Phenotypic plasticity and condition-dependent trait expression in sexual ornaments and body colour

in the alpine grasshopper *Gomphocerus sibiricus*

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Contents

Summary

This work focuses on two aspects of the variation in phenotypic plasticity in the alpine grasshopper *Gomphocerus sibiricus*, a widespread species inhabiting alpine regions. This species presents various traits which make it very amenable to assess how the interaction between genotype and environment drives phenotypic expression of important ecological and reproductive traits. Of special interest are the remarkable sexual traits present in the male, which are employed during the various stages of female attraction and courtship ritual. These traits constitute ideal targets to study individual condition and its effects on trait expression, since sexual traits are considered to be highly sensitive to condition, making them signals of male quality for the female.

First I assessed the effect which background colour and ambient temperature can have on the occurrence of colour morph switches and developmental darkening in *Gomphocerus sibiricus* (Chapter 2). This species presents a notorious green-brown colour polymorphism, and some species have the ability to switch between these two morphs in order to adapt to their environment. The analyses uncovered that while this species is not capable of switch between the aforementioned morphs, the nymphae are capable, under certain conditions, of darkening or lightening their colour morph. This finding hints at a possible strategy to maintain a good energy budget in an environment whose climatic conditions are in constant change and which are likely to hinder daily activity patterns due to reduced thermoregulation capacity.

In Chapter 3 I focused on the effect of individual condition on trait expression, and I specifically set out to test if sexually selected traits in *G. Sibiricus* are more strongly affected than non-sexually selected traits when condition is impaired. In order to test condition I employed an LPS-based immune challenge on three cohorts of nymphae, and I measured several traits of both types. I expected to observe a stronger decrease in size of sexual versus non-sexual traits in individuals whose condition was impaired due to the immune challenge. Still this was not observed, and instead traits showed an overall similar effect, with a trend for sexually selected traits to grow bigger than non-sexually selected traits. Based on personal observations in the field as well as similar approaches to test condition dependence in similar species, I discussed the significance of these findings for the evolution of the conspicuous sexual traits in this species.

On my final assessment I used a theoretical approach to analyse the relation between genetic quality and both sexual and non-sexual traits using a meta-analytic method. According to theory, sexually selected traits are expected to show higher inbreeding depression than non-sexually selected traits. The results of my meta-analysis encountered that contrary to the expectation, sexually selected traits show less inbreeding depression than non-sexually selected traits. I discussed a possible scenario for the evolution of sexually selected traits where their ability to signal individual quality is directly related to how little cheater males in poor condition have noticed their function as signals to attract females. It is these traits which still retain their function as signals of good quality the ones which would present higher inbreeding depression than non-sexually selected traits. On the other hand, those traits which were noticed by males in poor condition, cheater males, would be expressed by these males in spite of their poor condition because of the advantage that they provide to attract females.

Chapter 1

"I conclude that the musical notes and rhythms were first acquired by the male or female progenitors of mankind for the sake of charming the opposite sex."

— **Charles Darwin**—
Descent of Man

General Introduction

The plastic phenotype, a concept which was frowned upon and neglected at some point in the history of biological research, constitutes now an integrative concept of how the environment and the genotype can interact to produce more than one phenotype (Agrawal 2001). This flexibility of the genotype may allow the phenotype to adapt to changes in the environment in order to survive, for example by changing colour in order to camouflage better and reduce predation. On the other hand, it may also allow the genotype to go beyond its limits in order to produce conspicuous phenotypes, some of which can evolve to attract females (Andersson 1994). Still the plasticity of the genotype may also be influenced by temporal and spatial variation, and it may also evolve itself (West-Eberhard 1989). All these factors combine to make the genotype a flexible component which has allowed the evolution of the diversity of phenotypes which can be found not only between but also within species.

In this work I will explore some avenues of the phenotypic variation in a very conspicuous animal model. First I will introduce this flashy being which accompanied me through all these years both in the field and in the laboratory. After this I will introduce the concept of phenotypic variation in colour, also known as colour polymorphism, which is widespread in the animal kingdom (Darwin 1859). This variation can occur on various levels in a population, and their occurrence can be dynamic, changing as individuals grow, or ultimately being flexible enough as to allow individuals to adapt to their environment. Following this I will explain the concept of sexually selected traits and mate choice, since a big part of this work deals with those two concepts. I will enlighten how these traits appear in nature and what is their function in the process of mate choice. I will explain

what these traits convey to those individuals of the opposite sex, and how this process is vital to the evolution of these traits and to the process of mate choice as well. Finally, I will pose a theoretical question about the relation between the genetic quality of an individual and the expression of sexual and non-sexual traits. This question will allow the reader to interpret the contribution of this work to the field of behavioural ecology under a much broader light.

Introducing, from far Siberia: the Club-legged Grasshopper

The club-legged grasshopper, *Gomphocerus sibiricus*, is an orthopteran which can be found above the 1000 m.a.s.l. line, inhabiting the alpine and mountainous regions of Siberia, various countries of Eurasia, as well as central and south-east Europe (Sergeev *et al.* 1995, Bellmann 2006, Bellmann & Luquet 2009). Like several other high altitude orthopterans, their life cycle is intricately connected to the coming of spring. Hatchlings of *G. sibiricus* appear during the spring-early summer time, their timing varying with the altitude. Development into adults takes approximately four weeks, after which the reproductive season begins. After mating, females lay eggs in the ground, which will guard the eggs through the winter until the next spring. Most of the remaining individuals will die during the autumn season.

The habitat where one can find *G. sibiricus* consists of pastures as well as grasslands, and it shows a preference for heterogeneous mixtures of open terrain. Here *G. sibiricus* can be readily found in areas strewn with rocks and stones, which also host diverse types of herbaceous plants and dense clumps of grass. The assorted vegetation in this habitat provides individuals of *G. sibiricus* with a profusely motley substrate which no doubt has help promoting the occurrence of colour polymorphism in this species. This habitat feature could also potentially provide individuals with the ability to easily camouflage themselves and better escape predators. The variability present in the environment is additionally modified by the temporal fluctuations in climatic conditions, which in mountainous environments is very unpredictable.

Besides providing an environment which allows individuals to hide and forage in safety due to the heterogeneity of the substrate, this habitat also provides a stage for the sexual display of the males of *G. sibiricus*. Mixed among the vegetation patches males are often found sitting on bare rocks, performing an advertisement song used to attract the females. The song is composed in its majority by a high energy thrill which can be heard up to a distance of six meters from the location of the male. Besides performing this energy demanding song, the male normally presents his forelegs, which constitute his most

Figure 1. Habitat and variation in foreleg size between the sexes in *Gomphocerus sibiricus*. The upper panel shows the type of habitat where G. Sibiricus can be found. The middle left panel shows a typical male, in contrast to a female which is shown in the middle right panel. The lower panel shows a male singing and displaying his clubs.

conspicuous sexual trait (Figure 1). The forelegs of males are swollen, taking the form of a pear, and which are also referred to as clubs. These swollen appendices develop in the male during the last moult, and their size as well as their overall condition (dents, wrinkles) are could be involved in signalling the overall condition of males to females.

Besides the aforementioned sexual traits, males of *G. sibiricus* possess two behaviours which are used at close quarters when trying to seduce the females. First, males use their antennae, waving them back and forth in front of the females when these are close to the males. The antennae have a swollen tip which is presumed to emanate a pheromone to attract the females. After the antenna waving, males approach females, all the time singing their advertisement song, and all of a sudden males jump onto the females. Once on top of the females, males once again make use of their remarkable club-shapped forelegs, using them to repeatedly drum on the sides of the female thorax at a very high rate whilst intenting to copulate. This behaviour has been thought to be both a necessary mean to convince the female to copulate (the rate of striking could be a clue for females to assess the male's condition) and also the reason behing the occurrence of the swollen forelegs (the drumming behaviour may not be such an effective stimulant if performed with regular sized forelegs).

Colour Polymorphism

The term colour polymorphism indicates the occurrence of more than one type of colour morph in a species at a frequency that is too high to be explained by recurring mutations (Huxley 1955). This phenomenon is widespread among many taxa such as birds, frogs, mammals, insects, and spiders, among others, and the meaning for their occurrence and maintenance is in many cases not yet understood (Whiteley *et al.* 1997, Oxford & Gillespie 1998, Hoffman & Blouin 2000, Mundy 2005, Bond 2007). There is evidence that an individual's genotype can interact with the environment and help in determine the individual's colour. This feature is rather convenient in cases where the climatic conditions in a species habitat are unpredictable and fluctuate rapidly, such as in alpine and mountainous regions, since it allows individuals to better adapt to their surroundings (Rowell 1971). Very interestingly, it has been documented that several species are capable of changing their colour in response to diverse environmental cues such as temperature, substrate or predation threats, among others (Umbers *et al.* 2014).

Multiple example of colour polymorphism can be found among arthropods, particularly among orthopterans, where it is possible to find two or more colour morphs in the same species (Rowell 1971, Dearn 1990). A very well-known form of colour polymorphism

present in orthopterans is the so called phase polymorphism, which occurs primarily in locusts. This type of polymorphism, which induces a change in colour from brown or green to black, has an specific trigger, which is a change in population density. The change in colour is accompanied by substantial changes in the morphology, physiology and life history of the individuals (Uvarov 1966, Pener 1991, Pener & Yerushalmi 1998, Simpson & Sword 2008, Song 2011). Another type of very widespread colour polymorphism in orthopterans is the green-brown polymorphism, which is present among bush crickets and grasshoppers.

Among the Orthoptera it is possible to find substantial variation in the types of morphs that occur within populations, with species displaying only one of the two morphs from the green-brown polymorphism, while other are polymorphic, displaying a more complex array of coloration in their morphs (Rowell 1971). Variation in morphs can either be continuous, with morphs showing fine-scale in their colour. In other cases variation in colour can be more discrete, which allows individuals to be easily assigned to a few discrete colour morph categories (Nabours 1929, Forsman *et al.* 2002). In *G. sibiricus* the green-brown polymorphism is present, yet the nymphae also present small amounts of variation in their green or brown tones, displaying darker or lighter tones of each colour morph (Chapter 2, Valverde & Schielzeth 2015). Still the variation does not wander far from the dichotomy of green and brown, making the classification of either morph straightforward.

A phenomenon closely associated with colour polymorphism and which needs to be addressed in relation with it is homochromy. Homochromy denominates the matching of body colouration with the background patter found in an individual's habitat (Rowell 1971, Fuzeau-Braesch 1972, Booth 1990, Dearn 1990). Various mechanisms can drive homochromy in a species (see chapter 2), but perhaps the most interesting among these are those in close relation with developmental plasticity during the nymphal stages. Developmental switches belong to these type of mechanisms, and are particularly well suited to change an individual's colour morph in order to adapt to a specific background. These mechanisms are also very useful since the change occurs during an individual's lifetime, which is particularly important for species where the habitat changes its structure within a single reproductive season. Orthopterans are known to be able to use developmental switches, in species which present a fine-scale level of colour polymorphism as well as in species which display the green-brown polymorphism (see Table 1 in chapter 2) (Ergene 1952, Karlsson *et al.* 2008).

"The sparrow is sorry for the peacock at the burden of its tail."

— **Rabindranath Tagore**—

Sexually Selected Traits & Mate Choice

The evolution of sexually selected traits is a dynamic process which is still being understood because of its complexity, encompassing not only the direct function of the traits, but also its viability, its effect on reproduction and also on kin (Cornwallis & Uller 2010). Back in the time when Darwin became famous, over one a half decades ago, he had no explanation as to why sexually selected traits existed at all (Darwin 1860, Hunt 2017). Yet he developed the theory of female choice, based on the premise that female would have preferences for specific traits, nonetheless why were there preferences in the first place and how this led to the evolution of extravagant ornaments remained a question which he could not fully address at the time (Darwin 1871, Jones & Ratterman 2009). It was not until Sir Ronald Fisher that light was finally shed upon how female preferences for specific male traits could actually arise.

Fisher described a model by which females display a genetically inherited preference for an ornament in males. The preference to females mating with the males which present the ornament, and the offspring thus would inherit genes to produce the ornament as well as genes for the preference for the ornament. Once the inheritance of the preference and the ornament is linked, there will be a reinforcing cycle which will be responsible for the exaggeration of the ornament. This model was known as Fisherian runaway selection, and at the time when it was proposed it served to explain all the conspicuous traits which Darwin had failed to explain (Fisher 1915, Fisher 1930). Still this model did not incorporate concepts such as the cost for the male to produce the ornament, or whether there could be a point where the reinforcement cycle would stop and the ornament would not develop any further (Wiens 2001). Perhaps even more important was the fact that the model could not explain the evolution of two strategies to attract females based on how much males invest in their offspring after copulation has taken place (Jones & Ratterman 2009).

In nature one comes across two types males, regarding their contribution to the female's direct reproductive output. First there are those males which provide females with resources, such as parental care, food resources in the form of a territory or higher fertility, and these are known as direct benefits, because they are fecundity-enhancing. Those males which, in contrast to those previously mentioned, do not provide the female with any kind

of resources, are said to provide only indirect benefits, and these are by definition not fecundity-enhancing, but rather refer only to the benefits gained in the offspring generation from beneficial allelic variation (Rowe & Houle 1996, Kokko *et al.* 2003, Hill *et al.* 2008). Therefore, it is important that choosy females which do not receive resources make sure they receive good alleles from their mating partners. This idea was precisely included in a model which not only included the Fisherian runaway selection, but also a function for the ornaments in connection with the male genotype. The model stated that only those males in good condition, which depended on males having good genes, would be capable of fully developing the ornament preferred by the females. Thus, choosy females would be able to choose those males in the best condition based on the ornament's quality (Zahavi 1975, Kokko *et al.* 2003).

A particular mechanism, known as condition dependence, ensures that males expressing a sexual trait maintain honesty in the ornament signalling genetic quality. This honesty is ensured by the fact that condition is determined by the sum of genetic, epigenetic and environmental effects on vital processes, as well their effect on life history, behavioural and morphological traits, among others (Rowe & Houle 1996, Hill 2011). Females will therefore benefit the most when using those traits which can most efficiently capture the genetic variance which contribute to overall condition. In return, males having these traits will become more chosen by females, and this mechanism will turn these traits into sexual traits. This concept which allows traits to be better able at reflecting individual genetic variance and therefore being good proxies for condition is known as genic capture (Rowe & Houle 1996, Tomkins *et al.* 2004). But despite the fact that sexual traits expression is to a great extent influenced by individual condition, other factors which are not heritable can also affect both individual condition and sexual trait expression.

Environmental variation can be a very significant modifier of an individual's phenotype, and it can therefore influence development, which in turn has the potential to affect condition and therefore trait expression, specially the expression of sexual ornaments (Monaghan 2008). Changes in the environment can significantly influence things like feeding opportunities, modify the spaces where an individual hides and influence daily activity budges, among others. All this makes the interaction between condition and environmental cues the most significant driver of phenotypic expression, and it is expected that ornaments, which primarily serve as signals to advertise individual quality will be more sensitive to this interaction than non-sexual traits (Cotton *et al.* 2004). Finally, a rather meaningful factor that can affect condition is the genotype, i.e. the combination of alleles at individual loci. Inbreeding in particular influences the association of alleles at individual loci, typically increasing homozygosity beyond what is expected from random

association of alleles in the population. The degree of inbreeding (or its empirical substitute, genome-wide heterozygosity) displayed by any individual is expected to affect condition as well. Condition is expected to be lower in inbred individuals as well as in individuals that through inbreeding or other processes have reduced mutilocus heterozygosity, which I expect should again be more strongly reflected in sexually selected traits than in non-sexually selected traits (Cotton *et al.* 2004, Kempenaers 2007, Jennions *et al.* 2012).

This Thesis

The current work is focused on two broad topics in the context of phenotypic plasticity in *G. sibiricus.* The two areas which I studied had not been studied up to now. The ecology of the species has been studied in Siberia (Sergeev *et al.* 1995), while it has also been used for studies of toxicity (Bouchard 1998, Bouchard & Bouchard-Madrelle 2000) and cytology (Gosálvez & López-Fernández 1981, Gosálvez *et al.* 1982, Garcialafuente *et al.* 1983, Gosálvez *et al.* 1986, López-Fernández *et al.* 1986). My work attempted to shed light on the interaction between genotype and environment, and how traits are moulded after these factors in order to allow individuals to adapt to their habitat. The challenges imposed by the environment during development could also have particular effects on individual condition and therefore on sexually selected traits. Furthermore, I address the effects which variation on the genotype, an integral part of the interaction previously mentioned, would have in trait expression, both sexual and non-sexually selected.

Chapter 2

In this chapter I use nymphae hatched in the laboratory to test the effects of background colour and ambient temperature on the occurrence of colour morph switches and developmental darkening in *Gomphocerus sibiricus*. For this I monitored individually housed nymphae through three of their four developmental stages and during the first week of their imago stage. The results uncovered a fascinating ability which hints at a possible survival strategy for small nymphae of this species, in particular when the environmental conditions in their habitat are not favourable during development.

Chapter 3

In this chapter I use an immunological challenge on three different cohorts of nymphae to test if the expression putatively sexually selected traits are more strongly affected than the expression of non-sexually selected traits. For this I used both individuals collected in the field as well as individuals hatched in the laboratory, and included several types of traits, included morphological and song traits. The results contradict my initial predictions since the conspicuous putative ornaments in this animal model do not comply with the type of

traits being particularly sensitive to impaired condition. I discuss the significance of these findings and possible additional tests in the same context of condition dependence and sexual traits.

Chapter 4

In this final chapter I approach the subject of sexually selected traits and their expectancy to be highly sensitive to changes in the genotype, an integral component of an individual condition. I use a meta-analytical methodology to synthesize results from studies in the field which involve both sexually selected traits as well as a measure of genetic quality, this being either the degree of individual heterozygosity or the degree of inbreeding depression. I obtained a very interesting result from of the relationship between sexually selected traits and the degree of individual genetic quality, which I frame in the broad context of the evolution of sexually selected traits. This framing provides a better overview into the work of the previous chapter and provides and provides a good base for further exploration in this topic.

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Chapter 2

What triggers Colour Change? Effects of Background Colour and Temperature on the Development of an Alpine Grasshopper

J. Pablo Valverde & Holger Schielzeth

Abstract

Colour polymorphisms are a fascinating facet of many natural populations of plants and animals, and the selective processes that maintain such variation are as relevant as the processes which promote their development. Orthoptera, the insect group that encompasses grasshoppers and bush crickets, includes a particularly large number of species that are colour polymorphic with a marked green-brown polymorphism being particularly widespread. Colour polymorphism has been associated with the need for crypsis and background matching and background-dependent homochromy has been described in a few species. However, when and how different environmental conditions influence variation in colour remains poorly understood. Here we test for effects of background colour and ambient temperature on the occurrence of colour morph switches (green to brown or brown to green) and developmental darkening in the alpine dwelling club-legged grasshopper *Gomphocerus sibiricus*. We monitored individually housed nymphae across three of their four developmental stages and into the first week after final ecdysis. Our data show an absence of colour morph switches in *G. sibiricus*, without a single switch observed in our sample. Furthermore, we test for an effect of temperature on colouration by manipulating radiant heat, a limiting factor in alpine habitats. Radiant heat had a significant effect on developmental darkening: individuals under low radiant heat tended to darken, while individuals under high radiant heat tended to lighten within nymphal stages. Young imagoes darkened under either condition. Our results indicate a plastic response to a variable temperature and indicate that melanin, a multipurpose pigment responsible for dark colouration and presumed to be costly, seems to be strategically allocated according to the current environmental conditions. Unlike other orthopterans, the species is apparently unable to switch colour morphs (green/brown) during development, suggesting that colour morphs are determined genetically (or very early during development) and that other processes have to contribute to crypsis and homochromy in this species.

Introduction

Colour polymorphism has fascinated biologists since the time of Darwin, and its evolutionary meaning is still being revealed (Darwin 1871, Fisher 1930, Mayr 1942). Colour polymorphism, defined here as within-species phenotypic variation, occurs throughout the animal kingdom in several taxa of birds, fish, mammals, frogs, molluscs, spiders, several insect orders and also in plants (Darwin 1859, Whiteley *et al.* 1997, Oxford & Gillespie 1998, Hoffman & Blouin 2000, Mundy 2005, Bond 2007). The occurrence of colour polymorphisms in natural populations can result from biased mutation, pleiotropy and trade-offs, gene flow, spatially and/or temporally fluctuating selection and negative frequency-dependent selection that can counter loss of variation by genetic drift (Majerus 1998, Punzalan *et al.* 2005, Gray & McKinnon 2006, McKinnon & Pierotti 2010). Furthermore, developmental plasticity and phenotypic flexibility, if they do not invoke significant cost, might allow the maintenance of polymorphisms. This can be particularly advantageous in unpredictably variable environments.

Insects offer a multitude of examples for the coexistence of two or more colour morphs in groups such as grasshoppers, mantoids, cicadids, damselflies, lepidopterans and beetles (Rowell 1971, Dearn 1990, Majerus 1998). There is ample evidence for genetic and environmental effects, as well as genotype-by-environment interactions in colour determination (Key 1954, Rowell 1971, Dearn 1990, Bellmann 2006, Bellmann & Luquet 2009). Several species appear capable of modifying their colour in response to various environmental cues such as temperature, predation threats, behaviour stimulus (e.g. crab spiders which try to blend with their background to ambush prey, (Oxford & Gillespie 1998)), among others (Umbers *et al.* 2014). Within Orthoptera, colour polymorphism is present in dozens of species (reviewed in Dearn 1990, see also Booth 1990, Umbers *et al.* 2014). Two particularly eye-catching forms of colour polymorphism in orthopterans are a widespread green-brown polymorphism in grasshoppers and bush crickets and the famous phase polymorphism in locusts (Rowell 1971, Song 2005). Phase polymorphism is triggered by changes in population density which induces changes in colour (typically black patterns in gregarious versus pale green or brown colours in solitary phases) as part of more complex changes in morphology, physiology, behaviour and life history (Uvarov 1966, Pener 1991, Pener & Yerushalmi 1998, Simpson & Sword 2008, Song 2011).

The green-brown polymorphism is far more widespread among orthopterans than phase polymorphism and does not correlate with obvious changes in morphology and/or behaviour. Many families and genera of orthopterans comprise species that display either green or brown morphs, while other species are polymorphic (e.g. in genera *Decticus*, *Metrioptera*, *Oedaleus*). In some species, one of the morphs is very rare (such as brown

morphs in *Decticus verrucivorus*), while in others the ratios are far more even (as in *Metrioptera roeselii*) (Rowell 1971). With respect to environmental effects, green morphs seem to develop primarily under high humidity, while brown morphs are favoured under dry environmental conditions (Rowell & Cannis 1971, Dearn 1990, Lecoq & Pierozzi 1996). Besides the two very striking forms of colour polymorphism mentioned before, there is a range of more fine-scaled within-species variation in colour pattern and colouration among orthopterans (Rubtzov 1935, Richards & Waloff 1954). Groundhoppers, for example, differ substantially in their colour patterns, which can be categorized into variable numbers of discrete morphs (Nabours 1929, Forsman *et al.* 2002). In other species, differences in colour are more continuous such as with colouration of species in the genus *Oedipoda*. Such fine-scaled variation seems to be partly under genetic, partly under environmental control (Key 1954, Ergene 1955, Dearn 1990). Many species also show occasional pinkish, purple, yellow or blue colour morphs (Ingrisch & Köhler 1998), further illustrating the diversity of colour in orthopterans.

A very interesting phenomenon associated with colour polymorphism is homochromy, which describes matching of body colouration with variation in the background pattern of the local habitat (Rowell 1971, Fuzeau-Braesch 1972, Booth 1990, Dearn 1990). Such matching might arise for four different reasons: (i) local adaptation due to multi-generational history of selection on genetic polymorphisms, (ii) selective mortality within generations, (iii) individual-level choice of matching habitat patches (Edelaar *et al.* 2008), and (iv) developmental plasticity of body colouration to match local conditions (Rowell 1971). Developmental switches are particularly intriguing, because they allow individual-level matching, which is likely advantageous if habitats are unpredictably variable across generations, but predictable from environmental cues over the lifetime of individuals. Developmental matching has been reported in orthopterans for species with fine-scale variation in colour pattern (Ergene 1952, Karlsson *et al.* 2008), but also for species which present the green and brown colour polymorphism (Table 1).

Orthopterans are preyed upon by a large diversity of species, including birds, lizards, amphibians, spiders and other insects and are frequently parasitized by parasitic flies and mites (Uvarov 1966, Ingrisch & Köhler 1998). Visually hunting predators might constitute a force that can favour homochromy and crypsis, since survival to the imago stage is critical to individual fitness. Predators might also impose frequency-dependent selection if they develop search images and preferentially prey upon the most common morphs (Bond 2007). However, there are other influences that might affect body colour and this may or may not be in conflict with crypsis. For example, body colour is likely to affect the absorption of radiant heat and therefore play an important role in thermoregulation

(Kingsolver & Wiernasz 1991, Majerus 1998, Forsman 2000, Hegna *et al.* 2013). It has repeatedly been reported that orthopterans raised under cool conditions are darker than those raised under warm conditions (Duck 1944, Key 1954, Okay 1956, Abushama 1969, Umbers *et al.* 2014).

The club-legged grasshopper *Gomphocerus sibiricus* is a highly sexually dimorphic alpine dwelling grasshopper that exhibits the green-brown polymorphism present in many other orthopterans. Green individuals are rarer than brown morphs in most populations. Despite substantial fluctuations in population density (Uvarov 1966, Sergeev *et al.* 1995), the species does not show any typical phase polymorphism (Song 2011). It inhabits alpine pastures and grassland with a very heterogeneous composition of open terrain strewn with stones and mottled by various types of short grasses and herbaceous plants. Climate conditions in the mountains are very unpredictable and variable within and between years. The maintenance of the green-brown colour polymorphism could be aided by the heterogeneous habitat and/or temporal variability in climate conditions in the native habitat of *G. sibiricus*.

In the present study we aimed to test the effect of two known factors on developmental colour changes in *G. sibiricus*. First, we assessed the effect of background colour (green or brown) on colour morph development across almost the entire ontogeny. We were particularly interested in whether individuals are able to switch colour morphs to achieve homochromy as it has been described in other species (Table 1). We predicted that if individuals were able to switch colour morphs, then individuals whose colour morph mismatched the background colour would be capable of matching their background at an advanced developmental stage. Second, we assessed the effect of temperature by means of a radiant heat treatment on developmental darkness, while controlling for humidity, population density and food moisture content. Here we predicted that if individuals were capable of manipulate the degree of melanin in their cuticle, thermoregulation needs would promote a colouration darkening under conditions of low radiation. We followed individuals from the second nymphal stage through to the imaginal stage during two independent rounds of trials with two different radiant heat regimes. Individuals were exposed to experimental treatments from the second nymphal stage onwards. The long exposure to experimental conditions allowed us to evaluate if colour changes occur exclusively in connection with moults or if changes were possible even within nymphal stages.

Table 1. Studies on the effects of background colouration on the occurrence of colour morph switches in green-brown polymorphic (upper section) and other polymorphic (lower section) orthopterans.

Species	Background Material	N		Proportion of Switches		Stages with Switches	Reference
		matched	non-matched	matched	non-matched		
a) Green-Brown Polymorphism							
Acrida turrita	fresh/dry grass	62	145	4%	86%	after ecdysis	Ergene 1950
Acrida turrita	painted sawdust	14	15	0%	100%	after ecdysis	Ergene 1950
Acrida turrita	painted sawdust		32		0%	imago	Ergene 1950
Oedaleus decorus	NA	10	66	0%	88%	after ecdysis	Ergene 1955
Schistocerca americana	coloured paper		74		0%	across stages	Tanaka 2004
Schistocerca gregaria	coloured paper	58	312	0%	63.5%	within stages	Tanaka *et al.* 2012
Locusta migratoria	coloured paper	58	237	69%	24%	across stages	Hertz & Imms 1937
Rhammatocerus schistocercoides	habitat background		3000 >		90% >	imago	Lecoq & Pierozzi 1996
Chorthippus biggutulus	coloured paper		257		30%	across stages	Helfert 1978
b) Other Polymorphisms							
Oedipoda sp.	earth, clay, coal, stone, chalk	12	92	0%	80%	after ecdysis	Ergene 1952
Tetrix subulata	sand		312		16%	across stages	Hochkirch *et al.* 2008
Tetrix ceperoi	sand		228		16%	across stages	Hochkirch *et al.* 2008

The proportion of switches was calculated for various studies based on multiple assays either on matched or non-matched background colour. Studies do not indicate precise time of colour morph switch occurrence, only final results of repeated colour assessments across nymphal stages are stated (in the case of Ergene all switches occur after an ecdysis event). Percentages reflect amount of individuals from the total amount in any given category – matched or non-matched – which switched colour, therefore they are not expected to add up to 100%. Most studies used low densities of individuals (≤5) per cage, but one study (Ergene 1955) housed up to 10 individual per cage. NA = information not available.

Methods

Subjects

Last-instar individuals of *G. sibiricus* were collected in the field (near Sierre, Valais, Switzerland) in July 2013 and brought to the laboratory where they moulted into imagoes. We housed imagoes in separate cages (dimensions 22 x 16 x 16 cm^3) containing one male and one female and provided a cup of sand-vermiculite mixture as substrate for egg

laying. Eggs were collected once per week, kept for approximately 6 weeks at room temperature and subsequently stored at 4°C for diapause. After seven months in the refrigerator, eggs were taken out of their diapause and kept at room temperature until hatching. In total, 116 hatchlings hatched from 37 egg pods. Full-siblings from the same egg pod were housed together in the same cage (white cages lined with black mesh, dimensions as above) throughout the first nymphal stage, but were separated after the first nymphal stage (i.e. after about one week) and thereafter housed individually. We refer to the different nymphal stages as N2 (second nymphal stage), N3, N4 and IM (imago stage).

Experimental setup

The experiments were conducted under artificial full-spectral light conditions (Biolux L 58W/965, OSRAM, Munich, Germany) with a 14:10 H light dark cycle (7:00 till 21:00). Average humidity was kept at 70% by humidifiers. In order to maintain a high moisture content in the food, we provided fresh grass in small plastic vials (height x diameter: 5.8 cm x 2.1 cm) filled with water, and replaced the grass every other day in order to avoid withered or yellowing grass. Experiments were conducted in two rounds (we refer to the two rounds as R1 and R2) that differed in details of the radiant heat treatment, because of high nymphal mortality under one of the conditions in R1. Experimental setups consisted of blocks of four cages (11 and 10 replicate blocks in the R1 and R2, respectively) flanked on both sides by isolating dividers (22cm x 55 cm x 1 cm) (Figure 1). The dividers were installed to isolate alternating blocks exposed to different experimental conditions. Individuals were transferred to experimental setups on the day of moulting into the N2 and assignment to treatments was done at random. All individuals remained under the same experimental conditions until one week after moulting into IM (unless they died before completing the experimental period). Only three individuals reached the last nymphal stage under low radiant heat conditions in R1, two of which died shortly after, and therefore the experiment was stopped at this point due to lack of one treatment group. We did not score the sex of individuals during early nymphal stages because this is easily visible during the N4 stage. In the case of the R1, individuals died rapidly while the experiment was running, and sex scoring was not assessed in time. For this reason data on colour morph frequencies for both sexes is not presented in the results.

Background colour treatment

The first component of our experimental design involved an assessment of homochromy. Specifically we tested for changes in colour morph from green to brown or brown to green in response to differences in background colour. Each cage (same type as described above) was lined on two of its inner sides as well as the cage's floor with coloured paper (Coloured drawing paper, green = #ADFF2F, brown = #8B4513, Folia Paper Bringmann,

Wendelstein, Germany). Each experimental block consisted of two cages lined with brown colour and two cages lined with green colour. Individuals had visual contact to only one neighbouring cage that was ensured to be of the same colour as their own cages (Figure 1).

Temperature treatment

The second component of our experimental setup consisted of a temperature treatment and was applied to entire blocks. Grasshoppers regulate their body temperature behaviourally, elevating it substantially above ambient temperature by sun-bathing (Uvarov 1966). We aimed to simulate high (sunny) and low (overcast) radiant heat conditions, both of which occur in the natural habitats of *G. sibiricus*. Therefore, we placed 150 W infra-red heat bulbs (IOT/90, Elstein, Northeim, Germany) at the vortex of each block. In the R1, blocks were exposed to either 5 hours (high treatment) or 1 hour (low treatment) of radiant heat, but due to high mortality, this was increased to 4+6 hours (high treatment) or 2 hours (low treatment) per day in the R2 (Figure 1). The hours of exposure in the R1 for the high treatment were from 9:00 until 12:00, and again from 13:00 until 15:00, while the exposure time for the low treatment was from 12:00 until 13:00. The hours of exposure in the R2 for the high treatment were from 8:00 until 12:00, and again from 14:00 until 20:00, while the exposure time for the low treatment was from 12:00 until 14:00. The temperature at exposed locations was around 44 °C when heat lamps were active, in
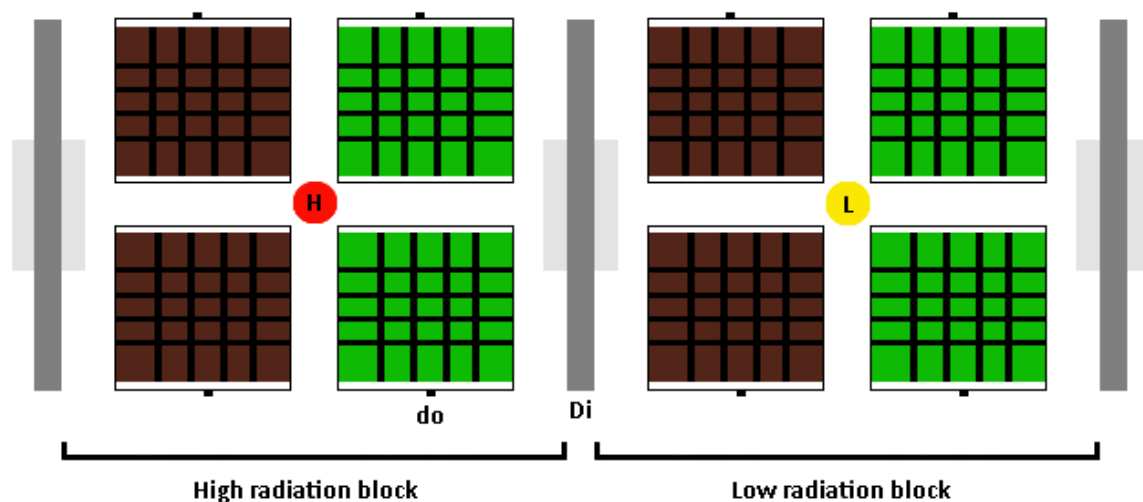
Figure 1. Experimental setup. Two blocks are shown, one exposed to the high radiant heat (orange dot) and one to the low radiant heat (yellow dot) treatment. Blocks are separated by dividers (Di). Each cage has its floor and two of its sides lined in green or brown paper, and each block has two cages lined with each colour. Time of exposure to radiant heat varied between rounds (see details in Temperature treatment section). The doors (do) on the sides allow easy access to cages.

contrast to the 25 °C when heat lamps were switched off. In the high radiant heat treatment, individuals were able to adjust the amount of radiant heat that they received by positing themselves at the mesh ceiling or by moving away from it or also by hiding behind the beams of the cage.

Scoring of body colour

Colour morphs are identifiable from the third day of the second nymphal stage. Both morphs vary in the degree of colouration darkness (e.g. from very light green, across a discreet range of green tones, to very dark green morphs). Pale versions (which presumably have reduced bile pigments, ommochromes and melanin – responsible for green, brown and black colouration, respectively; (Fuzeau-Braesch 1972, Oxford & Gillespie 1998)) as well as melanic versions (very dark in colouration) occur in the lab and in the field. We defined two intermediate categories among those extremes, based on a collection of photographs of individuals taken either in the lab or in the field on previous years. The scoring was done every third day, assigning the individuals a colour grade based on the degree of darkness in the area under study. The categories where defined as follows: 1. Very light = < 10% of black; 2. Light = 30-40% of black; 3. Dark = 60-70% of black; 4. Very dark = > 90% of black. Our analysis is based on a total of 433 scorings from the R1 and 306 scorings from the R2.

Statistical analysis

Since our sample size is necessarily finite, it is at least possible that despite an absence in our sample, colour morph switches occur with a low probability. We therefore estimated the lowest probability of colour morph switches that would still be consistent with our data. We used the following approach to assess the highest colour change probability that is consistent with our data. We define c as the probability of colour change in unmatched backgrounds, $(1-c)$ as the probability of no change and k as the number of individuals exposed to unmatched backgrounds. Based on binomial sampling, we expect to observe no colour morph switches among k individuals with a probability of $(1-c)^k$. We defined a probability threshold of $\alpha = 0.05$ and searched for the value of c at which the expectation of no-colour morph switches is equal to α. This gives the highest possible switching probability that is still considered reasonably consistent with our data.

To test for the effect of the temperature treatment in the body colour we used a random-slope mixed effects model. We chose this model because the possible response to the treatment could vary physiologically from individual to individual, making it necessary to account for this variability in our analysis (Schielzeth & Forstmeier 2009). The p-values for the fixed effects in the mixed models were approximated by a t distribution with degrees of freedom equal to the number of individuals minus the number of fixed effect

parameters estimated in the model. This approach is conservative in that it considers only individuals as independent datapoints. However, such random-slope models depend on assumptions about the linearity of the response-covariance relationship and about the distributions of random-deviations from the population mean and population slope. Therefore, we applied additional, simpler tests for the same question in order to verify the robustness of the results (see below for details).

In addition to the analysis of the data using random slope models, we considered using alternative ways of analysis to confirm the robustness of the results on colouration darkening. First, we identified those individuals who showed an unambiguous lightening or darkening in colour (individuals with bidirectional changes in colour darkening within a nymphal or imago stage were left out: R1, n = 10 nymphae + 1 imago; R2, n = 2 imagoes). Then we counted the number of cases of darkening and lightening in each stage, and compared them using Fisher's exact tests. Second, we compared treatments per observation time (early or late observations within nymphal stages) between treatments using two sample t-tests. Third, we compared the darkness between the earliest and latest observations per treatment within nymphal stages using paired t-tests. This was only possible for those individuals that had at least two observations per nymphal stage. All analysis were conducted in R 3.1.1 (R Core Team 2014), and we used the lme4 package (Bates *et al.* 2012) for mixed model fitting.

Results

A total of 78 individuals survived the first nymphal stage and entered the experimental setup in two rounds (referred to as R1 and R2). From this total, 34 individuals matched their cage background, while 44 did not match. Sixty one individuals were brown (78%, 36 in R1 and 25 in R2) and 17 green (22%, 6 in R1 and 11 in R2). The distribution of morphs in the R2 differed significantly between the sexes with brown individuals of both sexes (11 males = 44%, 14 females = 56%), but only green females (11 individuals) (X^2 = 5.05, df = 1, p = 0.025).

Colour morph switches

No colour morph switch was observed among the 34 individuals in cages with matched background colours (6 green individuals in green cages and 28 brown in brown cages in total for both rounds). Forty-four individuals (33 brown and 11 green) were exposed to unmatched backgrounds, but no colour morph switches were observed among these 44 individuals. Reasoning based on binomial sampling (see methods section) suggests that if *G. sibiricus* is capable of switch colour, the rate of colour morph switches is well below

Table 2. Number of individuals of *G. sibiricus* on mismatched backgrounds, all of which did not switch colour morph during development.

Round 1			
Morph & Background	**Temperature**		
	High	**Low**	**Sum**
Green on Brown	n = 3 c ≤ 0.63	n = 0 NA	n = 3 c ≤ 0.63
Brown on Green	n = 9 c ≤ 0.28	n = 9 c ≤ 0.28	n = 18 c ≤ 0.15
Sum	n = 12 c ≤ 0.22	n = 9 c ≤ 0.28	n = 21 c ≤ 0.13
Round 2			
Morph & Background	**Temperature**		
	High	**Low**	**Sum**
Green on Brown	n = 4 c ≤ 0.53	n = 4 c ≤ 0.53	n = 8 c ≤ 0.31
Brown on Green	n = 7 c ≤ 0.35	n = 8 c ≤ 0.31	n = 15 c ≤ 0.18
Sum	n = 11 c ≤ 0.24	n = 12 c ≤ 0.22	n = 23 c ≤ 0.12
Round 1+2			
Morph & Background	**Temperature**		
	High	**Low**	**Sum**
Green on Brown	n = 7 c ≤ 0.35	n = 4 c ≤ 0.53	n = 11 c ≤ 0.24
Brown on Green	n = 16 c ≤ 0.17	n = 17 c ≤ 0.16	n = 33 c ≤ 0.09
Sum	n = 23 c ≤ 0.012	n = 21 c ≤ 0.13	n = 44 c ≤ 0.07

Table depicts number of individuals for each of two rounds under two sets of radiant heat regimes and values for the two combined rounds. The c value shows the range of colour switch probabilities that are consistent with the data at $\alpha = 0.05$.

Table 3. Results of the random-slope mixed effects model used to test for effects of radiant heat treatment and larval age on colouration darkness in both green and brown coloured morphs.

	Round 1				Round 2			
	slope	SE	t	p	slope	SE	t	p
	N2 (n=42)				**N2 (n = 35)**			
Intercept	1.79	0.25	7.16	< 0.0001	1.92	0.33	5.77	< 0.0001
High radiant heat treatment	0.28	0.34	0.82	0.42	1.02	0.52	1.98	0.057
Larval age	0.063	0.017	3.78	0.00054	0.18	0.061	2.92	0.0065
Treatment x Age	-0.067	0.027	-2.49	0.022	-0.38	0.12	-3.29	0.0025
	N3 (n=34)				**N3 (n = 34)**			
Intercept	2.23	0.28	8.04	< 0.0001	2.55	0.2	12.77	< 0.0001
High radiant heat treatment	0.23	0.35	0.66	0.51	0.39	0.31	1.27	0.21
Larval age	0.036	0.014	2.65	0.013	0.035	0.045	0.79	0.43
Treatment x Age	-0.088	0.023	-3.82	0.00062	-0.12	0.076	-1.62	0.11
	N4 (n=22)				**N4 (n = 32)**			
Intercept	2.03	0.19	10.62	< 0.0001	1.85	0.2	9.06	< 0.0001
High radiant heat treatment	NA	NA	NA	NA	1.04	0.3	3.42	0.0019
Larval age	0.033	0.015	2.21	0.039	0.078	0.034	2.26	0.032
Treatment x Age	NA	NA	NA	NA	-0.22	0.061	-3.54	0.0014
	IM (n = 21)				**IM (n = 29)**			
Intercept	NA	NA	NA	NA	1.88	0.36	5.21	< 0.0001
High radiant heat treatment	NA	NA	NA	NA	0.34	0.45	0.76	0.45
Larval age	NA	NA	NA	NA	0.21	0.066	3.26	0.0032
Treatment x Age	NA	NA	NA	NA	-0.092	0.082	-1.13	0.27

Panels on the left and right side of the table show results for the first and second round, respectively. The number of individuals present in the experimental setup is depicted at the top of each block. The analysis of N4 in the first round only includes the test for the effect of age because almost all individuals in the low radiant heat treatment had died at the start of the nymphal stage. NA = not available.

values reported in previous studies ($c \leq 0.07$, Table 2). When we concentrate on the subset of the data where colour morph switches were most likely, given both non-matched background and temperature treatment (brown individuals on a green background under high radiant heat treatment), the probability of colour morph switch is still well below expectations ($c \leq 0.17$, Table 2).

Temperature-cued colouration darkening

We observed a significant change in colouration darkness in both rounds of trials. Individuals in the second and third nymphal stages (N2 and N3) of the round 1, and in the N2 and N4 stages of the round 2 experienced a change in darkness which depended on the direction of the temperature treatment. Individuals in the low radiant heat treatment became darker in colour, while those in the high radiant heat treatment became lighter in colour (Table 3, Figure 2). Individuals in the N3 stage in the R2 did not show a significant change in colour, yet the sign of the point estimate is the same as in stages N2 and N4. Individuals in the N4 stage of the R1 and under a high radiant heat treatment got significantly darker with age. Most of the low radiant heat treatment individuals from the R1 had perished, with the single remaining individual becoming lighter. Unlike the situation in nymphal stages, individuals in the imago stage undergo a darkening in colour in both treatments within the first week after final ecdysis (Figure 2).

The analysis of unambiguous cases of lightening or darkening using Fisher's exact tests confirmed a significant colour change for the N2 and N4 stages in the R2 (N2 stage: n = 14, p = 0.023; N3 stage: n = 8, p = 0.14; N4 stage: n = 10, p = 0.015; IM stage: n = 12, p = 0.47, see also Figure 2). Yet after exclusion of ambiguous cases, there was not significant effect in the R1 (N2 stage: n = 12, p = 0.091; N3 stage: n = 7, p = 0.28; N4 stage: n = 12, p = 0.33, see also Figure 2).

The comparison of treatments per observation time (early or late observations within nymphal stages) also confirmed a significant effect of the treatment in the colour of nymphae (upper p values in Figure 3). Individuals in the N2 stage in both rounds, and in the N3 stage of R1 show a significant difference in colouration darkness at the end of the nymphal stage. Individuals of the N4 stage differ at the start of the nymphal stage, but at the end colouration darkness had converged.

Finally the comparison between the earliest and latest observations per treatment within nymphal stages also found a significant effect of the treatment in the colouration darkness of nymphae (lower p values in Figure 3). Individuals in the N2 stage of both rounds (statistically significant only in the low radiant heat treatment), N3 stage of R1 (statistically significant only in the high radiant heat treatment) and N4 stages of R2 change their colouration darkness within nymphal stages. The colour change in the IM stage is also significant and the change in both groups is towards a darker coloration.
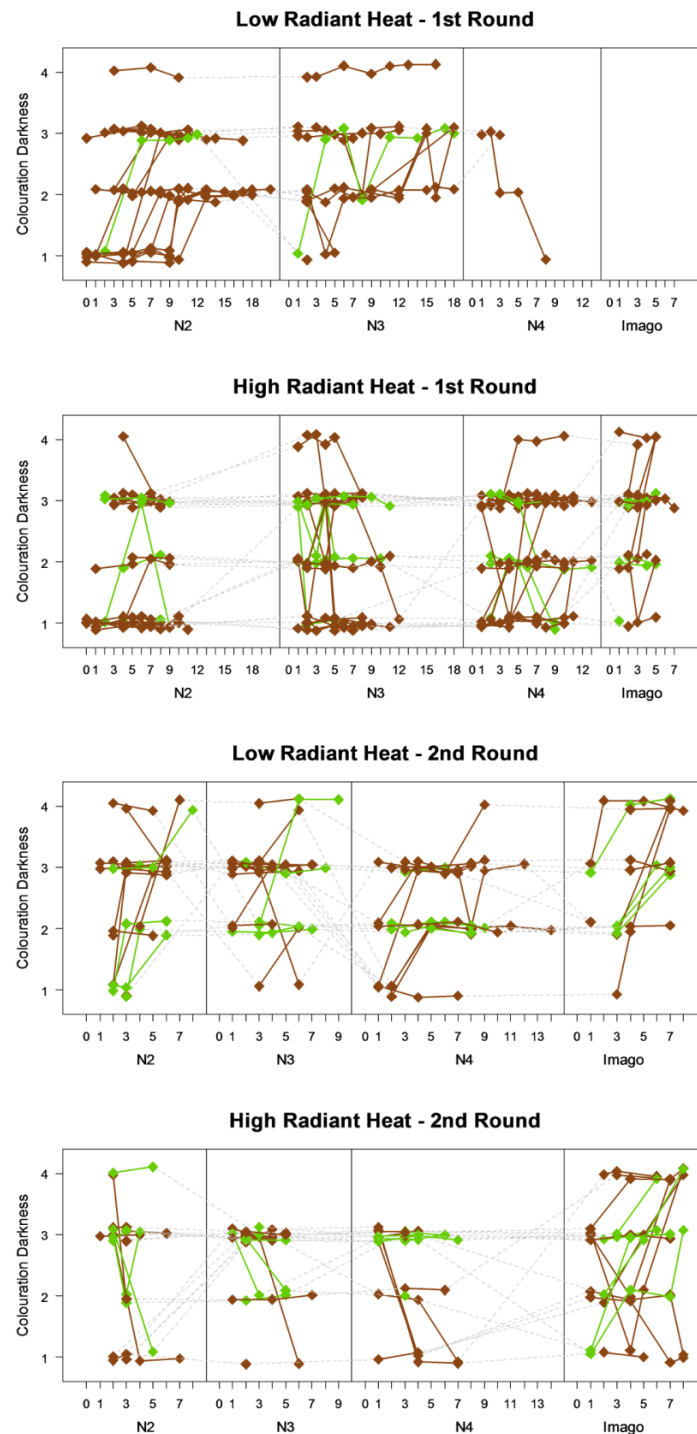
Figure 2. Changes in darkness across nymphal stages exposed to two different sets of radiant heat regimes (first round R1, and second round R2). In each set of panels, upper panels show low radiant heat treatments, bottom panels show high radiant heat treatments. Each line represents the trajectory of an individual (some individuals have only one observation per nymphal stage, and therefore appear only as a dot without line). Brown lines represent brown morphs, while green lines represent green morphs. Nymphal stages are measured in days since the start of each nymphal stage. Darkness has four levels, with 1 representing the lightest colour morph and 4 representing the darkest colour morph.
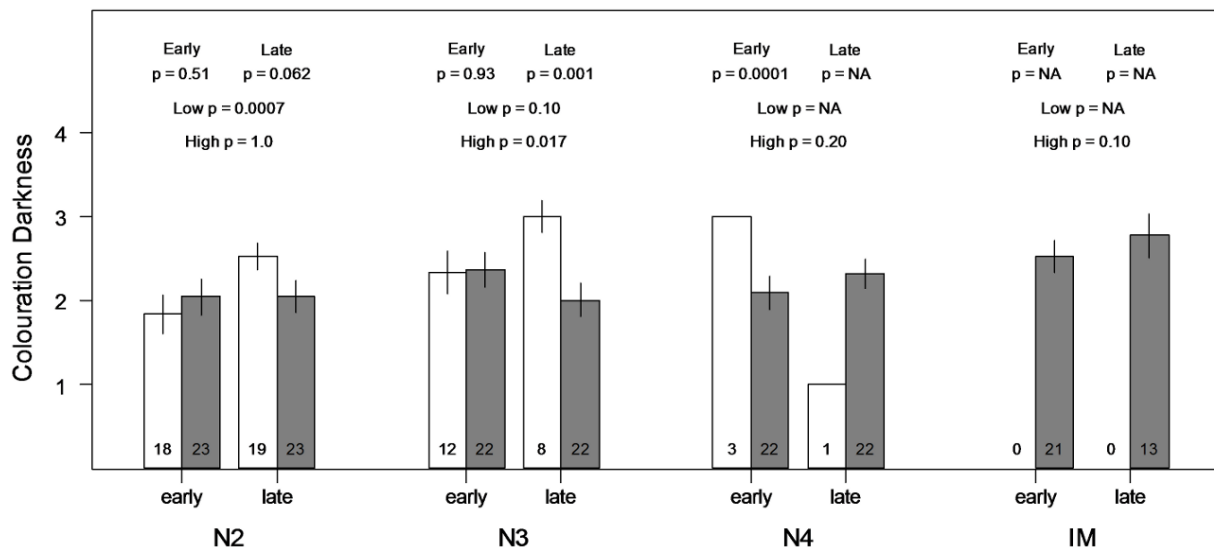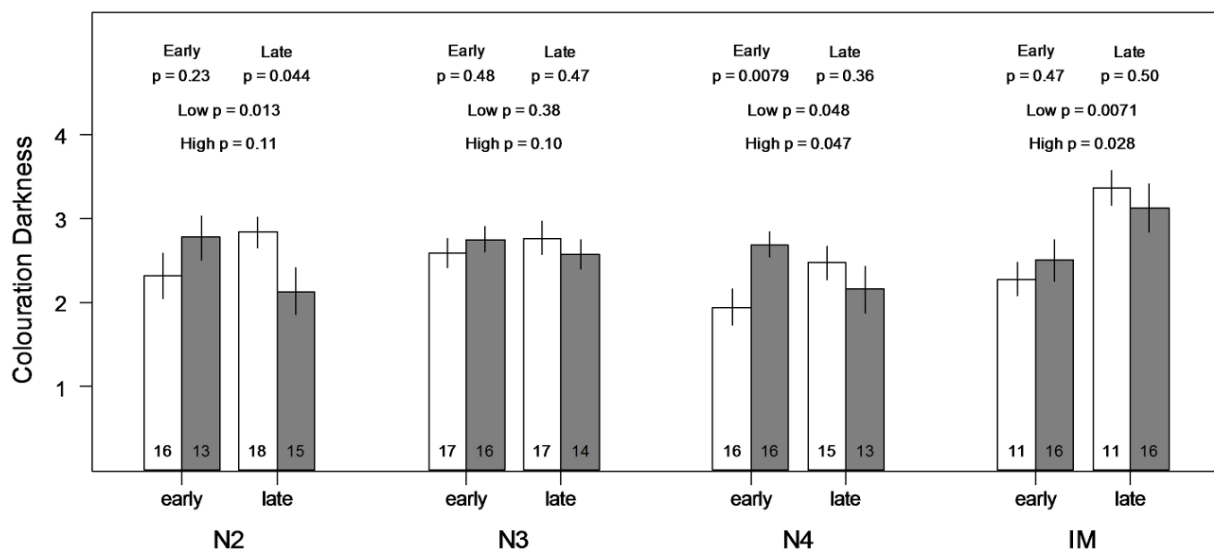
Figure 3. Mean darkness per nymphal stage at the earliest and latest observation point, separated per radiant heat treatment for two different sets of radiant heat regimes. Upper panel shows the first round (R1), lower panel shows the second round (R2). White bars represent low radiant heat treatments, grey bars represent high radiant heat treatments. Numbers inside the bar show the number of individuals per observation point. P-values for two-sample t-tests comparing treatments within observation points are shown on top (labeled as "Early" and "Late"). P-values for paired t-tests comparing observation points within treatments are shown below (labeled as "High" and "Low").

Discussion

We here show the effect of background colour and temperature on colour changes and darkening in *G. sibiricus*. There were no colour morph switches among 78 individuals tested, neither when exposed to matched nor to unmatched background. Colour morph switches have been widely reported in other orthopterans (Table 1), hence it is interesting that we cannot confirm this for our species. If this species is capable of switching colour at all, the probability of colour morph switch in individuals that mismatched their cage background was certainly very low for both rounds of trials (≤ 7%). Additionally, we find that the amount of radiant heat affected colouration darkening within nymphal stages, with darkening at low amounts of radiant heat and lightening at high amounts of radiant heat. Imagoes tended to darken within the first week after final ecdysis independent of radiant heat treatment, suggesting that imagoes may face different life-history trade-offs than nymphae.

Green-brown switches

Colour morph switches have been previously reported, with several observations of the phenomenon in a diverse array of species (Table 1). Several of the species for which colour morph switches have been reported are members of the Acrididae (the family that also includes *Gomphocerus*), but none of them is a member of the subfamily Gomphocerinae (Zhang *et al.* 2013). It is possible that the capability for colour morph switches has been lost somewhere in the branch of Gomphocerinae, but this remains speculative in the absence of information about other species. The lack of a mechanism for switching colour during nymphal stages in *G. sibiricus* may also be due to the really fine-grained structure of the habitat inhabited by the species. It would be very costly for an individual to move at all within the matrix of colours of the habitat if this would require an active colour switching to match their background, even if this could be done in a relatively short time window.

Background colour was visually perceivable to the developing individuals and based on previous studies, we assume visual perception to be the main input that triggers colour change to match the background (see Rowell 1971 and references therein). However, it might be argued that our coloured paper was not of the right kind for triggering colour changes. Previous studies have used a large diversity of materials for the background manipulation, such as stones, sawdust, sand, coal, clay, paper and paint (Table 1). These different materials have typically elicited colour morph switches, which suggests that the effect does not depend on the exact kind of materials used as background. We consider it

unlikely (albeit possible) that our paper type was so substantially different from previously used materials that it would not be suitable for triggering colour switches.

In previous experiments, individuals have been tested for different periods of time in order to assess colour morph switches, usually starting the experiments at early nymphal stages (Ergene 1950, Rowell & Cannis 1971, Forsman *et al.* 2002, Tanaka 2004, Hochkirch *et al.* 2008). Colour morph switches have typically been reported to occur across nymphal stages, often quantified a few days after ecdysis, though detailed information on the exact timing of switches is usually lacking. The only exception to colour morph changes occurring within nymphal stages are changes to black colouration, which have been reported to occur during the imaginal stage (Rowell 1971). We started with our experimental treatment very early in the life of the grasshoppers (also in comparison with previous studies), giving scope for switches within and/or between developmental stages. Therefore it is rather unlikely that the duration of the treatment prevented colour switching.

Switches might also have been expected due to the radiant heat treatment, since brown individuals tend to be darker on average than green individuals and we would expect them to be better able to heat up if radiant heat is limiting. High temperature, as well as high humidity, high food moisture content and low individual density, are known to drive green body colouration in grasshoppers (Rowell 1971, Dearn 1990). We expect that in our experiment high radiant heat conditions would have served to cue individuals of a green habitat. High alpine habitats are typically characterized by high humidity regimes due to high condensation of air humidity at night, which is available as dew drops early in the mornings, and also due to high precipitation regimes (Beniston 2006). Under these conditions, high temperature and high humidity will promote vegetation growth and produce greener habitats than conditions of low temperature, where vegetation would have weaker growth. Such conditions could have promoted colour morph switches from brown to green, possibly as a means for habitat matching. In contrast, low radiant heat conditions would have served to cue individuals of a browner habitat with less flourishing vegetation. These conditions, in the case of green individuals, could have promoted colour morph switches from green to brown, either for habitat matching and/or to improve thermoregulatory capacity.

Green individuals on a brown background and under the low radiant heat treatment thus constitute the subgroup for which colour morphs switches from green to brown appear particularly advantageous. It is possible that the combination of relative high humidity, high food moisture content and individual housing (i.e. low population density)

counteracted the effect of the background, hampering the occurrence of the colour morph switch (Rowell 1971, Dearn 1990). This is different for brown individuals on a green background and under the high radiant heat treatment, since for those individuals the combination of low density, high humidity, background mismatching and no need for improved heat absorption are all expected to favour colour morph switches towards green. Yet none of the 16 individuals under this suit of conditions switched colour.

Our limited sample size does not allow us to exclude the possibility that *G. sibiricus* is capable of colour morph switches under some conditions. Still it strongly points against frequent, general developmental switches in response to background colour and temperature. We had expected that if colour morph switches occurred in *G. sibiricus*, they would occur at the nymphal stages, given that matching the habitat background is expected to improve survival in natural conditions. It is possible that nymphs of *G. sibiricus* achieve homochromy even in the absence of developmental switches by actively seeking out matching (micro)habitats (Edelaar *et al.* 2008). Habitats of *G. sibiricus* are spatially highly heterogeneous and this might distinguish them from many of the other species that show developmental colour morph switches. Microhabitat variability might favour behavioural over developmental homochromy, while more global (temporal) variability in less structured habitats might favour developmental switches.

Colouration darkening

While the temperature treatment in our experiment did not elicit colour morph switches, it elicited a more subtle response in colouration, causing darkening under low radiant and lightening under high radiant conditions. Radiant heat can limit behaviour of individuals by hampering thermoregulation and this in turn can constrain activity levels, growth and development, and ultimately fitness (May 1979, Willott & Hassall 1998, Ellers & Boggs 2004). An increase in the amount of melanin under the cuticle surface would improve thermoregulatory capability due to a difference in heat absorbance between black and brown or green colours, and this would help counteract the effect of a short window of radiant heat exposure. Hence the darkening that we found in the low heat treatment is in line with what would be expected for improved thermoregulation (Kingsolver & Wiernasz 1991, Forsman 2000, Hegna *et al.* 2013).

The lightening in colouration in response to the high radiant heat treatment can result from a trade-off between melanin as a colour pigment and other functions of melanin. Melanin plays a role in several functions in insects, such as immune defence, integumental colouration, wound healing and cuticle sclerotisation, among others (reviewed in

Sugumaran 2002). It has also been documented that melanin production in insects can be costly, mostly because of the many possible functions of melanin, but also because of dietary limitations of melanin precursors or lack of enzymes necessary to process precursors (Ellers & Boggs 2004, Stoehr 2006, Stoehr 2010, Roff & Fairbairn 2013). Therefore lightening of colouration can be seen as an option to avoid investing melanin in body colouration when it is not necessary for absorbing more radiant heat. This reasoning might give an adaptive explanation for the lightening in our high radiant heat treatments.

A change in darkness within nymphal stages implies a mechanism which allows individuals to adjust the amount of visible melanin in their epidermis during the relatively short time spanned between moults. Such a mechanism would include cells at the epidermis capable of spreading pigment granules under the cuticle surface, but also capable of withdrawing the pigment granules under proper stimulation (Chapman 1998, Umbers *et al.* 2014). Relatively little is known about the physiology of colour changes in grasshoppers, but different physiological and morphological mechanisms have been described in other arthropods.

A very intuitive mechanism which could explain the changes in darkness under different radiant heat regimes is pigment dispersal and concentration within chromatophores (Fingerman 1970, Umbers *et al.* 2014). This type of cell is known to be present in several taxa, such as fish, reptiles, amphibians, crustaceans and bacteria Fingerman 1965. The shape of chromatophores is typically highly branched, allowing for pigment to disperse to the branches or contract to the centre to achieve colour change (Fingerman 1970, Keeling & Archibald 2008, Umbers *et al.* 2014). Another physiological mechanism involved in colour change is granule migration. Here granules of pigment are transported along microtubules which are perpendicular to the cuticular surface, and which branch distally, allowing the pigments to spread and therefore causing colour change. A striking example of this plastic mechanism is observed in the temperature-controlled daily changes in the colour of the chameleon grasshopper *Kosciuscola tristis*. In this case granules of pigment migrate from the epidermis when the grasshopper is exposed to temperatures above 25 °C, giving males a bright turquoise colouration (Umbers *et al.* 2014). A similar mechanism is used in the stick insect *Carausius morosus* (Berthold 1980). The colour change that we found in *G. sibiricus* is much slower, but since granule migration might simultaneously explain changes in darkness in both directions, it might contribute to our observations.

The unpredictable climate in the habitat of *G. sibiricus*, characterized by long spans of time with few favourable climatic conditions, might explain the occurrence of a darkening and lightening mechanism. In this environment, given the duration of each nymphal stage

(about one week, albeit likely to be substantially longer in the field) nymphae may need to adjust colouration even within nymphal stages to be able to cope with climatic variability. In species dwelling in habitats where radiant heat is a limited factor, energy balance and thus activity levels during early developmental stages could be hampered by limited sun exposure conditions. Being able to adjust colouration darkness could greatly improve the use of resources by an individual, in this case melanin which is a multipurpose and apparently costly pigment (Burtt 1981).

Conclusions

Colour polymorphism is a striking feature of several species, which involves variation in the types of morphs and different environmental triggers and genetic determinants which combine to help define the colour morph of an individual (Rowell 1971, Karlsson *et al.* 2008). Here we report the absence of colour morph switches in the alpine grasshopper *G. sibiricus*. The lack of switches could be driven by the heterogeneity of the habitat of *G. sibiricus*. Additionally we found that nymphae of *G. sibiricus* are capable of modifying the amount of colouration darkness in response to radiant heat. This capability could help nymphae adjust colouration within nymphal stages according to the unstable climatic conditions characteristic of their habitat, and in turn improve allocation of pigment resources to other physiological processes. The complex net of interactions between fine-scaled variation in polymorphisms, unpredictable and highly variable environments, and melanin pathways which may be costly and also shared by different developmental processes are not only a very promising avenue of study in behavioural ecology, but they will also change the way in which we assess colour polymorphism in natural populations.

List of abbreviations

N1: first nymphal stage
N2: second nymphal stage
N3: third nymphal stage
N4: fourth nymphal stage
IM: imaginal stage
R1: first round
R2: second round

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Chapter 3

Condition-dependence and sexual ornamentation: Effects of immune challenges on a highly sexually dimorphic grasshopper

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Abstract

Sexual dimorphism constitutes an important aspect of phenotypic diversity and is ultimately driven by sex-specific selection. In the context of mate choice, sexual ornaments can assume the function of signals of quality that the choosy sex uses to evaluate potential mating partners. In many species there are no obvious direct benefits and investment into mate choice is primarily rewarded by beneficial alleles that are inherited to the offspring. Inter-sexual communication via sexual ornaments requires honesty of the sexual signal, yet the question of what maintains honesty remains only partially solved. One solution is that honesty is maintained by trait expression being dependent on individual condition, since condition-dependent trait expression offers an effectively inexhaustible source of genetic variability. Here we test in the highly sexually dimorphic club-legged grasshopper *Gomphocerus sibiricus* if putative sexual ornaments, in particular the striking front-leg clubs, are more strongly affected by a lipopolysaccharide (LPS) immune challenge than putatively not sexually selected traits. Our results show overall little condition-dependent expression of morphological and song traits, with sexually selected traits exhibiting effects comparable to non-sexually selected traits (with the possible exception of stridulatory file length and syllable-to-pause ratio in advertisement songs). Interestingly, field observations of individuals of lethally parasitized individuals suggest that a very strong environmental challenge can specifically affect the expression of the front-leg clubs. The presence of a few percent of individuals with missing or heavily deformed clubs in natural populations furthermore indicate that there are risks associated with the development of this ornament during final ecdysis and this might act as a filter against severely deleterious alleles.

Introduction

Sexual dimorphism is one of the most conspicuous attributes of many species of animals and plants, and its relevance to phenotypic evolution by sexual selection was already known to Darwin (Darwin 1871, Andersson 1994). Ultimately sexual dimorphism results from selection acting differentially on homologous traits in the two sexes. Inter- and intra-sexual selection are two particularly important components of sex-specific selection that will often favour an exaggeration in colour, size or shape, thus giving rise to conspicuous sexual ornaments (Andersson 1994, Fairbairn 1997). Since choosiness is expected to be costly in terms of time and energy investment (Jennions & Petrie 1997), important benefits need to be gained from mate choice to compensate the costs (Booksmythe *et al.* 2008, Bonachea & Ryan 2011) and in species with no direct benefits there can only be good alleles for the offspring as indirect benefits to the choosy sex (Candolin 2003, Hettyey *et al.* 2010). Mate choice in this context would depend on reliable cues about genetic quality and a central question in behavioural and evolutionary ecology is what maintains honesty in mating signals for indirect benefits.

One mechanism that maintains honesty is condition-dependent expression of sexual ornaments (Cotton *et al.* 2004). Overall condition is determined by the sum of the genetic, epigenetic and environmental effects on numerous vital processes as well as in morphological, behavioural and life history traits (Rowe & Houle 1996, Hill 2011) and it is the heritable component of these influences that are the target of mate choice. The genetic variation that is contributing to overall condition likely offers a large mutational target and thus a perpetual source of indirect genetic benefits to being choosy in mate selection. Condition-dependent ornament expression therefore ensures a lasting source of genetic variation even in face of strong directional selection (Tomkins *et al.* 2004, Connallon 2010). Choosy individuals benefit most from relying on traits that can most efficiently capture the genetic variance contributing to overall condition and these traits are expected to (co)evolve with mating preferences and become sexual signalling traits. We expect sexual ornaments to be a non-random selection of traits in that they particularly well reflect overall condition. This concept of traits being selected for reflecting condition-dependence is known as genic capture (Rowe & Houle 1996, Tomkins *et al.* 2004).

Even if the heritable component of overall condition is the target of choice based on indirect genetic benefits, environmental variation during various developmental stages constitutes a very potent modifier of the phenotypic expression of many traits (Monaghan 2008). The early and the current environment can both affect overall condition, and condition-dependent trait expression will thus partly reflect this environmental variation. This source of variation in condition-dependent traits cannot be the target of mate choice

for indirect genetic benefits, since environmental perturbations are not inherited to the offspring. However, environmental effects on condition make condition-dependence amenable to experimentation. While most traits are expected to respond to environmental manipulations of condition, sexual ornaments that have evolved as honest signals of genetic quality mediated by their tight connection to overall condition are expected to show particularly strong responses to manipulations. Sexual ornaments are therefore predicted to show heightened condition-dependence when compared to non-sexual traits (Rowe & Houle 1996, Cotton *et al.* 2004, Tomkins *et al.* 2004, Gosden & Chenoweth 2011).

One important component contributing to an individual's condition is the immune defence system (Schulenburg *et al.* 2009). Individuals will facultatively upregulate their immune defence in response to the presence of pathogens. This upregulation will maximize fitness, given that the individual is environmentally challenged, but this comes at a cost since the plastic immune defence invests valuable resources that could otherwise be allocated to trait development (Sheldon & Verhulst 1996, Kurtz & Sauer 1999, Schmid-Hempel 2003). The connection between condition and the costs of immunity has been commonly tested by experimental immune challenges (e.g. Jacot *et al.* 2005a, Vainikka *et al.* 2007, Kelly 2011, Zanchi *et al.* 2012) that allow assessing if potential sexual ornaments are indeed better at reflecting individual quality than other traits. This framework constitutes a very versatile tool to study the development of secondary sexual traits and honesty in signal evolution (Rolff & Siva-Jothy 2003, Schulenburg *et al.* 2009).

We here use a lipopolysaccharide (LPS) immune challenge assay to test for condition-dependent expression of an extreme ornament in a grasshopper species. LPS activates the immune response, but does not have pathogenic effects on the individual, and it is cleared from the system of the individual within hours after entering the body (Mathison & Ulevitch 1979, da Silva *et al.* 2000, Jacot *et al.* 2005b). While the LPS is inside the body it causes the release and activation of phenoloxidase (normally stored in cells as the precursor prophenoloxidase) as well as antimicrobial substances such as lysozymes, both of which are key components of the humoral immune system (Cerenius *et al.* 2008, Eggert *et al.* 2014). LPS thus allows an easy manipulation of individual condition by simulating a bacterial infection. There are alternative options for manipulating condition in insects, e.g. manipulations of diet quality (Punzalan *et al.* 2008, Dmitriew & Blanckenhorn 2014, Johns *et al.* 2014), surgical insertion of a foreign body, usually a nylon filament which is inserted through the insect thoracic cavity (Tregenza *et al.* 2006, Stange & Ronacher 2012), or larval density manipulations (White & Rundle 2015). However, the LPS treatment is particularly appealing because it triggers ecologically relevant immune defence cascades.

Our model species is the club-legged grasshopper *Gomphocerus sibiricus*, an alpine dwelling acridid grasshopper that exhibits an outstanding degree of sexual dimorphism (Bellmann 2006, Bellmann & Luquet 2009). In this species the fore tibia of males (but not females) are proximo-distally enlarged to form conspicuous, pear-shaped clubs (Figure 1a-b). This striking clubs are readily presented to females during courtship, during which males continuously move to remain in front of the female and present the clubs from their broadest side. Furthermore, males perform "push-ups" as part of the final stages for the courtship display. The species also possesses clubbed antennae, and in males the tip of the antennae are somewhat more enlarged compared to females. Antennae are used during sexual display and during antagonistic interactions between males. In these situations, males quickly swing their antenna backwards and erect them over their thorax, which is followed by a slow forward sweep.

Male club-legged grasshoppers also produce undirected advertisement songs to attract females and directed display songs during courtship. In the field, males usually search for open areas with stones where they broadcast a highly energetic song which can last up to one minute. The song is produced by strokes of the stridulatory file against an elevated vein on the forewings (Uvarov 1966). The stridulatory file consists of a row of pegs on the inside of the hind femur (Uvarov 1966). Little is known on what features of the song are important to females of *G. sibiricus*, but it is possible that properties of the song serve also to signal male quality (Berger & Gottsberger 2010). In other orthopteran species songs convey information about different aspects of male quality, such as immunocompetence and overall health (Stange & Ronacher 2012), and are correlated to other proxies of condition such as fluctuating asymmetry (Simmons & Ritchie 1996) and energy invested in sound production (Klappert & Reinhold 2003, Rebar *et al.* 2009, Simmons *et al.* 2013).

In the present study we aim to test if the conspicuous sexually dimorphic traits of the club-legged grasshopper are more strongly affected by an environmental manipulation of condition during development than traits which are not involved in courtship and display. To assess this we employed an immune challenge with bacteria-derived LPS on three different cohorts of individuals. We test for effects on six putative sexual morphological traits, six non-sexual morphological traits, five aspects of the advertisement song and two developmental traits. We followed individuals from the penultimate nymphal stages into the imaginal stage (when ornaments are first fully expressed), and combined both field and laboratory raised individuals in order to complement the results of the experiments.

Typical Individuals

a) male

b) female

Males with club deformations

c)

d)

e)
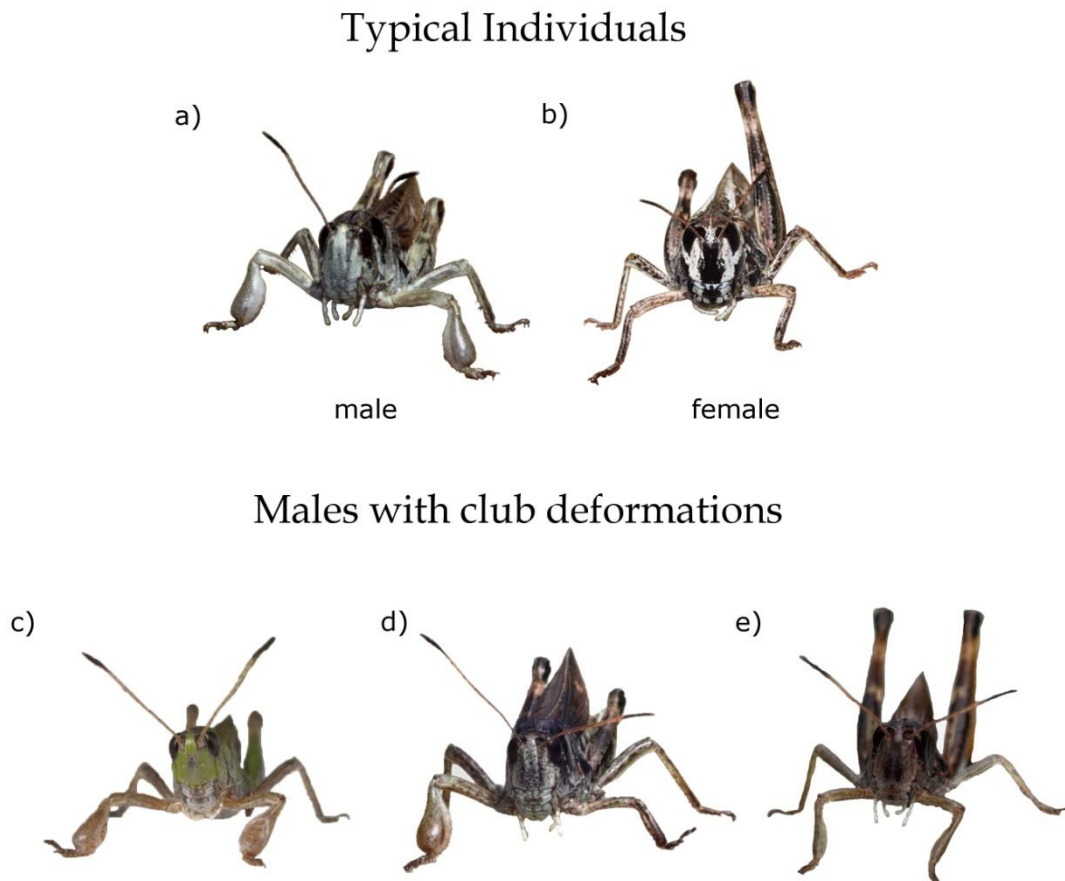
Figure 1. Variation in fore tibia development between of *Gomphocerus sibiricus* in the field. The upper part shows typical phenotypes of (a) males and (b) females. Note the striking dimorphism in the shape of the front legs. The lower part show variation in integrity of clubs that occur at low frequencies in natural populations: (c) male with dents on both clubs, (d) male with only 1 developed club, (e) male with both clubs undeveloped.

Methods

We performed three rounds (R1-3) of immune challenge experiments over three consecutive years (Table 1). R1 and R2 were intended as partial replicates with slight modification due to logistic reasons (e.g. field vs. lab raised stocks) as well as purposeful changes in response to results from R1 (in particular increased LPS dosage, choice of traits). The third round (R3) was implemented primarily for immunological assays. We did not conduct immunological assays in R1 and R2, because this would have required additional subjects, which we considered unnecessary initially. All subjects were housed with fresh grass provided as food in small plastic vials (height x diameter: 5.8 cm x 2.1 cm) filled with water, and the grass was replaced every other day in order to avoid withered or

yellowing grass. Throughout the study we used LPS derived from the Gram-negative bacterium *Serratia marcescens* (L6136, Sigma-Aldrich, Missouri, USA) and Grace's insect medium (G8142, Sigma-Aldrich, Missouri, USA) as carrier medium.

Immune Challenge on Field Collected Individuals

We collected 83 third nymphal stage (N3) males of *G. sibiricus* in the field (near Sierre, Valais, Switzerland) in July 2012 and brought them to our field station. Nymphae were housed in groups of seven individuals per cage (dimensions 22 x 16 x 16 cm^3; this type of cage was used throughout all three rounds unless otherwise stated). On the day following moult into the penultimate life stage (nymphal stage 4, from here on N4), individuals were weighed ("pre-treatment mass", Sartorius analytical balance, Quintix 124-1S, Sartorius, Göttingen, Germany), anesthetised with CO_2 and then challenged with either 1.0 μl of 0.1% LPS dissolved in Grace's insect medium (n = 42) or 1.0 μl of pure Grace's insect medium as control (n = 41). Solutions were injected between the 3rd and 4th sterna using a Hamilton syringe (SYR 10 μl 1701 RN - ga26s/51mm/pst2, Hamilton, Bonaduz, Switzerland) under a stereo microscope. Still under anaesthesia, we removed either the two right or two left distal segments of the middle tarsus as a marking of the treatment. After the injection N4 individuals were housed in two large cages (dimensions 47.5 x 47.5 x 93 cm3; BugDorm-44590F, MegaView Science, Taichung, Taiwan) until final ecdysis. In one of the two communal cages LPS injected individuals had the middle tarsi removed on the left side while controls had the right side tarsi removed, and on the other cage the sides were reversed. We specifically chose the middle leg because this treatment did not impair overall body balance and did not prevent individuals from singing. By a combination of treatment marks, inter-individual variation in colour marks and frequent checks for moulting individuals, it was possible to track all individuals across final ecdysis. Individuals were weighted again on the first day after final ecdysis ("imago mass 1") and on the fourth day after final ecdysis ("imago mass 4"), after which they were collected for morphological measurements.

Immune Challenge on Lab-reared Individuals

A total of 42 egg pods were collected from field-caught individuals (near Sierre, Valais, Switzerland) that were temporarily housed in individual cages in July 2012 (for details of the housing and mating design see Valverde & Schielzeth 2015). We used the laboratory-reared male offspring from those egg pods after overwintering in refrigerators at approximately +4°C. Hatchlings were housed individually through all nymphal stages. On the day following moult into N4, individuals were challenged with either 1.0 μl of 0.25% LPS dissolved in Grace's insect medium (n = 40) or 1.0 μl of pure Grace's insect medium as control (n = 38). Solutions were applied as in R1, but this time after chilling them for

Table 1: Summary of important aspects of the experimental design.

	Round 1	Round 2	Round 3
Purpose	To test for condition dependent expression of traits	To test for condition dependent expression of traits	To assay the effect of LPS in immune parameters
Year	2012	2013	2014
Origin of subjects	Field (collected as N3)	Lab-reared offspring	Field (collected as N3)
Housing	Communal cages	Individual cages	Individual cages
Timing of LPS challenge	Day 1 after ecdysis to N4	Day 1 after ecdysis to N4	Day 1 after ecdysis to N4
Application of LPS	1 µl of 0.1% LPS	1 µl of 0.25% LPS	1 µl of 0.25% LPS
Experimental marking	Clipping of middle-leg tarsi	None	None
Measurements on alive subjects	Pre-treatment mass, imago mass 1 and 4	Pre-treatment mass	None
Song recordings	None	2x 1 hour	None
Sampling of hemolymph	None	None	20h after treatment
Collection/Death	Collected on day 4 after moult to imago stage	Natural death	Natural death
Post-mortem measurements	Antenna, clubs, stridulatory file, wing, hind femur, pronotum	Antenna, clubs, stridulatory file, wing, hind femur, pronotum, eye span	None
Samples size	N = 83	N = 78	N = 48

3 minutes at -10 °C instead of CO_2 anaesthesia. After the immune challenge, N4 individuals were housed until final ecdysis in individual cages (such that no marking was necessary). One week after final ecdysis we recorded songs of individuals during two recording sessions of one hour each (see song recording section). After song recording individuals were once again housed individually and were maintained until they died. This gave us data on total lifespan. Recently deceased individuals were collected for morphological measurements.

Immune Challenge for Immunological Assay

In July 2014 we collected 48 N3 individuals in the field (again near Sierre, Valais, Switzerland) and transferred them to the laboratory where they were housed individually. On the day following moult into N4, individuals were challenged with either 1.0 µl of

0.25% LPS dissolved in Grace's insect medium (n = 25) or 1.0 μl of pure Grace's insect medium as control (n = 23). Solutions were applied as in R2 after chilling for 3 minutes at -10 °C. Twenty hours after the immune challenge we extracted 2 μl of haemolymph from each individual with a Hamilton syringe and added them to 3 μl of insect Ringer solution. The samples were stored in -80 °C for an analysis of two aspects of the immune parameter phenoloxidase (PO) (e.g. Jacot *et al.* 2005b). These assays were not employed during both R1 and R2 to avoid additional pricking of the individuals, which could have confounded the effect of the immune challenge. After the extraction individuals were kept in cages and were maintained until they died.

Morphological Trait Measurements

We measured antenna length, eye span (R2 only), pronotum length, fore wing length (R1 only), hind wing length (R2 only), club width and length, femur length and stridulatory file length (continuous and total, see below, R2 only) on dead individuals under the stereo-microscope and a cold light source. For bilateral traits (antennae, hind wing, femur, club and stridulatory file) we measured both sides and used the average of the two measurements in further analyses. Blind replicated measurements were taken on a random subsample of 23 individuals from the R2 in order to assess technical repeatability.

Since the stridulatory file consists of a row of continuous pegs, gaps in this structure could create gaps in the song and possibly affecting song attractiveness. Therefore we defined two types of lengths: first we quantified the longest stretch of file which had less than three pegs missing (stridulatory file continuous length). Lack of three and more pegs in a row produced a clearly visible gap in the stridulatory file. Second we quantified the total length of the stridulatory file including gaps (stridulatory file total length).

Beside trait-wise tests for treatment differences, we inspected broad categories of putatively sexually or putatively not sexually selected traits in order to compare if the immune challenge had a stronger effect on sexual traits than in non-sexual traits. The criteria we used for this classification are: (1) Use of the trait in sexual displays or during copulation and/or (2) strong dimorphism between the sexes beyond allometric size differences. Among traits that we classified as non-sexually selected are eye span, pronotum length, wing length and femur length. By contrast antenna length (which is used during display and could also have a function in individual CHC recognition), club width and length (both part of the club structure, which is involved in courtship displays), and stridulatory file length (the structure with which the song is produced) were classified as sexually selected traits. Furthermore, we classified song rate and song spectral and

Table 2: Phenotypic means (± SE) and phenotypic standard deviations of the analysed in the current study.

Trait	Round 1			Round 2		
	mean	SD	n	slope	SD	n
Antenna length (mm)	7.89 ± 0.09	0.452	56	8.01 ± 0.12	0.721	63
Club ratio	0.47 ± 0.01	0.041	56	0.45 ± 0.01	0.065	69
Club length (mm)	3.38 ± 0.05	0.255	56	4.12 ± 0.17	0.279	69
Club width (mm)	1.58 ± 0.03	0.165	56	1.86 ± 0.06	0.319	59
File total length (mm)	-	-	-	4.44 ± 0.07	0.396	69
File continuous length (mm)	4.16 ± 0.04	0.204	56	4.28 ± 0.06	0.365	69
Song rate (songs/h)	-	-	-	3.59 ± 1.15	6.834	57
Femur length (mm)	9.60 ± 0.07	0.359	56	9.80 ± 0.08	0.463	69
Wing length (mm)	12.18 ± 0.16	0.787	52	12.08 ± 0.13	0.717	66
Pronotum length (mm)	4.11 ± 0.04	0.193	56	4.40 ± 0.04	0.266	69
Eye span (mm)	-	-	-	1.24 ± 0.01	0.074	69
Mass 1 (g)	0.16 ± 0.00	0.020	46	-	-	-
Mass 4 (g)	0.16 ± 0.00	0.017	45	-	-	-
Syllables per bin	-	-	-	18.87 ± 0.59	2.641	173
Carrier frequency (kHz)	-	-	-	7.34 ± 0.33	1.559	140
Max power	-	-	-	86.09 ± 1.60	6.415	174
Song duration (sec)	-	-	-	14.99 ± 1.66	6.664	57

attributes as sexual traits, although we analysed the latter separately due to differences in sample size and data structure.

Morphological traits were obviously related to overall body size, but the trait correlations were only moderately related to body size, and did not differ between the two classes of traits (average phenotypic correlation among putative sexual traits r = 0.41, among non-sexual traits r = 0.46 and between the two categories r = 0.44 , Table S1).

Song Recording and Analysis

Advertisement songs were recorded for individuals from the R2 during two sessions on two non-consecutive days. To record the songs we designed recording cages (dimensions 28 x 27 x 28.5 cm³) from KömaCel panels (KömaCel Panels, Kömmerling, Pirmasens, Germany). Inside of the recording cages three of the sides were lined with soundproof material (1.8 cm thick) while the door was lined with fine mesh. An LED spotlight (Ce Led Lamp 6W 3000k 40D Dimmable, Philips, Eindhoven, The Netherlands) built in the ceiling illuminated the floor and motivated subjects to stay in the centre. The centre of the ceiling was equipped with an omnidirectional condenser microphone (Behringer C-2 condenser

microphone, Behringer, Makati City, Philippines). The microphones were connected with XLR cables (Cordial CCM 5FM, Cordial GmbH, Dachau, Germany) to an audio interface (PreSonus AudioBox 1818VSL, PreSonus, LA, USA) and the audio interface was connected to a PC. Incoming signals were continuously monitored for grasshopper songs using the Sound Analysis Pro 2011 software (Tchernichovski *et al.* 2000) and files were written to disk when song signals were detected (sampling rate 48000 Hz). Up to 16 individuals could be recorded simultaneously in separate boxes using two computers with 8 incoming channels each. Each recording session lasted one hour, and we calculated the song rate per hour from the two recording sessions of each individual.

The advertisement song of *G. sibiricus* can be separated into three main sections (Figure 2). The song starts with a rather soft introduction characterised by syllables of low energy and with variable inter-syllable distance (IPD) (Figure 2a, #1). The following middle part is characterised by a high energy trill composed of several syllables with a very uniform IPD (Figure 2a, #2). The middle part typically represents the longest part of the song. The variable ending section is composed of syllables with both low and high energy and with highly variable IPD (Figure 2a, #3). We used one to three songs per individual to quantify several spectrographic parameters which were measured using an evaluation version of Raven 1.4 (Raven 1.4, Cornell Lab of Ornithology, New York, USA) based on discrete Fourier transform (DFT) with the default window size of 256 samples and 50% overlap between windows. To quantify the parameters we divided the middle part of the song into bins of 4 seconds. Within each bin we measured the following parameters: (1) Number of syllables per bin (syllables occurring on the bin frame were accounted as half a syllable); (2) Max power (maximum power in the bin, measured in dB); (3) Carrier frequency(frequency at which max power occurs within the bin, measured in kHz). For all songs we quantified an additional parameter, (4) Song duration (without intro and ending section,measured in seconds). Both carrier frequency and max power were quantified in the range of 0-12 KHz, which contains the local frequency maximum, a component of advertisement songs in grasshoppers known to play a role in female response to singing males (von Helversen & von Helversen 1997, Lampe *et al.* 2012).

Immunological Assays

Phenoloxidase (PO) is the final enzyme in the melanisation cascade associated with antimicrobial activity in invertebrates. It is the key component of immune reactions and wound healing, as well as important in the melanisation of the insect exoskeleton. As it is cytotoxic, most invertebrates store PO in the inactive form prophenoloxidase (proPO). We therefore measured activity of both proPO and PO (V_{max}) in the haemolymph (Stanley & Kim 2011). From each treatment we prepared 20 samples. The haemolymph was collected
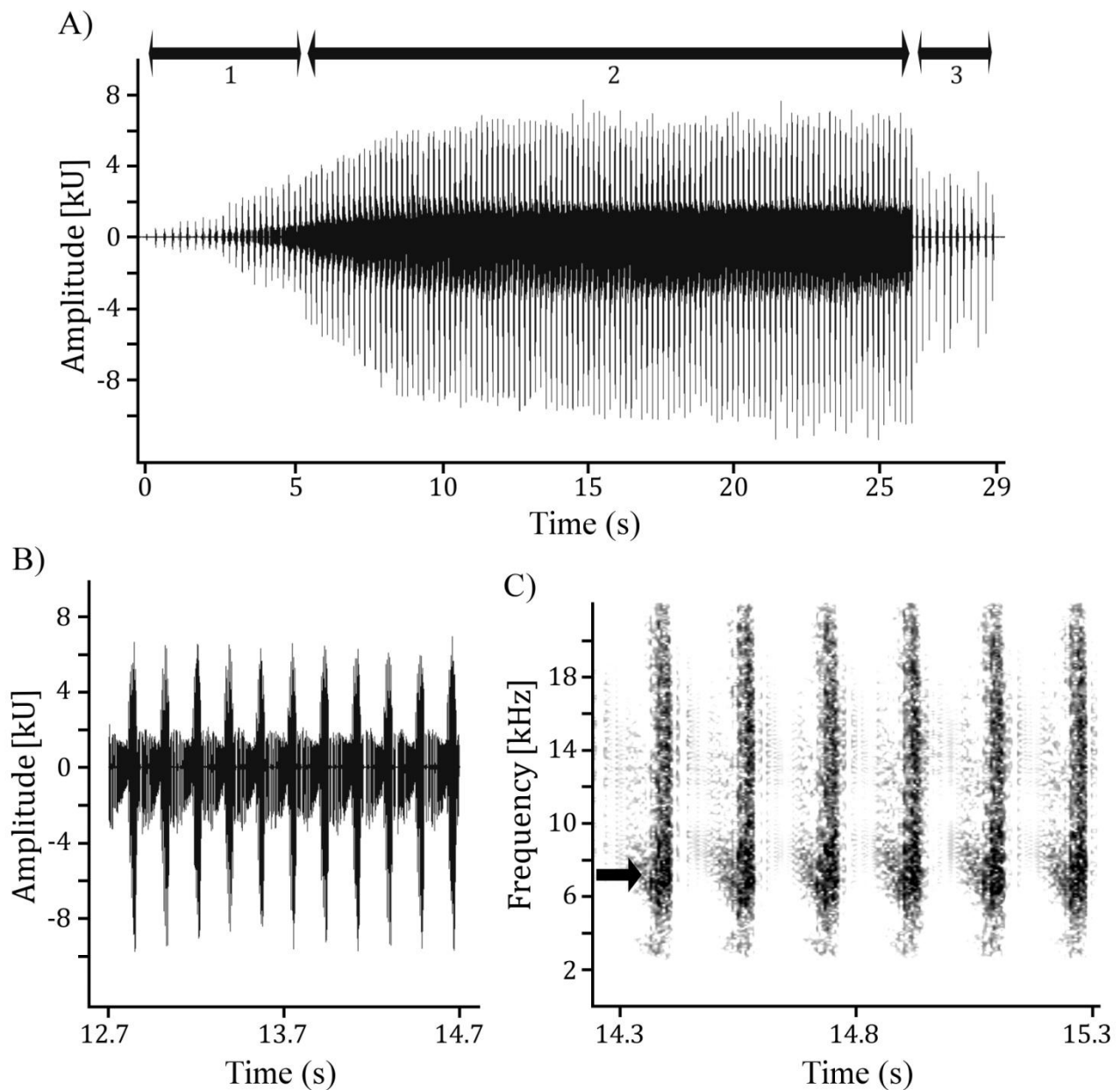
Figure 2. Structure of the advertisement song of *Gomphocerus sibiricus*. (A) The figure in the top shows the waveform view of a complete song, with the three sections (introduction, middle section, ending) marked respectively with numbers 1-3. (B) The bottom left figure shows 2 seconds of the waveform, belonging to the middle part of the song, composed by syllables of regular frequency. (C) The bottom right figure shows 1 second of the spectrogram of the middle part of the song; the black arrow in the first syllable of this figure signals the location of the carrier frequency.

by puncturing the pleural membrane between pronotum and occiput with a sterile hypodermic needle. Haemolymph samples were handled and measured as described by Ardia and colleagues 2012. Briefly, for each sample, we collected 0.5 µl of haemolymph, flushed it into in a 0.5-mL centrifuge tube in 20 µl Bis–Tris buffer (0.1 M, pH 7.5) and gently vortexed each tube and stored at -80°C.

To determine PO activity, 80 μl of *aqua dest* and 50 μl Bis–Tris buffer were given into wells of a 96-well plate (flat bottom) with 20 μl of haemolymph in Bis–Tris buffer, prepared as described above. After adding 50 μl of L-Dopa (4mg/ml L-Dopa dissolved in Bis–Tri buffer), absorbance was measured on a Tecan Infinite M200 plate reader (Tecan Group Ltd., Männedorf, Switzerland) at 490 nm and 37°C for 90 min, once every minute. Phenoloxidase activity was determined as the fastest change in absorbance over 15 minutes (V_{max}). Blanks as negative controls were included on each plate. To assay proPO, we thawed 20 μl of haemolymph in Bis–Tris buffer and mixed it with 145 μl distilled water in a chilled 96-well plate before adding 5 μl chymotrypsin (Sigma C4129 5 mg/ml in distilled water) (Sigma, St. Louis, Missouri, USA) and incubated samples at room temperature for 10 min. We then immediately added 20 μl of L-Dopa and measured the absorbance on a Tecan Infinite M200 plate reader at 490 nm and 37°C for 90 min, once every minute. Phenoloxidase activity was determined as the fastest change in absorbance over 15 minutes (V_{max}). Blanks as negative controls were included on each plate.

Field Sampling of Naturally occurring Deformations

In August 2014 and in July/August 2015 we sampled a total of 42 field populations from 29 regions in Switzerland and Austria for their conspicuous colour polymorphisms. In the context of sexual ornamentation, we here present the proportion of club deformations that we found in these natural populations. We here only briefly describe the sampling design, since details of this field sampling will be presented elsewhere (manuscript in preparation). We aimed to collect 100 individual from each of the sites by slowly walking across suitable habitat patches in search for resting and escaping individuals. Individuals were caught by hand or using a small net and were inspected for their phenotypes in hand. Individuals were released after phenotype scoring. In total we sampled 3963 individuals (84.3 ± 35.9 per site). In 2014 we focused on colour morphs, but while doing so noticed several severe malformations in the sample of males (N = 1326). Since we did not systematically record minor deformations, data from 2014 can only serve as minimum numbers. In 2015 we systematically inspected all males (N = 757) for club deformations, including minor indentations.

Statistical Analysis

All analysis were conducted in R 3.1.1 R Core Team 2014. We used the repeated measurements of 23 individuals from the R2 and estimated the technical repeatability of the morphological measurements using ANOVA based repeatabilities from the rptR package in R (Nakagawa & Schielzeth 2010). The remainder of the analyses were done on first measurements only. All morphological and song traits were standardised and centred prior to the analyses (Schielzeth 2010). We analysed N4 developmental time and imaginal

lifespan by Cox proportional hazard models as implemented in the flexsurv R package (Jackson 2014).

We tested the effect of the immune challenge on the morphological measurements and song rate using fixed effect linear models. The four song properties (number of syllables per bin, max frequency, max power and song duration) had unequal numbers of repeated observations per individual and therefore required to be analysed using mixed effect models controlling for variation between songs of the same individual and (for all song properties except song duration) between bins within songs by random effects. For the max frequency we fitted the model including only the first three bins, since these bins included the highest frequency in the song for the majority of the observations. Mixed models were fitted using the R package lme4 (Bates *et al.* 2012). For all traits we fitted two versions of the models, one version with treatment as the only fixed-effect predictor and another version including pre-treatment mass as a covariate. We focus on the analysis without control for pre-treatment mass in the main text and highlight differences if required (all results are included in the supplementary materials).

Our prediction was that sexual ornaments (especially the striking foreleg clubs) particularly well capture condition-dependence, but this does not necessarily have to apply to all sexual traits, since just one or few key traits might be sufficiently informative for choosing individuals. Nevertheless, it is possible that sexual traits in combination reflect condition-dependence better than non-sexual traits. We therefore use principal component analysis (PCA) to our three sets of data (sexual traits, non-sexual traits, song properties), using the prcomp() function in R to extract the dominant axis of shared variation. For those cases where data were missing in any trait, we used imputation by the mean of the specific variable to avoid losing data in the PCA. Alternative handling of missing values such as dropping the cases with missing data or imputation by regression by pre-treatment mass yielded similar results to those obtained by imputation by the mean.

Since R2 was implemented as a partial replicate of R1 (Kelly 2006) with some technical but no conceptual differences (see Table 1), we conducted a mini meta-analysis, and estimated effect sizes across the two rounds using the R package meta (Schwarzer 2015). The package meta uses the raw data to estimate individual effect sizes for each trait using Glass's Delta. This estimation allowed a global view of the net effect of the treatment on all traits assessed in both rounds.

Ethical Note

All the field samples were taken from populations with very high individual densities. No field permits were required either to perform the experiments or to transfer the animals to the laboratory. The animals in this study were maintained, housed and handled in the most gentle and humane conditions possible. Manipulation of individuals occurred only during the administration of the immune challenge and during haemolymph extraction. Apart from the marking inflicted on individuals from the R1, no other harm was done to any individual in the R1 or in any other round. The marking during the R1 was done under CO_2 anaesthesia to reduce suffering. Individuals behaved naturally shortly after injections and after marking and did not show increased mortality. However, we recorded increased mortality after extracting haemolymph, a procedure that was limited to a smaller sample of individuals. Only the individuals from the R1 were euthanised in 70% ethanol, while individuals from the R2 and R3 were maintained beyond experimental need and died naturally.

Results

A total of 175 individuals (90 LPS, 85 control) from all three rounds were used for the statistical analysis. From rounds R1 and R2 58 individuals (30 LPS, 28 control) and 69 individuals (35 LPS, 34 control) reached the imago stage (IM), respectively. There were no differences in pre-treatment mass between control and treatment individuals in the R1 or the R2 (paired t test: R1: $t_{48.8} = -1.067$, $p = 0.29$; R2: $t_{63.8} = 1.49$, $p = 0.14$).

Repeatability, Phenotypic Correlations and Directional Asymmetry

We used the repeated measurements of 23 individuals in the R2 to assess the technical repeatability of our morphological measurements. Technical repeatability exceeded $R = 0.96$ for all morphological traits measurements, except for eye span (R = 0.89) ($p < 0.0001$ for all traits). We also assessed the phenotypic correlations between left and right measurements of bilateral traits. We expected high correlations between left and right measurements due to the nature of bilateral traits to present only minor deviations in size in individuals which developed under good conditions (Lens *et al.* 2002). Phenotypic correlations were also large and significant for both the R1 and the R2, with correlations from R2 being slightly higher than those of the R1 (Table S2). Pre-treatment mass affected mass after final ecdysis in the R1 (imago mass 1 and 4), illustrating the fact that large nymphae moult into large imagoes.
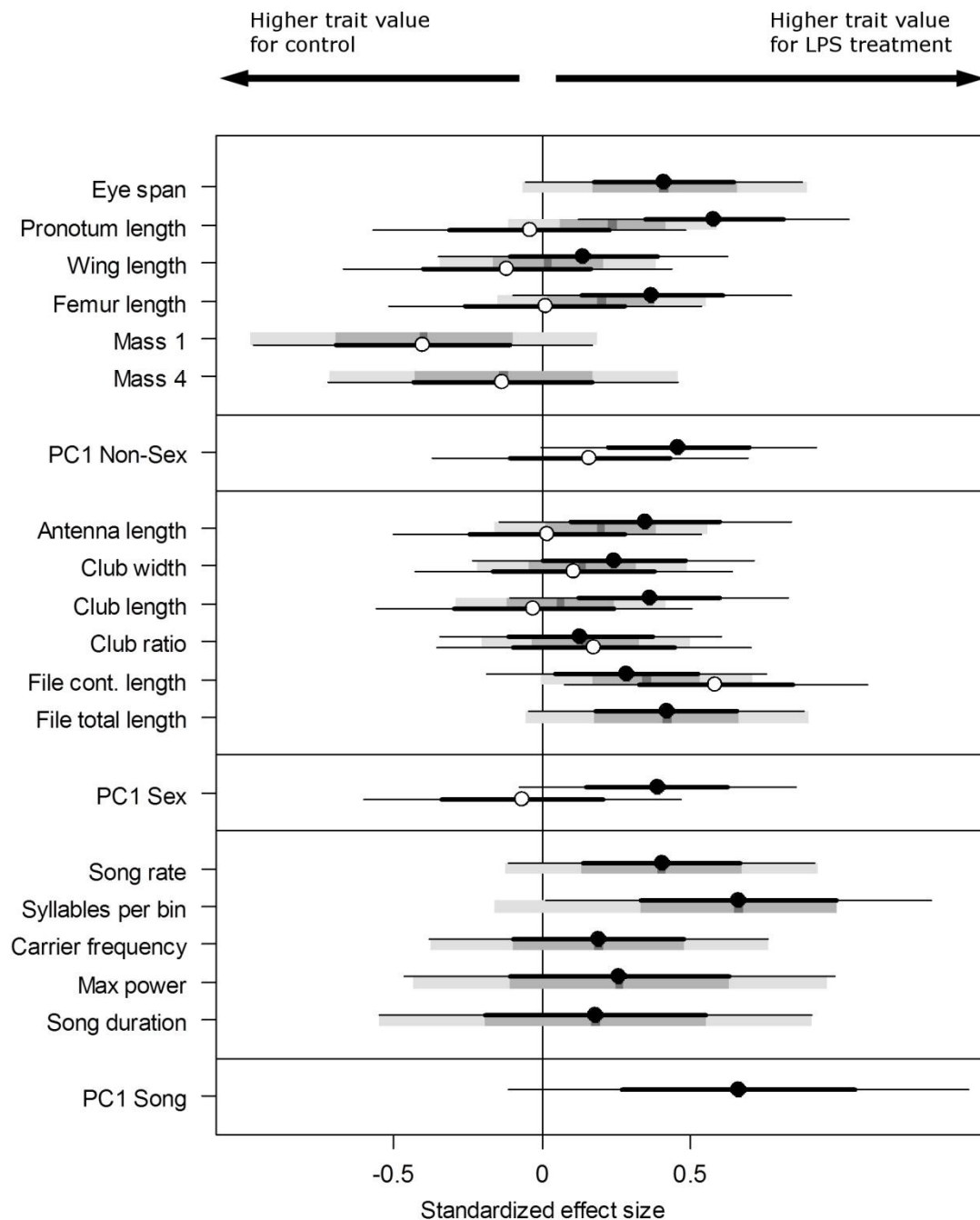
Figure 3: Standardised effect sizes of an immune challenge for sexual, non-sexual and spectrographic song traits. Below each set of traits is the standardised effect size of a PC1. Positive values indicate higher trait values for the LPS treated individuals. Open dots refer to effects sizes of the first round (R1) and filled dots to effect sizes of the second round (R2). Effect sizes of rounds and of the mini meta-analysis show standard error bars (thick short bars for effect sizes of the rounds, medium grey bars for effect sizes of the mini meta-analysis) and 95% confidence intervals (long whiskers for effect sizes of the rounds, light grey rectangles for effect sizes of the mini meta-analysis).

Life History Traits and Immunological Assays

We predicted that the LPS treatment would cause individuals to have a longer developmental time as well as a shorter total lifespan due to the disruption in condition created by the immune challenge. Still we observed no treatment differences in developmental time (N4 to IM) as assessed by likelihood ratio tests comparing alternative cox proportional hazard models (R1: X^2_2 = 3.63, p = 0.16; R2: X^2_2 = 1.55, p = 0.46) (Fig S1). Similarly, there were no differences between LPS and control individuals in adult total lifespan (R2: X^2_2 = 0.25, p = 0.88). We expected to see an increase in the activity of the two immunological parameters assessed in the R3, PO and proPO, due to the effect of the LPS treatment. However, we did not observe significant differences in activity of either of these parameters in the haemolymph of the individuals from the R3 due to the LPS treatment (t-test: PO: $t_{24.5}$ = -1.34, p = 0.19; proPO: $t_{46.0}$ = 0.005, p = 0.94).

Immune Challenge Effects on Putative Sexual and Non-Sexual Traits

We analysed the effect of the LPS treatment on a total of 17 morphological and song traits in imagoes. We expected to see a stronger effect of the LPS treatment effect on sexual traits than on morphological traits, especially on the clubs and on song traits. Yet only three traits showed indications of being affected by the LPS treatment (Figure 3, Table S3). First, LPS treated individuals had a longer pronotum in the R2, but the effect was absent (estimated even opposite in sign) in the R1. It thus likely represents a false positive. Second, LPS treated individuals had a significantly longer (continuous) stridulatory file in the R1, and since the point estimate of the effect was the same in the R2, the effect size estimated with the mini meta-analysis was almost significantly different from zero (p = 0.051, Figure 3). Third, there was a borderline non-significant trend (p = 0.058) for LPS treated individuals to produce a higher density of syllables in the middle part of the song (Figure 3, Table S3).There was no convincing evidence that the front-leg clubs were more strongly affected by the treatment than other traits. While it is possible that only one or few traits show heightened condition-dependence, we also tested for a combined effect across trait classes by PCA. We find a borderline non-significant trend in the PC1 of non-sexual traits in R2 (p = 0.056, LPS treatment tended to be associated with larger size), but clearly non-significant in all other comparisons (Figure 3, Table S3).

Naturally Occurring Deformations

Samples from the field in the Alps showed 14 out of 1326 (1.1%) males with missing, severely deformed or undeveloped clubs in 2014 and 6 out of 757 (1.4%) males with major deformations in 2015 (Figure 1c). Minor deformations were noted in 23 out of the 757 individuals (5.2%) in 2015 (Figure 1d).

Discussion

We here report the effects of two independent immune challenges on sexually selected (morphological and song traits) and non-sexually selected traits, in the club-legged grasshopper *Gomphocerus sibiricus*. Pre-treatment mass in the N4 had a significant positive effect on imago mass, demonstrating that large nymphae moult into large imagoes. The treatment did not significantly affect imago mass, timing of the final moult or imaginal lifespan. Effects on morphological traits were somewhat variable, but there was an overall tendency of LPS treated individuals to show positive effect sizes. With all caution required for interpreting individually non-significant tests, this means that immune challenged individuals were, if anything, larger rather than smaller than controls. Few of the sexual traits stood out as showing significantly elevated condition-dependence and this was particularly true for the size of the front-leg clubs. Notable trends remained for longer file length and higher syllable rate in LPS-treaded individuals, both of which are contributors to advertisement songs, a sexually selected trait, but the trends were unexpected in their signs. Hence, we conclude there is currently little evidence for elevated effects of immune challenges in putative sexual traits of the club-legged grasshopper.

Several factors could have influenced the observed lack of condition-dependent trait expression observed in our experimental manipulations. The point of time during ontogeny when individuals are challenged is very likely capable of influencing individual condition. The timing of our manipulation was set at the start of the fourth nymphal stage (N4) based on two conditions: (1) At this point males do not yet show signs of club development. (2) Earlier nymphal stages are very sensitive to handling, with high mortality occurring between N1 and N3. Several studies on condition-dependence trait expression and immune challenge have worked on adult individuals, which did not suit our hypothesis for testing the expression of the striking ornament in *G. sibiricus*, because once developed during final ecdysis, the most striking sexually dimorphic features of the species are fixed for life. In contrast to this, in the few studies where nymphae were challenged and where condition-dependent expression in sexually selected traits was uncovered, individuals were challenged at a late nymphal stage (da Silva *et al.* 2000, Jacot *et al.* 2005b). Even though we chose the timing of the manipulation carefully, it is certainly possible that the biologically relevant time window lies earlier or later.

Despite the abundance of condition-dependent studies in insects, few of them have worked with nymphae and it is not clear what the optimal dosage of an immune challenge would be in nymphae of our species. Our model species is relatively small (average 0.104 g in N4) in comparison to species used in other studies (Ahtiainen *et al.* 2004, Scheuber *et al.* 2004, Tregenza *et al.* 2006, Contreras-Garduno *et al.* 2008). Because of this we needed to

downscale the dosage of LPS and control solutions in our immune challenge to suit the size of the nymphae. Following this reasoning we decided to inject 1.0 μl of either LPS or control solution. A commonly used concentration of this solution in the literature is 0.1%, which we employed in our R1. After assessing the results of the dosage used in the R1 we decided to change the treatment, and changed to a concentration of 0.25%, which seemed appropriate for the size of the nymphae. This increase in concentration did not have the expected effect in the expression of the traits under study. It is also possible that one single challenge was not sufficient to elicit the relevant effect in condition. On the same line of arguments, LPS solutions with low concentrations appear to be susceptible to stability problems due to storage, with possible separation of the LPS molecules from the solvent and additional binding to the container (Safety and Documentation data sheet for L6136, Sigma-Aldrich, Missouri, USA), but this should not have too much of an effect with the comparatively high concentrations that we used.

A final factor to consider which may have hindered our treatment is the pricking involved in the immune challenge. The pricking that is unavoidable when injecting the individuals during the immune challenge constitutes a possible source of injury to the individuals. This injury, in combination with a weak LPS treatment due to the low dosage, could have levelled the effect of both our treatment and control. Phenoloxydase is particularly prone to react to wounding and after injecting the animals the reaction to the wound could have helped in masking differences between the LPS and the control treated individuals. The lack of support from the immunological parameters obtained from the R3 was initially surprising, yet it is consistent with the idea that the pricking may have levelled the overall immune challenge in both treatments. Still we studied two aspects of a single immune parameter (PO/proPO), which might not show an activation of immunity whereas other parameters might have done so (in particular antimicrobial responses such as AMP's or lysozyme). Still it is possible that the time point when we measured immune activation was too long after the treatment, since PO responds fast and high concentrations of the molecule in the haemolymph might level off quickly. Hence the absence of a demonstrable effect on PO/proPO does not necessarily mean that the treatment had no effect on the immune system.

The striking clubs exhibited by adult males of *G. sibiricus* are only fully developed once the individual reaches the adult stage. Before this stage male individuals in the N4 stage only show a very subtle swelling of the fore tibia while N3 male individuals show no visible signs of the clubs. The cuticle which forms the clubs is very thin (< 0.1 mm), and in adults the volume of the clubs is occupied by liquid and possibly fat bodies (own observations). We suspect that clubs attain their exaggerated volume during final ecdysis via

haemolymph which is pumped into the structures during ecdysis. This process occurs while the individual is still inside the old skin, because newly moulted imago males emerge out of the skin with their clubs fully developed, though still soft during the first few hours after ecdysis.

Even if we do not find an effect of the LPS challenge on club size, there is evidence that the risk of injury during final ecdysis poses a cost associated with fully developed clubs. In the field it is not unusual to find individuals which lack one or even both clubs entirely, with about 1% of the naturally occurring males showing severe and an additional 5% showing less severe deformations. These observations hint to the difficulties which must involve easing out of the old skin while fully developing the ornament. We have observed individuals that do not manage to emerge from the N4 skin, get trapped within the previous skin and die. It is probable that part of the challenge of developing clubs is not only being in condition to build them, but also being able to moult with fully developed clubs during the last ecdysis. Further observations suggest that development can be affected by condition, at least when condition is extremely impaired. We quite regularly find field caught individuals being affected by nematomorphs and these individual typically die in the last nymphal stage (when worms emerge). In a handful of cases, field-caught males survived final ecdysis but did not develop their front-leg clubs. This suggests that severe impairment of conditions can specifically affect this ornament.

Acknowledgements

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Supplementary Materials

Table S1. Correlation coefficients and p-values observed among all morphological and sexual traits measured to test for effects of an immune challenge. Correlations are shown below the diagonal and p-values are shown above the diagonal. Estimation of both indices is done pairwise, with individuals lacking the measurement for a specific trait being removed from the data set used for the calculation.

	1	2	3	4	5	6	7	8	9	10	11	12	13
1. Eyespan	-	<0.001	NA	<0.001	<0.001	NA	NA	0.008	0.66	0.06	0.57	0.001	0.001
2. Pronotum length	0.68	-	0.002	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	0.70	<0.001	<0.001
3. Fore wing length	NA	0.41	-	NA	0.02	0.59	0.42	0.003	<0.001	0.002	0.003	0.89	NA
4. Hind wing length	0.41	0.59	NA	-	<0.001	NA	NA	<0.001	0.03	<0.001	0.54	<0.001	<0.001
5. Femur length	0.57	0.70	0.31	0.60	-	0.15	0.02	<0.001	<0.001	<0.001	0.36	<0.001	<0.001
6. Mass 1	NA	0.50	0.09	NA	0.22	-	<0.001	0.65	0.27	0.007	0.76	0.26	NA
7. Mass 4	NA	0.54	0.13	NA	0.35	0.77	-	0.65	0.31	0.12	0.84	0.16	NA
8. Antenna length	0.33	0.50	0.39	0.42	0.54	-0.07	0.07	-	<0.001	<0.001	<0.001	<0.001	0.001
9. Club width	0.05	0.58	0.54	0.26	0.46	0.17	0.16	0.54	-	<0.001	<0.001	<0.001	<0.001
10. Club length	0.23	0.74	0.42	0.51	0.54	0.40	0.24	0.39	0.74	-	0.96	<0.001	<0.001
11. Club ratio	-0.07	0.03	0.40	0.08	0.08	-0.05	0.03	0.36	0.67	0.00	-	0.63	<0.001
12. Str. file cont. length	0.38	0.48	0.02	0.48	0.71	0.17	0.22	0.31	0.29	0.33	0.04	-	<0.001
13. Str. file total length	0.38	0.61	NA	0.47	0.81	NA	NA	0.40	0.40	0.56	0.21	0.91	-

Table S2. Sampling sites for the quantification of deformations. The sample size N refers to the number of males inspected.

Site	Latitude	Longitude	Date	N
Fafleralp	46.43641	7.847391	24/07/2014	63
Crans-Montana - Les Violettes I	46.32664	7.491467	25/07/2014	72
Crans-Montana - Cry d'Err	46.33568	7.481776	25/07/2014	66
Crans-Montana - Les Violettes II	46.34098	7.498748	25/07/2014	71
Torrent-Alp	46.35592	7.646620	27/07/2014	72
Torrent-Alp/Rinderhütte II	46.35919	7.652634	27/07/2014	74
Derborence	46.27321	7.210985	28/07/2014	34
Böse Platte/Heiligenblut	47.06476	12.77200	28/07/2014	43
Upper Laggin Valley	46.15744	8.056610	30/07/2014	51
Lower Laggin Valley	46.16586	8.065235	30/07/2014	73
Egga	46.20320	8.107659	30/07/2014	64
Torrent-Alp/Rinderhütte I	46.37002	7.650307	31/07/2014	51
Mitterbach Gemeindealp	47.81130	15.24680	04/08/2014	50
Ochsenkaralm/Bergbau	47.44917	14.42128	07/08/2014	6
Spielkogel	47.51855	14.56530	08/08/2014	57
Rotgraben/Tonner Alm	47.52490	14.56530	08/08/2014	53
Mödlingerhütte/Treffner Alm	47.52785	14.55353	08/08/2014	34
Millstätter Alm	46.84245	13.59378	10/08/2014	25
Millstätter Hütte	46.84855	13.59630	10/08/2014	44
Millstätter Törl	46.85037	13.59665	10/08/2014	43
Thurntaler Rast/Sillian	46.77378	12.40163	14/08/2014	51
Zettersfeld/Hochstein	46.82415	12.69260	15/08/2014	45
Eggalm/Finkenberg	47.16293	11.70770	17/08/2014	57
Penkenjoch/Finkenberg	47.17178	11.79398	17/08/2014	22
Mountain station Halsmarter/Tulfes	47.24037	11.54243	18/08/2014	4
Gaislachkogel/Sölden	46.95167	10.98467	19/08/2014	14
Timmelsjoch	46.91735	11.07898	20/08/2014	28
Silvretta Bielerhöhe	46.92000	10.09922	22/08/2014	7
below Silvretta Bielerhöhe	46.92138	10.06820	22/08/2014	40
Birkhahnkopf/Wirl	46.96268	10.14392	22/08/2014	12
Sedrun/Tgom	46.66460	8.766530	27/07/2015	53
Oberalppass/Canals	46.65115	8.686650	28/07/2015	37
Bernina Pass	46.44300	9.980270	02/08/2015	25
Corviglia-Marguns-Piz Nair II	46.51533	9.823280	02/08/2015	10
Corviglia-Marguns-Piz Nair I	46.51555	9.772930	02/08/2015	44
Julier Pass	46.47065	9.756150	03/08/2015	50
Pontresina/Alp Languard	46.48838	9.923000	03/08/2015	63
Samedan/Upper Muottas Muragl	46.51820	9.916080	04/08/2015	47
Samedan/Lower Muottas Muragl	46.52587	9.902170	04/08/2015	54
Fuorn Pass	46.63938	10.29060	05/08/2015	59
Albitzen/Heiligenblut I	47.06649	12.78056	06/08/2015	25
Albitzen/Heiligenblut II	47.06700	12.77800	29/08/2015	290

Table S3. Phenotypic correlations and directional asymmetry between left and right sides of bilateral morphological traits. Measurements were made on dead individuals under the stereo-microscope.

Phenotypic Correlations	r	Lower CI	Upper CI	t	df	P
			Round 1			
L&R Antenna length	0.75	0.59	0.85	7.84	49	0.0001>
L&R Fore wing length	0.95	0.91	0.97	19.30	42	0.0001>
L&R Club width	0.58	0.36	0.74	4.98	48	0.0001>
L&R Club length	0.50	0.25	0.68	3.96	48	0.00025
L&R Femur length	0.90	0.84	0.94	14.85	49	0.0001>
L&R Str. file continuous length	0.66	0.47	0.79	6.19	49	0.0001>
			Round 2			
L&R Antenna length	0.69	0.50	0.82	6.38	44	0.0001>
L&R Hind wing length	0.94	0.90	0.96	20.45	58	0.0001>
L&R Club width	0.92	0.87	0.95	18.54	61	0.0001>
L&R Club length	0.92	0.87	0.95	18.76	62	0.0001>
L&R Femur length	0.91	0.85	0.95	16.82	58	0.0001>
L&R Str. file continuous length	0.74	0.59	0.83	8.29	58	0.0001>
L&R Str. file total length	0.80	0.68	0.87	10.027	58	0.0001>
Directional Asymmetry	difference	Lower CI	Upper CI	t	df	P
			Round 1			
L&R Antenna length	0.025	-0.082	0.13	0.47	50	0.64
L&R Fore wing length	-0.10	-0.18	-0.023	-2.62	43	0.012
L&R Club width	0.012	-0.036	0.060	0.51	49	0.61
L&R Club length	0.034	-0.024	0.092	1.17	49	0.25
L&R Femur length	-0.024	-0.072	0.025	-0.98	50	0.33
L&R Str. file continuous length	0.062	0.0088	0.12	2.34	50	0.02
			Round 2			
L&R Antenna length	0.12	-0.098	0.33	1.089	45	0.28
L&R Hind wing length	-0.0067	-0.073	0.060	-0.2	59	0.84
L&R Club width	0.022	-0.011	0.055	1.34	62	0.19
L&R Club length	0.048	0.020	0.077	3.35	63	0.0014
L&R Femur length	-0.011	-0.062	0.040	-0.43	59	0.67
L&R Str. file continuous length	-0.002	-0.075	0.071	-0.055	59	0.96
L&R Str. file total length	0.060	-0.0067	0.13	1.80	59	0.077

Table S4. Results of the linear models (for morphological traits and PC1) and linear mixed models (for song traits) fits to test for effects of the immune challenge. Numbers in the sample size for the four song properties refer respectively to the number of individuals, songs and bins (for song duration bins can not be defined). V_I, V_S and V_B give the proportion of the phenotypic variance (after controlling for treatment) explained by variation among individuals, among songs within individuals and residual variation among bins within songs. Samples sizes vary slightly due to missing values when individuals had missing or deformed body parts.

	Round 1				Round 2			
	slope	SE	t	p	slope	SE	t	p
Antenna length		n = 56				n = 63		
Intercept	7.89	0.09	90.45	<0.0001	8.01	0.12	64.32	<0.0001
LPS treatment	0.01	0.12	0.07	0.95	0.25	0.18	1.39	0.17
Club ratio		n = 56				n = 69		
Intercept	0.47	0.01	58.07	<0.0001	0.45	0.011	40.20	<0.0001
LPS treatment	0.01	0.01	0.64	0.52	0.01	0.016	0.53	0.60
Club length		n = 56				n = 69		
Intercept	3.38	0.05	67.05	<0.0001	4.12	0.17	87.12	<0.0001
LPS treatment	-0.01	0.07	-0.10	0.92	0.10	0.07	1.51	0.14
Club width		n = 56				n = 69		
Intercept	1.58	0.03	48.13	<0.0001	1.86	0.06	-0.71	<0.0001
LPS treatment	0.02	0.04	0.40	0.69	0.08	0.08	1.00	0.32
File total length						n = 69		
Intercept	-	-	-	-	4.44	0.07	66.51	<0.0001
LPS treatment	-	-	-	-	0.17	0.09	1.76	0.083
File continuous length		n = 56				n = 69		
Intercept	4.16	0.04	107.27	<0.0001	4.28	0.06	68.72	<0.0001
LPS treatment	0.12	0.05	2.25	0.029	0.10	0.09	1.19	0.24
Song rate						n = 57		
Intercept	-	-	-	-	3.59	1.15	3.12	0.003
LPS treatment	-	-	-	-	2.52	1.64	1.54	0.13
PC1 Sexual Traits		n = 56				n = 69		
Intercept	0.03	0.29	0.11	0.91	-0.37	0.32	-1.16	0.25
LPS treatment	-0.09	0.40	-0.24	0.81	0.72	0.44	1.63	0.11
Femur length		n = 56				n = 69		
Intercept	9.60	0.07	135.88	<0.0001	9.80	0.08	124.64	<0.0001
LPS treatment	0.00	0.10	0.04	0.97	0.17	0.11	1.55	0.13
Wing length		n = 52				n = 66		
Intercept	12.18	0.16	76.45	<0.0001	12.08	0.13	94.72	<0.0001
LPS treatment	-0.09	0.22	-0.42	0.68	0.10	0.18	0.56	0.58
Pronotum length		n = 56				n = 69		
Intercept	4.11	0.04	108.41	<0.0001	4.40	0.04	100.12	<0.0001
LPS treatment	-0.01	0.05	-0.15	0.88	0.15	0.06	2.50	0.015

	Round 1				Round 2			
	slope	**SE**	**t**	**p**	**slope**	**SE**	**t**	**p**
Eye span						**n = 69**		
Intercept	-	-	-	-	1.24	0.01	99.32	<0.0001
LPS treatment	-	-	-	-	0.03	0.02	1.73	0.09
Mass1		**n = 46**						
Intercept	0.16	0.00	37.64	<0.0001	-	-	-	-
LPS treatment	-0.01	0.01	-1.37	0.18	-	-	-	-
Mass4		**n = 45**						
Intercept	0.16	0.00	44.45	<0.0001	-	-	-	-
LPS treatment	0.00	0.01	-0.44	0.66	-	-	-	-
PC1 Non-Sexual Traits		**n = 56**				**n = 69**		
Intercept	-0.16	0.31	-0.52	0.61	-0.39	0.28	-1.38	0.17
LPS treatment	0.25	0.42	0.60	0.55	0.76	0.39	1.94	0.056
Syllables per bin					**n = 24, 57, 173** ($V_I = 0.43$, $V_S = 0.31$, $V_B = 0.26$)			
Intercept	-	-	-	-	18.87	0.59	31.92	<0.0001
LPS treatment	-	-	-	-	1.75	0.87	2.01	0.058
Carrier frequency					**n = 24, 57, 140** ($V_I = 0.18$, $V_S = 0.52$, $V_B = 0.30$)			
Intercept	-	-	-	-	7.34	0.33	22.58	<0.0001
LPS treatment	-	-	-	-	0.32	0.48	0.67	0.51
Max power					**n = 24, 57, 174** ($V_I = 0.59$, $V_S = 0.22$, $V_B = 0.19$)			
Intercept	-	-	-	-	86.09	1.60	53.96	<0.0001
LPS treatment	-	-	-	-	1.64	2.36	0.70	0.49
Song duration					**n = 23, 57** ($V_I = 0.61$, $V_S = 0.39$)			
Intercept	-	-	-	-	14.99	1.66	9.01	<0.0001
LPS treatment	-	-	-	-	1.17	2.45	0.48	0.64
PC1 Song Traits						**n = 24**		
Intercept	-	-	-	-	-0.40	0.36	-1.14	0.27
LPS treatment	-	-	-	-	0.88	0.53	1.68	0.11

Table S5. Results of the linear models (for morphological traits and PC1) and linear mixed models (for song traits) fits, with control for pre-treatment mass, to test for effects of the immune challenge. Numbers in the sample size for the four song properties refer respectively to the number of individuals, songs and bins (for song duration bins can not be defined). V_I, V_S and V_B give the proportion of the phenotypic variance (after controlling for treatment) explained by variation among individuals, among songs within individuals and residual variation among bins within songs. Samples sizes vary slightly due to missing values when individuals had missing or deformed body parts.

	Round 1				Round 2			
	slope	SE	t	p	slope	SE	t	p
Antenna length average	n = 54				n = 63			
Intercept	7.90	0.09	90.93	<0.0001	8.05	0.12	67.92	<0.0001
LPS treatment	0.00	0.12	0.016	0.99	0.12	0.18	0.66	0.51
Mass	0.10	0.06	1.75	0.087	0.26	0.09	2.89	0.0054
Club ratio	n = 54				n = 69			
Intercept	0.47	0.01	58.25	<0.0001	0.45	0.01	40.74	<0.0001
LPS treatment	0.00	0.01	0.38	0.71	0.00	0.16	0.22	0.83
Mass	0.01	0.01	0.97	0.34	0.01	0.01	1.79	0.079
Club length	n = 54				n = 69			
Intercept	3.37	0.05	64.92	<0.0001	4.15	0.04	98.74	<0.0001
LPS treatment	0.02	0.07	0.23	0.82	0.05	0.06	0.89	0.38
Mass	0.05	0.04	1.34	0.19	0.14	0.03	4.53	<0.0001
Club width	n = 54				n = 69			
Intercept	1.58	0.03	48.36	<0.0001	1.89	0.05	36.46	<0.0001
LPS treatment	0.02	0.04	0.44	0.66	0.04	0.07	0.48	0.63
Mass	0.04	0.02	1.89	0.06	0.12	0.04	3.20	0.002
File total length					n = 69			
Intercept	-	-	-	-	4.48	0.06	75.51	<0.0001
LPS treatment	-	-	-	-	0.10	0.08	1.16	0.25
Mass	-	-	-	-	0.19	0.04	4.52	<0.0001
File continuous length	n = 54				n = 69			
Intercept	4.14	0.04	108.49	<0.0001	4.32	0.06	76.75	<0.0001
LPS treatment	0.15	0.05	2.87	0.0060	0.04	0.08	0.56	0.58
Mass	0.07	0.03	2.68	0.0098	0.17	0.04	4.22	<0.0001
Song rate					n = 57			
Intercept	-	-	-	-	3.70	1.17	3.17	0.003
LPS treatment	-	-	-	-	2.28	1.69	1.35	0.18
Mass	-	-	-	-	0.57	0.89	0.65	0.52
PC1 Sexual Traits	n = 54				n = 69			
Intercept	-0.01	0.29	-0.03	0.98	-0.19	0.27	-0.71	0.48
LPS treatment	-0.08	0.39	-0.20	0.84	0.38	0.39	0.99	0.33
Mass	-0.36	0.20	-1.84	0.07	0.96	0.20	4.94	<0.0001

	Round 1				Round 2			
	slope	SE	t	p	slope	SE	t	p
Femur length average			n = 54				n = 69	
Intercept	9.58	0.06	151.03	<0.0001	9.84	0.07	147.86	<0.0001
LPS treatment	0.07	0.09	0.76	0.45	0.08	0.09	0.86	0.40
Mass	0.17	0.04	3.82	0.0004	0.26	0.05	5.38	<0.0001
Wing length			n = 51				n = 66	
Intercept	12.21	0.15	80.70	<0.0001	12.14	0.12	104.87	<0.0001
LPS treatment	-0.10	0.21	-0.47	0.64	-0.02	0.16	-0.10	0.92
Mass	0.26	0.11	2.45	0.018	0.33	0.08	4.02	0.0002
Pronotum length			n = 54				n = 69	
Intercept	4.11	0.03	133.00	<0.0001	4.43	0.04	121.40	<0.0001
LPS treatment	0.01	0.04	0.27	0.79	0.10	0.05	1.96	0.054
Mass	0.09	0.02	4.42	<0.0001	0.15	0.03	5.74	<0.0001
Eye span							n = 69	
Intercept	-	-	-	-	1.25	0.01	109.12	<0.0001
LPS treatment	-	-	-	-	0.02	0.02	1.18	0.24
Mass	-	-	-	-	0.03	0.01	3.93	0.0002
Mass1			n = 46					
Intercept	0.15	0.00	42.90	<0.0001	-	-	-	-
LPS treatment	0.00	0.00	-0.99	0.33	-	-	-	-
Mass	0.01	0.00	5.07	<0.0001	-	-	-	-
Mass4			n = 45					
Intercept	0.15	0.00	61.71	<0.0001	-	-	-	-
LPS treatment	0.00	0.00	0.25	0.80	-	-	-	-
Mass	0.02	0.00	7.63	<0.0001	-	-	-	-
PC1 Non-Sexual Traits			n = 54				n = 69	
Intercept	-0.04	0.23	-0.15	0.88	-0.21	0.23	-0.92	0.36
LPS treatment	-0.04	0.32	-0.12	0.91	0.41	0.32	1.28	0.20
Mass	-1.08	0.16	-6.74	<0.0001	1.00	0.16	6.25	<0.0001
Syllables per bin					n = 24, 57, 173 ($V_I = 0.45$, $V_S = 0.31$, $V_B = 0.25$)			
Intercept	-	-	-	-	18.84	0.65	29.18	<0.0001
LPS treatment	-	-	-	-	1.81	0.99	1.82	0.08
Mass	-	-	-	-	-0.061	0.43	-0.14	0.89
Carrier frequency					n = 24, 57, 140 ($V_I = 0.19$, $V_S = 0.52$, $V_B = 0.29$)			
Intercept	-	-	-	-	7.40	0.35	21.22	<0.0001
LPS treatment	-	-	-	-	0.21	0.54	0.39	0.70
Mass	-	-	-	-	0.13	0.25	0.50	<0.0001
Max power					n = 24, 57, 173 ($V_I = 0.61$, $V_S = 0.21$, $V_B = 0.18$)			
Intercept	-	-	-	-	86.18	1.75	49.30	<0.0001
LPS treatment	-	-	-	-	1.50	2.69	0.56	0.58
Mass	-	-	-	-	0.14	1.16	0.12	<0.0001
Song duration					n = 24, 57 ($V_I = 0.62$, $V_S = 0.38$)			
Intercept	-	-	-	-	15.48	1.80	8.61	<0.0001
LPS treatment	-	-	-	-	0.23	2.76	0.083	0.93
Mass	-	-	-	-	0.91	1.19	0.77	0.45

	Round 1				Round 2			
	slope	**SE**	**t**	**p**	**slope**	**SE**	**t**	**p**
PC1 Song Traits					**n = 24**			
Intercept	-	-	-	-	-0.39	0.39	-1.00	0.33
LPS treatment	-	-	-	-	0.84	0.61	1.40	0.18
Mass	-	-	-	-	0.04	0.31	0.13	0.90

Figure S1. Effect of the LPS immune challenge on the duration of the N4 developmental stage. The left plot depicts the R1 while the right plot depicts the R2. Grey and black are used for LPS and control individuals respectively. Dotted lines indicate the best fit model for each data set.
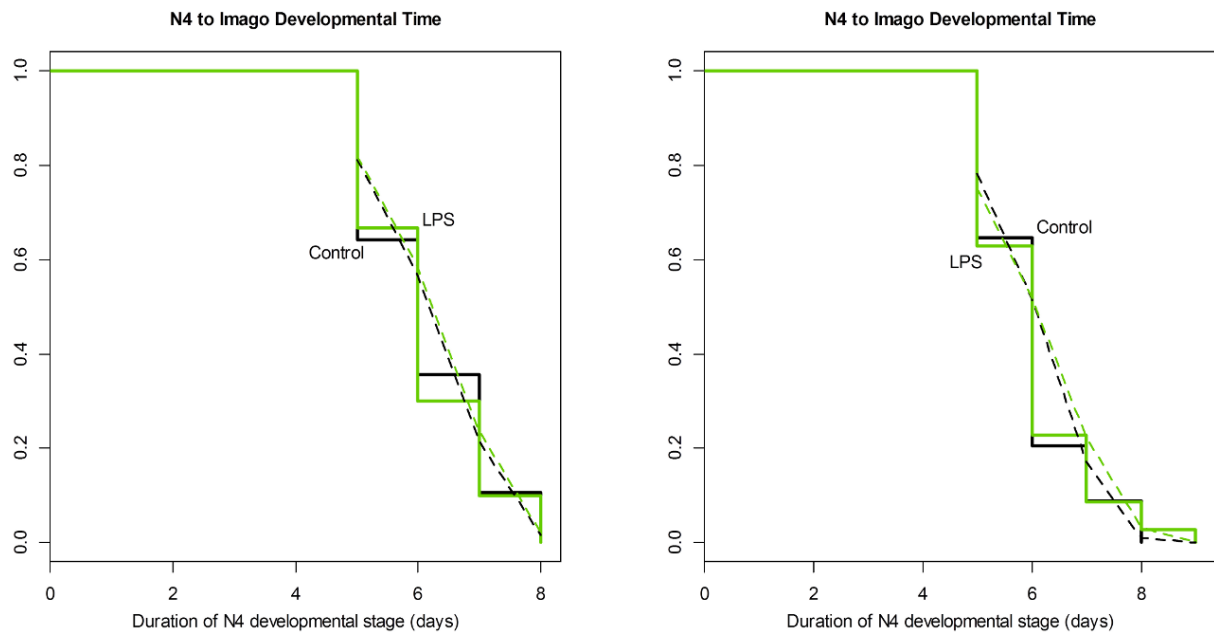
Figure S2. Standardised effect sizes of sexual and non-sexual traits across two rounds of immune challenges with correction for pre-treatment mass. Positive values indicate higher trait values for the LPS treated individuals. Open dots refer to effects sizes of the first round (R1) and filled dots to effect sizes of the second round (R2). Each effect size shows standard error bars (thick short bars on each side of the effect sizes) and 95% confidence intervals (thin long whiskers extending on each side of the standard error bars).
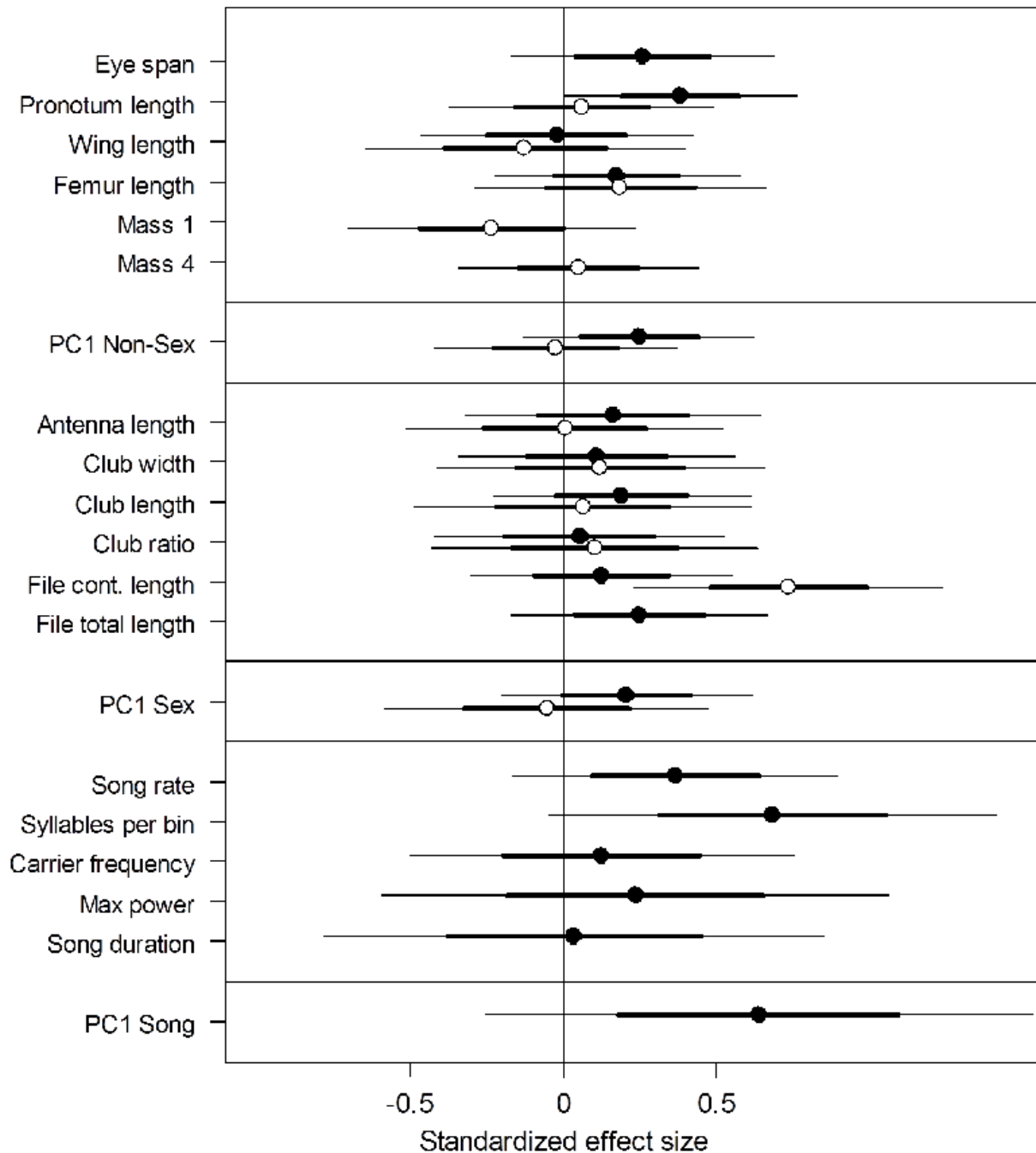
Chapter 4

Do sexual ornaments reflect inbreeding better than other traits? A meta-analysis of the effect of inbreeding and heterozygosity

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Abstract

Exaggerated sexual ornaments are often used in mate choice and are then likely to be the result of inter-sexual selection. While in some species males provide direct benefits to females, in other species there are only indirect benefits to mate choice. Unlikely direct benefits, indirect benefits are only realized as better performance in the offspring generation and therefore rest on heritable genetic variation. Still the evidence for indirect benefits are ambiguous, posing the question why females have evolved these preferences in the first place. Ornament expression is known to be sensitive to individual condition, which is influenced by many loci, and which can be modified by environmental factors as well as the degree of inbreeding of an individual. Here we test the relationship between ornament expression and inbreeding using a meta-analytic approach. We searched and compiled studies that present a measures of genetic quality, either the degree of heterozygosity or artificial inbreeding, as well as at least one sexually selected trait. After filtering the bulk of the articles we were left with 28 studies which were included in the meta-analysis. The analysis shows that sexually selected and non-sexually selected traits do not differ significantly in the strength of inbreeding depression, and point estimate even suggests a trend contrary to the initial prediction. The results were qualitatively similar when analyzing two non-overlapping subsets of the data, the studies based on heterozygosity and those based on artificial inbreeding. A visual assessment of the data set via a funnel plot did not provide evidence of publication bias. This finding might suggest a scenario where the function of sexually selected traits and their link to overall condition decays with time, due to males which, despite being in poor condition, allocate a disproportional amount of resources into the development of the ornament. If the phase of a strong link between condition and ornament expression is transient than the timing of the study in the processes of the evolution of an ornament is critical and many studies may fail to find an association if the ornaments are mostly the remnants of past episodes of selection.

Introduction

The continuous and sustained evolution of mating preferences for extravagantly ornamented males, in particular in the absence of direct and tangible benefits, constitutes an enduring puzzle in evolutionary biology (Qvarnström 2001, Neff & Pitcher 2005). While the evolution of ornaments in connection with direct benefits is straightforward, evidence of indirect benefits in connection with males bearing elaborate ornaments is still ambivalent (Kotiaho & Puurtinen 2007). Direct benefits are by definition fecundity-enhancing and these benefits do not depend on whether or not the source of benefits has a genetic basis. Indirect benefits, in contrast, are benefits that are only realized in the offspring generation and are necessarily genetic in origin (Andersson 1994). The question is therefore, whether sexual ornaments have evolved to reflect the genetic markup of the carrier particularly well and this question is particularly important if indirect benefits are the only benefits to mate choice in a particular system. This question can potentially be answered by a detailed look of the evidence published in the scientific literature.

Putatively costly ornaments were already noticed by Darwin (1871), and he suggested that the reason behind their occurrence was due to mating preferences in the other sex. It was Fisher's (1915, 1930) insightful idea which bridged the gap of how sexual ornaments could evolve and be exaggerated in a population. He suggested that if a preference for a particular trait exists (one of the sexes is choosy), this would confer a mating advantage to individuals of the chosen sex that possess the trait. If the trait is heritable, then individuals of the choosy sex who mate with individuals possessing the trait (or a more extreme versions of the trait) would gain a mating advantage by producing sexy offspring. The offspring from such a mating would be similarly ornamented as the chosen sex which would also inherit the preference for the trait, provided that preference and ornament both have a genetic basis. The genetic coupling can lead to a runaway process, and its validity for the evolution of ornaments has been supported even in absence of any selective advantages of the mating preference (Kirkpatrick 1982).

Mate choice for flashy ornaments has been known to be sensitive to developmental variation which may alter the outcome of the genotype. In particular if honesty of an ornament is maintained by condition-dependence (Hunt *et al.,* Rowe & Houle 1996), we expect strong sensitivity to environmentally induced, but also non-heritable genetic variation. Environmental manipulations of condition have shown that sexually selected traits are apparently more sensitive to changes during development than non-sexually selected traits, supporting in part the function of ornaments as signals of individual quality (Cotton *et al.* 2004). But since individual condition is expected to be influenced by a large number of loci (Hunt *et al.,* Rowe & Houle 1996), it is also likely to be affected by the degree of inbreeding that has genome-wide consequences in the form of reduced heterozygosity (Szulkin *et al.* 2010). It is an open question whether sexual ornaments indeed show increased inbreeding depression as they seem to show increased dependence on environmental variation (Cotton *et al.* 2004).

In contrast to an environmental manipulation of condition, an individual's degree of inbreeding can provide a deeper insight of the relation between ornament signal honesty and indirect benefits. First, inbreeding can lead to inbreeding depression in ornament expression; second, inbreeding in the parental generation can be transferred to the offspring, and this can also be seen in the degree of heterozygosity (Radwan 2008, Nietlisbach *et al.* 2016). Among possible indirect benefits gained by the choosy sex, additive benefits (good alleles) and nonadditive genetic benefits (compatibility alleles), both types are affected under inbreeding, and this in turn can also affect the signal of individual quality, the ornament. Therefore, the employment of genetic manipulations of condition (inbred vs outbred individuals) provides a direct assessment of both the effects on the signal and the source of benefits for the choosy sex. This reasoning also poses another dilemma: under any scenario in which the choosy sex gains indirect benefits in the form of good alleles, good alleles for the ornament will always be present. This present an evolutionary spin on the evolution of mating preferences for elaborated ornaments, which very likely will always be highly developed in the presence of good alleles.

In the present manuscript we employ a meta-analytic approach to test the relation between ornament expression and inbreeding. The use of meta-analysis allows to perform a quantitative systematic review, which goes beyond the scope of a simple review by including a body of statistical work which complements the literary analysis done in a literature review. Meta-analysis allow testing for context-dependence of biological effects, by testing if moderators (the equivalent of covariates in usual linear models) explain heterogeneity in estimates. Furthermore, random-effect meta-analysis allow testing for heterogeneity in research findings, which may be indicative of yet unexplored context-dependence that needs to be further studied. Due to the diversity of study systems that researchers work on in the fields of ecology and evolution, meta-analysis faces a special problem that is less prevalent in other fields (such as the medial or social sciences). Apart from a few well studied model species, most studies in ecology and evolution are typically taxonomically very diverse, but for a meta-analysis the synthesis of phylogenetically heterogeneous studies requires phylogenetic control (Lynch 1991).

Different reviews and meta-analysis have analysed the relationship among different components of the scenario of mate choice and sexual selection. Among these we find the relation between ornaments and heightened condition dependence (Cotton *et al.* 2004), mate choice and heterozygosity (Kempenaers 2007), heterozygosity and fitness (Chapman *et al.* 2009) and attractivenes and fitness (Prokop *et al.* 2012). Despite the large body of theory supporting the relations among these components of mate choice and sexual selection, these studies did not reported substantially positive results. However, the relation among inbreeding and ornaments, which is a form of heightened condition dependence, was not formally tested by Cotton in his review, most likely because of the lack of studies employing inbreeding in experimental designs.

The relevance of the connection between ornament development and individual genetic quality, expressed by the degree of inbreeding, makes the present review in a sense long due. The main question that we want to answer is whether sexually selected traits (ornaments) are more sensible

to inbreeding than non-sexually selected traits. If this condition is met, we expect to see a stronger effect due to inbreeding in ornaments than in traits which are not employed during display rituals or courtship. It has also become common to find in the literature the use of the degree of multilocus heterozygosity (MLH) as a proxy to assess inbreeding (Szulkin *et al.* 2010). The degree of interchangeability between inbreeding and MLH has been highly debated, in particular because the evidence varies as to its suitability. High variation in the number of markers employed as well as the nature of the markers, neutral or functional, in addition to variation in the effects of individual markers within individuals (introducing another source or error) are all possible shortcomings of its use (Balloux *et al.* 2004). Regardless of these limitations, several studies have gathered substantial evidence for the effect or either MLH or *f* in the evolution of mate choice for exaggerated ornaments.

Methods

Literature Search & Data Collection

The main literature search was carried out using the online search engines ISI Web of Science (https://webofknowledge.com/) and Scopus (https://www.scopus.com). We used the following keyword combination for both searches: (TS=((genetic AND challenge) AND (sexual AND trait*)) OR TS=(inbreed* AND sexual AND trait) OR TS=((genetic AND challenge) AND (sexual AND character*)) OR TS=(inbreed* AND sexual AND character*)) as well as the combination TS=(heterozygosity AND sexual AND trait). Our search of the databases was set to include studies in English, German, Spanish, French and Portuguese. We tried to adhere to the PRISMA protocol as thoroughly as possible (Liberati *et al.* 2009). All the titles and abstracts of the studies obtained from the searches were screened in order to assess whether they would meet our criteria, which were:

1. The study needed to include an assay on a classical sexual trait (we aimed to use sexual traits which had been already tested to attract females in order to avoid possible ambiguity in the interpretation of the results).
2. The study needed to include a measurement of the degree genetic quality of the individuals been tested, preferentially the degree of inbreeding or the degree of heterozygosity of the individuals.

Besides the aforementioned two criteria, we decided from the start not to include studies based on: 1. MHC measures of heterozygosity; 2. Fluctuating asymmetry (FA) as a sexually selected trait; 3. Studies on humans. Despite the fact that MHC studies assess heterogeneity in a set of markers, often the set used is small and therefore can not realistically be used as a proxy of multilocus heterozygosity (Roberts *et al.* 2005). Also the assessed genetic quality of MHC markers constitutes a relative assessment – it depends on the MHC constitution of the potential mate – and therefore can not be used to compare against objective measures of genetic quality. On the case of FA studies,

the relationship between FA and sexual traits is not always clear (Rowe *et al.* 1997), and therefore the use of these studies in the analysis could impose constraints on the interpretation of the results.

As a mean to find additional studies relevant to our search we also performed backward searches on seminal papers related to the topic (Sheridan & Pomiankowski 1997, Cotton *et al.* 2004, Chapman *et al.* 2009, Jennions *et al.* 2012). Furthermore we searched the websites of the authors of the seminal papers previously mentioned as well as the websites of authors for which we had selected relevant studies for the meta-analysis (this website search was done on the websites of the authors whose studies were selected for a full-text screening).

Exclusion Criteria

After screening all the abstracts we selected those studies which appeared to be relevant for a further full-text screening. During this stage we excluded articles based on the following criteria:

1. Studies which used postcopulatory sexual traits, which are basically sperm traits, as well as those dealing with genitalia were excluded due to the incomplete knowledge/possibility for dual interpretation which may arise from their inclusion, in constrast to classical traits known as ornaments.
2. Studies where the genetic quality was indirectly assessed through measures of individual immunity. Despite the existing evidence of the relation between sexual traits and immunocompetence (Zahavi 1975, Hamilton & Zuk 1982), the immunity assessment does not provide a measure of an individual's genetic constitution, which is a necessary element for our hypothesis testing.
3. Studies where the sexual trait grows throughout the whole life of the animal, such as horns, and where the quality being measured on the trait is horn width increase as a function of season. Despite the fact that this trait appeared to be a typical ornament, its function is more that of an armament, and their immediate function in several cases is the defense of territories from conspecifics (Janis 1982, Packer 1983). It is also not straightforward, on a study where several measurements were taken over a span of multiple years, which year or years should be included in the analyses.
4. Studies where the sexual trait is a male's size or its ability to defend/own a territory. In several species the biggest males or those which provide resources, which incidentally often also is associated with an individual's body size, are the ones which are chosen by females.
5. Studies where the sexual trait is copulation time, behaviour during copulation or reproductive success. In these cases, despite them being directly related to the female mating preferences, they either relate to the mating per se, which occurs after a male has gained access to the female, and did not involve a quantification of the sexual trait, or in the case of reproductive success, is a fitness trait and not a sexual trait.
6. Studies where attractiveness is based on cuticular hydrocarbons (CHC's). Here it is again a situation similar to the MHC studies, where CHC's can be a subjective measure dependent on the female's own genetic constitution.

7. Studies where the individuals used were genetically modified and therefore possessed specific mutations which rendered them handicapped, which could have a side effect on an ornament or sexually selected trait assessed by the female.

8. Studies where a dataset was being reassessed using a model and the original data belonged to an already selected article in our pool.

9. Studies where the sexual trait under assessment belonged to the female and not to the male.

10. Studies where the data could not be extracted/converted to an effect size which could be further used in the analysis.

In various cases we tried to contact authors in order to obtain data which was missing from the study and which was necessary to estimate a suitable effect size which could be included in the meta-analysis. We contacted 11 authors and received replies from six of them. In five of those six cases the author provided us with the original data from the study, which we were able to use to estimate effect sizes for our own analysis.

Data Extraction

After the full-text screening and exclusion of studies based on the above mentioned criteria (see Fig. S1), we were left with 28 studies from an original preselection of 92 studies. We assigned all traits from the studies which were relevant for the analysis to either one of two categories, sexual or non-sexual traits, which constitutes the main interest of our research. Since the traits within these two categories are rather diverse, we made use of another set of categories which would then be used as moderators in the analysis. Traits were categorised as belonging to: 1. morphology; 2. colour (here we included all traits which reflected the expression of colour, whether they measured colour or measured an area which qualitatively was related to colour expression e.g. breast band size); 3. song; 4. behaviour; 5. attractiveness (here we included male traits which were evaluated by the female); and 6. physiology (in this category we included glandular secretions which were specifically quantified separately from the female evaluation of the trait).

Data which we would use as moderators during the analysis were further assigned to other categories. We assigned the studies to either of two categories which specified whether the contrast of the sexual trait (the assessment of either a non-sexual trait on males or of the sexual trait in females) had been carried on males or females. We also assigned the studies to broad taxonomical categories as a mean to control for phylogeny during the analyses (Nakagawa & Santos 2012). Our categories were: bird, fish, insect, mammal and lizard. Finally we included the year of publication of the study as a possible moderator because of what is known as "effects fade with time", by which studies with either small effect sizes or non-significant results take longer to be published than studies with big effect sizes or significant results (Jennions & Møller 2002).

During our study search we included two types of keywords in regard to the genetic quality, inbreeding and heterozygosity. Our searches retrieved studies with both kinds of measures of genetic quality, and for each measure we encountered various degrees of inbreeding as well as

different measures of heterozygosity (Table 1). We adjusted all effect sizes derived from inbreeding studies so as to reflect the effect of one generation of full-sib mating (f=0.25) on the traits under study. As to the measures of heterozygosity, even though there are various types of measures among our dataset (see Table 1), all reflect similar estimates of heterozygosity, and therefore we assume that their discrepancies to be negligible (Szulkin *et al.* 2010). Their respective effect sizes are, in cases where necessary, scaled so as to reflect a similar comparison to that of the inbreeding coefficient (this is done mostly when converting correlation coefficients to Cohen's *d*, since the correlation assesses a relation among the predictor and the predicted variable which goes between 0 and 1).

We converted all the extracted estimates to Cohen's *d*. From the relevant studies we obtained correlation coefficients, Mann-Whitney Z scores and standardised regression coefficients β as well as slopes from various types of models and sample means. We consistently extracted only group data, since in some studies assays were also done at the individual level. From the slopes we obtained Z scores, and these were used in equation 11 from (Nakagawa & Cuthill 2007) to obtain a correlation coefficient, and this estimates were then transformed in the same way as with the other correlations coefficients. Variances for the correlation coefficients as well as for the β scores were estimated using equation 7.6 from chapter 7 from Borenstein *et al.* (2009). To obtain Cohen's *d* we used an online effect size calculator, which can be found under the following link https://www.psychometrica.de/effect_size.html.

Effect Size Adjustment

Before adjustment of effect sizes was done we revised the relationship betweeen the independent and the dependent variable so as to make sure that it was linear. This was necessary since an adjustment involving exponential or other non-linear relationship would have probably required certain in-depth knowledge of the study subjects. We also adjusted the standard error of the effect size as well as the 95% confidence intervals. Given that we did not simply divide or multiply as would be the case for a simple adjustment, we choose to explain each of the four cases briefly as a way to help clarity in our methodology.

1. Aspi 2000: here the inbreeding coefficient was equal to one, so we simply divided the value of the effect size by four to achieve a f = 0.25.

2. Ketola & Kotiaho 2010: here the average inbreeding coefficient equaled 0.081, therefore we multiplied by 3, which brings the inbreeding coefficient to almost 0.25.

3. Reid *et al.* 2005: here a strong relationship between inbreeding and repertoire size was reported, where f=0.034. Since adjustment due to the small inbreeding coefficient would have implied a correlation very close to 1.0, we opted for a middle value between 1.0 and 0.5, which is the area where a correlation is considered to be strong (Cohen 1988), as a manner of being conservative in the adjustment of the effect size.

Table 1. Studies included in the meta-analysis. The column n-effsiz refers to the number of effect sizes per study, while the reported data column refers to the original data reported in the study, from which effect sizes for the analysis were estimated. The column Inb/Het refers to whether the study worked with inbreeding or heterozygosity, whereas the column Gen. Qual. Measure specifies the type of measure used.

Author	Year	Taxon	Inb/Het	Gen. Qual. Measure	n-effsizes	Reported Data
Aparicio *et al.*	2001	*Sturnus unicolor*	het	H	4	correlations
Araya-Ajoy *et al.*	2009	*Phaenostictus mcleannani*	het	HL	2	correlations
Aspi	2000	*Drosophila montana*	inb	f=1.0	18	means+SD/SE
Bolund *et al.*	2010	*Taeniopygia guttata*	inb	f=0.25	25	means+SD/SE
Charpentier *et al.*	2008	*Lemur catta*	het	Ho	2	correlations
de Boer *et al.*	2016	*Serinus canaria*	inb	f=0.25	24	means+SD/SE
Drayton *et al.*	2007	*Teleogryllus commodus*	inb	f=0.25	6	Hedges d
Drayton *et al.*	2010	*Teleogryllus commodus*	inb	f=0.25	6	Hedges d
Ferrer *et al.*	2015	*Cyanistes caeruleus*	het	HL	18	slopes GLM
Foerster *et al.*	2003	*Cyanistes caeruleus*	het	HS	4	correlations
Forstmeier *et al.*	2012	*Taeniopygia guttata*	inb	f=0.25	1	correlations
Frommen *et al.*	2008	*Gasterosteus aculeatus*	inb	f=0.25-0.375	5	means+SD/SE
Herdegen *et al.*	2014	*Poecilia reticulata*	het	MLH	4	correl. & slopes GLM
Ilmonen *et al.*	2009	*Mus musculus*	inb	f=0.25	2	means+SD/SE
Janhunen *et al.*	2011	*Salvelinus alpinus*	het	IR	3	correlations
Ketola & Kotiaho	2010	*Grylloides signatus*	inb	f=0.08	3	correlations
Mariette *et al.*	2006	*Poecilia reticulata*	inb	f=0.25	6	means+SD/SE
Marshall *et al.*	2003	*Acrocephalus schoenobaenus*	het	mean d2	1	correlations
Polkki *et al.*	2012	*Tenebrio molitor*	inb	f=0.25	4	means+SD/SE
Prokop *et al.*	2010	*Teleopsis dalmanni*	inb	f=0.25	6	means+SD/SE
Reid *et al.*	2005	*Melospiza melodia*	inb	f=0.034	1	correlations
Reid	2007	*Melospiza melodia*	het	k	1	correlations
Runemark *et al.*	2013	*Podarcis gaigeae*	het	HE	11	beta (stand.regr.coeff.)
Seddon *et al.*	2004	*Monias benschi*	het	HS	6	correlations
Sheridan & Pomiankowski	1997	*Poecilia reticulata*	inb	f=0.25	38	means+SD/SE
van Bergen *et al.*	2013	*Bicyclus anynana*	inb	f=0.25	6	means+SD/SE
Van Oosterhout *et al.*	2003	*Poecilia reticulata*	inb	f=0.25-0.5	6	slopes GLM
Weatherhead *et al.*	1999	*Agelaius phoeniceus*	het	AV	10	means+SD/SE

H: homozygosity index; HL: homozygosity by loci; Ho: neutral heterozygosity; HS: standardized heterozygosity; MLH: multilocus heterozygosity; IR: internal relatedness; mean d: squared difference in repeat number in a locus; k: coefficient of kinship; HE: Nei's expected heterozygosity; AV: allozyme variation.

4. Van Oosterhout *et al.* 2003, Frommen *et al.* 2008: in these two cases the inbreeding coefficient of the individuals included in the study ranged between 0.25 to 0.375 and from 0.25 to 0.50 respectively. When looking at the figures and the relation between the predictor

and predicted variable it was not clear what type of response the predicted variable displayed. Therefore, we decided to settle for the middle point of the effect size, and divided the effect size by two.

Statistical Analyses

All analyses were conducted in R 3.1.2 (R Core Team 2014). We employed the metafor package because it allowed us to fit a model which included both moderators and random effects (Viechtbauer 2010) as implemented in the rma.mv function. This function implements a mixed-effects model that adds weights to all estimates, where weights are inversely proportional to the uncertainty with which they are estimated. Studies with precise estimates therefore gain higher weight in the estimation of overall effects compared to studies with low precision. We fitted a model including six moderators: year of publication, taxon (classified into five groups), type of original effect size, trait type (see above), whether the study dealt with heterozygosity or inbreeding, and finally if the trait was putatively sexual or not. We included study identity as a random effect to account for variability among studies, since studies provided multiple estimates. Model fitting was done by restricted maximum likelihood (REML), and significance of the individual moderators (except for the heterozygosity/inbreeding and the sexual/non-sexual trait moderators) was done via likelihood ratio tests (for which we used maximum likelihood fits). Asymmetry of the data set was assessed using funnel plots.

We predicted that if sexual traits indeed are linked to condition and therefore to the genetic constitution of individuals, we would see stronger inbreeding depression in sexual traits than in non-sexual traits. If the whole dataset confirms this prediction, a further step towards asserting this pattern would be to use meaningful subsets of the data set to fit models similar as that used for the whole data set. An obvious criterion for subsetting are the two types of genetic measures, artificial inbreeding versus heterozygosity-trait correlations, since the experimental and correlations studies may well produce different outcomes. Given the lack of knowledge of the PRISMA protocol during the search stage of the articles by one of us (PV) our protocol lacks the exact number of studies which were duplicates in both searches of a specific keyword (heterozygosity or inbreeding, see Fig. S1). Yet, assuming that the searches using the keyword heterozygosity include all those articles found in the searches using the keyword inbreeding (the former search had fewer hits in both search engines), and adding the search with the higher number of hits (the Scopus search) plus the articles found through other sources, one can safely say that a minimum of 636 articles were excluded after browsing the title and abstract.

Results

We screened the abstracts from over 729 articles obtained through various searches. From that total we retained 92 for a further full-text screening, and after excluding 64 articles based on whether they met our criteria for inclusion in the meta-analysis, we were left with 28 studies (Table 1). We obtained 215 effect sizes, from which 162 belonged to sexual traits and 53 belonged to non-

sexual traits. Also 58 effect sizes assessed genetic quality in the form of heterozygosity, while 157 assessed the degree of inbreeding. The best model fit was that which included only the moderators trait type and study number, since the reduced models without the type of original effect size or the taxon moderators were significantly better than the full model (taxon: χ^2_3 = 0.97, p = 0.80; type of original effect size: χ^2_{11} = 18.7, p = 0.06; trait type: χ^2_5 = 54.4, p < 0.0001; study number: χ^2_1 = 105.6, p < 0.0001). A test of heterogeneity found significant residual heterogeneity in the data set (QE = 1288.5, df = 191, p < 0.0001). Yet given the high diversity of studies and taxa as well as traits assessed in them, it is expected that the data set would show high heterogeneity.

Based on our model we found that the prediction that sexually selected traits would present stronger inbreeding depression than non-sexually selected traits is non-significant (b = -0.38, SE = 0.25, p = 0.12). With all caution required for the interpretation of non-significant results, the data indicates that, if anything, non-sexually selected traits appear to be under stronger inbreeding depression than sexually selected traits (Fig. 1), a trend that goes against the initial expectation. This result was qualitatively similar in two subsets of the data, namely the studies based on heterozygosity measures and those based on inbreeding depression assessments (Heterozygosity: b = -0.47, SE = 0.31, p = 0.13; Inbreeding: b = -0.30, SE = 0.41, p = 0.47). From inspection of the funnel plots we did not find strong evidence of publication bias except for three estimates which appeared to be outliers (Fig. 2).

Discussion

We here report the results of a meta-analysis on a set of studies on inbreeding depression in sexual and non-sexual traits. We included studied that manipulated inbreeding and those that estimated inbreeding indirectly via various measures of heterozygosity. We found a full model fit including all moderators, and comparison of this model with reduced models were significantly worse at explaining variation in the data set than the full model. Our working hypothesis, that sexual traits would show stronger inbreeding depression than non-sexual traits due to their connection to individual condition was non-significant, and very surprisingly even hinted at an opposite trend. By analyzing non-overlapping subsets we show that the estimated was negative in the subset of inbreeding as well as in the subset of heterozygosity studies. Our data set did not yield evidence of publication bias, which reasserts our search for publications by not finding evidence of unpublished data which may be missing from our data set.

Ornament evolution and its dependence on individual condition has been found to occur on several taxa and on several traits, yet this study appears to challenge this general notion. Sexual traits, given their precondition as indicators of individual quality, would seem to be necessarily under major pressure than non-sexual traits if the individual undergoes inbreeding depression. Yet this scenario appears to be reversed in our sample of studies form the published literature, indicating that it may be non-sexual traits that are experiencing stronger condition-dependence rather than sexual traits. At least there is no evidence for our initial prediction of a stronger inbreeding depression in sexual ornaments. Our data set includes several examples of classical

animal models, many of which have been under study due to their striking ornaments for some time already. If the striking ornaments have been present in a species and their function as a condition-dependent signalling trait has been long under use, perhaps they are no longer reliable as signals of condition, and perhaps this is why our data set cannot find inbreeding depression in the sexual traits in our data set.
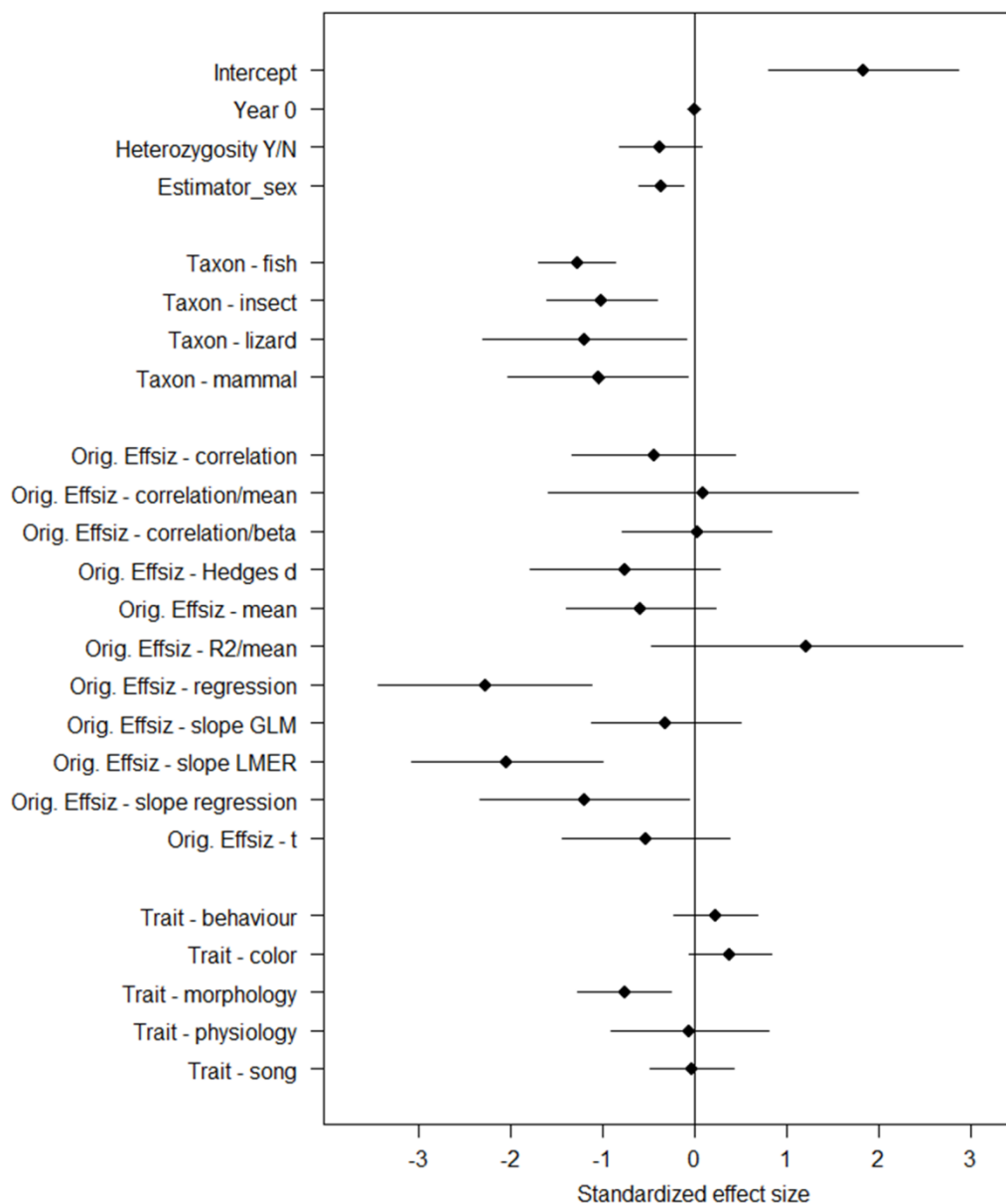
Figure 1. Forest plot of the standardised effect sizes and their corresponding for the full model fit. Moderators are grouped according to taxon, trait type and type of original effect size. Standard errors are shown as thin bars branching out of the effect sizes.
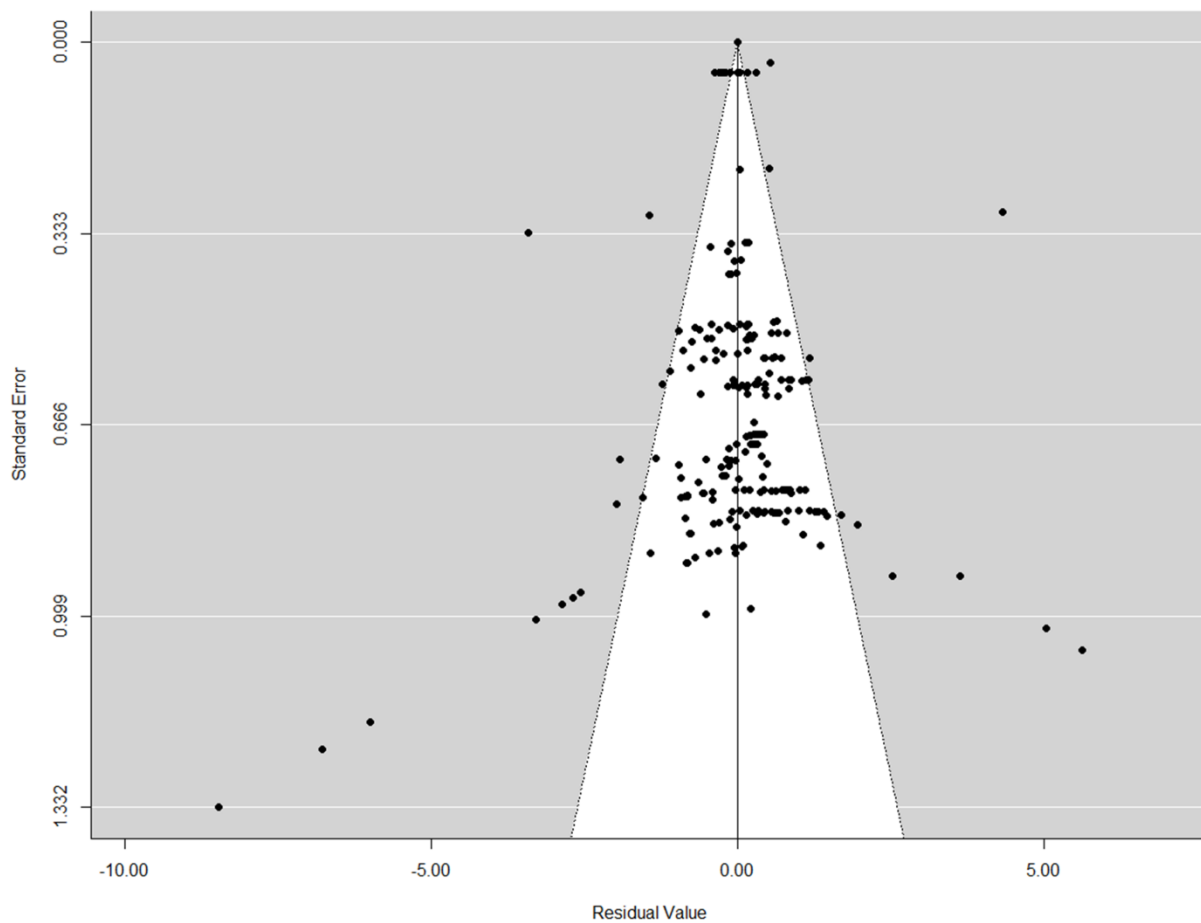
Figure 2. Funnel plot of the standardised effect sizes included in the meta-analysis used to assess publication bias. On the lower right corner of the figure are visible the only three effect sizes which could indicate some publication bias. Still these effect sizes were obtained through conventional estimations, and were therefore retained for the meta-analysis.

We suggest the following scenario for the evolution of sexual ornaments. The scenario involves a stage at which traits are condition-dependent and therefore honest signals of quality, but this link decays at alter stages. At an initial stage there is a, perhaps coincidental, association between trait expression, overall condition and genetic quality. Traits that are conspicuous and particularly well correlated, can become the target trait of choice for genetic quality, because they make it particularly easy to discriminate among males in good and bad genetic quality. Yet there may be males that are of poor overall quality, but may allocate a disproportional amount of resources into the development of the very same ornament. These are effectively cheater males that are preferred without providing the expected amount of indirect benefits. In the following, most males will show expression of the ornament, but the association with genetic quality and ultimately also condition will break down. Thus we expect only traits in a transient phase of recent or current exaggeration (or traits that absolutely cannot be produced cheaply) to show increased inbreeding depression.

In light of this reasoning, it is expected that only sexual traits in their early evolutionary steps will consequently show stronger inbreeding depression than non-sexual traits. Contemporary evolution of sexually selected traits would provide an ideal window to assess their condition-dependent expression, but this appear to be rather difficult to observe. Criteria such as the time span that it takes for them to be recognised by females as quality signals as well as the rate and direction of the selection appears to be very arbitrary, and its occurrence could either take only a few generations or many (Svensson & Gosden 2007). The scarcity in the observation of these phenomena, coupled with the experimental effort of many studies (a few months at most) hints at a rather narrow and unpredictable window to observe true quality signalling sexual traits. It is likely that in our data set some traits do signal quality in males, but if several sexual traits have become arbitrary traits then their contribution to the overall effect in the meta-analysis won't be effectively noted.

A meta-analysis on the relation between heterozygosity and various types of traits seems to provides support to our findings. The study assessed the intensity of the correlations among various types of traits, morphological, physiological and life history traits (which have been argued to be as sensitive to individual condition as sexual traits), and heterozygosity. The authors found that even though all traits were positively correlated with heterozygosity, there were no differences among the effect sizes regarding the type of trait (Chapman *et al.* 2009). Even though our findings show a trend for sexual traits towards less inbreeding depression, the difference between effect sizes is not significant, and in reflects in part the lack of difference among effect sizes found the heterozygosity-fitness correlations study.

Finally, however good our search might have been for the articles included in this study, a few articles could not be included due to lack of data presented in the original publication, or due to lack of information on the role of an specific trait as a putative sexually selected character. It is also very surprising that over a span of 20 years since the publication of the oldest article included in our study, only a little less than 30 studies have been published which could fit the criteria to be included in our meta-analysis. In a field as prolific as the study of sexually selected traits it is hard not to think that there is some unpublished data which would complete our data set. It is definitely necessary for the field to observe contemporary development of sexual traits, since only this path will allow to shed more light upon a question which we think still remains partly unanswered.

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Supplementary Materials

Figure S1. PRISMA scheme, showing our article search process as well as the results from the screening steps.
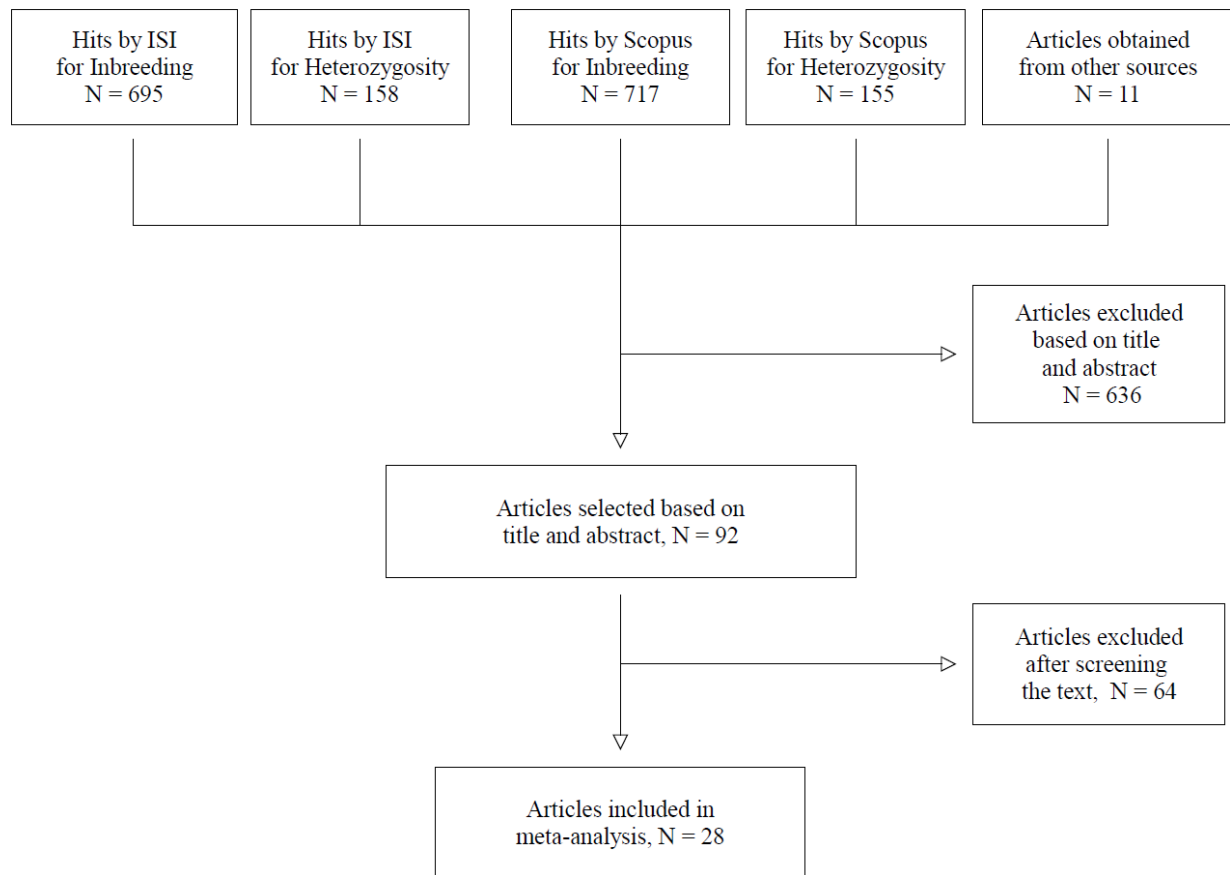
Chapter 5

General Discussion

The approach to the interaction between genotype and environment allowed me to achieve a broad view on the effects of different changes in both members of the interaction on trait expression in a very fascinating and novel animal model, the club-legged grasshopper *Gomphocerus sibiricus*. The assessment of the developmental plasticity of colour polymorphism found that, unlike other species of grasshoppers, this species appears to lack the capability to switch between colour morphs during the nymphal stages. However, I discovered that nymphae of *G. sibiricus* appear capable of modifying the amount of melanin in their integument, allowing them to either darken or lighten their colouration regarding the amount of radiant heat in their habitat. This ability appears to be exclusive to nymphal stages, since all imagoes in this experiment darkened during the first week after final ecdysis. This change in colour strategy hints at clear differences between different life stages, probably those related to thermoregulation and activity budget.

The second experimental assay allowed me to explore the effects of an LPS-based immune challenge on individual condition of nymphae from *G. sibiricus*, as a mean to assess the differential effects on sexual versus non-sexually selected traits. First I observed a trend in the opposite direction of the what I expected, with effects on sexually selected traits being bigger rather than smaller than non-sexually selected traits. I had expected that individuals which were immune challenged would find themselves in poorer condition, which would lead to a decrease in size of the sexually selected traits, yet this was not observed. Second, I found that overall effects were similar on both types of traits. Based on these findings I came to the conclusion that condition in *G. sibiricus* may not be so sensitive to immune challenges, which likely led to the lack of a strong effect in the expression of the sexually selected traits.

The third approach which I took was very different and made use on the primary literature on variation in trait expression. This way I aimed to broaden the view on the effects of condition on the expression of sexually selected traits. I analysed the relation between genetic quality and both sexual as well as non-sexual traits using a meta-analytic method. I encountered that contrary to the prediction sexually selected traits show less inbreeding depression than non-sexually selected traits. I considered the reason which

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could cause this result, and arrived at the conclusion that it is likely that sexually selected traits which display lower inbreeding depression have been literally cheated upon by males in poor condition and which discovered the usefulness of those traits to attract females. Even males in poor condition would likely allocate resources to sexual traits, possibly at the expense of other traits, ensuring that they produce the ornament, which ultimately may secure mating and increase their overall fitness. Therefore only sexually selected traits which have recently evolved and have not yet been noticed by males in poor condition would show higher inbreeding depression than non-sexually selected traits.

On the following sections I briefly go through each topic and point towards new avenues of research in the topic, while also expanding on variations for the experiments presented here which may help to further elucidate the topics which were explored on this work.

Colour Polymorphism: A Palette of Possibilites

The colour variation observed in *G. sibiricus* in the field constitutes a source with many possible outlets for research. In this study I only tapped into one aspect of the green-brown colour polymorphism, based principally on theoretical accounts which had been formulated a few decades ago (Uvarov 1966, Rowell 1971, Dearn 1990). I discovered the absence of developmental colour switches in *G. sibiricus*, but as discussed in chapter 2, I only manipulated 2 conditions, radiation and colour background, and other conditions may have thrown this assessment off. It would be very interesting to work in isolation with the variables which, according to theory, are the ones which trigger these switches, namely temperature, humidity, food moisture content and individual density. Additional factors such as the use of free ranging enclosures, the use of real substrate as a background, and the inclusion of individuals from different populations could certainly provide a more satisfying answer to the question of whether or not *G. sibiricus* has lost the ability to switch between the green and brown colour morphs.

The finding that nymphae have the ability to adjust their degree of darkness (likely the amount of melanin in the epidermis, below the cuticle) open an interesting path to investigate the physiology of darkening which no likely has repercussions on various life stages. Given the type of habitat inhabited by *G. sibiricus*, this discovery fits pretty well under an evolutionary scenario of adaptation to the unpredictable weather in the high elevations. The small nymphae, probably used to the cold weather and often clouded sky, would have evolved to assess during the first or second day of each instar whether or not to invest in adding melanin to their epidermis, just below the procuticle. This change, which in this experimental setting was non-reversible, could indeed be an adaptation to

help nymphae thermoregulate efficiently. If during the first days of a specific instar the climatic conditions are not deemed good, a nymphae may probably decide to switch to a "darker colour mode", or if the opposite is true, then they may decide to switch to a "lighter colour mode".

If indeed this capability is a necessary adaptation to survive, it takes the shade of a viability trait (Cornwallis & Uller 2010). Since it would allow better acquisition of radiant energy, and therefore a better energy budget, this would mean more foraging time, which can translate into better development and therefore in better condition. Because of this ramification, it would be very interesting to further explore if nymphae are really exploiting this capability, under which conditions, and even whether different populations differ in this trait. In addition to an evolutionary approach, a physiological approach to the causes and mechanisms implied in this case of developmental plasticity would also benefit research in other study areas. If it is assumed that melanin is the likely candidate implied for the colour darkening mechanism (Sugumaran 2002), then producing melanin to darken the epidermis will certainly not be cost free. Given the various roles of melanin in insect physiology, a probable trade-off would probably take place, and this is likely to have effects on other aspects of development as well as condition and trait expression.

How can we assess heightened condition dependence in *G. sibiricus*?

In the assessment of condition dependence in *Gomphocerus sibiricus* I included various aspects of three putative sexually selected traits. Among these traits I measured four parameters of the advertisement song of the male as well as morphological measures of the antennae and the swollen forelegs. From these set of traits, the clubs (from here on I use this term to refer to the forelegs) appear to be a very suitable candidate to advertise male condition. Still, despite using two cohorts, field and lab individuals, and two different dosages, I could not really detect a strong effect of the treatment in the traits included in the analysis. And even though there may be some things which can be changed in order to improve the immune challenge, it could possible that *G. sibiricus* is too resilient and also that the clubs are simply too important to not be developed. In order to validate this claim, a different approach is needed to test whether the clubs are an indispensable trait, which regardless of the type of condition assessment will always develop.

The clubs in *G. sibiricus* are specially employed when males perform displays in front of the females. Still the clubs appear to be an essential tool to convince females too copulate once they manage to jump on the back of females. It is then that males rhythmically drum

on the sides of the thorax of the female, whose function is unknown yet but which hints to a stimulation to copulate. Perhaps it is during this part of the courtship ritual that one could assess the true usefulness of the clubs. An experiment where one would employ pads on both the male clubs and the sides of the female's thorax could provide a buffer to the drumming rate or strength, which may affect the outcome of the courtship for the male. Since it is possible to encounter males with a club which is not fully developed, one could pair males with fully developed and with undeveloped clubs, and perform mate choice trials. This experiment would be a non-invasive version of the previously suggested experiment involving pads.

The drumming rate at which males strike the female is another promising source of information regarding the function of the clubs as a signal of individual condition. To assess this rate one could take either a visual or a audible approach. A possibility could be to video-tape the behaviour, which occurs at very high speeds, and from the recording one could assess the drumming rate. A similar results could perhaps be obtained by using a substrate microphone, which could, when placed under the female, measure the vibration caused by the male during the drumming behaviour. All these methods, which are aimed at measuring an obviously important behaviour of *G. sibiricus* where the clubs are employed could certainly shed a clearer light into their function and also their relative importance. If the clubs indeed are so important as to resist condition manipulations, the slightest variations in either the drumming rate or on the drumming per se (pad experiment), would very likely yield variation in mate choice trials.

In addition to the clubs, which appear to have a vital function in the courtship behaviour, the function of both the advertisement song as well as the antennae are two straightforward traits which no doubt have a function in the male attractiveness, but up to remains unknown. The advertisement song has a definite function in attracting the females in the field, since males purposefully seek areas more or less devoid of vegetation and which have stones, on which they climb to sing their advertisement song. Following the start of the singing it is common to observe females approach singing males. Still, what attributes of the advertisement song are preferred by females is remains an open question. The advertisement song of *G. sibiricus* is composed of three parts, where the middle part of the song is composed by a high-energy thrill. If one reviews studies based on other grasshoppers and crickets (Cotton *et al.* 2004, Jacot *et al.* 2004, Tregenza *et al.* 2006, Drayton *et al.* 2010, Stange & Ronacher 2012), the carrier frequency, the number of elements in the thrill per time unit, and the singing rate could all probably signal male condition during the singing bouts of *G. sibiricus*.

Finally, I also want to mention the possibility that the antennae could also be under selection as a signal of condition in *G. sibiricus*. This is based on observations made during courtship. Males use their antennae as mentioned previously, waving them in a complex movement which starts in the back of the body and swing the antennae forward, in from of the female. Similar antenna displays are shown in other grasshoppers (Dumas *et al.* 2010). But perhaps it is not the movement which helps to woo the female. During the measuring of the antennae for the LPS manuscripts, I observed that the antennae possess several pores of significant size on its tip, which is also very broad. I discussed the possibility that the antennae may be releasing a pheromone during the antennae waving behaviour. This seems also likely given that the movement comes from the back of the male's body, whipping the antennae forward, which would help in pushing the pheromone towards the female. This component of the courtship behaviour seems to hold another possibility to assess condition in *G. sibiricus*, but first one needs to go beyond this speculation and determine whether or not a pheromone is involved in the courtship behaviour.

How and where can we find sexual traits which signal good condition?

It is very challenging to imagine that probably several of the sexually selected traits under study in various animal models are not true signals of condition anymore. Additional complications arise when one realises that the development from arbitrary trait to sexual trait can last any amount of time, and could occur at any time, and also almost any trait can potentially become a sexual trait. All these reasons make the hunt of truly condition dependent sexual traits a daunting task. Part of the answer to the question of how can we find these traits lies in examples set by the few studies available which have found them (Svensson & Gosden 2007). The key element in chasing the contemporary evolution of these traits is the duration of the studies, since it is likely that a few generations may be needed for the development of a novel sexual trait to occur. It would be interesting to concentrate on those species included in the data set and which show lower inbreeding depression for sexual traits, since perhaps in these systems new correlations between condition and arbitrary traits could now be on the making.

A complement to long studies is to improve the assessment of the relation between genetic measures of quality and sexual traits. I tried to cast a wide net for studies by using broad terms during the article search phase. Still when I look at the data, it is likely that more data exists on various of the classic animal models included in this study which I could not find because of the focus of the search. Any study which, for example, dealt with either of the two measures of genetic quality which I used, but which did not include a reference to

sexually selected traits in their text would not have been found by the search. By pointing this out I want to call attention to the need to eventually zoom in on these species separately, given that it is likely to find the data if the search is aimed specifically at them. This could also help to assess more specifically in which phase are sexual traits in regards to their condition signalling quality, and this in turn could help explain results in other type of studies which often appear to have no clear explanation.

Regardless of shortcomings from the amount of data, this meta-analysis provides a step to try to reconcile theory and data. In my view this study has provided two clear statements about the relation between sexually selected traits and condition based on measures of genetic quality. On one side there are traits which have reached a point where they are no longer signalling male quality, turning them once again into arbitrary traits. On the other side there is a path to try to find sexually selected traits just after the Fisherian runaway selection process took place. It is very exciting to think about the many possibilities which are provided in systems where generations occur fast, and where the species reside in taxonomical groups where related species show conspicuous sexual traits, and it is therefore likely that an extravagant sexual trait may suddenly arise. I trust that new data as well as new assessments on classical animal models will shed even more light in the evolution of sexually selected traits in a non-distant future.

A few final words

I hope that my findings in *G. sibiricus* will provide an interesting point of comparison and prompt similar assessments in the fields of colour polymorphism and condition dependent sexually selected traits. Variation in phenotypic plasticity in this species regarding colour allowed me to uncover two aspects of its response to the environment, both of which shed light in the behaviour and adaptation of this species to its environment. In contrast to these findings in the phenotypic plasticity of colour, the manipulations of condition generated several new questions regarding the interaction of various sexual traits in a very elaborated courtship behaviour. Finally, I tested the validity of a long standing theoretical expectation with the data at hand, further creating more questions, and so promoting the advance of science. I hope that my contribution to this field will benefit other researchers which no doubt, just like myself, also marvel at the endless variation in phenotypic expression in nature.

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Statement of Contribution (Beitragsnachweis)

This cumulative dissertation involves collaborative work with my supervisor, Prof. Dr. Holger Schielzeth, and two external scientists, Dr. Hendrik Eggert and Prof. Dr. Joachim Kurtz. My contribution to the various aspects of the following chapters such as the conception of ideas, data collection, analysis, and finally the writing of the manuscripts is detailed in the section below. My collaborators have seen the present statement and have given their consent that my contribution is accurate. The present publications and the unpublished manuscript have not been submitted as part of any other doctoral thesis or dissertation in any other university.

Chapter 2

J. Pablo Valverde and Holger Schielzeth. 2015. What triggers colour change? Effects of background colour and temperature on the development of an alpine grasshopper. *BMC Evolutionary Biology*. doi: 10.1186/s12862-015-0419-9.
Conception: 50%, Data Collection: 100%, Analysis: 60%, Writing: 60%.

Chapter 3

J. Pablo Valverde, Hendrik Eggert, Joachim Kurtz and Holger Schielzeth. 2017. Condition-dependence and sexual ornamentation: Effects of immune challenges on a highly sexually dimorphic grasshopper. *Insect Science*. doi: 10.1111/1744-7917.12448.
Conception: 50%, Data Collection: 80%, Analysis: 60%, Writing: 60%.

Chapter 4

J. Pablo Valverde and Holger Schielzeth. Do better genes signal better? A meta-analysis of the effect of inbreeding and heterozygosity on sexual vs non-sexual traits. (*unsubmitted manuscript*).
Conception: 50%, Data Collection: 100%, Analysis: 80%, Writing: 80%.
This chapter will be submitted for publication in a peer-reviewed journal.

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Declaration of Originality/ Selbstständigkeitserklärung

By presenting this thesis in fulfilment of the requirements for a doctoral degree (Dr. rer. nat.) at Bielefeld University, I here by confirm that I am aware of the doctoral degree regulations of the University. I also confirm that the present work, entitled "Phenotypic plasticity and condition-dependent trait expression in sexual ornaments and body colour in the alpine grasshopper *Gomphocerus sibiricus*" is genuine and that I have written it myself. The current work has not been submitted for a higher degree to any other university or institution. Finally I confirm that I have cited all references used in the present work.

Juan Pablo Valverde Monge

Bielefeld, July 2017

Hiermit versichere ich, dass mir die geltende Promotionsordnung der Universität Bielefeld bekannt ist. Dazu versichere ich dass die vorliegende Arbeit zur Erlangung des Doktorgrades (Dr. rer.nat.) mit dem Titel „Phenotypic plasticity and condition-dependent trait expression in sexual ornaments and body colour in the alpine grasshopper *Gomphocerus sibiricus*" original ist und dass ich die Arbeit selbstständig verfasst habe. Die Arbeit hat in dieser oder ähnlicher Form keiner anderen Prüfungsbehörde vorgelegen. Ich versichere dass ich alle benutzten Hilfsmittel und Quellen kenntlich gemacht habe.

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Bielefeld, Juli 2017