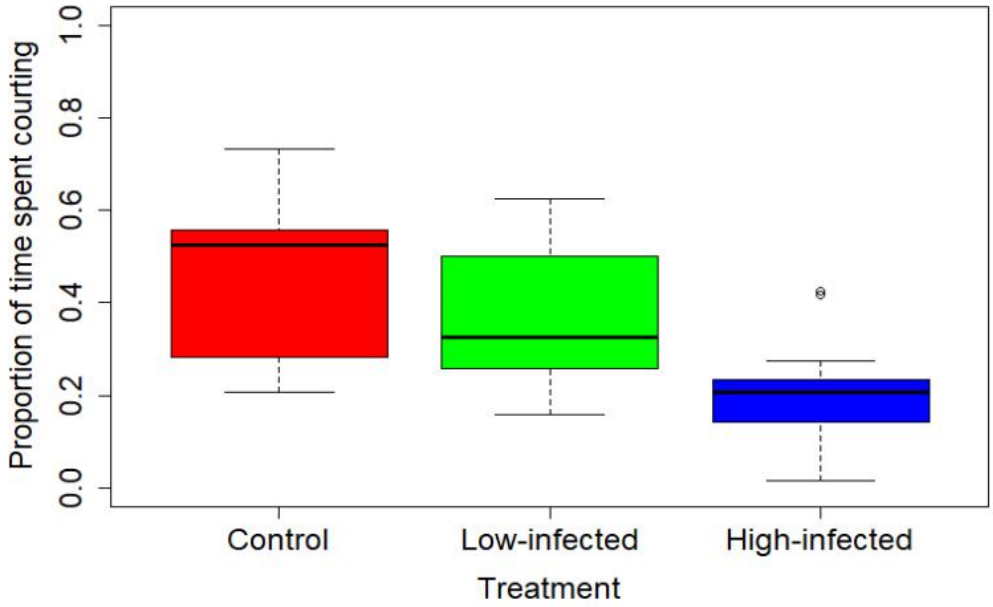


Figure 6: Boxplots of the proportion of time spent courting for each treatment in the two male assays. In Red Control, in Green Low-infected and in Blue High-infected.

The overall proportion of time spent courting, indicator of courting intensity, for each treatment for the two male assays (Fig. 6) show the same trends as in the single male assays. Control has a higher overall mean (~0.5), followed by Low-infected (~0.3) and High infected has the lowest (~0.2). The effect of the treatment on the courtship intensity is highly significant according to the GLMM (*Chisq* = 14.74, *df* = 2, *p* = 0.0006). The post-Hoc tests performed to compare the treatments between each other show that there is a significant difference between Control and High-infected (*z-ratio* = 4.244, *df* = inf, p = 0.0001) and between Low-infected and High-infected (*z-ratio* = -2.670, *df* = inf, *p* = 0.0207). Indicating that the High-infected flies court less than the two other groups. There was no significant difference between Control and Low-infected treatments (*z-ratio* = 1.375, *df* = inf, *p* = 0.3539). In

addition, the overall means are lower for each of the treatments than they are in the single male assays. The proportion of individual that court for a given proportion of time for each treatment for the two male assays gives details about the distribution among individual males of courtship intensity between each treatment. These proportions are different than the ones from the single male assays. Whereas the control treatment had high proportion of individual from 0.6 to 1, here it shows no individuals on the extremities. The higher proportions of individuals are between 0.5 and 0.6 (~40%) and between 0.2 and 0.3 (~30%). For the Low-infected treatment, there is a difference with the single male assays as well. The higher proportion of individuals is between 0.2 and 0.4 (~20- 35%) and there are no individuals between 0.7 and 1. Finally, the High-infected treatment is different as well. It shows no individuals between 0.5 and 1 and between 0.3 and 0.4. The highest proportion of individuals is found between 0.2 and 0.3 (~40%).

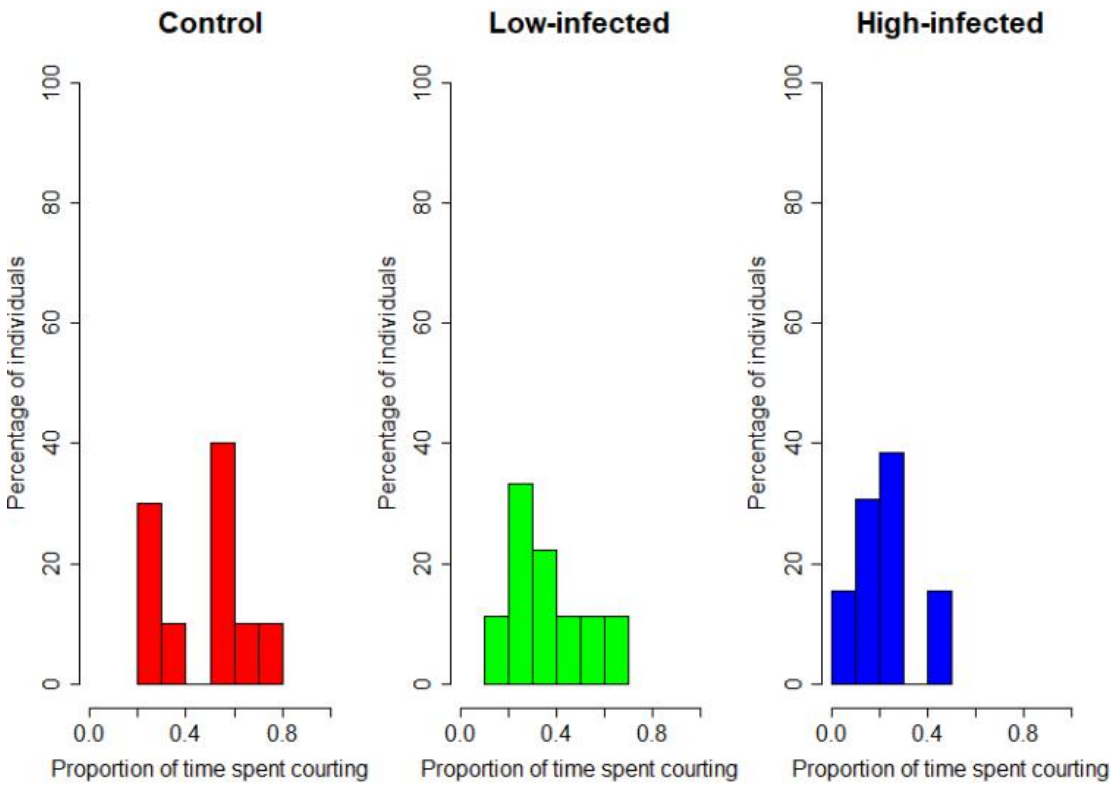


Figure 7: Histograms of the proportion of individual by the proportion of time spent courting in the two male assays. From left to right: Control, Low-infect, and High-infected. In Red Control, in Green Low-infected and in Blue High-infected. On the x-axis, the percentage of individuals. On the y-axis the proportion of time spent courting

Discussion

Single male assays

The Hamilton-Zuk hypothesis states that sexual traits can be condition-dependant and are influenced by the pathogenic environment. Males that are resistant to the pathogen present in the environment at the time of mating choice should have a better reproductive success. Therefore, the courtship intensity (here indicated by the proportion of time spent courting in a set amount of time) and by extension, the courtship behaviour should be affected by infection. This study shows that infection indeed does affect the courtship intensity. Highly infected males court less than lowly infected and control ones. There is however no clear explanation as to why there is less courtship intensity in highly infected flies. This effect could be explained in two different ways. One would be that the courtship intensity as a sexual trait is indeed condition-dependant. The reduction in intensity observed in highly infected flies could be because of a trade-off happening between the sexual trait and the fight against the pathogen. The second explanation could be that the flies are incapacitated by the infection, not able to mate or court.

This study shows that the lowly infected males does not have a significant different courtship intensity from the control and the highly infected ones. This, and the sus mentioned difference between highly infected and control flies, show no evidence of terminal reproductive investment. It would be expected that flies under the reproductive investment effect court more than the control ones. These results are to be taken with care as they come from small samples and some trends might only appear because of the size of the samples.

Two males assays

Concerning the two male assays, the Significant difference between the control and highly infected flies further confirm the effect of infection on courtship intensity. Competition does not seem to be a stimulant enough to push the highly infected flies to court more. However, it is not sufficient evidence to say that these flies cannot court more in any other conditions. The case of the Lowly infected fly is also interesting to look at, it is here significantly different form the highly infected flies. This could be an effect of the stimulation brought by the competition settings. However, the smaller number of individuals used in this experiment could be the reason behind the difference between the two experiments. We observed again no evidence of terminal reproductive investment in this study as none of the treatment showed more courtship intensity than the control. A trend was observed in the two male assays where the flies have an overall lower courtship intensity than in the single male assay but that can be explained by the competition setting. Half of the time the fly would spend courting is taken by the competitive fly.

Further perspectives

This study is a first look at how infection can impact courtship behaviour. The proportion of time spent courting in a set amount of time used to indicate courtship intensity is only one of many aspects of the courtship behaviour in *D. melanogaster.* These other aspects need to be investigated as well to be able to assess the real impact of infection on the courtship behaviour. Some aspects of the courtship behaviour might show signs of terminal reproductive investment even if there were none in the courtship intensity.

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