SECONDARY CONTACT IN THE EUROPEAN WALL LIZARD

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Abstract

A critical mechanism underpinning current biological diversity is the extent to which one species mates with, or avoids mating with, another. However, little is known about the factors that mediate hybridisation, especially during the initial and rarely observed stages of secondary contact when interspecific interactions have not responded to selection. In particular, whilst hybridisation is ultimately a behavioural phenomenon, the role of behaviour in mediating hybridisation and how it is influenced by environmental and circumstantial factors is rarely investigated. Recently introduced species provide us with unequalled opportunities to study these factors. In this thesis I examine the role of behavioural mechanisms, in particular male-male competition and mate choice, in mediating mating patterns between two genetically and phenotypically distinct lineages of European wall lizard (*Podarcis muralis*) that have come into recent secondary contact through human introductions.

In Chapter Two, I investigated how sexual selection during allopatry is responsible for creating stark differences in phenotypic traits such as body size and weapon performance evident in the two lineages today, ultimately explaining the strong biases in dominance during territorial disputes between males. However, I also show that even given this asymmetry in male competitive ability, the extent to which it extrapolates into greater access to females in naturalistic, outdoor enclosures depends strongly on the spatial clustering of basking sites, a critically important resource for many ectotherms.

In contrast to initial predictions suggested by asymmetries in male competition outlined in the previous chapter, in Chapter Three I show that both paternity and courtship behaviour was strongly assortative in the outdoor enclosures. Further investigation through staged experiments on olfactory mate choice, mating trials and analyses on specific behavioural data obtained in an enclosure experiment, I show that lineage based dominance actually contributes to assortative mating patterns in conjunction with weak conspecific male choice. In contrast, female choice seems to play no role in mediating the mating patterns observed between the two lineages.

In Chapter Four I had the rare opportunity to examine the morphological and behavioural factors that predict why animals should hybridise in the first place, using the data obtained in the enclosure experiment above. I found that hybridisation was particularly common between small individuals of the larger lineage and large individuals of the smaller lineage; a result that corroborates the mechanisms determining the assortative patterns uncovered in Chapter Three. Additionally, hybridisation rates were particularly high in less dominant individuals, which I suggest is due to subordinate males having reduced opportunities for courting conspecific females due to male-male

competition, requiring them to become less ‘choosy’ and therefore more likely to mate with heterospecifics.

Finally, secondary contact cannot occur without at least one lineage coming into a new environment, and yet relatively little attention is paid to how this environmental change can affect the signals involved in intraspecific communication and mate choice. In Chapter Five I show that a change in the amount of time male lizards spend thermoregulating (a likely consequence of arriving in a new environment) significantly changes the chemical composition of their scent marks. However, whilst female lizards were able to detect these effects, they did not seem to base their mating decisions on them. Nevertheless, this result raises interesting questions about the potential function and consequences of this plasticity, and highlights the importance of considering plasticity in chemical communication in heterogeneous environments.

Overall, this thesis shows the critically important role of behaviour in mediating intra- and interspecific mating patterns during recent secondary contact. In particular, it highlights how the direction and extent of hybridisation and competition are influenced by the degree to which differing morphological and behavioural phenotypes interact over a heterogeneous environment, particularly during the initial stage of secondary contact when mate choice has not had the chance to respond to the selective pressures of hybridisation.

For Fi

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Author contributions

I confirm that this work is solely my own, except in the following instances:

The morphometric and colouration data used in this thesis was collected in conjunction with Tobias Uller and Geoffrey While for all chapters. Fiona Moultrie and James Stroud also helped with collecting a substantial proportion of the lizards used in Chapters 5, and those used in the staged male-male interactions in Chapter 2.

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CHAPTER ONE

INTRODUCTION

Unpublished manuscript

1.1 What is secondary contact, and why is it important?

For millennia, organism populations across the globe have been endlessly cleaved and blended together by physical processes such as the rise and fall of mountain ranges and the ebb and flow of glaciers. Secondary contact refers to the critical stage whereby these populations, after being separated, can once again reproduce after a physical barrier is removed. When populations become isolated, they accumulate genetic and phenotypic differences, and it is these differences that make the processes and consequences that follow secondary contact so important for understanding the evolution of biodiversity. Ultimately these consequences are defined by the various degrees to which genetically different lineages compete (Schoener 1974; Grether et al. 2009), interact with and respond to novel environments (Agrawal 2001; Ghalambor et al. 2007), or hybridise (Mayr 1963; Harrison 1993).

In this introduction, I will set the scene for the rest of my thesis by firstly giving a brief overview of just how important secondary contact is for evolution, focussing specifically on the consequences that arise from interspecific hybridisation. Hopefully, this will be convincing enough that my following consideration of why it is important to study the mechanisms that ultimately determine these consequences will then become obvious. I will then move onto what these mechanisms are, with a particular focus on behavioural interactions (hybridisation is, after all, ultimately a behavioural phenomenon in animals (Grant and Grant 1997)) and how they are mediated by the environmental and circumstantial factors related to the phenotypic divergence that occurred during allopatry. I will end on the specific topics that I will be addressing in this thesis, and then tell you about the lizards.

1.2 The varying consequences of hybridisation

"The grossest blunder in sexual preference, which we can conceive of an animal making, would be to mate with a species different from its own and with which the hybrids are either infertile or, through the mixture of instincts and other attributes appropriate to different courses of life, at so serious a disadvantage as to leave no descendants."

-R.A. Fisher (1930)

As soon as two lineages come back into contact after a short period of allopatry, they are likely to utilise similar resources in similar ways. Much attention has been paid to the consequences of the competition that inevitably results from this similarity in resource use, in particular its role in causing character displacement (e.g. Brown and Wilson 1956; Schluter 2001). Character displacement is generally predicted to occur when two lineages compete through exploitation of shared ecological resources (Schluter and McPhail 1992; Adams and Rohlf 2000), or even direct agonistic interactions (Grether et al. 2009), and the resulting costs that arise provide the selection pressure that drives further diversification (Brown and Wilson 1956).

However, the evolutionary consequences of secondary contact also depend largely on the varying degree of another critical phenomena, and the focus of this thesis: hybridisation. Throughout this thesis, I refer to 'hybridisation' without any reference to taxonomic labelling, but simply as the interbreeding that occurs between individuals from genetically distinct populations, (*sensu* Barton and Hewitt 1985). To

start with, I will address the direct evolutionary consequences of one extreme degree of hybridisation in animals that can follow secondary contact; specifically, when there is a lot of it. Botanists have long-appreciated the importance of hybridisation in evolution as a method of generating genetic novelty. For the most part, this was due to the frequent observation of polyploidy (i.e. chromosome duplication) that frequently occurs following interbreeding between different plant lineages. Polyploid hybrid offspring are usually reproductively incompatible with either parent clade, and therefore can be considered, in essence, a totally separate species (Adams and Wendel 2005). In fact, it has been estimated that more than half of globally distributed angiosperm species are the direct product of historical hybridisation (Arnold 1997). Almost unbelievably, zoologists have been slower on the uptake. Traditionally, animal hybridisation was seen as the consequence of rare and costly recognition errors during reproduction (Fisher 1930; Mayr 1963), and as such was often assumed to be unimportant in evolution. Unlike plants, polyploidy is rare in animals, and thus its consideration has not prompted a great deal of research on the generic evolutionary importance of hybridisation. However, recent surveys on the extent of hybridisation across multiple species suggest that between 10-30% of all animal species regularly hybridise (Mallet 2005). Also, it actually represents the only mechanism, along with genetic mutations, by which new alleles can be introduced into populations in the first place, and thus is a major supplier for the raw building blocks of evolutionary adaptation. In many instances it can even play a *more* 'important' role in introducing genetic variation to populations compared to mutations (I apostrophe 'important' since all genetic material introduced by hybridisation still ultimately originated from mutations) (Grant and Grant 1994; Kim and Rieseberg 1999; Kunte et al. 2011; Abbott et al. 2013). For instance, human-introduced populations can exhibit higher

genetic variation than their parental or native ranges due to the admixture that occurs from multiple introductions (Kolbe et al. 2004; Genton et al. 2005; Roman and Darling 2007), and hybridisation in Darwin's finches introduces genetic variation at a rate of two to three orders of magnitude above that of genetic mutation (Grant and Grant 1994).

Biologists are now starting to accumulate examples of just how important the repercussions of this increased genetic variation can be. For example, the novel allelic combinations in hybrid progeny can facilitate adaptation to novel environments (Ellstrand and Schierenbeck 2000; Sakai et al. 2001), and introduce novel traits into lineages that can influence mate choice and sexual selection (Stein and Uy 2006). Additionally, several reproductively isolated animal lineages/species are now known to originate exclusively from hybrid matings (Mavarez et al. 2006; Grant and Grant 2009). Hybridisation may even play an important role in facilitating adaptive radiations, where the cyclical sympatry and allopatry experienced during repeated range shifts can create new and adaptive genetic combinations, but also facilitate divergence due to the costs associated with hybridisation (Seehausen 2004).

This raises a critical point. Hybrid offspring often have intermediate phenotypes 'untested' by natural selection, and as a consequence are often less fit compared to the parental lineages. In addition to creating genetic variation, hybridisation therefore plays an important evolutionary role at a different scale, notably by providing the costs that can result in selection pressures being generated to reduce it occurring in the first place (Fisher 1930; Mayr 1963). These costs vary from marginally reduced survivorship of hybrid offspring (e.g. Lancaster et al. 2007), to absolute reproductive

'failure', where embryos may die prematurely (Tyler et al. 2013), or where the offspring are totally sterile (Haldane 1922; Hewitt et al. 1987). Importantly, this generates selection against hybridisation following secondary contact, and the reduction in gene flow that follows is a critical requirement in allopatric speciation, generally accepted as being the major engine driving the generation of new species (Mayr 1963; Coyne 1992; Knowlton et al. 1993; Via 2001; Hewitt 2004; Weir and Price 2011).

The most widely acknowledged adaptive response to reduce hybridisation is 'reinforcement' (Paterson 1982; Noor 1999b), where the traits involved in species recognition and mate choice diverge, such as lineage-specific signals or heterospecific recognition ability (Dobzhansky 1940; Coyne and Orr 2004; Sobel et al. 2010; Servedio et al. 2011). Whilst a controversial subject for many years, partly because it was often assumed that an adaptive response to the costs of hybridisation required its level to be so high that divergence was impossible (e.g. Paterson 1982; Spencer et al. 1986), there is now considerable evidence for reinforcement (reviewed in Noor 1999a; Servedio and Noor 2003). This includes the widespread observation that individuals in sympatry show stronger mate avoidance of heterospecifics and/or more divergent intraspecific signals compared to individuals of the same species in allopatry (Hoskin et al. 2005; Urbanelli and Porretta 2008; Yukilevich 2012). For example, two genetically distinct lineages of Australian tree frog (*Litoria genimaculata*) show greater differences in male vocalisation and body size, and better female discrimination against males from the other lineage, at the sympatric zone of their range compared to the allopatric zones (Hoskin et al. 2005).

As might be guessed from above, the costs and benefits of hybridisation, and the resulting degree of admixture or divergence, can result in a variety of distinctive natural systems that we can observe today. These vary between total reproductive isolation, introgression of specific traits into the genome of another lineage (e.g. Stein and Uy 2006) total introgression resulting in hybrid swarms where the identity of one lineage is totally lost to another (e.g., Avise et al. 1984) and the more commonly observed intermediate 'hybrid zones', where overlapping species ranges can maintain a stable degree of hybridisation via the costs of hybridising and the dispersal of individuals from each side of the species border (Barton and Hewitt 1985; Barton and Hewitt 1989). Given this diversity of possible scenarios following relative degrees of hybridisation, it is important to examine the specific biological and environmental factors that ultimately create them.

1.3 Mechanisms mediating hybridisation

The mechanisms mediating all reproductive patterns, of which hybridisation is but one, can be categorised into postzygotic and prezygotic factors (Dobzhansky and Dobzhansky 1937). The consequences of postzygotic mechanisms in general depend on whether the fate of hybrid offspring is environmentally determined (such as through reduced survivorship and competition for mates (Hatfield and Schluter 1999; Lancaster et al. 2007)), or the product of the genetic incompatibility between the two species (Haldane 1922; Hewitt et al. 1987; Tyler et al. 2013). Importantly however, since the costs of hybridisation are mostly realised at the postzygotic stage (although energetically expensive courtship displays can also be costly to heterospecifics (e.g.

Servedio and Noor 2003), the adaptive responses to reduce these costs are typically expressed at the prezygotic stage.

These prezygotic factors can be further split into those that occur before or after copulation. Postcopulatory mechanisms, i.e. the period after mating but before a zygote has formed, are mainly studied in respect to conspecific sperm precedence and cryptic female choice, and largely ignored from a hybridisation perspective. Postcopulatory isolation can occur through an inability to deposit sperm into the female reproductive tract (often due to mechanical reasons such as incompatible genital morphology), or differing abilities between heterospecifics and conspecifics in the ability of the sperm to reach the ova (reviewed in Birkhead and Brillard 2007). Likely mechanisms for the latter include species-specific differential female immune responses to the glycoprotein signature of spermatozoa (Steele and Wishart 1992), or differential abilities of conspecific sperm to traverse the female reproductive tract due to motility differences or seminal fluid composition (Price 1997; Birkhead and Møller 1998; Price et al. 2000; Birkhead and Brillard 2007). Given that many studies have shown mismatches between conspecific and heterospecific fertility rates following copulations (Gregory and Howard 1994; Sagga and Civetta 2011), postcopulatory mechanisms play an important, but as yet poorly understood, factor in mediating the rate of hybridisation.

Moving away from post-copulatory mechanisms, perhaps the greatest mechanistic focus on hybridisation has been precopulatory factors. As the ubiquitous mechanism that mediates all reproductive interactions in animals, behaviour plays a key role, arguably the most important role (Grant and Grant 1997), in mediating the degree and

direction of hybridisation (Servedio and Noor 2003; Abbott et al. 2013; Willis 2013). This point is particularly well illustrated by the observation that many sympatric species that rarely, if ever, hybridise in the wild can produce viable hybrid offspring in captivity or through artificial insemination, when the behavioural barriers mediating hybridisation are removed (references in Willis 2013). In particular, as the most immediate factor that determines whether animals will try to copulate or not, mate choice has received a great deal of attention in studies on hybridisation. Indeed, all other precopulatory causal factors of hybridisation can be argued to occur simply through the degree to which they facilitate, circumvent, or reduce mate choice.

I will now give an overview of how mate choice mediates hybridisation between animals, arguing that the influence of other factors is realised largely through their secondary influence on mate choice. In particular, my thesis addresses the role of male-male competition and mate choice on determining patterns of hybridisation upon first contact between two divergent lizard lineages. Furthermore, I address how these interactions can vary across habitats and how they are influenced by the importance of phenotypic divergence accumulated in allopatry. Below, I will introduce these topics using a 'bottom-up' approach, arguing that mate choice represents the most immediate factor determining hybridisation. I suggest that male-male competition directly influences the degree to which mate choice can occur; the degree and nature of phenotypic divergence influences the outcome of male-male competition and mate choice; and environmental factors ultimately mediate the degree to which all aspects of phenotype influence individual interactions.

1.4 - Mate choice as the primary mediator of hybridisation

There has been an extensive amount of research investigating the degree to which different forms of mate choice contribute to hybridisation. Females generally have a greater per-capita investment in each offspring compared to males and can therefore maximise their fitness by choosing the best male in terms of the indirect (genetic) and direct (material) benefits he will provide. Males on the other hand maximise their fitness through gaining access to as many females as possible (Williams 1966; Trivers 1972; Bateson 1983). This usually makes females the 'gatekeepers' of hybridisation, as the relative costs associated with making an incorrect mating decision (such as mating with a different species) will be higher for females compared to males, and hence select for their greater mate choosiness (Wirtz 1999). Consequently, it is a relatively common observation that males court heterospecific females more frequently than the latter consent (e.g. Noor 1996; Hettyey et al. 2009), and when males do show high heterospecific mating avoidance, they also tend to have high reproductive investment, such as providing paternal care (Sargent et al. 1986; Rosenqvist 1990; Kraak and Bakker 1998). Observations of greater than expected hybridisation rates therefore tend to occur in situations where the normal level of mate choosiness is reduced, either for adaptive reasons or when female choice is constrained.

1.4.1 Hybridisation as an adaptive strategy

Perhaps the most widespread explanation for why hybridisation occurs is 'Hubbs principle' (Hubbs 1955), whereby the frequency of hybridisation increases as the

probability of encountering a conspecific mate declines. Scenarios in which this occurs include the introduction of a small number of immigrants into a population of numerous heterospecifics (Huxel 1999; Mooney and Cleland 2001), when one species is at a low population density (Avise and Saunders 1984; Rhymer and Simberloff 1996), or when the operational sex ratio is skewed due to sex-specific seasonal differences in fertility (Brodsky and Weatherhead 1984; Nuechterlein and Buitron 1998).

This observation also raises the issue that rather than being a 'mistake', hybridisation may in fact represent one outcome on a spectrum of adaptive mating decisions, which includes accepting less desirable conspecific mates as well. As long as offspring are at least partially viable, then hybridising may represent a better option than foregoing reproduction completely, and therefore be under some degree of positive selection as a 'best of a bad job' strategy (Wirtz 1999; Randler 2002; Izzo and Gray 2011). In some instances, hybridisation has even been reported as being adaptive over mating with conspecifics, through, for example, greater food provisioning by a heterospecific mate, or facultatively choosing to hybridise if it is favoured by environmental conditions (Grant and Grant 1992; Veen 2001; Pfennig 2007).

1.5 Male-male competition

Competition for mates and resources following secondary contact is an important mechanism determining evolutionary diversity (Gause 1934; Connor and Simberloff 1979; Schoener 1983). Indeed, competition following secondary contact is likely to be

particularly fierce considering that closely related species often use similar resources (Hardin 1960; Vallin et al. 2012), and that isolation in allopatry is unlikely to have produced adaptive responses to heterospecifics. In particular, there is no guarantee that heterospecific recognition may be present at all, in which case competition can be enhanced competition through normally intraspecific processes such as male territorial behaviour and mate competition. Even though male-male competition is widely accepted as being of pivotal importance in defining mating patterns within species (Wong and Candolin 2005; Hunt et al. 2009), its role in determining patterns of hybridisation has received little attention. This is perhaps understandable, as any asymmetries in male-male competition would likely lead to an unsustainable system with rapid introgression from the dominant lineage, making actual observations of these processes in action relatively rare (e.g. Rhymer and Simberloff 1996). Nevertheless, lineage integrity can be maintained even in the face of extreme asymmetries in male dominance, for example if there is strong female rejection of dominant heterospecific mates (Goldsworthy et al. 1999; Stein and Uy 2006). Additionally, lineage integrity can exist with male competitive differences under more dynamic scenarios. For example, male Townsend warblers (*Dendroica townsendi*) are strongly dominant over male Hermit warblers (*D. occidentalis*) in territorial disputes. In some regions of their contact zone, this can result in extensive displacement of the subordinate male Hermit's, resulting in such a strong sex-ratio bias that female Hermit's are forced to hybridise due a dearth of conspecific mates (Pearson 2000; Pearson and Rohwer 2000). Nevertheless, species integrity is maintained through a combination of greatly reduced hybrid survivorship, which limits extensive introgression, displacement of subordinate lineages into less favourable habitats, and the creation of dynamic 'moving hybrid zones', where the dominant lineage

essentially ‘chases’ the subordinate at a geographical scale (Bronson et al. 2003; Woodcock et al. 2005; Reudink et al. 2006). Male-male competition can also interact with female choice more directly. In species where females adaptively choose the ‘best mates’ via observations of dominance disputes, this can result in females actively choosing a dominant heterospecific (Pearson 2000; Pearson and Rohwer 2000; Bronson et al. 2003; Woodcock et al. 2005; Reudink et al. 2006). For example, the preference of American black ducks (*Anas rubripes*) for mallard drakes (*A. platyrhynchos*) was originally suggested to be due to the latter’s more ornamented plumage (Johnsgard 1960). In actual fact, it seems that whilst female black ducks have an intrinsic preference for their own drakes, they will switch their mate preference to mallard drakes if they observe the latter dominating conspecific males in territorial contests (Brodsky et al. 1988).

1.6 Phenotypic divergence

The interactions I have discussed above depend to a large extent on the extent of phenotypic and genetic divergence that occurs during allopatry. The causes of this divergence can of course depend on any environmental difference that creates a differential change in morphology, for example adaptation to differing food sources (Ratcliffe and Grant 1983) and character displacement via interactions with heterospecifics (e.g. Grant 1972; McCormack et al. 2010).

Perhaps most importantly from a heterospecific mating context, differences in sexual selection during allopatry can result in diverging selection on the structure and

extravagance of sexual ornaments, and the effectiveness of status badges and weapons used in male combat (Uy and Borgia 2000; Rasner et al. 2004; Yeh 2004). In particular, the lineage under stronger sexual selection during allopatry may represent a more attractive option to heterospecific mates by exhibiting traits that exploit pre-existing female preferences (e.g. Jones and Hunter 1998). For example, female *Xiphophorus* fish show a pre-existing preference for both larger males and those that exhibit 'sword-tails' (Rosenthal and Evans 1998). As a result, females of the normally sword-less species, *X. pygmaeus*, show a greater preference for males of a closely related species (*X. nigrensis*) that is both more sexually dimorphic in body size and exhibits a 'sword-tail', suggesting a history of greater sexual selection (Ryan and Wagner Jr 1987).

As might be expected when phenotypes vary, hybridisation should also vary non-randomly between individuals once they come into secondary contact, specifically being higher between individuals that are most similar in terms of body size, signal type or courtship display (Grant and Grant 1992; Seehausen and van Alphen 1998; Qvarnström et al. 2006). However, there are relatively few studies that explicitly address how phenotypic variation within populations contributes to interspecific mating patterns upon secondary contact. One notable example is in sticklebacks where hybridisation is common between the larger individuals of the smaller benthic lineage and smaller individuals of the larger limnetic lineage (Nagel and Schluter 1998; Conte and Schluter 2013).

1.7 Environmental factors

So far I have discussed the more 'immediate' importance of mate choice in secondary contact, the manner by which this can be affected by male-male competition, and then how lineage-specific and individual phenotypic differences can influence both these factors. My final consideration is the role of environmental conditions in the secondary contact zone in mediating heterospecific interactions. Indeed, the fitness outcome of a behavioural, or indeed any phenotypic, trait depends on how it interacts with the environment. This is a particularly important consideration during secondary contact, which by definition can only come about when there has been a shift in a species' geographical and/or environmental distribution. Even so, the degree to which environmental factors mediate hybridisation through their effects on behaviour has received relatively little attention. A good example of how important these environmental factors might be are the many documented cases of 'mosaic hybrid zones', in which the relative direction and degree of introgression varies across the contact region between two species (Rand and Harrison 1989), and yet we have very little understanding about the causes of this variation (e.g. Ross et al. 2002). Some studies have inferred process from pattern and suggested that these mosaics are the result of varying degrees to which each lineage can introgress into the other due to environmentally-dependent competitive advantages of one lineage (Barton and Hewitt 1985; Spence 1990; Sperling and Spence 1991; Arnold 1997; Ross et al. 2002; Chatfield et al. 2010). Ecological studies that have addressed this phenomena usually argue that differences in the direction and degree of hybridisation in these areas is due to whether the local habitat favours high population abundances of one lineage over the other (MacCallum et al. 1998). However, population density is only one of several

factors that may be important. For example, mate choice almost always involves some degree of signalling between conspecifics, and the efficacy of these signals is strongly dependent on, and usually highly adapted to, local environmental conditions (e.g. Badyaev and Leaf 1997; Ord and Martins 2006). Therefore, any shift in range distribution or the local environment can alter the species-specific properties of these signals. For example, increases in pollutant levels or reductions in water clarity caused by human-disturbance, such as eutrophication and sewage runoff, can cause a breakdown in visual and chemical communication in some fish species. The decreased ability to identify and assess mates has resulted in reduced ability for females to choose mates based on secondary sexual characters and, in some cases, this has caused an increase in hybridisation rates (Seehausen et al. 1997; Järvenpää and Lindström 2004; Fisher et al. 2006). Additionally, species that inhabit heterogeneous and fluctuating habitats are also known to have a large degree of plasticity in their signal production (Ord et al. 2010; Wilgers and Hebets 2011), and yet we know almost nothing about how this plasticity influences mating patterns and behaviour in novel environments, both intra- and interspecifically.

1.8 Study systems for studying mating patterns in secondary contact, and human introductions

Up until this point, I have mostly considered the mechanisms driving heterospecific mating patterns in established hybrid zones, when they have themselves been the consequence of selection following the costs associated with secondary contact. However, what happens in those early stages of secondary contact when two lineages

do not respond adaptively to one another? Indeed, this stage of the contact period probably represents the most important for understanding the consequences of secondary contact, and is certainly the stage we have the least understanding of due the rarity by which it can be observed in natural systems. Will hybridisation be so extensive that neither lineage can respond rapidly enough to offset it occurring, resulting in the formation of a single hybrid swarm? Or will it be sufficiently slow to allow the adaptive evolution of mechanisms to reduce the extent of hybridisation? These questions are very difficult to address in ancient hybrid zones. Firstly, because they represent a biased subset that is stable enough to be observed in the first place, and secondly because the mechanisms that mediate mating patterns in these zones are in themselves evolutionary consequences of hybridisation. Indeed, it is only by comparing genetic similarities between allopatric and sympatric pairs of species that we can uncover the 'ghosts' of historical hybridisation if its current rates are low (Grant et al. 2005).

However, natural observations of range expansions where the processes responsible at the initial contact phase are rare, simply because the physical processes that facilitate them occur at time scales that minimise our chances of observing them (although see Grant and Grant 2007 for a rare exception). Nevertheless, they have been occurring recently at a rate totally unprecedented in the earth's history via human introductions. Whilst introduced species have attracted most attention in terms of the conservation issues they raise (Rhymer and Simberloff 1996; Fritts and Gordon 1998; Mack et al. 2000), they also offer unparalleled examples for addressing questions regarding the causes and consequences of hybridisation in 'real-time' (e.g. Phillips et al. 2006; Herrel et al. 2008), which were previously unfathomable. For example, how do

organisms initially respond to environmental changes? How do lineages respond to one another during the early stages of secondary contact? Human introductions can provide powerful systems in which to address these questions. For example, based on theoretical expectations and the current patterns evident in hybrid zones, we might assume that genetically distinct species should rapidly form assortatively mating populations. However, an important lesson learned from human-introductions is that extensive hybridisation often occurs before barriers to these mating patterns can form, leading to near total admixture (Mooney and Cleland 2001; Muñoz-Fuentes et al. 2007; Senn and Pemberton 2009). With hindsight, such patterns are perhaps not so surprising considering neither species will have evolved adaptive mate choice strategies to offset this hybridisation in allopatry. These human introductions therefore provide fantastic opportunities to corroborate our observations of initial mechanism with observed diversity in more established systems.

1.9 The European wall lizard (*Podarcis muralis*)

Some of the most well known examples of rapid evolution come from the reptiles, and in particular lizards. These include one of the best known explosive adaptive radiations in the anoles (Losos et al. 1998; Losos 2009), extensive population-level genetic changes in the Cuban anole (*Anolis sagrei*) (Kolbe et al. 2004), and spectacular adaptive evolution in novel environments from the Italian wall lizard (*Podarcis siculus*) (Herrel et al. 2008). In addition, lizards provide useful study systems in terms of recent secondary contact since many species have been spread across the globe by human introductions, usually via plant-nursery stowaways (e.g.

Case et al. 1994) or releases from the exotic pet-trade (e.g. Schlaepfer et al. 2005). For example, the Asian house gecko (*Hemidactylus frenatus*) is now found on every continent except Antarctica as a result of its ability to hitch-hike with human global movements (Roedder et al. 2008).

My thesis concerns another successful lizard coloniser: the European wall lizard (*Podarcis muralis*). This species is a member of the large Lacertidae family, a group with ~280 species distributed extensively throughout Africa and Europe (Arnold et al. 2007). The *Podarcis* genus itself underwent rapid diversification during the Miocene to form the currently existing 19 species (Poulakakis et al. 2005), and now ranks amongst the most commonly observed reptile genera found throughout the Mediterranean. In particular, *P. muralis* ('wall lizard' from hereon) has a wide native range across central and southern Europe (Appendix 1 Figure S1). Whilst the taxonomy of this species is currently debated (Schulte 2008; Bellati et al. 2011), conservative estimates suggest it is comprised of approximately eight genetically and phenotypically distinct lineages that differentiated during the Pleistocene (Schulte et al. 2012a), partly as a result of separation in ice-age refugia (Joger et al. 2007; Giovannotti et al. 2010). Of particular interest from a secondary contact perspective, several of these lineages show starkly contrasting phenotypes and are now coming into reproductive contact throughout their native and exotic range.

The wall lizard ranks amongst the most successful colonisers of any lizard species, and certainly of any reptile species introduced into Europe outside of the Mediterranean (Kraus 2009). Currently, there are around 200 non-native populations now documented throughout Europe and North America (Brown et al. 1995; Schulte

et al. 2012a; Michaelides et al. 2013). These introductions have occurred extensively through intentional releases for "environmental enhancement" in private gardens, beach fronts or quarries (Schulte et al. 2012a). Introduced populations date back to at least the mid 1870s in Europe (references in Schulte et al. 2012a) and are particularly widespread throughout Germany and the UK, extending well north of their native range (Schulte et al. 2012a; Michaelides et al. 2013). Additionally, multiple genetically and phenotypically diverse clades are now coming into secondary contact via these introductions, and population genetics studies in Germany and the UK have shown that hybridisation between these clades is possible, and in some cases might be extensive (Schulte et al. 2012a; Schulte et al. 2012b; Michaelides et al. 2013).

In this thesis, I have focussed exclusively on the introduced UK wall lizard populations distributed across southern England (Michaelides et al. 2013). Currently, there are at least 25 viable UK populations (see Appendix 1 Figure S2 for UK distribution map) that stem predominantly from five distinct clades in the native range, mostly originating from France (*P. m. brogniardi*) and Italy (*P. m. nigriventris*) (Michaelides et al. 2013) (see Appendix 1 Figure S1 for native distribution of these two clades). Importantly, UK populations are comprised of both single or multiple clades, with on-going and predicted future secondary contact in several places. This makes the wall lizard an excellent system to address questions concerning how phenotypic and genetic divergence and diversity influence hybridisation, and its consequences for population viability and future evolutionary responses. In particular, there are stark phenotypic differences between the French and Italian lineages, most notably the dorsal and lateral colouration, which is a bright emerald green in Italian animals and a dark brown-to-grey in the French animals (Appendix 1 Figure S3), and

Italian animals are also significantly larger in many morphological characters (Appendix 1 Table S1).

Wall lizards are exclusively diurnal, small lacertids (snout-vent-length of ~55mm, and a total length of ~150mm). They exhibit a territorial polygamous mating system, where both sexes mate multiply and females exhibit high degrees of multiple paternity (Oppliger et al. 2007). Males aggressively defend delimited territories through stereotyped displays and contests, the area of which primarily depends on the presence of their major environmental resources: shelters and basking sites (Edsman 1990; Font et al. 2012; this thesis). Males also extensively scent mark their territories with specialised glands on the underside of their hind legs (femoral glands), which are thought to function primarily in both female mate assessment of high quality males (Martín et al. 2008), and male rivalry assessment (Carazo et al. 2007; Font et al. 2012). Taken together, the combination of large lineage-based phenotypic differences, strong male-male competition, the likely presence of female choice, along with a reliance on specific environmental resources, make the wall lizard an ideal species to test how environmental factors interact with phenotypic traits to influence mating patterns in secondary contact.

1.10 Thesis Aims

In this thesis I examine the roles of phenotypic divergence and environmental variation on behavioural interactions in secondary contact between two major lineages of European wall lizard; *P. m. nigriventris* (from hereon 'Italians'), and *P. m.*

brogniardi (from hereon ‘French’) in the UK. In Chapter two, I address how relative differences in sexual selection during allopatry, by comparing sexual dimorphism of traits between lineages, have led to divergent phenotypic traits known to be important in male-male competition. I then investigate to what extent these trait differences influence asymmetric outcomes in staged male territorial interactions, and finally the degree to which this translates into relative courtship frequency of reproductive females across different habitats in outdoor enclosures. In Chapter three, I use paternity and behavioural interaction data from the same ‘contact zone’ enclosure experiment used in Chapter two to create social and genetic networks to determine the extent to which the two lineages hybridise with one another in a natural scenario. I subsequently determine the relative importance of male and female choice in driving these patterns by firstly characterising the chemical signatures of males, and then determining its relative importance in driving mate choice through male and female olfactory choice trials, and finally staged mating trials. In Chapter four, I determine the behavioural and morphological factors that predict varying levels of hybridisation in the outdoor enclosures. Finally, secondary contact cannot occur without at least one lineage experiencing a shift in environmental conditions. In this Chapter, I wanted to determine whether differences in basking opportunity may influence the chemical composition of scent marks in male lizards, and whether this may have effects on female choice and the subsequent implications for signalling in a variable environment. In summary, this thesis suggests that the reproductive and competitive outcomes of secondary contact cannot be predicted from a single viewpoint, but must be approached from a holistic perspective which incorporates both morphological and behavioural lineage differences, and how these interact in a variable environment.

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CHAPTER TWO

Historical strength of sexual selection and local resource distribution mediate male-male competition during recent secondary contact

Unpublished manuscript

ABSTRACT

The outcome of species interactions upon secondary contact is often influenced by competition for mates and resources. The outcome of such competition may depend on the history of selection for traits involved in male-male competition. Furthermore, competition for resources is often strongly mediated by how those resources are structured in time and space, and so the competitive advantage of one lineage over another may vary across habitats. Here we show that two previously allopatric lineages of wall lizard (*Podarcis muralis*) that have recently come into secondary contact differ substantially in traits associated with sexual selection for male competitive ability and in their degree of sexual dimorphism. As expected, this results in the more sexually dimorphic lineage strongly dominating in staged male-male competitive interactions (the more dimorphic Italian males won all 15 staged heterospecific interactions). However, the degree of this asymmetry in male competitive ability and the extent to which it leads to greater female access depends on the spatial clustering of important resources (basking sites). This study suggests that differences in sexual selection during allopatry play a pivotal role in determining the outcome of heterospecific competition. However, the extent to which these differences are realised in naturalistic settings, with potential consequences on factors such as directional hybridisation, may depend on the structure and distribution of key environmental resources.

2.1 INTRODUCTION

Uncovering the biological and environmental factors that mediate interspecies competition is important for understanding the consequences of secondary contact. In particular, the mechanisms that mediate 'interference competition', where species engage in costly interactions over shared resources, are particularly relevant for the long-term coexistence of lineages (Grether et al. 2009). These can include processes such as aggressive displacement of competitors (Minot and Perrins 1986), interspecific-induced infanticide (Palomares and Caro 1999; Tannerfeldt et al. 2002) and predation (Fritts and Gordon 1998; Meshaka 2001; Phillips et al. 2003; Pälvi et al. 2007). This competition is usually centred on a shared resource, such as similar feeding or nest sites (e.g. Linnell and Strand 2000; Vahl et al. 2005). However, under some circumstances these 'resources' can also include reproductive partners of both lineages (Groning and Hochkirch 2008; Grether et al. 2009). This is particularly likely during the early stages of secondary contact between closely related lineages, as isolation in allopatry often means heterospecific recognition has yet to evolve. As such, factors normally important in mediating within-species intra-sexual competition for mates, such as phenotypic differences in weaponry (Emlen 2008), ornament extravagance (Møller 1987), or environmental heterogeneity (Emlen and Oring 1977; Clutton-Brock and Harvey 1978) could also play an important role in mediating the outcome of between-species competition for mates (McGowan and Davidson 1992; Wirtz 1999; Garcia-Vazquez et al. 2002).

Perhaps the major factor determining the outcome of mate competition between species is the relative degree of sexual selection experienced during allopatry. For

example, males of the recently introduced sheepshead minnow (*C. variegatus*) are both dominant over males of the native Peco pupfish (*Cyprinodon pecosensis*) and more attractive to heterospecific females (Rosenfield and Kodric-Brown 2003). Whilst the differences that were responsible for dominance could not be identified in this study, it suggests that selection for mate competition was historically higher in the sheepshead minnow. Indeed, the consequences of interspecific differences in sexual traits can have strong effects on species coexistence. For example, hybridisation between introduced and native species is considered a major conservation priority (Rhymer and Simberloff 1996), and lineages that have previously experienced enhanced sexual selection can greatly accelerate asymmetrical introgression by outcompeting or displacing heterospecific males and thereby monopolising reproductive access to (both con- and heterospecific) mating partners (Brodsky et al. 1988; Pearson and Rohwer 2000; Rosenfield and Kodric-Brown 2003).

Rather than acting in isolation, the fitness consequences of sexually selected traits also depends on their interaction with a heterogeneous environment (Cornwallis and Uller 2010). In particular, the degree to which within-species differences in competitive ability can translate into differing mating success depends heavily on the physical arrangement and structure of the habitat. For example, local population densities are often strongly determined by the spatial clustering of resources key to female reproductive success, which in turn influences the potential for dominant males to monopolise exclusive access to females (Emlen and Oring 1977; Clutton-Brock and Harvey 1978). Thus, habitat structure should also be important for inter-specific interactions upon secondary contact. Furthermore, introduced populations

often occur around human-disturbed habitats, which are often characterised by heterogeneous environments with patchily distributed resources that can facilitate high population densities (Beissinger and Osborne 1982; Brown et al. 1995). However, the role of resource distribution on competition in recent secondary contact has rarely been investigated experimentally (although see Petren and Case 1998).

We aimed to address the role of phenotypic divergence and habitat structure on competition during recent secondary contact in the European wall lizard (*Podarcis muralis*). Previously allopatric subspecies of this lizard originating from Italy, France and Germany have been coming into secondary contact over the past 120 years through introductions and escapees from the herpetocultural pet trade (Schulte et al. 2012a; Michaelides et al. 2013). For example, introductions into southern England are comprised of two major lineages hailing from northern Italy and Western France (Michaelides et al. 2013). Additionally, this species is known to colonise a wide variety of habitats, which vary greatly in local density (Barbault and Mou 1988; Brown et al. 1995), making this an ideal study system to address how resource clustering can affect inter- and intra-specific competition and reproductive interactions. Genetic analysis of these multi-lineage populations has shown that hybridisation occurs, and that hybrid populations can show greater introgresson of Italian nuclear genes compared to mitochondrial DNA (Schulte et al. 2012b). This implies that Italian males may be either dominant over the French (*P. m. brogniardi*) in obtaining access to mates, or preferred by females of both lineages. In this study we addressed the potential importance of male-male competition in driving this potential asymmetry, by determining the contribution that differences in sexual dimorphism (and thus, differences in historical sexual selection) in traits known to be important in

male dominance may play in determining the outcome of territorial interactions between the lineages. Additionally, we also investigated the degree to which these lineage-differences in male dominance translated into greater access to females in semi-natural enclosure populations, and the extent to which this is mediated by the spatial distribution of resources.

2.2 MATERIALS AND METHODS

2.2.1 Study species

The European wall lizard *Podarcis muralis* is a diurnal, small (up to 75mm snout-to-vent-length (SVL)), primarily insectivorous lacertid with a widespread geographic range stretching across Western and Southern Europe. The taxonomy of this species is debated, but is generally considered to consist of 5-8 subspecies that are variable in colouration and morphology (Mayr 1963; Schulte et al. 2012a). Anthropogenic introductions via the pet trade have resulted in multiple populations throughout the UK, Germany and the US (Brown et al. 1995; Schulte et al. 2012a; Michaelides et al. 2013). The UK is home to at least 25 viable populations, which are composed from approximately 2-3 lineages originating from across France and northern Italy (Michaelides et al. 2013). Additionally, multiple haplotypes from both French and Italian lineages are found within the same populations in the UK (Michaelides et al, 2013), and preliminary ncDNA analyses suggest that many individuals within these mixed populations are hybrids (Michaelides et al, unpubl. data). Wall lizards are sexually dimorphic, seasonal breeders where males emerge from hibernation and aggressively defend delimited territories from other males, which females then settle within (Edsman 1990; Font et al. 2012). Outcomes of male agonistic encounters are strongly influenced by both the size and residency status of animals, with larger or resident males being more dominant (Edsman 1990; Sacchi et al. 2009) (this paper). Lizards of both sexes are promiscuous, and their mating system is characterised by high levels of multiple paternity (Oppliger et al. 2007).

2.2.2 Sexual dimorphism in traits important in male-male competition

All animals in this study originated from introduced populations in southern England, previously confirmed by both mtDNA and microsatellite analysis to be from only the Italian or French subspecies (Michaelides et al. 2013; Michaelides et al, unpubl. data). During April (the beginning of the reproductive season in wall lizards) in 2010, 2011 and 2012, we caught a total of 447 lizards by noosing from fourteen populations, consisting of the Italian subspecies (AB, BB, DL, PO, SH, SW, VB, WS, WW, and VT; *P. m. nigriventris*) or French subspecies (BU, WE, CWN, WB, CWS; *P. m. brogniardi*; see Michaelides et al (2013) for details on these populations).

To indicate any historical differences in sexual selection that may have led to trait divergence, we determined lineage differences in sexual dimorphism of key traits known to be important in male-male competition in lacertid lizards. These traits were recordings of snout-vent-length (SVL), mass, head length, head width, bite force, and dorso-lateral green colouration. We additionally recorded incidences of toe loss and conspecific-induced scarring in male lizards, since conspecific induced injuries have previously been linked to between-population differences in sexual selection and male aggression in the closely related *P. siculus* (Vervust et al. 2009).

Morphological measurements

SVL, mass, head width and head length were recorded from all lizards over the three years in a standardised manner as used in many lizard studies. SVL was measured between the edge of the ventral cloacal scale to the tip of the nose to the nearest using

a 300mm plastic ruler, and head length and width using electronic callipers to the nearest 0.1mm. Head length was recorded between the tip of the nose to the most posterior edge of the occipital scale, and head width from the widest part of the head (i.e. in line with the central position of the parietal scales).

We chose four different morphological variables known to be important in lizard behavioural interactions to determine sexual dimorphism between the lineages. These traits were SVL, mass, head length and a principal component between the three (positively loaded with all three, to generate an overall proxy for 'body size'). Each of these four variables were used as a separate response in four separate linear mixed models (LMMs). For each model, we included the predictors sex, lineage and their interaction. Additionally, lizard population was included as a random effect.

In all models in this paper, we chose the best morphological predictor between SVL, mass, head length, head width and a principal component of all four ('body size') by running separate maximal models, and using the predictor whose model had the lowest AIC (Akaike Information Criterion) value (<2 from the next best model). The single principal component explained 68.1% of the data variance, and was positively loaded (>0.68) for all four variables.

Due to issues of correctly calculating significance values from models that include random effects (because of ambiguities involved with determining the denominator degrees of freedom), the significance of factors in all mixed models was analysed using likelihood-ratio-tests on models with and without the factor (Valdar et al., 2006;

Öckinger et al., 2010). Higher order interactions were removed from all models to determine the significance of the lower order terms.

Bite force

Territorial disputes of many lizard species involve using the jaws for threatening and biting opponents, and bite force is therefore strongly correlated with male dominance, and often mating success in many lizard species (e.g. Lailvaux et al. 2004; Lappin and Husak 2005). We recorded lizard bite forces from all 117 animals caught in 2012 using a specially designed bite force meter, constructed from a modified Sauter FK 25N force meter (Appendix 2 Figure S1). Bite force was recorded by encouraging a lizard to bite on two metal 'biting plates' (with insulation tape attached so lizards did not damage their teeth). One of the plates was attached to the main body of the force meter, and the other attached to the recording rod, ensuring that the two plates had a maximum gap of 1mm. Lizards biting the two plates therefore allowed their bite force to be recorded by depressing the recording rod into the meter. In all recordings the position and angle of the lizard's head was kept as similar as possible in relation to the biting plates. Three maximum bite force recordings were taken from each lizard at 30-second intervals, which were then averaged to produce the bite force for that lizard that was used in our analysis. If any of the three recordings differed by more than 50% (which in all cases we could attribute to the lizard biting on the plates using a different head position), an extra recording was taken and the outlier discarded. Additionally, lizard bite force performance is relatively body temperature dependent (Anderson et al. 2008), and so all basking lights were switched off 30 minutes before recordings so that all lizard were at room temperature (22+/- 2°C) when being

recorded (preliminary trials showed that keeping the basking lights on gave highly variable results depending on if the lizard had recently been basking). Bite force recordings showed a left-biased skew, so were square root transformed to create a normal distribution. They were then used as a response variable with head length, sex, lineage, and sex interacting with lineage as predictors in a LMM, with lizard population as a random effect.

Colouration

For the colouration analysis, we used 80 Italian lizards caught in May 2010 (40 males and 40 females), which were used in the enclosure experiment detailed below, to quantify condition dependence and sexual dimorphism in the green colouration found in this lineage. Green colouration is totally lacking in French males, and so an interspecific comparison was not possible. We photographed the dorsal and lateral sides of each lizard using a Canon EOS 350D digital camera. The photographs were then analysed in Photoshop vC4 to quantify the intensity of green colouration ('greenness') on the dorsal and lateral surfaces. Greenness was quantified by calculating the proportion of green pixels on the dorsal surface of each lizard (by dividing the green pixel count by the total RGB pixel count). We controlled for differing lighting and photographic conditions across different photos by then dividing the proportion of green pixels on the lizard by the proportion of green pixels on a green standard of an X-rite Colour-Checker chart, which was present in all photographs. In the analysis, we constructed two separate LMMs with greenness as response variables in both. In the first model we included body condition (mass/SVL),

sex and its interaction, and in the second we used SVL, sex and its interaction as the predictors. Both models also included population as a random effect.

2.2.3 Evidence of male-male competition in the wild

To determine natural levels of male-male competition, we used a dataset that included 357 male lizards caught in 2010 and 2011 from the 14 UK populations detailed above. We took the standardised morphometric readings detailed above (with the exception of bite force). We also counted the number of toes that males had lost naturally, and the number of conspecific-inflicted scars present. A characteristic "V" shape on the ventral scales and head typifies scars obtained from male-male territorial fights, which typically last for four-five weeks before healing completely (R. Heathcote, pers. obs; Figure 1), and these are easily distinguished from scars inflicted by predatory attempts. Scar number and toe loss values were used as response variables in Poisson and binomial family generalised linear mixed models (GLMMs) respectively, with lizard population as a random effect, and SVL, lineage, and time of year each lizard was captured (which allowed us to account for how scars accumulated during a reproductive season) as predictors. Date of capture was normalised across 2010 and 2011 to allow the two years to be combined ('standard days'). In all non-Gaussian family models we controlled for overdispersion by including an observation-level random effect, which models the scale parameter and thus conformity to the binomial/Poisson distribution (Elston et al. 2001).

2.2.4 Staged territorial disputes between males

We staged territorial disputes between males of both lineages to determine how their different phenotypes may translate into behavioural differences in aggression and dominance between the two lineages. We collected 64 males in April 2012 from four populations (CWS, CWN, VT, VB), transferring them the same day to indoor laboratory cages. Laboratory conditions for the lizards included shelters and basking areas, and a 12h:12h light:dark cycle using overhead room lighting and specialist reptile basking lights. All animals were fed with crickets and mealworms, and given water *ad libitum*. Males were allowed to acclimatise for one week in their 'home' cages before being used in staged territorial disputes. All staged interactions were carried out in an 800x300x400mm (length x width x height) acrylic 'trial cage' (allowing full spectrum lighting to pass through, as glass blocks out the visible UV spectrum for lizards (R. Heathcote. pers. obs.) custom made by 3 Way Displays (http://www.3waydisplays.com) (see Appendix 2 Figure S2). The trial cage had sand as a substrate, and a single basking spot in the middle, with specialist artificial lighting that created a continuous light spectrum to account for the differing visual system of the lizards (using a Lucky Reptile 'Bright Sun UV' bulb, and an Iwasaki 'Eye Arc' lamp).

Before trials began, two male lizards were placed into opposite ends of the trial cage for 15 minutes, separated by opaque dividers along with the brick shelter from their home cage, which preliminary trials showed reduced stress-indicative behaviours such as perimeter pacing and encouraged territorial behaviour during the trials. After the habituation period, the dividers were removed allowing both lizards access to the

central basking spot, and the trials were observed through a one-way-plastic film to stop any observer disturbance. All observations were carried out by one of the authors. Behaviours during trials were scored in relation to an ethogram that included seven aggressive behaviours and three submissive behaviours (although of the submissive behaviours, only 'foot shakes' were observed frequently enough to analyse quantitatively). Each behaviour was assigned a score in terms of its relative intensity following Whiting (2010) (Appendix 2 Table S2). In the conspecific trials, the aggression scores for both individuals were then summed to obtain an overall 'aggression index' for each of the trials.

The aim of the staged trials was to compare the overall aggression levels for both lineages in the conspecific trials, and to determine which lineage was dominant in the heterospecific trials, rather than determine the morphological factors that led to a lizard winning an interaction. A trial was concluded when one of the lizards 'won' three interactions in a row, which our preliminary trials suggested was the minimum number required to accurately identify the overall dominant animal. A 'win' was assigned to the animal that caused its opponent to retreat in response to a directed 'approach' (with or without a 'display'). As such, all animals were size matched to the nearest 2mm in SVL and 2g in mass to minimise any effects of body size on dominance (preliminary trials suggested that if too large a size difference existed between the two male lizards then the smaller animal would invariably retreat before meaningful behavioural observations could be taken). None of the trials resulted in animals inflicting injuries of any description to one another, and in only one Italian-Italian trial did lizards attempt to bite one another; this was quickly interrupted and the trial finished before any injuries occurred.

Aggression indices between French and Italian conspecific trials were compared in a Poisson generalised linear model with lineage, absolute difference in head length (to control for the effects of body size on the interactions), mean mass between both competitors, and the interaction between head length difference and lineage. Additionally the number of foot shakes were analysed in a Poisson generalised linear model, with the same covariates for the aggression index analysis, and an observation-level random effect to control for overdispersion. Aggression indices in the heterospecific trials were analysed in a similar way, except that the aggression indices and foot shakes were calculated for each lizard (rather than each trial, as in the conspecific trials). These were then both analysed in a Poisson GLMM with 'trial number' as a random effect, aggression index as the response variable, and head length, lineage and their interaction included as the predictors.

2.2.5 Semi-natural population experiment

To determine how the lineage based phenotypic differences detailed above translated into population-level behaviours under different environmental conditions, we set up 10 large outdoor enclosures (~7m x 7m) (Appendix 2 Figure S3) in April 2010 to house semi-natural populations of lizards to simulate a 'contact zone' between the Italian and French lizards, as might be found in the hybridising populations in the UK and Germany. Lizards populating these enclosures were caught from nine UK populations that haplotype data suggests consists of only Italian or French lineages: BU; CWN; CWS; SH; WW; VT; VB; WE and WS (see Michaelides et al (2013) for

details on these populations). All lizards again had the same morphological traits as detailed above, bar the bite force recordings. Each enclosure housed 16 lizards with an equal number of males and females. Whilst there were an equal number of Italian and French males in all enclosures, four enclosures had equal numbers of females of each lineage, and six enclosures had more Italian females (five enclosures had five Italians and three French, and one enclosure had six Italians and two French).

Central to male wall lizard territory formation and competition is the presence and quality of refuges and areas enabling thermoregulation by basking in direct sunlight (Edsman 1990; R. Heathcote, pers. obs). In each enclosure we therefore constructed nine basking resources, made from wooden pallets (each $1m^2$) and breezeblocks, providing lizards with areas to both shelter and thermoregulate. We varied the spatial distribution of these basking sites to create our experimental treatment on resource distribution ('treatment' from hereon). In five enclosures, these resources were spaced equally apart in a 3x3 arrangement, each pallet approximately 80cm apart ('dispersed treatment'), and in the other five enclosures the 3x3 pallet arrangement was clumped together into the centre of the enclosures ('clumped treatment').

Before being released, lizards were marked with yellow and white tags (made from Tesa tape) on their dorsal surface, allowing them to be individually identified. Animals were released 8th May 2013, and observed daily for 26 days. Each observation period for an enclosure lasted one hour, and started when lizards first emerged in the morning and finished when the last lizard retired to its shelter in the evening, providing an accumulated observation period of ~510 hours. During observations, we recorded all agonistic and sexually related social interactions

between individually marked animals based on an ethogram (Appendix 2 Table S4). In particular, we recorded which males were courting which females, and the outcomes of all agonistic encounters (win/lose; defined by when an individual retreated from another that was showing an agonistic display).

Throughout the enclosure experiment we also observed multiple events of male lizards spending extended periods of time associating closely with a single female (total of 71 observations from 28 males). These associations frequently lasted several hours, and occasionally over two days of observations (n=6). They typically involved the male closely following the female (often maintaining direct body contact with a limb or the head), with the pair rarely exceeding a distance of one body-length from one another. This behaviour is in stark contrast to the short associations (less than one minute) that typified other interactions, including mating behaviour. We therefore suggest this is a form of 'mate guarding', as has been recorded for other lacertids (Olsson 1993a; Galán and Price 2000). Importantly, mate guarding in other lizard species provides opportunities for males to monopolise access to females, where only the largest and most competitively superior males are able to sustain extended periods guarding females (Olsson 1993b).

To reduce any biases that could arise through observing the same enclosure at the same time of day throughout the entire experiment, a different enclosure was observed at the start of each day. The male territorial encounters allowed us to calculate dominance scores for the males, based on the modified David's score, in SOCPROG v2.3 (Whitehead 2009). To determine the effect of body size on territorial interactions in males, we ran two models with either dominance, or the overall number of territorial interactions as two different response variables. For the

dominance model, we ran a LMM with lineage, body size (based on the principal component) and their interaction as separate predictors, with enclosure as a random effect. For the total male-male territorial interactions, we ran a Poisson GLMM with the same predictors as the dominance model.

To determine whether dominance and courtship success was correlated with the dorsal green colouration in Italian males, we ran two models with either dominance (in a LMM) or total observed courtships (in a Poisson GLMM) as separate response variables, and green intensity and mass as predictors, with enclosure as a random effect. For the mate guarding analysis we used the total counts of observed mate guarding events for each male as a response variable in a Poisson GLMM with the predictors male dominance, treatment, and male lineage, with enclosure as a random effect.

In addition to the behavioural observations, we recorded the locations of all visible lizards in all enclosures every hour throughout the activity period of the animals each day. This location data allowed us to calculate the core home range size in m^2 for each lizard using Ranges v8, with 'core home ranges' determined by calculating the 50% kernel cores for each lizard, using a least squares cross-validation smoothing parameter (Powell 2000). The 50% kernel was chosen since it encompassed the majority of an animal's social interactions, and secondly because 50% kernels delimited the start of the plateau between the kernel percentage isopleth and the home range size (m^2) correlation in our lizards, indicating this area denoted where the lizards were spending a concentration of their time. From this data, we could calculate

both the total home range area, and also the number of animals that were overlapping in another individuals core home range.

Home range areas were compared between Italian and French animals over the different experimental treatments in a LMM with lineage, treatment, and their interaction as predictors, and with the individual outdoor enclosure as a random effect. Additionally, we ran a further LMM to determine differences in home range overlap. This model had the number of overlapping home ranges per individual as a response variable, with the lizard's lineage, the treatment, and their interaction as the three predictors, and enclosure as a random effect.

Finally, to determine the importance of the experimental treatment interacting with male characteristics on behavioural interactions between the two lizard lineages, we created two separate models with either the total number of courtships he was observed making, or the number of individual females he courted (both GLMMs with Poisson distribution). In each model we included the predictors treatment, male body size (based on the principal component), male lineage, and their three-way interaction, with individual enclosure as a random effect. We included both courtship response variables in separate models to account for any potential differences where males might be courting multiple females a few times or a single female multiple times.

2.3 RESULTS

2.3.1 Sexual dimorphism and evidence of male-male competition in the wild

Italian lizards were larger (based on the principal component for body size) than French lizards and showed greater sexual size dimorphism in body size (LMM: lineage*sex: $F_{1,113}$=4.06, p=0.045; lineage: $F_{1,113}$=39.06, p<0.001; sex: $F_{1,113}$=43.77, p<0.001). This difference in overall body size was largely driven by body mass and head size, with no significant difference in SVL, or sexual dimorphism in SVL, (mean+/-SE: Italian male: 6.4g+/-0.1; Italian female: 5.2g+/-0.2; French male: 4.9g+/-0.1; French female: 4.4g+/-0.2; LMM: lineage*sex: $F_{1,113}$=4.12, p<0.045; lineage: $F_{1,113}$=9.92, p<0.002; sex: $F_{1,113}$=3.10, p=0.075). There was no sexual dimorphism in SVL for either lineage, and only a marginal difference in SVL between the Italian and French lizards overall (LMM: lineage*sex: $F_{1,113}$=2.23, p=0.138; lineage: $F_{1,113}$=3.88, p=0.051; Sex: $F_{1,113}$=0.01, p=0.966).

Additionally, Italian animals were more sexually dimorphic in head length compared to the French, and Italian lizards also had larger heads overall compared to the French lizards (mean+/-SE: Italian male: 15.7mm+/-0.1; Italian female: 12.6mm+/-0.1; French male: 14.4mm+/-0.2; French female: 12.0+/-0.1; LMM: lineage*sex: $F_{1,113}$=6.06, p=0.015; lineage: $F_{1,113}$=147.87, p<0.001; sex: $F_{1,113}$=8.47, p=0.004).

Head length, lineage and sex significantly explained 78.4% of the variation in bite force (LMM: lineage*sex: $F_{1,113}$= 37.90, p<0.001; head length: $F_{1,113}$=16.45, p<0.001; lineage: $F_{1,113}$= 10.05, p=0.002; sex: $F_{1,113}$= 0.07, p=0.792; Figure 2). When head

length was kept constant, Italian lizards had significantly stronger bite forces than French lizards (LMM: $F_{1,112}$=10.07, p=0.002). However, whilst Italian lizards were sexually dimorphic in their bite forces, French lizards were not when head length was controlled for in the model (LMM: Italian male vs Italian female: $F_{1,112}$=27.50, p<0.001; French male vs French female: $F_{1,112}$=0.07, p=0.79; Figure 2).

Male Italian lizards are more intensely green on average than female Italian lizards ($F_{1,81}$=12.86, p<0.001). Furthermore, greenness increased more rapidly with both body condition and SVL in males compared to females (condition: sex*condition: $F_{1,79}$=0.152, p=0.696; condition: $F_{1,79}$=26.73, p<0.001; sex: $F_{1,79}$=1.24, p=0.268; SVL: sex*SVL: $F_{1,79}$=4.90, p=0.036; sex: $F_{1,79}$=3.40, p=0.069; SVL: $F_{1,79}$=0.06, p=0.801; Figure 3).

None of the predictors of toe loss in the natural populations reached statistical significance (GLMM: lineage*SVL: χ^2=0.43, p=0.510; lineage: χ^2=0.32, p=0.574, standard days: χ^2=0.43, p=0.808, SVL: χ^2=0.11, p=0.740). However, contrary to expectations, males from French-origin populations had significantly more fighting-induced scars than males from Italian-origin populations (Italian: 1.54 +/-0.13; French: 3.88 +/-0.39; GLMM: χ^2=4.64, p=0.031). The number of scars also declined with increasing body size in both lineages (GLMM: χ^2=9.62, p=0.002) and later in the season (GLMM: χ^2=65.90, p<0.001).

2.3.2. Staged territorial disputes between males

In the conspecific trials Italian males showed many more aggressive displays compared to French males (LMM: Lineage*head length $F_{1,26}$=<0.01, p=0.993; head length: $F_{1,26}$<0.01, p=0.970; average mass: $F_{1,26}$=0.21, p=0.649; lineage: $F_{1,26}$=18.71, p<0.001) and French lizards also displayed approximately six times more submissive 'foot-shakes' towards one another compared to Italian males (GLMM: Lineage: χ^2=24.23, p<0.001; Figure 4). As expected from the conspecific trials, Italian males had a much higher mean aggression index directed towards French males than vice versa in the heterospecific trials (Italian mean+/- SE: 13.7+/-2.7; French mean+/- SE: 3.3+/-1.3; GLMM: Lineage*head length: χ^2=3.30, p=0.069; lineage: χ^2=11.42, p<0.001; head length: χ^2=0.28, p=0.594). However, whilst French males displayed a total of 23 foot shakes compared to only 1 observed by an Italian male, this was not statistically significant (Lineage*head length: χ^2=0.01, p=0.923; lineage: χ^2=2.32, p=0.128; head length: χ^2=0.03, p=0.873), although Italian males won all 15 of the staged heterospecific trials against French males (Sign test: χ^2=60, p<0.001).

2.3.3 Male-male interaction and monopolization of females in semi-natural enclosures

There was a significant interaction between lineage and treatment in the size of lizard core home ranges (LMM: Lineage*treatment: χ^2= 5.33, p=0.021; lineage: χ^2= 6.42, p= 0.011; treatment: χ^2= 2.50, p= 0.114). This was due to French males having particularly large home ranges in the dispersed treatment (Figure 5). As a

consequence, French males also overlapped their core home ranges with more individuals compared to Italian males overall, and both lineages had more overlapping home ranges in the clumped treatment compared to the dispersed (on average nine individuals in the clumped compared to five in the dispersed; LMM: Lineage*treatment: χ^2=1.62, p=0.204; lineage: χ^2=4.17, p=0.041; treatment: χ^2=7.87, p=0.005).

Corroborating the staged territorial interactions, Italian males were strongly dominant overall compared to the French males in the outdoor enclosures, and dominance was associated with increased body size in Italian males but not in French males (LMM: Lineage*body size: χ^2=4.31, p=0.038; lineage: χ^2=0.13, p=0.714; body size: χ^2=10.46, p=0.001; Figure 6). The total number of male-male territorial interactions was the same for both lineages, and positively affected by body size (GLMM: Lineage*body size: χ^2=0.21, p=0.645; lineage: χ^2= 0.14, p=0.704; body size: χ^2=7.64, p=0.006).

Male dominance was also strongly correlated with the number of females he was observed guarding in the enclosures (GLMM: lineage: χ^2=0.34, p=0.560; treatment: χ^2=0.08, p=0.774; dominance: χ^2=12.61, p<0.001). Neither dominance (LMM: mass: χ^2=12.83, p<0.001; greenness: χ^2<0.01; p=0.947) nor courtship success (GLMM: mass: χ^2=4.31, p<0.038; greenness: χ^2=0.49; p=0.483) was affected by greenness in males.

Interestingly, both of the models on courtship success (with number of courted females and total number of courtships as the separate response variables) had significant three way interactions between male body size, treatment, and lineage

(Table 1). This was due to dominance and courtship increasing more rapidly with body size in Italian males in the clumped treatment compared to the dispersed treatment, whereas there was no effect of body size for French males (Figure 7).

2.4 DISCUSSION

French and Italian lineages have diverged substantially in terms of morphology. Italian males are heavier, with proportionally larger heads, bite forces nearly three times those of French males, and the Italian lineage is more sexually dimorphic in these traits compared to the French lineage. Italian lizards also expressed a unique sexually dimorphic trait, green dorso-lateral colouration, which correlated with male body condition and body size, implying its role as a condition-dependent signal. Differences in sexual dimorphism between lineages have been used extensively as a proxy for evaluating relative intensities of historical sexual selection (e.g. Kenward and Holm 1993; Andersson 1994; Pälvi et al. 2007). Male traits such as body size, bite force and colouration intensity are associated with dominance and reproductive success in a wide range of lizard species (Tokarz 1985; Braña 1996; Anderholm et al. 2004; Lappin and Husak 2005; Whiting et al. 2006). The positive correlation of green dorso-lateral colouration with body size in Italian males suggests this trait has the potential to act as a condition dependent signal of competitive ability, as has been shown in other lizard species (e.g. Anderholm et al. 2004). However, green intensity did not significantly correlate with either dominance or courtship success, implying that its importance as a signal may act primarily in concordance with other cues such as body size (as has been shown in other lizard species with colour signals, e.g. Thompson and Moore 1991).

We suggest that these differences in weaponry and sexual dimorphism, likely brought about through differences in the strength of intra-sexual selection for male competitive ability during allopatry, explain why Italian males were both more

aggressive than, and strongly dominant over, French males. Indeed, this dominance seemed to explain the differences in home range size between the French and Italian males over the two resource treatments. Whilst the size of home ranges did not differ between the Italian males, French males had much larger home ranges in the dispersed compared to the clumped treatment. This pattern likely arose because territorial conflicts are initiated when competitors were within approximately one meter of one another. As such, only a single male was usually found on any pallet in the dispersed basking treatment. Subordinate animals therefore had to travel much further to reach another pallet following territorial displacement in the dispersed treatment compared to the clumped. Overall, the combination of small body size, less effective weaponry, low level of intraspecific aggression, and use of a large home range are strikingly similar characteristics to males that utilise a 'floating' territorial strategy (e.g. Morrison et al. 2002; Whiting et al. 2006). Indeed, the data from the outdoor enclosures shows that whilst French males engaged in more territorial confrontations with increasing body size (similarly to large Italian males), they did not obtain any benefits in terms of increased dominance or courtship success for doing so, suggesting that sexual selection under conditions similar to our enclosures may be weak in this lineage. An intriguing question that arises from these results is whether the home range and lack of body size effects we observed in our enclosures was attributed to the presence of the dominant Italian males. The role of heterospecific interactions on intra-sexual selection seems to have received very little attention. An additional enclosure-based study with pure Italian or French populations might disentangle the degree to which the effects we observed here are the result of plasticity in response to heterospecific interactions.

Perhaps surprisingly, given that they are more aggressive and have stronger bite forces, Italian males had fewer male-inflicted scars than French males, and there was also no difference in toe loss between the lineages. This contrasts with previous studies that show natural levels of conspecific-inflicted injury rates (toe loss) are correlated with the degree of intraspecific aggression and sexual selection across different populations of a single, closely related species, *Podarcis siculus* (Vervust et al. 2009). Our results are more consistent with game-theory models on weapon evolution, which predict that weapons are used less frequently the more dangerous they become (as has assumedly occurred in the Italian males with bite force), since signalling of motivation and weapon quality become more effective to reduce the likelihood of costly escalated conflicts (Maynard Smith and Harper 2003 pp. 67). Whilst Italian males displayed more agonistic signals to one another during the staged interactions, which might suggest an increased likelihood of conflict escalation, these agonistic signals are thought to provide a central role in confrontational-motivation assessment in lizards (Edsman 1990; Mack et al. 2000). Additionally, large males from both lineages had fewer scars, which could be attributed to their dominance, and therefore a lower likelihood of engaging in costly fights. If higher levels of agonistic signalling actually correlate with reducing the likelihood of a fight occurring, then we might therefore expect large male pairs to show high aggression indices. However, there was no effect of average mass on the aggression index in the staged interactions. The negative correlation between scarring and body size may therefore be attributed to larger adult males being less likely to receive injuries by simply being bigger than their neighbouring competitors, as escalated conflicts usually only occur between similarly sized animals, where superiority may be unclear by signalling alone (Maynard Smith and Harper 2003; R. Heathcote, pers. obs). Overall, since we did not

witness any sustained fighting in the staged interactions (bar one Italian-Italian interaction which was quickly interrupted), we cannot conclusively explain these observed scarring patterns in our data. Nevertheless, these results raise questions about the use of scar and injury rates in evaluating relative levels of sexual selection between populations of lizards.

Even though Italian males are substantially superior to French males in direct territorial confrontations, our results suggest that the degree to which this superiority translates into increased access to females (via courtship success and mate guarding) depends on the spatial distribution of basking sites. In the clumped-resource enclosures, larger Italian males were able to court more females compared to smaller Italian and/or French males, whereas there was a negligible benefit of being Italian and/or large in the dispersed-resource treatment. This result is likely attributed to increased intensity of competition due to greater core home rage overlap in the clumped-resource enclosures, allowing competitively superior animals to more efficiently exclude other males from gaining mating access to females (sensu Emlen and Oring 1977).

The distribution of resources can have strong effects on the mating systems of many animal species by altering local population densities, which consequently affects the economic feasibility of small numbers of competitively superior males being able to defend and monopolise access to females. In particular, female monopolisation is facilitated in patchy, predictable and/or rare resources (Emlen and Oring 1977; Clutton-Brock and Harvey 1978), as was simulated in the ‘clumped’ habitat in our experiment. However, there have been very few studies investigating this concept

from an interspecific perspective. A previous study on geckos showed that competitive superiority of the invasive Asian house gecko (*Hemidactylus frenatus*) over the native mourning gecko (*Lepidodactylus lugubris*) was facilitated in structurally simple habitats and when food resources are clumped (in the form of insect aggregations around artificial lights) (Petren and Case 1998). The effect of habitat structure, such as resource distribution, on competition for mates should be particularly exaggerated when lineages are capable of hybridising, as frequently occurs following artificial introductions (including *P. muralis*; e.g. Muñoz-Fuentes et al. 2007; Schulte et al. 2012b). As such, when hybridisation is possible introgression may be higher in habitats where males of one lineage are strongly dominant over another. These habitat effects on relative competitive ability between closely related lineages may provide an explanation for why there is often a large degree of geographical variation in introgression levels between lineages found in 'mosaic hybrid zones' (Barton and Hewitt 1985; Sperling and Spence 1991; Senn and Pemberton 2009).

Whilst we cannot completely rule out the possibility that female choice may at least contribute to male-female interactions in the outdoor enclosures, previous work on lizards and introduced species, and in particular our own data (see Chapter three), suggests it has limited potential to explain the results. In fact, female choice is notoriously rare in lizards (Olsson and Madsen 1995; Uller and Olsson 2008). The only sensory modality where it seems to be important is in olfactory communication (Mason and Parker 2010; Martín and López 2012), although our own work suggests female wall lizards do not discriminate between males of different lineages based on olfactory cues (see Chapter three). Furthermore, the importance of female choice in

mediating interspecific matings between introduced species is unclear, as selection for heterospecific recognition and mating avoidance cannot have directly occurred between previously allopatric lineages. The disparity in competitive ability between males of the two lineages reported here is therefore likely to drive patterns of both territorial displacement and male courtship.

In summary, we have shown that differences in the history of sexual selection between two closely related lineages predict their relative competitive ability in mate and resource competition. Importantly, the extent of this dominance is habitat-dependent, such that clumped basking resources increases the ability of the more aggressive lineage to dominate heterospecifics. We suggest that habitat structure may contribute to geographical variation in introgression found in natural hybrid zones.

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Table 1) Summary statistics showing factors that determined courtship success in the outdoor enclosure experiment. Significance values for each predictor were determined using likelihood-ratio-tests between a model with and without the focal factor (see methods). Non significant terms are included at the point of their deletion from the model.

Response variable	Factors	Estimate	χ^2	p
No. females courted	Lineage*body size*Resource structure	-1.56	4.29	0.038
	Body size*Resource structure	-0.51	2.06	0.152
	Lineage*Resource structure	-0.67	0.06	0.813
	Lineage*body size	0.03	4.27	0.039
	Prop. females of same lineage	-0.41	0.11	0.745
	Treatment	0.31	0.26	0.613
	Body size	0.92	4.22	0.040
	Lineage	-0.32	2.50	0.113
Total No. courtships	Lineage*body size*Resource structure	-2.56	5.48	0.019
	Body size*Resource structure	-0.14	2.15	0.142
	Lineage*Resource structure	-1.35	0.03	0.871
	Lineage*Body size	-0.14	7.31	0.007
	Prop. females of same lineage	-0.30	0.55	0.459
	Treatment	-0.84	0.01	0.930
	Body size	-1.65	4.45	0.035
	Lineage	-0.57	3.85	0.049



Figure 1) Photograph of male wall lizard showing the characteristic scarring that results from conspecific biting during territorial disputes.

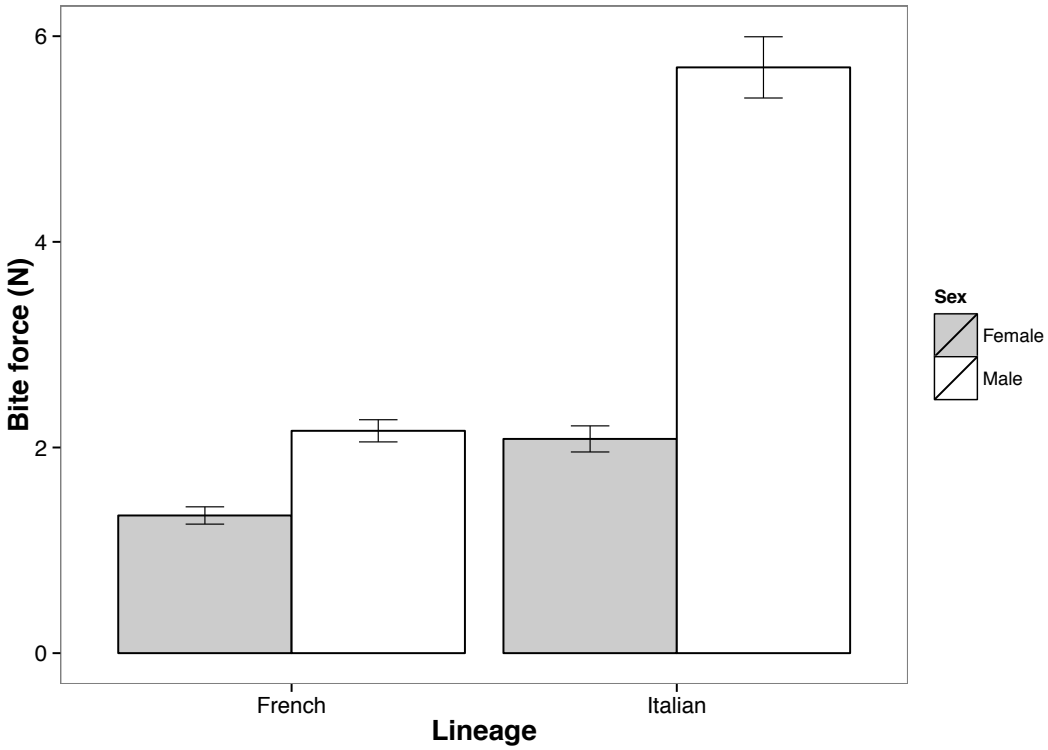


Figure 2) Average bite force (in Newtons) of male and female lizards from the French and Italian lineages. Error bars represent +/- standard error.

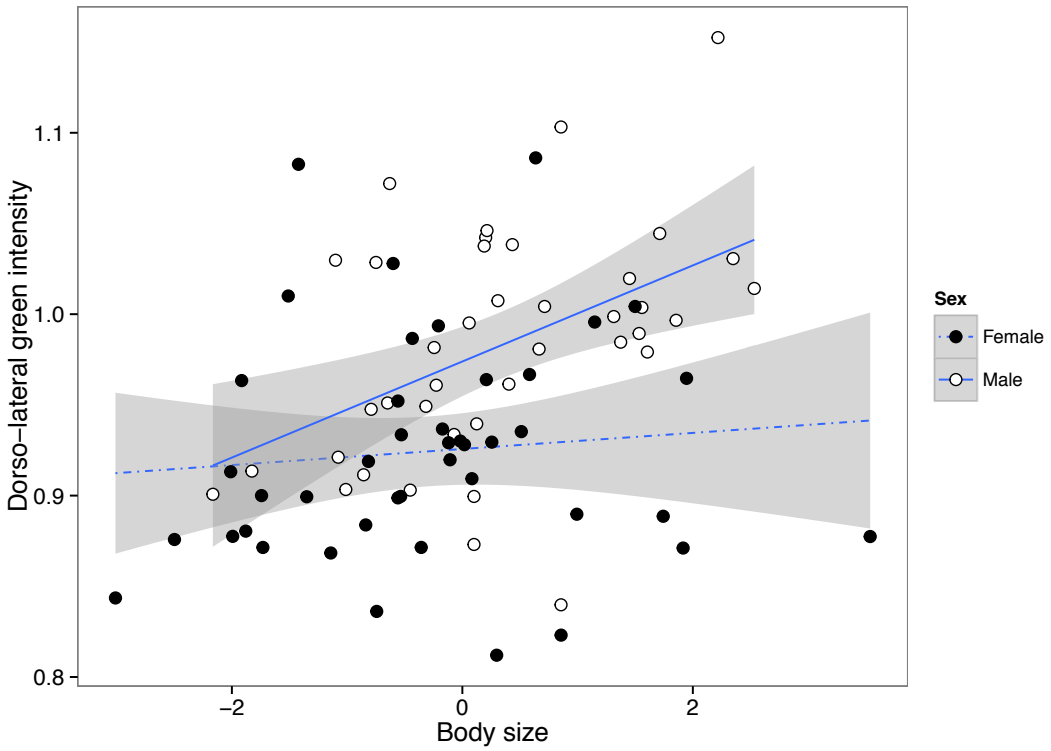


Figure 3) Correlation between body size (based on a principal component) and the intensity of green dorso-lateral colouration in Italian male and female lizards. Shaded areas represent the 95% confidence interval.

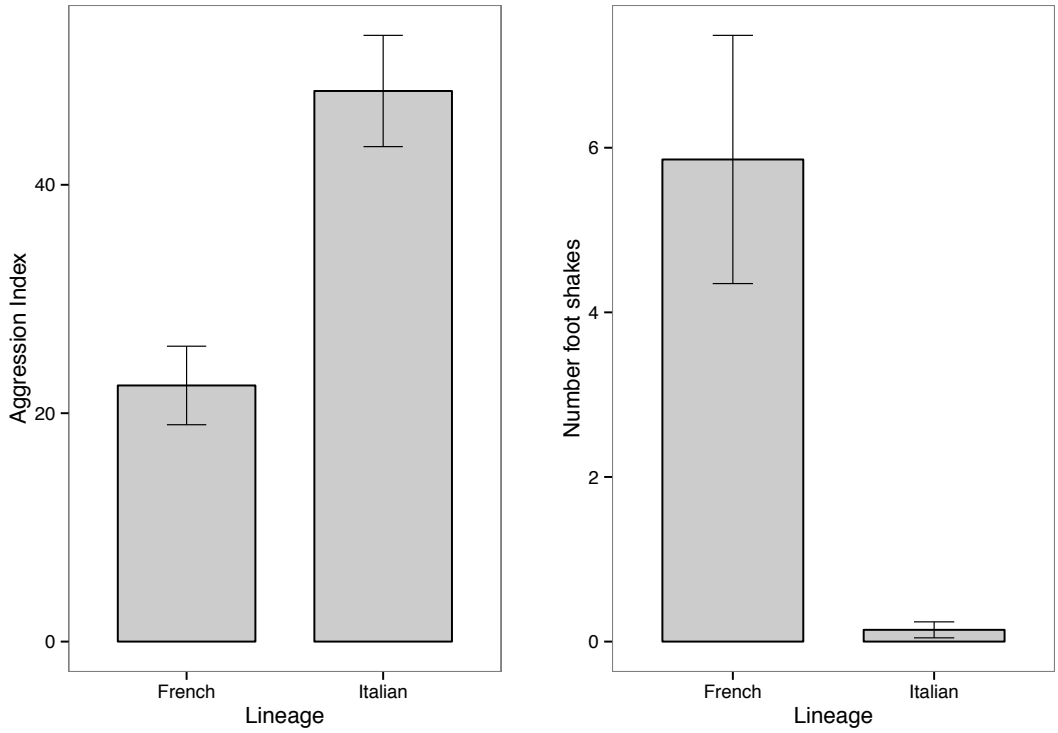


Figure 4) Aggression index (left-hand plot) and the number of 'foot shakes' (a submissive display; right-hand plot) for French and Italian males during staged conspecific territorial contests. Error bars represent +/- standard error.

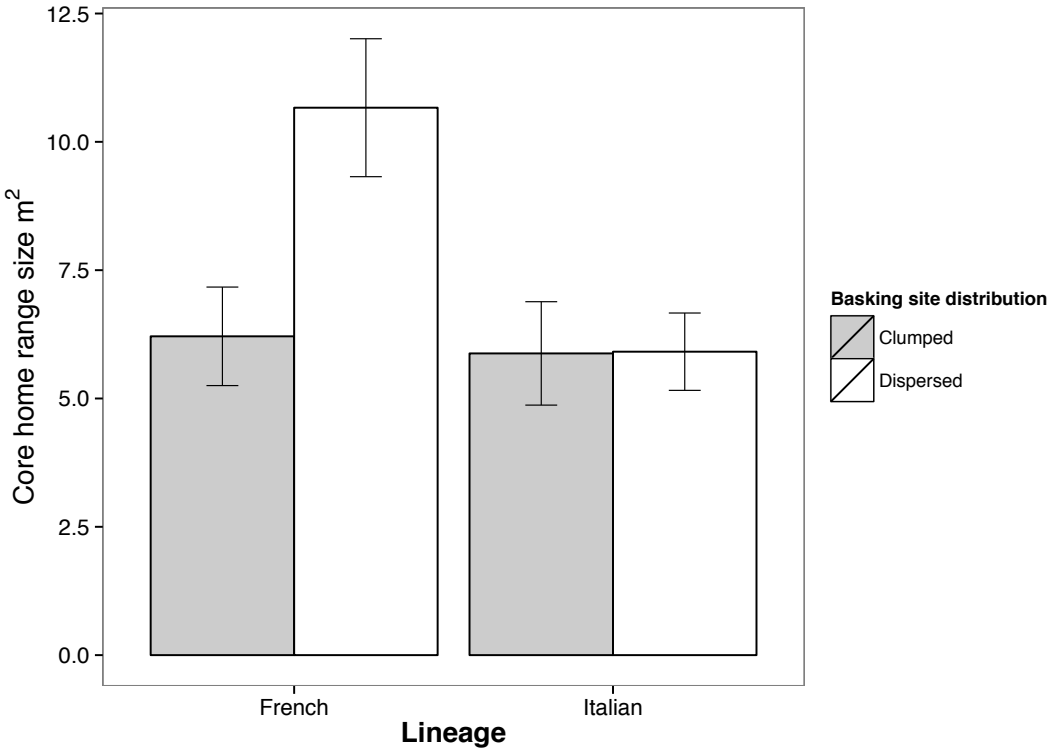


Figure 5) Core home range size for male French and Italian lizards in clumped and dispersed basking treatments. Error bars represent +/- standard error.

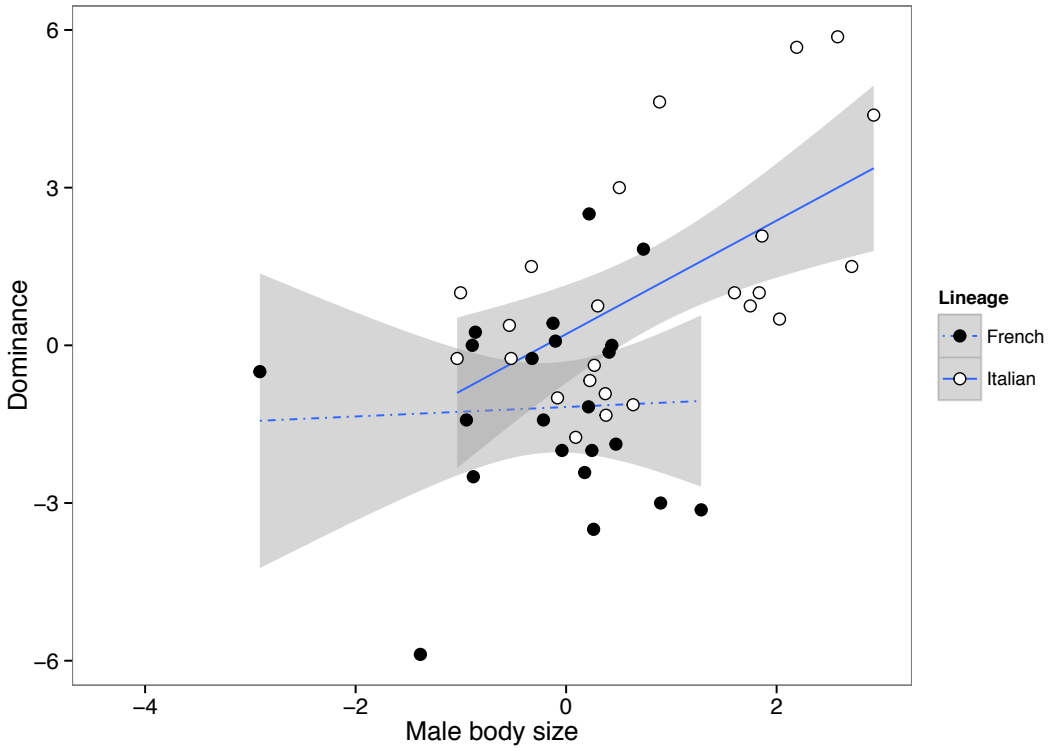


Figure 6) Interaction between male body size (based on principal component between SVL and mass) and lineage on dominance score in the naturalistic enclosures. Shaded areas represent the 95% confidence interval.

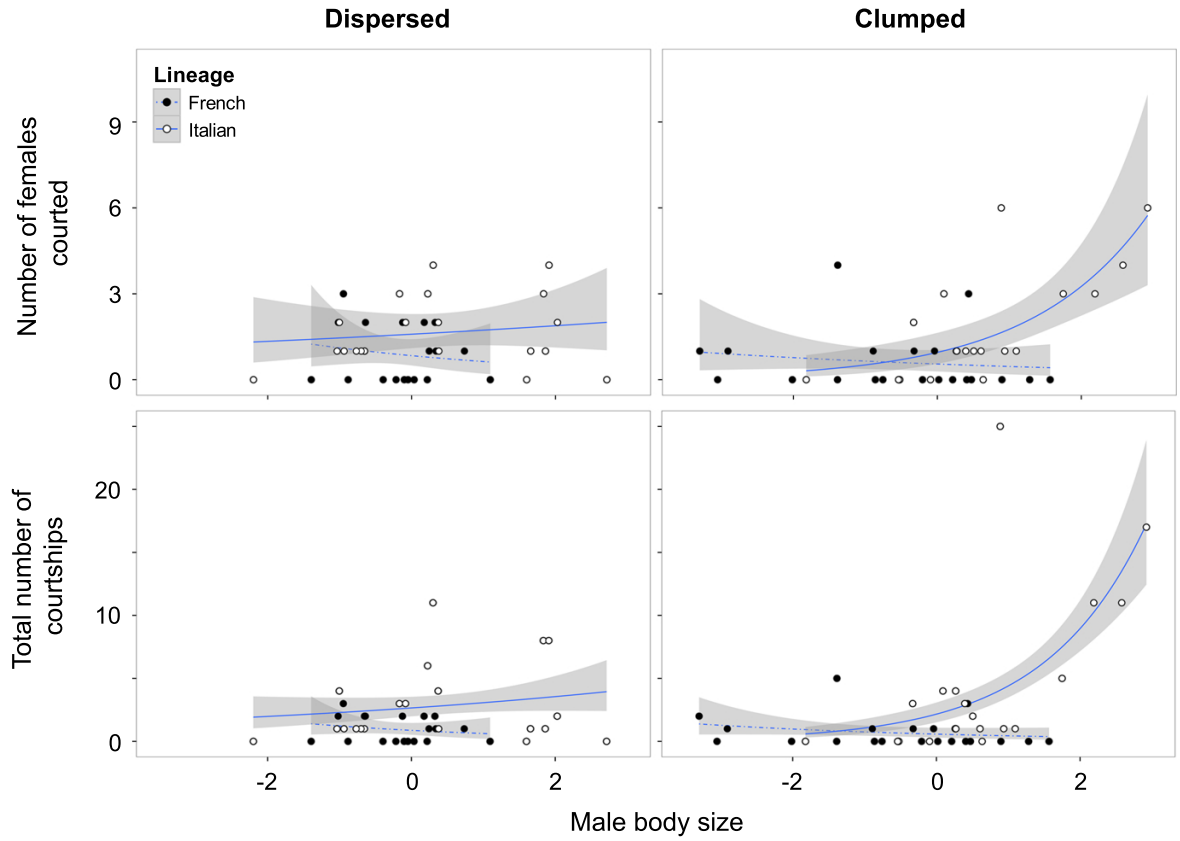


Figure 7) Effect of body size on the number of females a male was observed courting (top two figures) and the total number of courtships a male was observed making (bottom two figures) in the enclosures with dispersed (left-most figures) and clumped (right-most figures) basking sites. Shaded areas represent the 95% confidence interval.

CHAPTER THREE

Processes underlying assortative parentage during the initial stage of secondary contact in a lizard

Unpublished manuscript

ABSTRACT

The evolutionary consequences of secondary contact depend largely on the relative degree to which closely related animals hybridise. However, whilst the direction and extent of hybridisation depends on the processes that underlie behavioural interactions between individuals of different lineages, those interactions are rarely quantified, particularly in the early stages of secondary contact. Here we use an experimental approach to test the role of male-male interactions and female choice in driving patterns of assortative mating between two different lizard subspecies originating from France and Italy that have recently come into contact via human introductions. Despite substantial phenotypic divergence between lineages, there was no evidence for female choice. However, 83% of offspring produced in experimental 'contact zones' were from conspecific matings. Data from observed courtships and experimental tests of male and female responses to olfactory cues and interactions in staged mating trials suggest that this assortativity arises from two mechanisms. Firstly, dominant males preferentially monopolize large females. This creates assortative mating because Italian males are dominant over French males, and Italian females are larger than French females. Secondly, males have only a limited ability to discriminate between heterospecific females depending on their size. These results suggest that assortative paternity in secondary contact zones could arise even with no female choice and only limited preference for conspecifics.

3.1 INTRODUCTION

The mechanisms mediating the consequences of secondary contact following periods of allopatric divergence are critical determinates of biological diversity (Mayr 1963; Coyne 1992; Knowlton et al. 1993; Via 2001; Hewitt 2004; Weir and Price 2011). These consequences of secondary contact may include extensive hybridisation resulting in total admixture and thus lineage extinction (e.g. Rhymer and Simberloff 1996); the development of hybrid zones (Barton and Hewitt 1985; Barton and Hewitt 1989) and/or the rapid creation of assortatively reproducing populations following reinforcement, which represents a crucial stage in the speciation process (e.g. Mayr 1963; Grant and Grant 2009). However, elucidating the mechanisms behind these processes in the early stages of secondary contact is often difficult. Whilst we can draw tentative conclusions about some post-zygotic factors such as relative introgression levels using genetic techniques (Johnston et al. 2001; Ross and Harrison 2002; Jezkova et al. 2013), the importance of pre-zygotic mechanisms, in particular behavioural mate choice, are often lost to history. This is problematic as mate choice clearly plays a pivotal role in mediating gene flow between current sympatric species (Grant and Grant 1997; Veen 2001; Grant and Grant 2009), and is frequently the most important factor reducing hybridisation and maintaining assortative mating patterns (e.g. see references in Coyne and Orr 2004; Willis 2013). Studies of established hybrid zones may not be informative in this regard as heterospecific recognition and mate avoidance may only evolve in response to the selection associated with costly hybrid matings after secondary contact has already occurred (Brodsky et al. 1988; Wirtz 1999; Tynkkynen et al. 2004). In fact, lineages may only have a limited ability to discriminate between con- and heterospecifics during initial secondary contact, and

studies from invasive species suggest this can result in such rapid hybridisation that the genetic integrity between lineages is eradicated before heterospecific mate avoidance can evolve (Echelle and Connor 1989; Rhymer and Simberloff 1996; Huxel 1999; Muñoz-Fuentes et al. 2007).

A key challenge therefore is to determine the behavioural mechanisms that contribute to assortative reproduction in the early stages of secondary contact, in particular when direct conspecific mate choice is weak or absent. This may limit introgression enough to maintain lineage identity over at least part of the range, and allow populations to respond to selection associated with costly hybrid matings. Whilst this topic has received much theoretical attention in regards to the evolution of assortative mating and how this may create barriers to gene-flow in established sympatric populations (Kirkpatrick 2000; Ihara and Feldman 2003; Otto et al. 2008), empirical studies documenting the processes underlying assortative mating arising in recent secondary contact zones are rare (although see Grant and Grant 2009).

Contact zones created by human-introductions provide unparalleled systems to study these processes, as the initial mechanisms and processes mediating gene flow in the first stage of secondary contact can be observed 'in action' (Sax et al. 2007; Blackburn et al. 2009; Senn and Pemberton 2009). Within this theme, we studied introduced populations of the European wall lizard in the UK, which are comprised of multiple lineages introduced from throughout the native range (Michaelides et al. 2013). In particular, the UK is composed of two major subspecies, *P. m. brogniardi* and *P. m. nigriventris*, that hail from Western France and Italy respectively. The two subspecies are estimated to have diverged ~2 million years ago (Gassert et al. 2013),

and have starkly differing phenotypes, with Italian populations characterised by much greater sexual dimorphism including bright green dorsal colouration and larger male head sizes and bite forces (see Chapter 2). Accordingly, Italian males are dominant over French males during territorial interactions. We might therefore predict that any hybridisation between the lineages will be Italian-male biased. However, additional processes such as female choice and postcopulatory processes may limit the direction and extent of hybridisation. The low natural rate of hybridisation of lacertids (Carretero 2008), and well developed heterospecific recognition between sympatric species (Martín and López 2006a; Font et al. 2012) contrasts with the recorded levels of hybridisation reported between the Italian and French lineages in introduced populations (Schulte et al. 2012b). Introduced wall lizards therefore make an excellent study system for elucidating the mechanisms that mediate initial gene flow between lineages in the initial stages of secondary contact.

3.2 METHODS

3.2.1 Study species

The European wall lizard *Podarcis muralis* is a small (up to 75mm snout-to-vent-length (SVL)), primarily insectivorous diurnal lacertid that is comprised of multiple subspecies that extend across Western and Southern Europe (Mayr 1963; Schulte et al. 2012a). In addition, many non-native populations have become established in the UK and Germany via introductions through the pet-trade (Schulte et al. 2012a; Michaelides et al. 2013). The UK contains at least 25 viable populations constituting of both pure and mixed-origin animals from 2-3 lineages originating from Italy and France.

3.2.2 Outdoor enclosure experiment and behavioural analyses

This Chapter uses data from the semi-natural population experiment detailed in Chapter two, and so the setup and protocol is only mentioned here in brief for convenience. In April 2010 we created 10 outdoor enclosures (~7m × 7m) to house semi-natural populations of lizards to simulate a 'secondary contact zone' between the Italian and French lineages. Each enclosure had one of two different spatial arrangements of basking sites created from breezeblocks and wooden pallets for a concurrently running experiment (see Chapter two).

Lizards populating these enclosures were caught from nine UK populations confirmed to consist of animals from single origin (i.e., either Western France or Italy; see Michaelides et al. 2013). Each lizard had its snout-vent-length (SVL), mass, head length, and head width recorded, and a tissue sample taken (tail tip) for DNA analysis, before being placed into the enclosure. Each enclosure housed eight males and eight females with an approximately equal number of Italian and French origin animals. Males were introduced to the enclosures five-to-seven days earlier than females, simulating how males emerge from hibernation before females to establish territories (R. Heathcote pers. obs). Females were only introduced to the enclosures after they had laid their first clutch of eggs (this species lays multiple clutches per year). Because sperm storage does not occur in this species (Pellitteri-Rosa et al. 2012), this ensured that any resulting offspring would be fathered by males from the enclosure.

Prior to being released into the enclosure, each lizard was marked with circular stickers to allow individual identification. Behavioural interactions and lizard locations were recorded in each enclosure during hourly observation periods throughout the daily activity periods of the lizards for a total of 26 days. After this observational period, all lizards were captured and brought back to the indoor facilities in Oxford. Eggs laid by females were artificially incubated and tissue samples (tail-tips) were taken of all the resulting juveniles to use in the genetic analysis.

3.2.3 DNA extraction, genotyping and paternity assignment

All adults and their resulting offspring from the enclosure experiment were genotyped at nine highly variable, custom-developed microsatellite loci (primers used: C150, C168, C275-278, C164, C038, C028, C356, C109, C103; see Appendix 3 Table S1 for details of primers). DNA was first extracted using a standard ammonium acetate protocol. We used a PCR protocol where multiplex primer mixes (three multiplexes were used, with three primers in each) were added to Qiagen Mastermix, which was then added to 1µl of dry DNA and amplified in an MJ Research model PTC DNA Engine Tetrad thermal cycler PCR machine. The PCR conditions were set at 95°C for the first 15 minutes and then 35 repeated cycles of 94°C at 30 seconds, 57°C for 90 seconds, and 72°C for 60 seconds. Samples were genotyped with an ABI3730 48-well capillary sequencer using a ROX size-standard marker and alleles were categorised using Genemapper analysis software. All primers were tested for conformity with Hardy-Weinberg equilibrium. Paternity assignment (maternity was known since females laid eggs in the lab) was then undertaken with CERVUS v3.2 using 10,000 simulations, with the mother's allele conformation being set as the known parent, and all eight males she shared her enclosure with as the potential fathers. Paternity of the 296 juveniles from 61 clutches was then confirmed at >99% confidence. Overall thirteen females laid their clutch in the outdoor enclosures before we could transfer them to the lab. Seven of these clutches were subsequently recovered, for which both parents were identified in Cervus by assigning potential mothers as those females from the same enclosure who did not lay eggs in the lab.

3.2.4 Network analysis of social, genetic and spatial associations

For each enclosure, we created a 'courtship', 'genetic', and 'overlap' network using the courtship observations, parentage data, and home range overlap data detailed above. Network 'edges' (i.e. links between individuals (Croft et al. 2008)) in the courtship network were created between individuals observed in courtship behaviour. Edges for the genetic network were created between the parents of each offspring that was successfully genotyped and assigned parentage, and edges for the home range data were created between any two individual that had overlapping core home ranges.

We calculated assortativity levels for each network per enclosure between Italian and French lizards using a within-matrix Mantel test procedure using SOCPROG social analysis software (Whitehead 2009), to determine if associations within a class (in this case, lizard subspecies) are more frequent than between classes (Schnell et al. 1985). A significant assortativity level implies that lizards were interacting more frequently with their own subspecies rather than with the different subspecies. Using Mantel tests, we also examined if the courtship and home range overlap networks correlated with the genetic networks for each enclosure, which would indicate that pre-copulatory male-female behaviours predict resulting paternity patterns (which may not be the case, due to, for example, postcopulatory processes (e.g. Westneat 1987; Coltman et al. 1999)). Mantel t-statistics for the correlation between networks for each enclosure were calculated from 1000 permutation runs (determined by sequentially increasing the number of permutations until the t statistic stabilised). We assessed the overall support for a correlation between networks by combining the

results from each of the 10 enclosures using Fisher's omnibus test (Haccou and Meelis 1992).

3.2.5 Differences between males

As we have previously shown (see Chapter 2), we calculated the David's Score of dominance for 58 males that were observed in agonistic interactions. These showed that Italian males were significantly dominant over French males (χ^2=13.01, p<0.001). Indeed, out of 58 interactions observed between Italian and French males, we only observed five where the French animal won (8.6%).

3.2.6 Male mate choice determined from the enclosures

Throughout the enclosure experiment we observed multiple events (n=71) of male lizards spending extended periods of time associating closely with a single female. These associations frequently lasted several hours, and occasionally over two days of observations (n=6). These typically involved the male closely following the female (often maintaining direct body contact with a limb or the head), with the pair rarely exceeding a distance of one body-length from one another. This behaviour is in stark contrast to the short associations (less than one minute) that typified other interactions, including mating behaviour. We therefore suggest this is a form of 'mate guarding', as has been recorded for other lacertids (Olsson 1993a; Galán and Price 2000). Importantly, mate guarding in other lizard species provides opportunities for

male choice to be expressed, where only the largest and most competitively superior males are able to sustain extended periods guarding the largest and most desirable females (Olsson 1993b). To determine if there was a general assortativity by body size in this guarding behaviour, we therefore ran a LMM with a female's size (based on the first principal component of mass and SVL; eigenvectors of 0.71 for both), as a response variable, and male dominance as the predictor. Male ID was also included as a random effect as some males were observed to independently guard several females during the observation period. Additionally, we also ran a Poisson GLMM on the number of courtships a female received from males, with the female body size (based on the principal component), lineage, and their interaction as predictors.

3.2.7 Chemical signalling differences between the males

To test whether males from the different lineages differed in the chemical signatures of their scent marks, and to determine if females could discriminate these differences (and therefore potentially use this information in mate choice), we collected 30 additional males and 15 additional female lizards of each lineage (i.e. 60 males and 30 females in total) in May 2011 from seven populations of either Italian or French origin (VT, VB, CWN, CWS, WE, WS and DL; see Michaelides et al. 2013 for more details on populations). All lizards were captured by noosing, and transferred the same day to the facilities in Oxford, where they were kept individually in cages (500x300x400mm (length × width × height)). All cages contained a basking spot, two hides made from bricks, and sand as substrate. Lighting and heat was provided by a 60W spot bulb placed over a brick on one end of the cage to provide a surface thermal

gradient from 45°C to 22°C), an EXO-TERRA^{TM} 10.0 UVB fluorescent tube, and overhead fluorescent room lighting. All lizards were provided with food (live crickets and mealworms) and water *ad libitum*. Animals were sexed by the presence of sexually dimorphic characters such as larger heads and the presence of femoral pores and hemipenal bulges in males. Females were carrying eggs from their first clutch of the season at the time of capture, which they later laid in the laboratory (see below).

Each of the females was assigned a pair of males, one from each subspecies, which were matched to within 3mm SVL of each other. Because body size is a strong determinant of the chemical composition of male scent marks, and consequently of female behavioural responses (see Chapter 5), we ensured that equal numbers of trios had either the French or Italian male as being larger.

Samples of femoral pore secretions were taken within 2 days of capture. Scent mark secretions were collected by gently squeezing the femoral pores of males using sterilised forceps. Samples were then stored in sterile glass containers at -20°C until chemical analysis. Samples were dissolved into 200µL of pentane, and the lipophilic compounds within were then analysed by gas chromatography-mass spectrometry (GC-MS) by injecting 3µL of the dissolved femoral secretion into an Agilent Technologies 7890A gas chromatograph (capillary column: Agilent HP-5MS, 30m x 250µm x 0.25µm; split-splitless injector; carrying helium gas at 1ml/min) coupled with an Agilent 5975 mass spectrometer operated at 70 eV in the electron impact ionization mode. The temperature programme started at 50°C for one minute, increased to 180°C at 30°C/min, then to 250°C at 10°C/min and finally to 320°C at 5°C/min and kept for 5 minutes. The areas of GC peaks were integrated using the

Agilent Chemstation software, and the relative compound levels were then calculated with Aitchison's formula: $Zij = \ln(Yij / g(Yj))$, where *Zij* is the standardized peak area (*i*) for individual (*j*), *Yij* is the peak area *i* for individual *j* and $g(Yj)$ is the geometric mean of all peaks for individual *j* (Aitchison, 1986; Dietemann et al., 2003). Relative rather than absolute compound levels were used because synergistic chemical composition of pheromone cues is known to be particularly important in olfactory communication (Wyatt, 2003). Identification of compounds was made by initial comparison to the mass spectral library in NIST 2008, and confirmed by comparison to pure synthetic compounds obtained from Sigma-Aldrich (Milwaukee, WI, USA). Compounds that could not be identified are reported by their characteristic retention time and distinctive ion current signature.

A principal component analysis was done on the normalised peak area from compounds shared by all individuals. Samples that showed signs of contamination were excluded from the analysis. The first seven PCs, which together explained 87% of total chemical variation, included the main compounds previously shown to be important in lizard olfactory communication (Mason and Parker, 2010; see below).

We therefore used these seven principal components to determine the overall difference in chemical signature between the two subspecies of males by running a MANCOVA with lineage and body size (SVL) as the two explanatory variables. After obtaining a significant effect in the MANCOVAs, we did further 'Protected ANCOVAs' on each principal component as a response variable, with the same predictors as the preceding MANCOVA. Additionally, there was a single compound

(α-tocopherol) that differed in its presence/abundance likelihood between the lineages, for which we analysed using a sign test.

3.2.8 Female and male olfactory discrimination and mate choice

Behavioural trials were started between three and seven days after a female laid her first clutch of eggs. This corresponds to the timing of matings under these housing conditions and hence maximised the likelihood that females were receptive (R. Heathcote, G. While & T. Uller, pers. obs.). Each female was grouped with two males of different lineages to make a unique trio used throughout the olfactory and mating trials. Each trio underwent three different behavioural trials with her pair of males to determine firstly whether females could discriminate between the different subspecies (using a tongue flick protocol). Secondly, we determined whether these females also preferred to associate and settle within the territories of males from either subspecies. Lastly, we ran staged mating trials to determine whether courtship and mating patterns were affected by the different male-female lineage combinations.

3.2.8.1 Female tongue flick trials

We quantified a female's ability to discriminate between the olfactory cues of the two different males by counting the number of 'tongue flicks' directed at scented cotton-wool buds (Cooper 1994). Olfactory stimuli in actively foraging squamates are predominantly received through the Jacobson's organ, a specialised vomeronasal organ that opens into the roof of the mouth, transferred from a notched or forked

tongue via tongue flicking (Cooper, 1994). Quantifying 'tongue flicks' directed at different stimuli has been extensively used as a means of determining the level of discrimination and preference for food and mates (e.g. Labra, 2011; Runemark et al., 2011; reviewed in Mason and Parker, 2010). However, it is disputable whether the number of tongue flicks alone actually can be interpreted as 'preference', and we therefore restrict our interpretation of any differences only in terms of whether or not females are able to discriminate between olfactory stimulation.

Scent was collected from males in a standardised manner by rubbing a sterile cotton bud along the length of the femoral pores of one leg, which ensured approximately equal amounts of secretion were collected. These cotton buds were then immediately presented to females during the tongue flicking trials.

Each female underwent a separate tongue flicking trial for each of the two males at the same time of day on two successive days. Pilot experiments showed that this time period between trials was necessary to reduce the effect of female habituation to the scented cotton buds, and that presenting two cotton buds simultaneously resulted in females ignoring the second swab she encountered. The presentation order of the two treatments was randomised for each female, and all trials were done in the female's own cage, which was covered with one-way plastic film to eliminate observer disturbances (see Appendix 3 Figure S1). Each male-scented cotton bud was presented simultaneously with a clean cotton bud (5cm apart), which acted as a control to ensure that females were tongue flicking due to a male's scent mark rather than simply because of the cotton bud itself. Both cotton buds were attached to a 30cm long wooden 'kebab stick' which allowed placing them onto the brick under the

basking spot without disturbing the female, and removed any potential effects of an experimenter encouraging additional tongue flicks by moving the cotton bud once a female had seen it. The number of tongue flicks directed to each cotton bud was then counted over a 30-minute trial by an observer who was unaware of male lineage.

The difference in tongue-flicks towards the control and scented cotton buds was compared using a generalized linear mixed model (GLMM; to account for female as a random effect in tongue flick response), with a Poisson distribution, which confirmed that females responded much more strongly towards the scented buds (14.9 ± 1.7 (mean ± SE) tongue flicks per scented cotton bud vs 3.7 ± 0.8 for the control; LRT: χ^2=51.62, p<0.001). The total number of tongue flicks directed towards the scented cotton buds was then used as a response variable in generalized linear mixed models with Poisson distributions, with the predictors male SVL, male-female lineage combination (i.e. a factor with four levels), trial order (i.e. 1st vs 2nd), and an interaction between male SVL and lineage combination. Female ID was included as a random effect.

3.2.8.2 Female settlement trials

Trials testing female association preference were run two days after a female completed the tongue flick trials. All trials were run in the outdoor enclosures detailed above in the population-level experiment. Each enclosure had six basking sites, created from wooden pallets and breezeblocks. The bricks, sand and hides that were present in the cage of the same pair of males whose scent was presented to a particular female during the tongue flicking trials were used to artificially scent-mark

the different basking sites in the outdoor enclosures. Each male was randomly allocated to scent two basking sites, leaving the last two basking sites as unscented controls (which had sterile sand, bricks and slates added). A single female was then added to an enclosure, and her location was recorded every hour between her first emergence until she retired in the evening for four days, at which point she was captured and placed back in the lab. The total number of observations a female spent on each pallet was then pooled together and analysed with a GLMM, with a binomial distribution, with preferred basking site (i.e. Italian-scented, French scented or control) as a predictor.

3.2.8.3 Staged mating trials

Finally, after the two olfactory trials, each trio was used in a staged mating experiment. Only a single male was introduced at a time, and the order that males were introduced in was randomised. All trials were done in clean cages identical in dimensions to those used to house the females. Each cage had a basking rock and shelter at both ends and a divider in the middle. Males and females were introduced into the enclosure and allowed to acclimatise for 20 minutes. The divider was then lifted and the trial commenced. Trials ran for 60 minutes per male, during which time we recorded whether a male courted a female or not, whether the pair mated, the latency to mate and the duration of mating. The cages were covered with one way plastic so that behavioural observations could be undertaken without disturbing the focal animals.

We created three response variables (court or not, mate or not, mating length) that were used in three separate models with the lineage combination, female size (based on the principal component between SVL and mass), and their interaction.

3.2.8.4 Male tongue flicks

To determine the possibility that male choice based on olfactory cues might contribute to the observed mating patterns we recorded from the enclosure experiment, we conducted a male olfactory discrimination experiment, following the protocol developed for females. We captured 30 females and 15 males of both lineages from four populations (CWS, CWN, VB and VT) in May 2012, and brought them into the lab. Three days after females had laid their first clutch of eggs, we took standardised swabs along the posterior dorsal and lateral sides using sterile cotton-buds. These were then stored in a -80°C freezer until they were used in the male behavioural trials. Each male was then presented with the scent of a female in a similar protocol to the female tongue-flicking trials. However, because males (unlike females) seemed to rapidly habituate to the scented buds, where subsequent encounters after the initial exposure only elicited very few tongue flicks (between one and five tongue flicks), we only used the tongue flicks from the first encounter with the cotton bud in our analysis. To determine the effect of female body size on male tongue flicks we used the first principal component between female SVL and mass, which was positively loaded with both (eigenvectors of 0.71 for SVL and mass), and explained 89% of the total variation. Female body size, male-female lineage combination (as for the female tongue flicks), trial number, and the three-way

interaction were included as predictors in a Poisson GLMM with tongue flicks as the response variable.

In the maximal model, there was a highly significant three way-interaction between trial number, female body size and the male-female lineage combination in the tongue flick trials (χ^2=18.02, p<0.001), which was due to males habituating to the cotton swabs between the first and second trials (swabs presented first were tongue-flicked twice as often as the swabs presented second (First swab mean: 41.9±5.6, second swab mean: 27.8±3.7). To try and eliminate this habituation effect as much as possible (i.e. to obtain results that are biologically meaningful rather than artefacts of our experimental design), we therefore restricted our analysis to the male tongue-flicking responses of the first presented swabs.

3.2.9 General statistical protocol

In all analyses using Poisson and binomial responses distributions, we controlled for overdispersion in our data by including an observation-level random effect, allowing the scale parameter to be correctly modelled to validate the Poisson distribution (Elston et al. 2001). All models were simplified using a stepwise deletion of non-significant terms, where the significance of each term was determined using likelihood-ratio-tests on models with and without the factor (Valdar et al., 2006; Öckinger et al., 2010). Statistical values for non-significant terms are reported as they appear at their point of deletion.

3.3 RESULT

3.3.1 Mating and courtship assortativity

The genetic networks created from the paternity assignment showed that parentage was overall strongly assortative towards the same lineage (Fisher's omnibus test: df=20, χ^2= 88.61, P<0.001; Figure 1a). Overall, 53% (32 of 61) of clutches and 83% (247 of 296) of offspring were sired by conspecific males, with slight, but not statistically significant differences in the conspecific paternity rate between the subspecies (French: 12 of 28 (43%) clutches and 96 of 123 (78%) offspring; Italian: 20 of 33 (60%) of clutches and 151 of 173 (87%) offspring; binomial GLMM clutches: χ^2=1.92, p=0.166; offspring: χ^2=2.58, p=0.108)). Italian females also had slightly larger, but not significantly different, clutches compared to French females (Italian versus French clutch size (mean+/- standard deviation): 6.6+/-1.59 versus 6.1+/-2.20; t test: t=0.25, df=65, p=0.252).

The networks created from our behavioural observations showed that this assortativity was also reflected in the observed courtships (Fisher's omnibus test: df=20, χ^2=35.51, p=0.018; Figure 1b), which were strongly correlated with the genetic networks (Fisher's omnibus test: df=20, χ^2=55.66, p<0.001). Additionally, female body size significantly explained the number of courtships received, whereas lineage, and its interaction with body size, was non-significant (lineage*female body size: χ^2=0.08, p=0.773, female body size: χ^2=4.42, p=0.036; lineage: χ^2=1.11, p=0.292; Figure 2). The overlap networks (calculated from the home range data) in contrast were not

assortative (χ^2=18.77, df=20, p=0.536), nor were they correlated with the genetic networks (χ^2=14.45, df=20, p=0.807).

Our observations of mate guarding correlated closely with male paternity: 49 out of 71 observed 'mate guardings' (69%) resulted in a male siring some of the female's offspring. Additionally, 62 (87%) of these observations were by Italian males. There was a strong positive effect of a male's dominance score on the body size of the female he was observed guarding (χ^2= 38.32, p<0.001).

3.3.2 Chemical profiles of male femoral secretions

We characterised 28 different lipophilic compounds from all the male lizards, of which 15 compounds were shared by all individuals (see Appendix 3 Table S1 for chemical compounds and S2 for eigenvector loadings). These shared compounds were collapsed into seven principal components, which together explained 87% of the total variance, as response variables in a MANOVA to determine if there were any differences in male lineage or population in determining his chemical signature.

Both lineage and population (nested within lineage) had significant effects on the overall chemical composition of femoral secretions of male lizards (MANCOVA; lineage: $F_{7,44}$=22.75, p<0.001; Population: $F_{35,240}$=1.05, p=0.005). Protected ANOVAs show that population significantly explained PC2 and PC5, whereas lineage significantly explained PC1, with Italian males having considerably lower values for PC1 (see Appendix 3 Table S3 for summary statistics). PC1 had strong positive

loadings with cholesterol, hexedecanal, heptadecene, and octodecanoic acid, and strong negative loadings with squalene, cholecalciferol (provitamin D) and campesterol (Appendix 3 Table S2). Additionally, French and Italian males differed strongly in the presence of α-tocopherol (vitamin E), where only one French male was recorded producing the compound, whereas only one Italian male was found to not have it (for both French and Italian animals; sign test: $p<0.001$).

3.3.3 Female discrimination and choice analysis

In the female tongue flick results, there was no effect of male lineage ($\chi^2=3.61$, $p=0.306$), male body size ($\chi^2=1.94$, $p=0.164$) or their interaction ($\chi^2=3.70$, $p=0.354$). In the settlement trials, females preferred to settle on male-scented pallets over the controls ($\chi^2=13.30$, $p=0.02$), but showed no preference for visiting a pallet scent-marked by males of either lineage (Post-hoc Tukey's test: French females: $Z=1.54$, $p=0.12$; Italian females: $Z=0.77$, $p=0.44$), or any preference for a male based on body size ($\chi^2=1.90$, $p=0.17$).

3.3.4 Male olfactory discrimination

In the male tongue flick analysis there was a significant two-way interaction between lineage combination (male-female origin) and female body size on the number of tongue flicks ($\chi^2=10.71$, $p=0.003$; Appendix 3 Table S4). Males of both origins tongue flicked less for larger females, with the exception for French males that tongue

flicked more for larger French females. Additionally, whilst there was no difference in the slopes on tongue flicks with body size between the different female lineages for Italian males, there was a significant difference in French males (Appendix 3 Table S5; Figure 3).

3.3.5 Staged mating trials

Overall, there was no effect of lineage combination or body size of females on the total number of courtships observed during a trial or whether the pair mated or not. However, there was a borderline significant effect for female body size and lineage on whether a male courted her or not (Appendix 3 Table S6). Larger females were more likely to be courted than smaller females, and there was a borderline effect of Italian males being more likely to court Italian females than French females (Appendix 3 Table S7). Additionally, there was an overall difference in the lineage combination on the mating duration (Appendix 3 Table S6), which was due to Italian males mating with French females for longer than they mated with Italian females (Appendix 3 Table S8).

4.1 DISCUSSION

We have shown that strong assortative mating and parentage can occur upon first contact between two normally allopatric wall lizard subspecies. Such strong assortativity is perhaps surprising considering that males of the Italian subspecies are almost totally dominant over males of the French subspecies (see Chapter 2), and populations that diverge in allopatry are unlikely to show strong immediate heterospecific mating avoidance following secondary contact (Wirtz 1999; Rundle 2002). In fact, our data suggests that this difference in male competitive ability actually contributes to assortative breeding by enabling Italian males almost exclusive access to the larger, and hence more attractive, Italian female lizards. Additionally, males of both lineages appear to discriminate between certain sized females of the two lineages using olfactory cues, and Italian males show some evidence of preferring conspecific females even when the overall size difference is taken into account, which may contribute to assortative courtship and hence paternity. In contrast, female choice does not appear to contribute to patterns of paternity, despite the substantial phenotypic differences between males of the two origins.

The prevailing view of how assortative mating arises in secondary contact zones suggests female mate choice should play a central role, due to their greater per capita investment, and hence potential costs, in reproduction (Randler 2002). Although female choice is poorly documented in lizards in general, it seems to occur in lacertids based on characteristics in male scent marks (Martín and Lopez 2000; Martín and López 2006b; Mason and Parker 2010; although see Font et al. 2012). We therefore attempted to establish whether females could discriminate between femoral secretions

from males of different lineages and, if so, whether they preferred to associate with males of the same subspecies. The potential for this discrimination is certainly possible, since the lineages differed considerably in the composition of their scent marks. For example, α-tocopherol (vitamin E) was found in almost all Italian male femoral secretions but absent in all but one French male, and the two lineages differed significantly overall in the relative proportions of their shared chemicals. However, even though previous studies have suggested female lacertids are remarkably adept at discriminating between male phenotypes based on their scent marks (e.g. Lopez et al. 2003; López and Martín 2005), we found no evidence that the females of either lineage could discriminate between, or showed any preference for, either male subspecies. This complies with Font et al (2012), who showed females of two closely related species, *Podarcis hispanica* and *P. bocagei,* also did not discriminate between males of different species based on their olfactory cues. Additionally, there is no empirical evidence from either previous studies, or our own data, on female choice being involved in courtship behaviour. The lack of assortativity in the home range networks also negates the possibility that females may have been practicing mate conspecific mate choice by establishing a home range near to a preferred male. Overall therefore, our data provides no evidence for precopulatory female choice contributing to the assortative mating and parentage patterns we have shown in this study.

In contrast, male lizards seemed capable of discriminating between different sized females based on olfactory cues alone. Additionally, they may be able to at least partly discriminate between females from different lineages as tongue flick rates differed at certain size ranges. Whilst Italian males show differences in tongue flicks

between the two lineages when females are smaller, French males showed the opposite effect. In contrast to previous studies that assume a positive relationship between tongue-flick rates and the degree of mate choice (Labra 2011; Runemark et al. 2011), these results show an overall negative correlation between tongue flicking and female size, and yet our enclosure and staged mating-trial results strongly suggest that males in fact prefer larger females. Based on these results, we reiterate our previously argued stance that interpretations for differences in tongue-flick rates to olfactory stimuli should be limited to discrimination ability. Whilst this may potentially contribute to mate choice (since mate choice cannot occur without mate discrimination), tongue flick differences alone should not be assumed to automatically correlate with mate preference (see Chapter 5)

The ability of males to discriminate between different sized females, and potentially different lineages, may therefore have contributed to the differences in male mate choice we showed in our experiments. Our staged mating trials show that Italian males were more likely to court larger females, and a tendency to court Italian females over French females, although they showed no preference in the likelihood of mating or mating frequency. Male preference for larger, and therefore usually more fecund, females has been shown previously in several lizard species (Olsson 1993b; Whiting and Bateman 1999). Indeed, this generic preference for larger females, and potentially Italian male preference for courting conspecifics, based on the staged mating trials, appear to be the only aspects of mate choice that contribute to the assortative mating patterns we report here. Female choice in many species is often largely responsible for heterospecific mating avoidance as the greater per-capita investment females normally invest into each offspring make ‘incorrect’ mating

decisions (such as those that result in hybrid offspring with lower fitness) more costly relative to males (Parker and Partridge 1998; Wirtz 1999). Strong male choice is mostly expected when males experience high costs of mating (e.g. through predation risk), where mating opportunities are more limited, or if males show paternal care (Albert and Schluter 2004; Peterson et al. 2005; Svensson et al. 2007; Zoppoth et al. 2013). Additionally, it has been previously argued that male choice may be just as important in determining assortative mating patterns in secondary contact zones since there has been no previous selection for direct heterospecific recognition (Servedio 2007). Furthermore, previous authors have argued that male, rather than female, choice may be more important in mating patterns in lacertids (Olsson 1993a; Font et al. 2012). Whilst the biological reasons for a general lack of pre-copulatory female choice in lizards remain largely unknown, our study emphasises that this lack of choice may be significant for understanding the dynamics of mating patterns during secondary contact in this taxa.

Even though both male lineages preferred larger females, the competitive superiority of Italian males resulted in their monopolising mating access to the larger Italian females in the outdoor enclosures. This is particularly evident in our data on mate guarding, which showed that more dominant males guarded the largest females. This is likely because large males are able to sustain longer periods defeating rivals. Indeed, since mate guarding was mostly practiced by Italian males, this may provide an important contributing mechanism of assortative mating and paternity in this system. In the closely related sand lizard (*Lacerta agilis*), this size-based dominance in males has been shown to be particularly important in the size assortative mating observed during mate-guarding (Olsson 1993b; Olsson 1993a). In our system this

would ultimately ensure that the more dominant Italian males tended to mate with and court the generally larger Italian females.

The strength of assortative mating through the mechanisms we report here should be strongly dependent on the relative overlap in sizes and dominance differences between the two lineages; as phenotype overlap increases, assortativity should decrease. However, even a small degree of conspecific mate choice (as shown by the Italian males, and possibly French males since they were also able to discriminate between females) should contribute to the greater-than expected assortativity compared to the overlapping size-range between our lineages. However, it must be stressed that the assortative mating patterns in this study are the result of the first breeding season in a 'simulated' contact zone between these two lineages. Any hybridisation that subsequently occurs will likely degrade the morphological and behavioural disparity between the subspecies that seems critical for sustaining their assortative mating patterns. This suggests that the extent to which the mechanisms described here can contribute to long-term reduced gene flow will depend on both the degree of initial phenotypic divergence between the lineages, and the rapidity of reinforcement before the critical phenotypic differences between the lineages become blurred through hybridisation.

As argued elsewhere (see Chapter 2; Rice 1998; Tregenza et al. 2000), a likely reason for this initial divergence to occur is via differences in sexual selection in allopatry. The exposure to differing environments that occurs when a population splits via geographical boundaries will inevitably give rise to differing selection pressures, including sexual selection (Mayr 1963; Emlen and Oring 1977; Lande 1980; West-Eberhard 1983; Coyne 1992; Boughman 2001). This can give rise to the coupling of

male and female traits in ways that enhance assortativity even in the absence of preference. Most notably, sexual selection on male body size could lead to a correlated response in females (Hedrick and Témeles 1989). Assuming there is generic male choice for larger females across lineages, heterospecific recognition and mating avoidance may not be necessary to create assortative mating following secondary contact as competitively superior males will monopolise access to the larger females that happen to also be conspecifics.

In general, reinforcement following secondary contact is favoured when post-zygotic isolation is strong, mate recognition is well developed, and there is strong linkage between mate choice and the genes responsible for affecting hybrid fitness (Liou and Price 1994). Without direct mate choice for conspecifics, the likelihood of assortative mating being reinforced in this system before introgression becomes too extensive will require at least some costs associated with hybridisation, for which we currently have little information in our system. If the costs of hybridisation are high, such as hybrid progeny having particularly low fitness, then reinforcement should be rapid, which without the presence of heterospecific mate avoidance (although there does appear to be some level of female lineage recognition by males) will exaggerate the phenotypic differences in the two lineages. Either way, it is likely that the mechanisms we report here will require at least some level of post-copulatory mating costs to contribute to the reinforcement of assortative mating, and ultimately increase the potential for two populations to become reproductively isolated.

In summary, our data suggests a novel mechanism by which assortative mating between two previously allopatric clades can occur without strong heterospecific mate avoidance. Currently we can only speculate about the importance that an interplay

between correlated male competitive ability and female attraction can create long-term sustained assortative mating, as we have only provided data on the initial phase of a simulated secondary contact event. However, we suggest this is a formative step in identifying the mechanisms that may reduce rapid introgression during the early stages of secondary contact when heterospecific mate avoidance may not have evolved.

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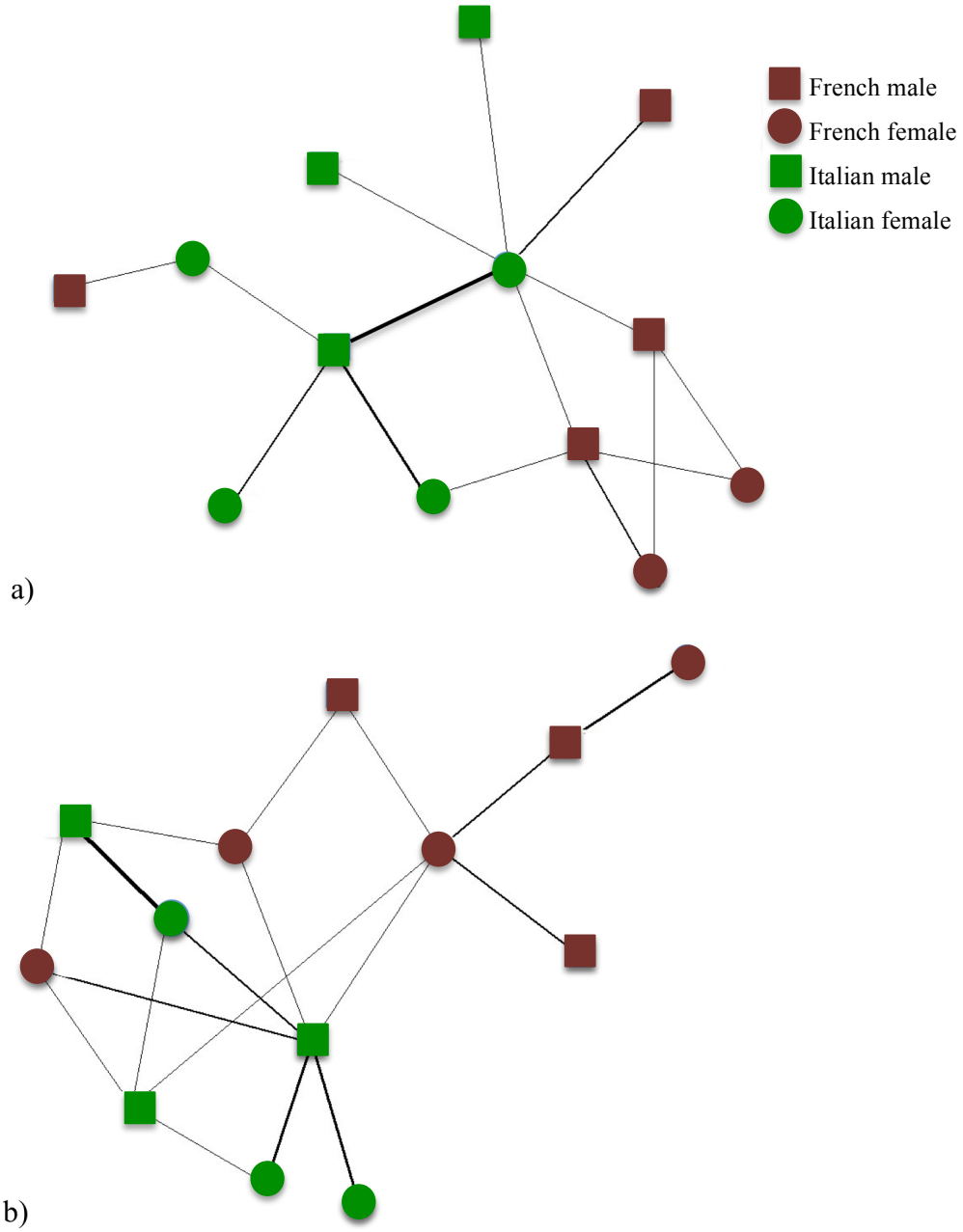


Figure 1) Example networks of male-female courtships (1a) and the parents of offspring sired in the outdoor enclosures (1b), both from the same enclosure. Line thickness denotes the number of interactions (1a) or offspring (1b) between two individuals.

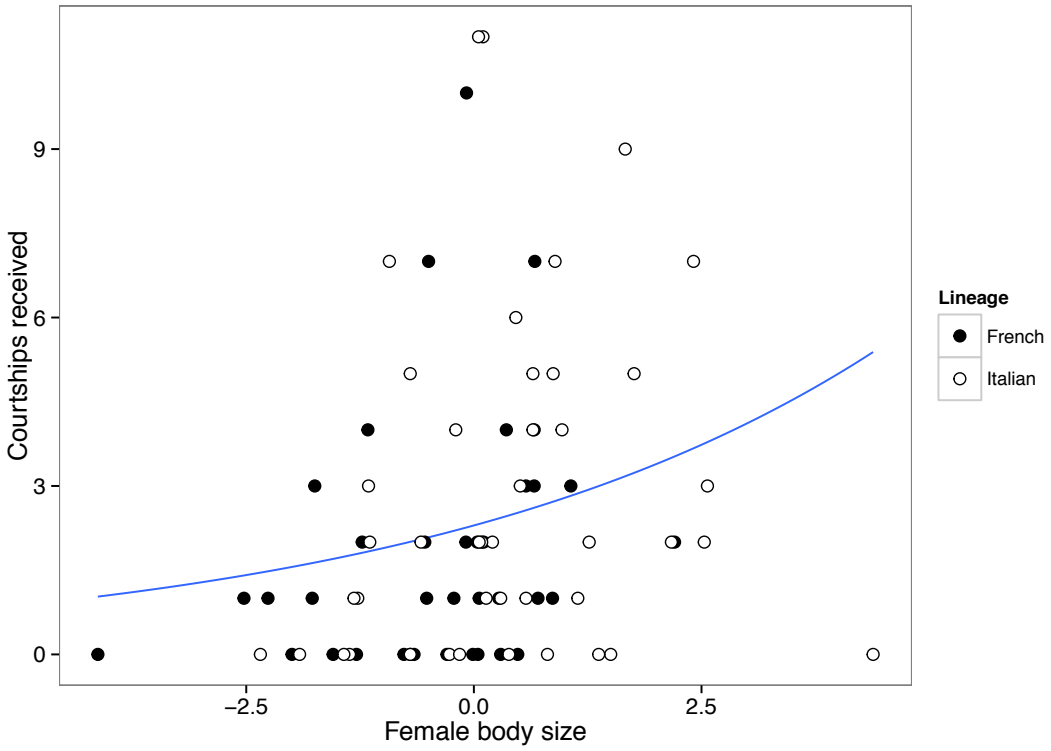


Figure 2. Relationship between female body size (principal component of SVL and body mass) and the number of total courtships received in the outdoor enclosure experiment.

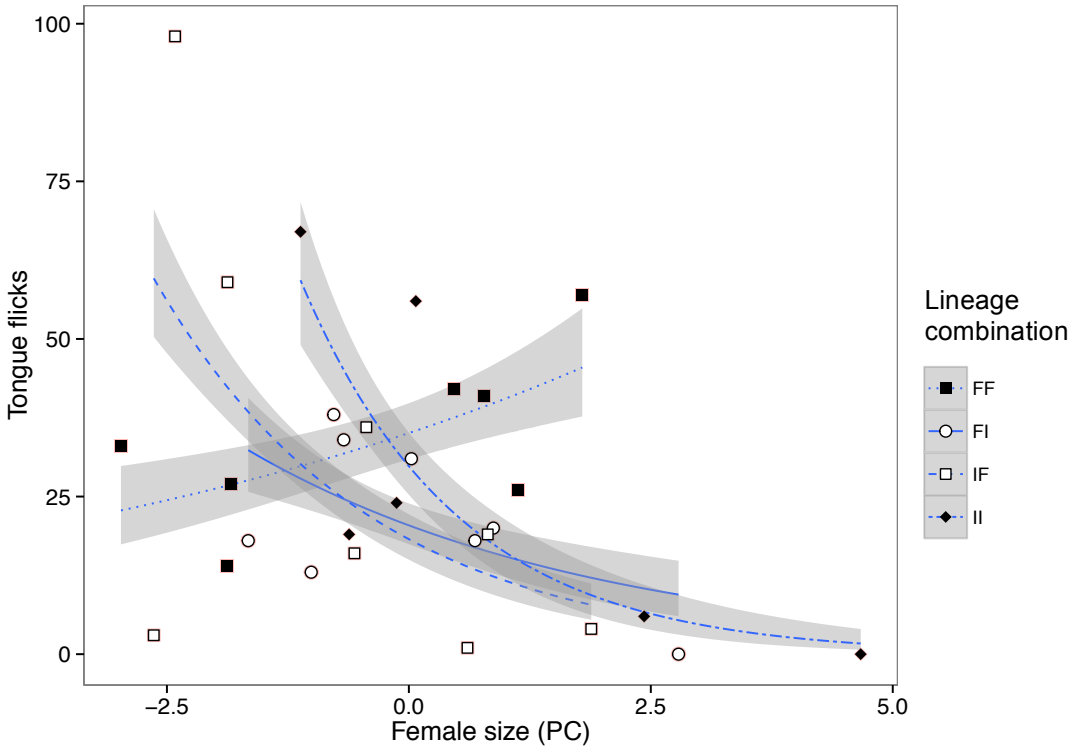


Figure 3. Tongue flicks from male lizards in response to different sized and lineages of female lizards. The legend indicates the male-female lineage combination (F=French; I= Italian). The shaded region around the lines represents the 95% confidence interval.

CHAPTER FOUR

Behavioural and phenotypic predictors of hybridisation between lizards in secondary contact

Unpublished manuscript

ABSTRACT

The degree and direction of hybridization upon secondary contact varies substantially between species and populations. Whilst we know much about the processes that determine hybridisation at these levels, we have a relatively poor understanding about how and why hybridisation varies between individuals within populations. We investigated the morphological and behavioural characteristics that predict individual hybridisation between two previously allopatric lineages of wall lizard (*Podarcis muralis nigriventris* and *P. m. brogniardii*) that exhibit divergent phenotypes. We found that more hybrid offspring were produced by smaller males from the generally larger lineage, and females with shorter heads (which correlates with body size) from the smaller lineage. Additionally, whilst there were no behavioural correlates of hybridisation in females, more dominant males with high reproductive success produced relatively fewer hybrid offspring. We suggest that subordinate males produce significantly more hybrid offspring because of reduced opportunities for courting conspecific females due to male-male competition, requiring them to become less 'choosy' and therefore more likely to mate with heterospecifics.

4.1 INTRODUCTION

Hybridisation is a major contributor to biological variation (Seehausen 2004; Bachtrog et al. 2006; Mavarez et al. 2006). Hybridisation can homogenise populations via introgression (Rhymer and Simberloff 1996; Seehausen et al. 1997), create genetically isolated reproductive lineages (Mavarez et al. 2006; Grant and Grant 2009), or generate allelic combinations that facilitate adaptation to novel environments (Ellstrand and Schierenbeck 2000; Sakai et al. 2001). Perhaps its most important and widely-cited role is in generating fitness costs that reinforce reproductive isolation between genetically distinct lineages; an integral component of the contact phase in allopatric speciation (Mayr 1963). These costs vary from marginally reduced survivorship of hybrid offspring (e.g. Lancaster et al. 2007), to absolute reproductive 'failure', where embryos may die prematurely (Tyler et al. 2013), or offspring are totally sterile (Haldane 1922; Hewitt et al. 1987). And yet given these costs, hybridisation is still surprisingly widespread; a conservative estimate has suggested approximately 10% of all animal species frequently hybridise (Mallet 2005). Given its importance in evolution therefore, a key focus in research has been to understand the processes and mechanisms that mediate hybridisation, and to understand why it often remains widespread in the face of extensive costs.

One of the most frequently cited reason for animals hybridising is due to 'Hubbs principle', where individuals hybridise due to a low availability of conspecific mates (Hubbs 1955). The causes of this can be varied, such as lineage differences in mating seasons (Nuechterlein and Buitron 1998), species occurring at the edge of their range distribution (Barton and Hewitt 1985), or through the anthropogenic introductions of

small numbers of exotics (Muñoz-Fuentes et al. 2007; Senn and Pemberton 2009). Overall, the general assumption is that mate 'choosiness' decreases as the availability of preferred mates declines due to the escalating costs associated with continued mate search and increasing chances of total reproductive failure, i.e. the animals are 'making the best of a bad job' (Anderson 1977; Baker 1996). Generally however, whilst we know much about generic population-level processes that mediate heterospecific mate avoidance, we have little understanding of the causes, and degree to which, hybridisation varies within populations, particularly in regards to how behavioural interactions between individuals might influence their likelihood of hybridising.

In this study, we investigated the causes of individual variation in hybridisation in a simulated 'secondary contact zone' between two subspecies of European wall lizard (*Podarcis muralis nigriventris* and *P. m. brogniardii*). These two subspecies diverged around 2 MYA (Gassert et al. 2013; Michaelides unpubl. data), and have recently come into secondary contact via anthropogenic introductions in Germany and the UK over the past one hundred years or so (Schulte et al. 2012a; Michaelides et al. 2013). Whilst the two lineages are known to hybridise in the wild (Schulte et al. 2012b), our own work shows that the two lineages show strong, male-mediated, assortative mating at ~80%, which is caused by a combination of male choice and asymmetries in phenotypic and agonistic traits with only limited direct conspecific male mate choice (Chapter 2 of this thesis). We therefore aimed to identify individual-level morphological and behavioural predictors of hybridization, and whether this differs between males and female of the two different lineages.

4.2 METHODS

4.2.1 Study species

The European wall lizard *Podarcis muralis* is a diurnal, small (up to 75mm snout-to-vent-length (SVL)), primarily insectivorous lacertid that is comprised of multiple subspecies that extend across Western and Southern Europe (Schulte et al. 2012b). Populations in the UK and Germany were established via introductions through the pet-trade (Schulte et al. 2012a; Michaelides et al. 2013). The UK contains at least 25 viable populations constituting of both pure and mixed-origin animals from lineages originating from Italy (*P. m. nigriventris*) and France (*P. m. brogniardii*). The lineages differ starkly in phenotype, characterised by bright green dorsal colouration, stronger sexual dimorphism, and dominance in Italian animals compared to the brown-grey dorsal colours in French lizards (see Chapter 2 of this thesis).

4.2.2 Outdoor enclosure experiment and behavioural analyses

This chapter is based exclusively on the outdoor enclosure experiment that has been detailed previously in this thesis (see Chapter 2), and so will only be covered briefly here. In April 2010 we created 10 large outdoor enclosures (~7m x 7m) to house semi-natural populations of lizards to simulate a 'secondary contact zone' between the Italian and French lineages. Lizards populating these enclosures were caught from nine UK populations that genetic data suggests consists of only the Italian or only French lineage (Michaelides et al. 2013), unpubl data. Each enclosure housed 16

lizards with an equal number of males and females, and an approximately equal number of Italian and French origin animals. Each lizard had its snout-vent-length (SVL), head length, head width and mass recorded. Males were introduced between five and seven days before females to establish territories, and females were introduced only after they had laid a clutch of eggs in the lab. Because this species lays multiple clutches (R. Heathcote, G. While, T. Uller pers. observation), and sperm storage is poorly developed, this ensured that any resulting offspring were likely to be fathered by males from the enclosure.

In each enclosure we created nine uniform basking sites created from breezeblocks and a wooden pallet. The basking sites were clumped together in half the enclosures, and distributed equally in the other half, used during a concurrently running experiment (see Chapter 1 of this thesis). Each lizard was marked with a unique sticker to allow individual identification. All behavioural interactions and lizard locations were recorded in each enclosure during hourly observation periods throughout the lizard activity period for 26 days. From the behavioural observations, we were able to determine a lizard's core home range (using the 50% kernel core; see methods section in Chapter 1 for the justification) calculated using Ranges 8 analysis software (Kenward et al. 2008) and also the number of courtships and territorial disputes each animal was involved in. The outcomes of territorial disputes were used to calculate the David's score for each male lizard (an indication of its relative dominance position within the enclosure) using SOCPROG social analysis software (Whitehead 2009).

After the 26-day observational period, all lizards were captured and brought back into the lab. Eggs laid by females were incubated artificially at 24°C, using an incubation medium of vermiculite and water at 5:1 volume ratio. Tissue samples (tail-tips) were then taken of all hatchlings and enclosure adults to use in the genetic analysis.

4.2.3 DNA extraction, genotyping and paternity assignment

All adults and their resulting offspring from the enclosure experiment were genotyped at nine highly variable, custom-developed microsatellite loci (primers used: C150, C168, C275-278, C164, C038, C028, C356, C109, C103; see Appendix 3 Table S1 for details on microsatellite primers). DNA was extracted using a standard ammonium acetate protocol. We used a PCR protocol where multiplex primer mixes (three multiplexes were used, with three primers in each) were added to Qiagen Mastermix, which was then added to 1µl of dry DNA and amplified in an MJ Research model PTC DNA Engine Tetrad thermal cycler PCR machine. The PCR conditions were set at 95°C for the first 15 minutes and then 35 repeated cycles of 94°C at 30 seconds, 57°C for 90 seconds, and 72°C for 60 seconds. Samples were genotyped with an ABI3730 48-well capillary sequencer using a ROX size-standard marker and alleles were categorised using Genemapper analysis software. All primers were tested for conformity with Hardy-Weinberg equilibrium. Paternity was assigned (maternity was mostly unnecessary since the majority of mothers were known due to laying their eggs in the lab) using CERVUS v3.2 with 10,000 simulations, setting the parameters to include a mother's allele conformation as the known parent and all eight males she shared her enclosure with as the potential fathers. Paternity of 296 juveniles was then

confirmed at >99% confidence. Overall, seven clutches were recovered from females that had laid in the enclosures, for which both parents were identified in Cervus by assigning potential mothers as those females from the same enclosure who did not lay in the lab.

4.2.4 Data analysis

For all analyses, we only compared individuals that produced successfully genotyped offspring (61 males and 62 females in total). The male analysis was complicated by the fact that more Italian females laid clutches compared to French females, meaning that average hybridisation rate could be higher for French males than for Italian males simply by there being more potential Italian offspring to sire by chance.

We therefore calculated male hybridisation levels as the 'deviation from expected hybridisation'. This variable was determined by firstly calculating the 'expected hybridisation level' for each male, determined by multiplying the total number of offspring he sired by the total proportion of eggs produced by heterospecific females in the same enclosure. This was then subtracted from the actual number of hybrid offspring a male produced to give the 'deviation' from expected. Thus, according to this index, any value greater than zero indicates a male that produced less hybrid offspring than expected, and less than zero indicates a male that produced more than expected. Since we had exactly the same number of males of each subspecies in each enclosure, we determined hybridisation rate for females as the number of hybrid and purebred offspring produced as a joint response variable.

Initially, we wanted to determine whether reproductive success correlated with hybridisation rate in both sexes, which would give some indication as to whether hybridising is an adaptive strategy by less attractive or reproductively unsuccessful individuals. We therefore ran models on both sexes with degree of hybridisation as a response variable, and total number of offspring as a covariate, with enclosure as a random factor.

We are not aware of any previous study that has investigated phenotypic correlates of hybridisation at the individual level in any lizard species. We predicted that the patterns of hybridisation would likely follow those that arose from the assortative mating results, where small Italian males and large French females were more likely to produce hybrid offspring. However, we had few strong *a priori* expectations regarding behavioural factors that were likely to predict hybridisation in our system. We therefore selected morphological variables collected on the different aspects of body size (see Chapters two and three), and behavioural aspects we expected to be important such as home range size and the ratio of overlapping conspecifics and heterospecifics in a home range.

To avoid over-parameterisation, we split our analyses so that morphological and behavioural predictors were analysed in separate models for each sex. For the morphological models, we included the continuous variables head length, head width, SVL and mass, after confirming that they had correlations <0.70 to avoid problems of multicollinearity. We also included lineage, and its interaction with each of these continuous variables. In the female model, we constructed a generalised linear mixed

model (GLMM) with the number of hybrid offspring and pure offspring as the binomial response variable. Enclosure number was included as a random variable, and we controlled for overdispersion in the female analysis using an observation-level random effect, which models the scale parameter and thus conformity to the binomial distribution (Elston et al. 2001). In the male morphological model, we used the 'deviation from expected hybridisation' variable detailed above as the response variable in a linear mixed model (LMM), with enclosure included as a random effect.

In the female behavioural model, we again used the number of hybrid offspring and pure offspring as the response variable, and included the following six predictors: basking-site treatment (i.e. the spatial organisation of the basking sites in the enclosures which was run for a concurrent experiment; Chapter 2); her 'courtship index' (see below); female core home range size; ratio of same/different subspecies of males overlapping her home range; lineage; and number of males siring offspring in a female's clutch. We also included the interactions between each of the behavioural traits and a female's lineage. The female's courtship index was calculated by combining the total number of courtships, and the number of males that courted a female, into a single principal component, which accounted for 87% of variation, and had strong positive loadings (0.93) for both. This was used to keep the number of predictors in our model down to a minimum.

For the male behavioural analyses we constructed a model that included the following variables hypothesised to be important in hybridisation: basking site treatment; dominance; courtship index; core home range size; ratio of same/different female overlapping home ranges; and lineage. We also included two-way interactions

between lineage and all the behavioural variables, and enclosure was again included as a random effect. Similar to females, 'courtship index' was the first principal component that combined the total number of females a male courted with the total number of courtships.

All models were simplified until only significant terms remained by step-wise backwards elimination, and checking if each term's removal caused a significant change in the model's fit using likelihood-ratio-tests. Due to issues of correctly calculating significance values from models that include random effects (because of ambiguities involved with determining the denominator degrees of freedom), the significance of each fixed factor was then analysed using likelihood-ratio-tests on models with and without the factor (Valdar et al., 2006; Öckinger et al., 2010).

4.3 RESULTS

Over the 26 day enclosure experiment we collected ~510 hours of behavioural observations between the 10 different enclosure populations. Of the total offspring that hatched in the lab, parentage was assigned to 296 juveniles at >99% confidence, and out of these a total of 49 hybrid juveniles were produced (17%). In addition, 28 out of 61 males and 29 out of 62 females produced at least one hybrid offspring. Overall, more hybrids were produced from Italian than French sires (48 hybrid offspring had Italian sires versus 31 with French sires), although this difference was only borderline significant (Pearson's chi squared test: χ^2= 3.66, p=0.056)

4.3.1 Female analysis

In female wall lizards, overall fecundity did not correlate with the relative number of hybrid offspring produced (χ^2=1.95, p=0.16). Additionally, there was no difference in the number of infertile eggs between females that produced at least one hybrid offspring compared to those that produced conspecific offspring (Wilcoxon test: Z=0.68, p=0.500). Female hybridisation (the proportion of her offspring that were hybrids) was significantly affected by an interaction between both head length and lineage (χ^2=22.48, p<0.001; Figure 1), and head width and lineage (χ^2=13.40, p<0.001; Appendix 4 Table S1). This was due to Italian females with shorter and wider heads and French females with longer heads being much more likely to hybridise than vice-versa (Figure 1). None of the behavioural observations we

collected for females were significant in predicting her level of hybridisation (Appendix 4 Table S2).

4.3.2 Male analysis

Males that sired more offspring produced relatively fewer hybrids than expected (χ^2= 23.83, p<0.001; Figure 2). There was a significant interaction between the male lineage and mass (χ^2=7.77, p=0.005), SVL (χ^2=5.60, p=0.018), and head width (χ^2=10.31, p=0.001) where smaller Italian males with small heads and larger French males with large heads produced more hybrid offspring (Appendix 4 Table S3; Figure 3). A male's relative dominance was strongly correlated to his degree of hybridisation, with more dominant males producing fewer hybrid offspring (χ^2=18.61, p<0.001; Appendix 4 Table S4; Figure 4).

4.4 DISCUSSION

We have shown that hybridisation in males occurs more frequently in less dominant individuals with low reproductive success, in particular small Italian males (with small head width, mass and SVL) and large French males. In addition, Italian females with small heads and French females with larger heads are also more likely to hybridise.

The increased lower reproductive success of males that produce more hybrid offspring supports the notion that accepting heterospecific mates is the 'best of a bad job', where hybridisation represents an adaptive strategy to ameliorate the costs associated with being less able to mate with conspecifics (Anderson 1977; Baker 1996; Wirtz 1999; Randler 2002). In contrast, the lack of a correlation between hybridisation and offspring number in female lizards suggests, as we have previously shown, that females do not exhibit any aspects of choice. Alternatively, this may be simply due to their lower variation in reproductive success and hence low statistical power.

In general, the morphology-related hybridisation patterns between the two lizard lineages we have shown here corroborate our previous findings that assortative mating is facilitated by a combination of general male preference for larger (more frequently Italian) females, which are then monopolised by the dominant Italian males (see Chapter 3), and at least some degree of male choice for conspecifics. Smaller and less dominant Italian males appear to compensate for their reduced reproductive success by choosing the 'next best option', i.e. the larger French females, their access

to whom is ensured since even small Italian males are still dominant over large French males (see Chapter 2).

In contrast, the effect of increased hybridisation in large French males could be a consequence of a shared preference for (large) Italian females. However, we believe an alternative explanation is more likely. Whilst larger Italian males win more heterospecific interactions (as shown by their greater dominance), and court more females as a result, larger French males do not win more interactions (assumedly due to their inferiority to Italian males), and in fact there is a trend towards them even courting fewer females of both lineages (see Figure 7, Chapter 2). We suggest that this increased time spent in costly territorial disputes, but without any benefits of increased female access, compromises the ability of these males to sire the offspring of preferred females compared to the smaller French males who engage in very few competitive interactions. This interpretation implies that males of both lineages show some degree of conspecific mate choice. Our previous work on this subject has returned mixed results. Whilst both lineages are capable of discriminating between females based on their olfactory cues, only Italian males showed a preference for conspecifics during staged mating trials (see Chapter 3). However, the strongly assortative courtship patterns suggest that at least some degree of male choice is present in both lineages. Male choice being present to some degree in both lineages is corroborated by the observation that increased dominance in both lineages was negatively associated with hybridisation rate in males. We suggest this is likely a consequence of a combination of mate guarding and direct harassment by more dominant males (see Chapter 3 for an overview of mate guarding in this system). As a result, less dominant males of both lineages appear to compensate by settling for

generally smaller and/or heterospecific females, leading to greater levels of hybridisation.

A lack of available preferred mates has been frequently cited as an explanation for increased hybridisation rates in animals. Known as 'Hubbs principle', the causes of this unavailability of mates is normally attributed to environmental factors, such as in rare species (Avise and Saunders 1984; Ribi and Oertli 2000), when individuals of one species disperse outside of their geographical range (Barton and Hewitt 1985), or following the introduction of small numbers of individuals into a non-native range (Muñoz-Fuentes et al. 2007; Senn and Pemberton 2009). However, the role of intra- and interspecific social interactions in causing hybridisation, and in particular the importance of male competition, has rarely been investigated. The few studies to consider its influence have shown it can indirectly encourage hybridisation by increasing female preference for dominant heterospecifics (e.g. Brodsky et al. 1988; Bronson et al. 2003) whereas the potential for male-male competition to directly affect patterns of hybridisation, without the influence of female choice, has never been shown to our knowledge.

The finding that head size is an important predictor of hybridisation in female lizards is surprising. Selection on morphology in lizards seems to favour large heads in males, due to the increased bite force and thus competitive advantage it provides them in territorial disputes, and on large abdomen sizes in females to facilitate increased egg production (Braña 1996; Cox et al. 2003; Lappin and Husak 2005). As such, many authors have found that male lizards often have a preference for generally larger females (measured by SVL) (Olsson 1993; Whiting and Bateman 1999), which we

have also shown in this system (see Chapter 2). Why then do Italian females with small heads and French females with large heads produce more hybrid offspring? Currently we can only speculate on this result. However, head length correlates positively with both aspects of body size in female lizards (both mass and SVL), and the hybridisation patterns we report here would be expected based on the predictions outlined in Chapter 2 (detailed above) on how assortative mating arises between these two lineages. Because Italian females are larger than French females (and have larger heads), they are generally preferred during courtship displays by the most dominant Italian males. As a consequence, the increased hybridisation of smaller Italian females may be a product of their being less preferred by conspecifics, and thus more likely to be mated by French males. Why head length should be a stronger predictor of this expected pattern than body size itself is currently uncertain, but we note that while the experiment was set up to keep differences in SVL minor (and hence there is limited statistical power to detect differences based on SVL), differences in head size was not considered. However, information on the exact morphological characteristics that determine mate choice in lizards are needed to assess whether head size itself is important.

Several studies have shown that hybridisation between divergent lineages is most common between individuals in the overlapping phenotype range. In particular, smaller individuals of larger species tend to mate with larger individuals of smaller species, for example through 'phenotype matching', or simply the increased chance of making recognition errors (e.g. Nagel and Schluter 1998; Schmeller et al. 2005; Conte and Schluter 2013). Indeed, the costs associated with this non-random hybridisation is an important cause for the necessary disruptive selection that results in character

displacement and continuing divergence between heterospecifics following secondary contact (e.g. Hoskin et al. 2005; Nosil et al. 2007).

In regards to the wall lizard system, we can only speculate about the evolutionary consequences of the patterns shown here because the costs of hybridisation are currently unknown. Assuming some degree of fitness cost to hybrids, disruptive selection could operate on the opposite extremes in body size in the two lineages, with the potential that the current phenotypic differences are further exaggerated. Indeed, reinforcement on body size is a common consequence following the costs of hybridisation during secondary contact (Nagel and Schluter 1998; Hoskin et al. 2005). A study investigating the extent of these costs is currently underway, which should provide insights into the likely evolutionary consequences of the patterns we report here.

In summary, our results show that hybridisation rate varies non-randomly in respect to the morphological variation expressed by phenotypically divergent lineages. In addition, we have shown that hybridisation rate is higher in less dominant males, likely due to their requirement to offset the costs of reduced reproductive success with conspecifics due to their lower competitive ability. More generally, the importance of male-male competition in driving hybridisation levels, and the relative contribution that mate choice may play in mediating these effects in other taxa, provide interesting avenues for future studies.

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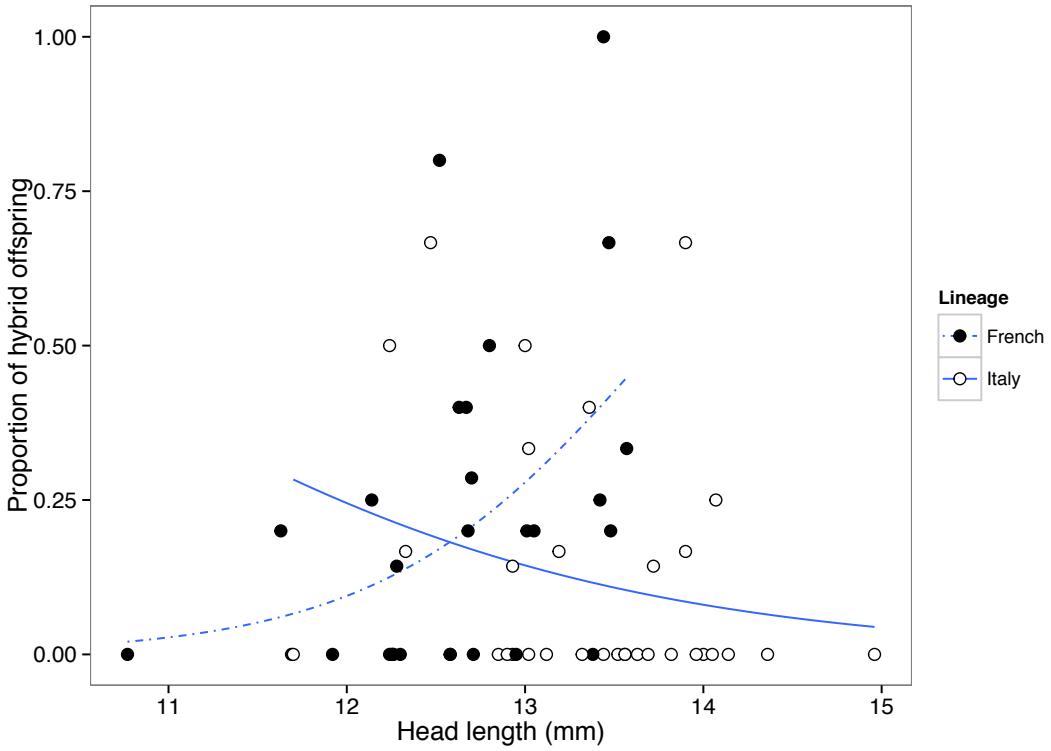


Figure 1) Proportion of a female's offspring that were hybrids as affected by her subspecies and head length.

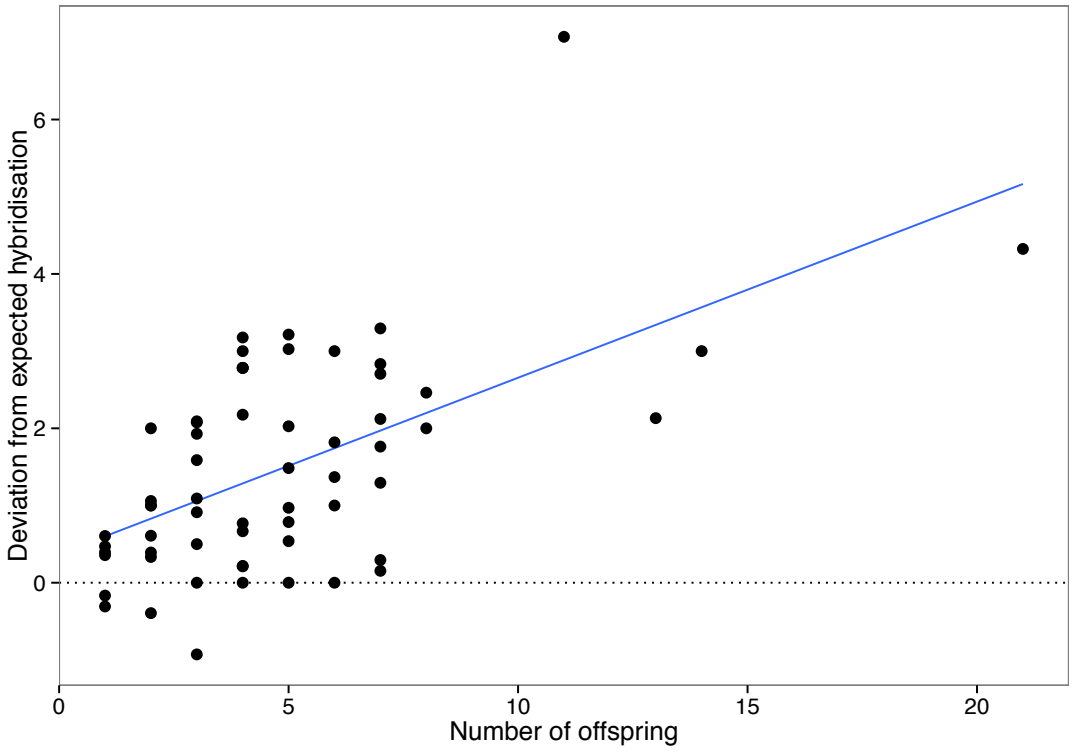


Figure 2) Correlation between deviation from expected hybridisation level and the number of offspring a male sired. The horizontal dashed line indicates where there was no deviation, i.e. when the proportion of hybrid offspring equalled the proportion of heterospecific eggs available to fertilise.

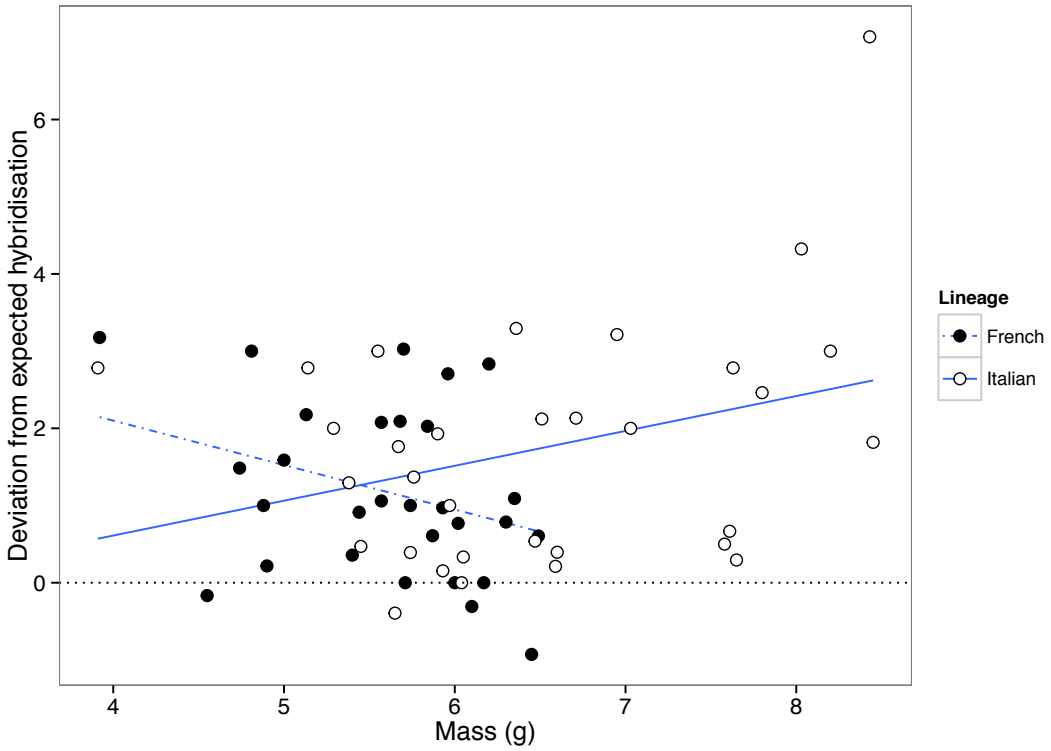


Figure 3) Interaction plot showing how the deviation from expected level of hybridisation changes with mass for males of both subspecies. The horizontal dashed line indicates where there was no deviation from expected hybridisation.

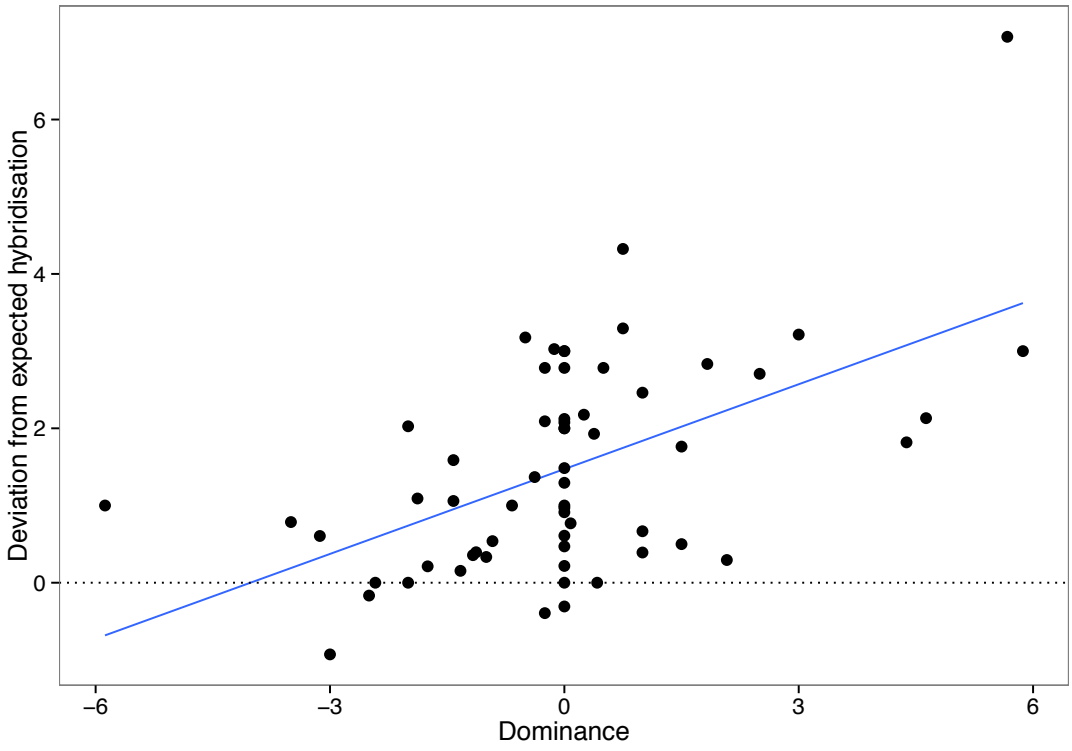


Figure 4) Relationship between a male's dominance level (David's score) and how much his hybridisation level deviated from expected. The horizontal dashed line indicates where there was no deviation from expected hybridisation.

CHAPTER FIVE

The scent of sun worship: basking experience alters scent mark composition in male lizards

Accepted by Behavioral Ecology and Sociobiology

ABSTRACT

Signals used in female choice should honestly advertise the benefits that males can provide, with direct benefits often argued as being more important than indirect benefits. However, the nature of direct benefits in species without paternal care or nuptial gifts is poorly understood. Previous studies on lizards suggest that females decide where to settle and assumedly who to mate with based on information contained in scent marks from territorial males. Access to high-quality thermal resources is crucial for female reproductive success. Females may therefore be able to detect and exploit variation in the chemical composition of male scent marks caused by differing thermal habitats when assessing the quality of his territory. We show that the amount of time male wall lizards (*Podarcis muralis*) are allowed to bask significantly alters the chemical composition of their femoral secretions used in scent marking. The direction of the change is consistent with adaptive plasticity to maintain signalling efficacy under warm conditions that increase evaporation of femoral secretions. The compounds affected by basking experience included those previously associated with male quality or shown to mediate male-male competition in lizards. However, whilst female lizards could discriminate between scent marks of males that had experienced different basking conditions, they did not preferentially associate with the scent from males from high-quality thermal conditions. These results highlight the potential importance of a previously neglected environmental effect on chemical signalling. We suggest thermal effects may have significant consequences for scent-mark composition in variable environments, with potential repercussions on olfactory communication in lizards.

5.1 INTRODUCTION

Understanding when and how males should signal their ability to provision direct or indirect benefits to females is a fundamental question in sexual selection. In general, selection should favour males that advertise their provisioning of the key components that are limiting for female reproductive success. For example, in species that use patchily distributed and/or rare resources, male access to females can be enhanced by monopolising these resources (e.g. Emlen and Oring, 1977; Clutton-Brock and Harvey, 1978). Female choice may therefore promote the evolution of honest male advertising of the availability of these resources (Kirkpatrick and Ryan, 1991; Andersson, 1994; Kirkpatrick, 1996). However, whilst examples of male advertisement of direct benefits such as parental ability and nuptial gifts are well established (Dussourd et al., 1991; Hill, 1991; Vahed, 1998; Candolin, 2000), there are relatively few examples of species where males signal the quality of environmental resources in their territory (although see e.g. Trumbo and Eggert, 1994; Hoi-Leitner et al., 1995; Eisner et al., 1996).

A possible reason for this lack of research is that females are assumed to be capable of assessing the quantity and quality of these resources themselves. However, males will likely have more information than females regarding the quality of their own territorial resources. Thus, signalling of food and shelter availability may be particularly likely in cases where mate choice costs are high and reliable immediate assessments of territory resources are difficult for females (for example, in species that inhabit temporally variable environments). Additionally, some aspects of territory quality may also affect male signals in ways that females can exploit when deciding

where to settle or who to mate with. Olfactory signals may be particularly important in this regard as they are typically produced continuously throughout the reproductive season, and many species produce semiochemicals (i.e. compounds important in communication) in proportion to the availability of particular environmental resources. Signalling of food availability has been particularly well studied in this context, especially in insects, where many species sequester and emit chemical compounds found in the diet directly as pheromones (Landolt and Phillips, 1997). For example, females of several beetle species preferentially mate with males that emit greater levels of chemicals obtained from their dietary plants (Eisner et al., 1996; Tallamy et al., 2000).

The few studies on vertebrates that have examined signals of resource quality have frequently focused on food availability. For example, male lizards artificially supplemented with dietary vitamins have correspondingly higher levels of the same vitamins in their femoral secretions, which females may use in mate choice (Martín and López, 2006b; Kopena et al., 2011). However, food is rarely a limiting resource in mainland lizard populations, where feeding rates, particularly in temperate species, often equal laboratory *ad libitum* levels (Avery, 1971, 1978; Edsman, 1990), making the relative importance of dietary derived olfactory cues questionable in the natural context of mate choice within this group. Instead, a more important environmental resource for temperate-zone lizards is the availability of areas that allow optimal thermoregulation (Huey et al., 1989; Calsbeek and Sinervo, 2007). In diurnal species for example, variation in basking site availability and relative quality can have strong effects on conspecific interactions, with repercussions for male and female reproductive success (Fox et al., 1981; Calsbeek and Sinervo, 2002; Calsbeek and

Sinervo, 2007; Olsson et al. 2011). Despite this, there have been no studies to our knowledge addressing whether males attract females through signals that reflect the quality of the thermal environment.

Olfactory signals are likely to play an important role in this context as males of many reptiles, particularly lizards, extensively scent mark their territories using chemically-diverse oily secretions from specialised glands (Mason, 1992; Houck, 2009). In lizards these scent marks have a diversity of functions such as male-directed signals of individual identity and competitive ability (e.g. Carazo et al., 2007; Font et al., 2012), and potentially as female-directed signals of male quality used in female choice (e.g. Martin and Lopez, 2000; Martín and López, 2006b). Male territories, which often encompass several female home ranges, are strongly defended throughout the mating season (Edsman, 1990; R. Heathcote, unpubl. data),. Females will settle within a currently existing male's territory, and these settlement decisions seem strongly mediated by the presence and characteristics of male scent marks (Carazo et al., 2011; Martín and López, 2012). Male scent marks may thus provide an efficient means for females to assess the quality of resources available to her in a male's territory, particularly thermal resources that are critical for female reproductive success but whose long term reliability might take time to assess (Calsbeek and Sinervo, 2007). Cues about thermal quality could be transmitted in male scent marks because variations in average metabolic rates, as determined by total time spent at optimal operating body temperature, might create differences in the relative thermal-dependant rates that chemical compounds can be sequestered, metabolised and secreted as scent marks.

In this study we used introduced populations of the European wall lizard (*Podarcis muralis*) in the UK to test whether the chemical composition of male femoral secretions are affected by the opportunity for basking, and whether females discriminate and show any preference for males with good basking resources. Introduced wall lizards are particularly relevant in this regard, as they are at the northern range limit for this species and experience substantial heterogeneity in thermal opportunity both within and between years (R. Heathcote, T. Uller & G. While, pers. obs.). Indeed, many populations have undergone extremely low range expansion, implying that basking sites and additional thermal resources are limiting for this species in the UK. The quality of basking sites within populations thus varies considerably, which should promote male-male competition and potentially female choice for access to areas that allow optimal thermoregulation.

5.2 METHODS

5.2.1 Study species

The European wall lizard *Podarcis muralis* is a diurnal, small (up to 75mm snout-to-vent-length (SVL)), primarily insectivorous lacertid with a widespread geographic range across Western and Southern Europe. Additionally, this species has been introduced extensively throughout the southern United Kingdom (Michaelides et al., 2013), Germany (Schulte et al., 2012) and in a number of locations in North America (Kwiat and Gist, 1987). In the UK, more than 25 extant introduced populations are known with an introductory pathway mediated mainly by escapees and deliberate releases from the herpetocultural trade (Frazer, 1964; Lever, 1977; Michaelides et al., 2013)

5.2.2 Captivity conditions

Thirty-four males and 17 female wall lizards were collected in May 2011 from two locations (town centre and the Botanic Gardens) in Ventnor on the Isle of Wight, United Kingdom. Microsatellite and mtDNA data confirm that these populations originate from northern Italy, with reported sightings going back to at least the 1930's (Michaelides et al., 2013). All lizards were captured by noosing, and transferred the same day to animal facilities at Oxford, where they were kept individually in cages (500x300x400mm (length x width x height)). Animals were sexed by the presence of sexually dimorphic characters such as larger heads and the presence of femoral pores

and hemipenal bulges in males. All animals had their SVL (snout-vent-length), mass, head length and head width measured. We calculated the overall 'body size' for each male by combining his SVL, mass, head length and head width into a single principal component, which explained 79.9% of the total variation, and was positively associated with all four variables (eigenvector loadings; mass: 0.51; SVL: 0.50; head length: 0.52; head width: 0.47). Females were carrying eggs from their first clutch at the time of capture, which they later laid in the laboratory (see below). All cages contained a basking spot, two hides made from bricks, and sand as substrate. Lighting and heat was provided by a 60W spot bulb placed over a brick on one end of the cage to provide a surface thermal gradient from 45°C to 22°C), an EXO-TERRA^{TM} 10.0 UVB fluorescent tube, and overhead fluorescent room lighting. All lizards were provided with food (live crickets and mealworms fed only orange slices following weekly delivery from a breeding facility) and water *ad libitum*.

5.2.3 Basking experiment treatment

Male pairs matched to within 3mm SVL were assigned to each of 17 females to create 17 unique trios used throughout the behavioural experiments. Each male within a trio was then randomly allocated to either a 'high-quality basking' or a 'low-quality basking' treatment. High-quality basking treatment males received 10 hours per day of basking opportunity from the 60W tungsten spot-lamp, and 4 hours per day of UVB light. The low-quality basking treatment males received 5 hours of basking opportunity and 2 hours of UVB light exposure. Both of these treatments allowed for lizard activity levels that fall within the natural variation found in the UK and native

lizards, as determined from sustained observations of semi-natural populations in outdoor enclosures (R. Heathcote, unpubl data). The ambient light regime was 12 hours of overhead room lighting for both treatments. All female lizards in the trios received the same lighting treatment as the 'high-quality treatment' males. Lizards were maintained under these treatments for at least 21 days until female behavioural trials commenced.

5.2.4 Collection and analysis of femoral pore secretions

Samples of femoral pore secretions were taken within 2 days of capture, and were then extracted again three weeks later within four days of behavioural trials commencing. Scent mark secretions were collected by gently squeezing the femoral pores of males using sterilised forceps. Samples were then stored in sterile glass containers at -20°C until chemical analysis.

Samples were dissolved into 200µL of pentane, and 3 µL of the extract were analysed by gas chromatography-mass spectrometry (GC-MS) using an Agilent Technologies 7890A gas chromatograph (capillary column: Agilent HP-5MS, 30m x 250µm x 0.25µm; split-splitless injector; carrying helium gas at 1ml/min) coupled with an Agilent 5975 mass spectrometer operated at 70 eV in the electron impact ionization mode. The temperature programme started at 50°C for one minute, increased to 180°C at 30°C/min, then to 250°C at 10°C/min and finally to 320°C at 5°C/min and kept for 5 minutes. The areas of GC peaks were integrated using the Agilent

Chemstation software, and the relative compound levels were then calculated with Aitchison's formula: $Zij = \ln(Yij / g(Yj)$, where *Zij* is the standardized peak area (*i*) for individual (*j*), *Yij* is the peak area *i* for individual *j* and $g(Yj)$ is the geometric mean of all peaks for individual *j* (Aitchison, 1986; Dietemann et al., 2003). Relative rather than absolute compound levels were used because synergistic chemical composition of pheromone cues is known to be particularly important in olfactory communication (Wyatt, 2003). Identification of compounds was made by initial comparison to the mass spectral library in NIST 2008, and confirmed by comparison to pure synthetic compounds obtained from Sigma-Aldrich (Milwaukee, WI, USA). Compounds that could not be identified are reported by their characteristic retention time and distinctive ion current signature.

Principal component analysis was performed on the normalised peak area from compounds shared by all individuals. Samples that showed signs of contamination were excluded from the analysis. The first six PCs, which together explained 74% of total chemical variation, included the main compounds previously shown to be important in lizard olfactory communication (e.g. Mason and Parker, 2010; Martin et al., 2007; Martín et al. 2008; see below).

Because we were interested in determining the effect of our experimental treatment on both the overall chemical signature of our test subjects, and also in identifying the individual chemicals that constitute this overall difference, we took a sequential 'top-down' approach in our chemical analysis. Firstly, we used the principal components as response variables in two MANCOVAs for the pre- and post-basking treatment femoral pore secretion samples. In the pre-basking treatment samples, male body size

was used as the sole predictor, and in the post-basking treatment samples the predictors were male body size and basking treatment. These MANCOVAs allowed us to determine if the basking treatment and male body size influence the overall chemical composition of our test animals. After obtaining a significant effect in the MANCOVAs, we did further 'Protected ANOVAs' on each principal component as a response variable, with the same predictors as the preceding MANCOVA. Finally, following significant effects of male treatment on the different principal components, we then identified those chemicals that had relatively high factor loadings (+/- 0.25; more than one standard deviation from the mean factor loading) as response variables in separate linear models, with the same predictors as before. Even though statistical correction for multiple testing has been considered excessive for studies with small sample sizes (Nakagawa, 2004), inflation of Type I errors is still an issue with multiple response variables, and our top-down approach allows informed further testing of only those variables deemed important from the 'higher-order' test. We therefore provide details on the magnitude of the effect for the individual-level analyses in Supplementary Tables.

5.2.5 Female behavioural analysis

Behavioural trials were started between three and seven days after a female laid her first clutch of eggs (this species lays multiple clutches a year; R. Heathcote, G. While, T. Uller unpubl. data). This corresponds to the timing of matings under these housing conditions and hence maximised the likelihood that females were receptive and therefore would exercise mate choice (R. Heathcote, G. While & T. Uller, pers. obs.). Each female underwent two different behavioural trials to determine, first, whether

females could discriminate between males of the two treatments ('tongue flick trials') and, second, if females preferred to associate with scent from males of a particular treatment ('association preference trials').

5.2.5.1 Tongue flick trials

We quantified a female's potential ability to discriminate between the olfactory cues of the two different males by counting the number of 'tongue flicks' directed at scented cotton-wool buds (Cooper, 1994). Olfactory stimuli in actively foraging squamates are predominantly received through the Jacobson's organ, a specialised vomeronasal organ that opens into the roof of the mouth, transferred from a notched or forked tongue via tongue flicking (Cooper, 1994). Quantifying 'tongue flicks' directed at different stimuli has been extensively used as a means of determining the level of preference for food and mates (e.g. (Labra, 2011; Runemark et al., 2011; reviewed in Mason and Parker, 2010). However, it is disputable whether the number of tongue flicks alone actually can be interpreted as 'preference', and we therefore restrict our interpretation of any differences only in terms of whether or not females may be able to discriminate between olfactory stimulation.

Scent was collected from males in a standardised manner by rubbing a sterile cotton bud along the length of the femoral pores of one leg, which ensured approximately equal amounts of secretion were collected. These cotton buds were then immediately presented to females during the tongue flicking trials.

Each female underwent a separate tongue flicking trial for each of the two males at the same time of day on two successive days. Pilot experiments showed that this time period between trials was necessary to reduce the effect of female habituation to the scented cotton buds. The presentation order of the two treatments was randomised for each female, and all trials were done in the female's own cage, which was covered with one-way plastic film to eliminate observer disturbances (see Appendix 3 Figure S1). Each male-scented cotton bud was presented simultaneously with a clean cotton bud (5cm apart), which acted as a control, both cotton buds were attached to a 30cm long wooden 'kebab stick' which allowed placing them onto the brick under the basking spot without disturbing the female, and removed any potential effects of an experimenter encouraging additional tongue flicks by moving the cotton bud once a female had seen it. The number of tongue flicks directed to each cotton bud was then counted over a 30-minute trial by an observer who was unaware of the male's basking treatment.

The difference in tongue-flicks towards the control and scented cotton buds was compared using a generalized linear mixed model (GLMM; to account for female as a random effect in tongue flick response), with a Poisson distribution, which confirmed that females responded much more strongly towards the scented buds (23±6.7 (mean±S.E.) tongue flicks per scented cotton bud vs 11±2.6 for the control; LRT: X^2=22.8, p<0.001). The total number of tongue flicks directed towards the scented cotton buds was then used as a response variable in generalized linear mixed models with Poisson distributions, with the predictors male body size, basking treatment and their interaction, and trial order (i.e. 1st vs 2nd trial). Female ID was included as a random effect. The significance of each fixed factor was analysed using likelihood-

ratio-tests on models with and without the factor (Valdar et al., 2006; Öckinger et al., 2010). There was a single outlier, but its removal did not affect the significance of the results and we therefore present the results including all data points.

5.2.5.2 Association preference trials

Trials testing female association preference were done two days after a female completed the tongue flick trials. The trials were carried out in an opaque cage (1500x300x500 mm) (length x width x height) containing three bricks and shelters with basking lights provided by a 60W tungsten bulb placed over each brick. Two bricks were scent marked with the same pair of males each female was exposed to in the tongue flick trials; a third brick acted as an unscented control. Bricks were scent marked by extracting a standardised amount of femoral pore secretion from males using sterilised forceps, which was then rubbed over a brick using a cotton bud. The control brick underwent an identical 'scenting procedure' with a clean cotton bud. Females were placed in the cage for 10 hours, and the brick she was basking on was recorded every 20 minutes (giving 28 observations per female), after which she was returned to her original cage.

Female association trials were analysed in two ways. Firstly, we wanted to determine if females had a preference for basking on any of the three bricks (i.e. the two scent marked or the single unscented control brick). The second analysis was restricted to observations where females were only seen basking on male-scented bricks, with observations on the control removed, to determine how male and female characteristics determined female association preference. In both analyses the total

number of times females were observed basking on a brick was used as the response variable in a generalized linear mixed model with female as a random factor, and with a poisson distribution. The only independent variable in the first analysis was basking treatment (with control, high-quality basking treatment and low-quality basking treatment as the three levels). In the second analysis the independent variables used were the number of tongue flicks directed towards the same male in the tongue-flicking trials; male body size, basking treatment; and the interaction between male body size and treatment. All statistical analyses were done using R (R Development Core Team, 2012), with the mixed models conducted using the lme4 package (Bates et al. 2012).

5.3 RESULTS

5.3.1 Pheromone composition and analysis

In total, we found 26 different lipophilic compounds that regularly occurred in the chemical extract from femoral secretions in the male lizards, of which 16 could be identified (see Appendix 6 Table S1 for a full list of compounds and their diagnostic ions and retention times). All males shared sixteen of these compounds and their normalised peak areas were consequently used as variables in the Principal Component Analysis. Of these shared compounds, the most common by relative abundance were Cholesterol (55.56%), α-Tocopherol (vitamin E) (13.55%), Cholesta-5,7 dien-3-ol (pro-vitamin D) (10.20%), Ergosta-5,8-dien-3-ol, (3β) (3.48%), an unknown compound with a retention time of 20.09 min (3.25%), and Squalene (3.05%).

The six main principal components (relative eigenvalues of principal components and eigenvector loadings of the different compounds are shown in Appendix 6 Table S2 and S3 respectively) were used in a MANCOVA on the femoral gland samples extracted from freshly caught lizards (before they began their basking treatments). This revealed a significant effect of male body size ($F_{6,21}$=2.91, p=0.032). The MANCOVA on the six main principal components of femoral gland secretions collected at the end of the basking treatment confirmed the effect of body size and also revealed a significant effect of the basking treatment on semiochemical composition (Treatment: $F_{6,23}$=3.01, p=0.025; body size: $F_{6,23}$=4.66, p=0.003). Subsequent ANOVAs with each principal component as a separate response variable

showed that male body size primarily affected PC1, and basking treatment significantly affected PC6, with borderline effects on PC2 and PC5 (see Appendix 6 Table S4 for summary statistics).

The follow-up linear models done on the individual chemical compounds on the second extracted samples showed the basking treatment effects were mostly attributed to differences in heptadecene, campesterol, octadecenoic acid and, to a lesser extent, hexedecanal (Appendix 6 Table S5; means for treatments +/- S.E.: heptadecene: high-quality: -2.00 +/- 0.10; low-quality: -1.87 +/- 0.11; campesterol: high-quality: 0.21+/- 0.11; low-quality: -0.42 +/-0.12; octadecenoic acid: high-quality: -0.52 +/-0.25; low-quality: -0.87 +/- 0.14; hexedecanal: high-quality: -0.99 +/-0.13; low-quality: -0.80 +/-0.10). Additionally, the negative correlation between PC1 and male body size in the first extractions was largely due to effects on heptadecene, hexedecanal, and campesterol (Figure 1; Appendix 6 Table S6).

5.3.2 Female behavioural responses

5.3.2.1 Tongue flick data

There was a significant interaction between male body size and basking treatment on tongue flicks (LRT: χ^2=5.65, p= 0.017), which was caused by larger males in the low-quality basking treatment receiving more tongue flicks than similarly large males in the high-quality basking treatment (Figure 2). Additionally, the changing relationship between the two treatments as male body size differed suggest that females tongue

flicked more for low-quality basking treatment males than for males from the high-quality treatment.

5.3.2.2 Settlement trials

In the female association experiment, females did not prefer to associate with bricks scented with males from either treatment, or indeed over the control brick (χ^2=1.01, p=0.60). Using GLMMs on observations of females limited to male-scented bricks (which allowed the inclusion of male-specific traits in the analysis) also showed that females did not show a preference based on male body size (χ^2=0.12, p=0.73) or male basking treatment (χ^2=1.22, p=0.27).

5.4 DISCUSSION

Our results show that the amount of time male wall lizards spend thermoregulating can have significant effects on the chemical composition of their femoral pore secretions. To our knowledge, this is the first study showing plasticity in the composition of a vertebrate's chemical signature in relation to the thermal environment (see Rouault et al., 2004; Wagner et al., 2001; Greene, 2010 for invertebrate examples of pheromone production).

The lack of attention to thermal effects on chemical communication is perhaps surprising considering that access to thermal resources by many ectotherms is highly correlated with suites of characters typically believed to be important in communication and sexual selection (Calsbeek and Sinervo, 2002). Indeed, several of the compounds affected by basking conditions in this study have been associated with male-male competition and male 'quality' in previous work on lizard chemical communication. For example, increased levels of hexedecanal appear to elicit male aggressive behaviour in the closely related Iberian rock lizards (*Podarcis hispanica*) (Martín and López, 2007; Martin et al., 2007). However, our study suggests that hexedecanal levels had a tendency to be higher in femoral secretions from both small males, which is opposite to the results in Iberian rock lizards (Martin et al. 2007), and those from the low-basking treatment. Additionally, differences in the amount of octadecenoic acid esters have been associated with parasite infection and colouration in male *P. muralis* (Martín et al. 2008). Given these effects, thermal plasticity of femoral secretion composition certainly has the potential to affect the dynamics of chemical communication in wall lizards. Below we discuss some potential

mechanisms underlying plasticity in the composition of wall lizard femoral secretions and the potential consequences for female mate choice and male-male competition.

Plasticity in male chemical profile composition may be an immediate consequence of the relative differences in the thermal-dependent rates of metabolising and secreting the diverse range of chemical compounds found in male scent marks, and therefore have no adaptive function from the signaller's perspective. If so, we might expect compounds that undergo the least modification between consumption and secretion (such as dietary derived compounds) to be least affected by basking opportunity. There were not a sufficiently large number of compounds that were strongly affected by the basking treatment to evaluate this hypothesis, but out of two major diet-derived compounds we identified, α-tocopherol and campesterol, campesterol levels were greater in males from the high-quality basking treatment. Alternatively, changes in chemical composition could also be an active process that maintains signal stability under variable environments. As with other signals, environmental conditions are likely to affect the efficacy of scent marks, which should in turn exert selection on their composition and design to best match the local environment (Alberts, 1992; Escobar et al., 2003; Martín and López, 2006a). For example, hotter and more humid environments should select for scent marks consisting of longer-chained compounds with lower volatility to reduce the costs of increased evaporation and assumedly shorter signal lifespan (Alberts, 1992; Escobar et al., 2003). This is consistent with our results because two of the four compounds that were most affected by the basking treatment, heptadecene and hexedecanal, were also the two most volatile chemicals that we identified, and were both found at lower levels in males from the high-quality basking treatment. Additionally, long-chain sterols and oily secretions such as

cholesterol have been suggested to function as ‘carrier molecules’, which encapsulate more volatile compounds and increase the functional lifespan of scent marks by decreasing evaporation (Alberts, 1992; Escobar et al., 2003; Martín and López, 2006a). This may explain the increase in abundance of campesterol (a sterol with similar properties to cholesterol) we found in the femoral secretions from high-quality basking males. Hexedecanal and heptadecene were also negatively associated with male body size in wild lizards, which is consistent with larger males monopolizing the best thermal habitats in the wild (Calsbeek and Sinervo, 2002). We therefore suggest the shift in chemical composition with basking opportunity may promote the stability of scent marks in thermally variable environments. This is important since a recent study showed that high temperatures decrease the lifespan of lizard scent marks, with suggested negative repercussions on mate choice (Martín and López, 2013). Further work is required to determine if the compositional plasticity we report here might to some degree ameliorate the direct effect of temperature on signal efficacy.

Thermal effects on semiochemical composition through physiological constraints or to maintain signalling effectiveness may ensure honesty by being an index signal, i.e. one that cannot be faked due to physical or physiological constraints on its production or efficacy (e.g. Maynard Smith and Harper 2003). Male lacertids emerge early from hibernation to aggressively defend large delimited territories which are extensively scent marked, and the later emerging females then establish smaller home ranges often totally encompassed within those of the males (Damme et al., 1987; Kwiat and Gist, 1987; Edsman, 1990; Olsson and Madsen, 1996; Font et al., 2012; R. Heathcote, G. While & T. Uller pers. obs.). Femoral secretions from male lacertids appear to provide information about the size, competitive ability, identity and potential threat

level of males through a complex mixture of compounds (Martín and López, 2007; Martin et al., 2007; Font et al., 2012). If this signal composition changes due to physiological constraints, or to maintain effectiveness over differing environments, then information about the local habitat conditions may also be inadvertently provided for other individuals, such as females, who could use this information in settlement decisions. This could imply that whilst female choice might play a minor role in the evolution of scent marks from the signallers perspective, as has been argued by others (e.g. Font et al., 2012), selection may nevertheless favour females that are able to exploit cues about habitat or individual quality that are left by male scent marks. If so, we would expect females to be able to discriminate between males from different thermal treatments and to prefer or associate with scent from males from high-quality basking conditions.

Consistent with this hypothesis, the tongue flick trials in this study show that females can differentially respond between different sized males (large size is typically associated with male 'quality' in lizards; e.g. Tokarz (1985)) based on tongue flicks, and those that had experienced different degrees of basking opportunity. However, females tongue flicked more for the low basking treatment males. This sheds doubt on the assumption that tongue flicks and female preference should be positively correlated (assuming that females should prefer males with good basking resources or habitats) (e.g. Martín and López, 2006b; Runemark et al., 2011; reviewed in Houck, 2009). If tongue flicking is viewed simply as the collection of olfactory information, then differing levels of tongue flicking rates could arise for a number of reasons independent of female preference of high quality males (Cooper, 1994; Mason 1992). For example, objects or individuals perceived as novel or unusual by many animals

often receive increased sensory exploration, a concept underlying classic habituation-dishabituation paradigms (Falls, 1982; McGregor, 1993; Carazo et al., 2008). Interestingly, the difference in tongue flicks between the basking treatments was more exaggerated with larger male pairs. Whilst some compounds were affected by both male body size and thermal opportunity (e.g. hexedecanal, heptadecene), there were also several that were uniquely affected by either male body size (pro-vitamin D and two identified compounds: 22.241 and 23.65) or basking treatment (campesterol) alone. Females may therefore be able to perceive a mismatch between different aspects of the chemical signature that usually correlate since dominance and territory quality are usually strongly associated with body size in territorial lizards (including *P. muralis*) (e.g. Fox et al., 1981; Edsman, 1990; Haenel et al., 2003). Since large males are unlikely to be found in poor basking habitats, the females may have directed more tongue flicks to obtain more olfactory information about these 'unusual' individuals. While this explanation is speculative, we stress that greater tongue flicking does not necessarily imply preference, and that differences in tongue flicking should be used to infer a potential ability to discriminate between scents and not as an index of preference.

Despite the differences in tongue flick response, females did not preferentially associate with basking spots scented with femoral secretions from large males with a good basking treatment (in fact, females did not preferentially associate with male scented bricks over the control either). Female spatial associations using this experimental design are commonly assumed to approximate mating decisions in lizard olfactory studies (e.g. Martín and López, 2000; López et al., 2002; López and Martín, 2005). If this assumption is correct, our study suggests that whilst female *P.*

muralis can detect olfactory cues that reflect male body size and basking experience, they do not appear to choose males based on these characteristics. However, caution must be taken when dismissing this possibility as the methodologies commonly used to assess settlement behaviour as a proxy for mate choice have not yet been shown to reflect actual mating decisions. Indeed, Carazo and colleagues (2011) showed that females might even prefer to take refuge in territories of smaller males, opposite to the mating patterns seen naturally. Since females respond differently to male scent marks depending on the male's basking experience, we feel the potential that females may use this information in mating decisions is still an open question.

A major function of male lizard scent marks is to provide information to competing males as well as females (Gosling et al., 1996; Haenel et al., 2003; Carazo et al., 2007; Font et al., 2012). If olfactory cues indicating access to resources, such as good basking sites, are detectable by other male lizards, this could increase the costs of territory defence by residents (e.g. Bushmann and Atema, 2000). Therefore, if females do not choose males based on their scent marks, why should males reveal the quality of their territory resources if they do not obtain mating benefits in doing so? Whilst we cannot currently answer this question, it is possible that thermally-influenced olfactory cues in this species are a by-product arising from the necessity to secrete chemically diverse scent marks that have a multitude of important functions, such as indicators of male competitive ability, and species and mate recognition (Cooper and Trauth, 1992; Shine et al., 2002; Barbosa et al., 2006; Moreira et al., 2006; reviewed in Johansson and Jones, 2007). As argued previously, one potentially important factor therefore is the need to moderate scent mark composition to maintain the signalling of these important functions over differing environments. Thus, scent

mark plasticity in response to different environmental factors, such as temperature, may not be under strong direct selection as a signal component in itself, and potentially even create costly repercussions on signallers, for example due to indicating ownership of poor territories which females may avoid. Experiments testing this could focus on decoupling intrinsic male quality, scent mark, and territory resource prevalence and quantifying the resulting mating patterns (*sensu* Calsbeek and Sinervo, 2002).

The effects of the thermal environment on the composition of femoral secretions may also explain some of the previously observed population differences in semiochemical composition, which are often attributed to genetic divergence (e.g. Escobar et al., 2003; Runemark et al., 2011; Gabirot et al., 2010a; Gabirot et al., 2010b). Future studies using *in situ* sampling of lizard (and other ectotherm) semiochemicals may need to take plasticity in relation to the local thermal environment into account when attributing the cause of population differences in chemical profiles. With respect to the present study species that has a very broad geographic range, including introduced populations (Schulte et al. 2011; Michaelides et al. 2013; Salvi et al. 2013), both direct climatic effects and adaptive plasticity may contribute to geographic variation in chemical profiles. We suggest that comparative analyses of chemical composition of femoral secretions would be useful to begin to assess the extent to which they have diverged in response to local climatic conditions.

In summary, we have shown that the chemical signature of male wall lizards can be affected by basking opportunity, providing the interesting possibility that lizards are capable of dynamically modifying their scent marks to enhance signalling stability

across differing thermal habitats. Whilst females are able to detect these differences, there is little evidence they use this information in mate choice. We suggest that the thermal environment may have important effects on the generation and use of olfactory information by conspecifics, which may have interesting consequences on social behaviour in ectotherms.

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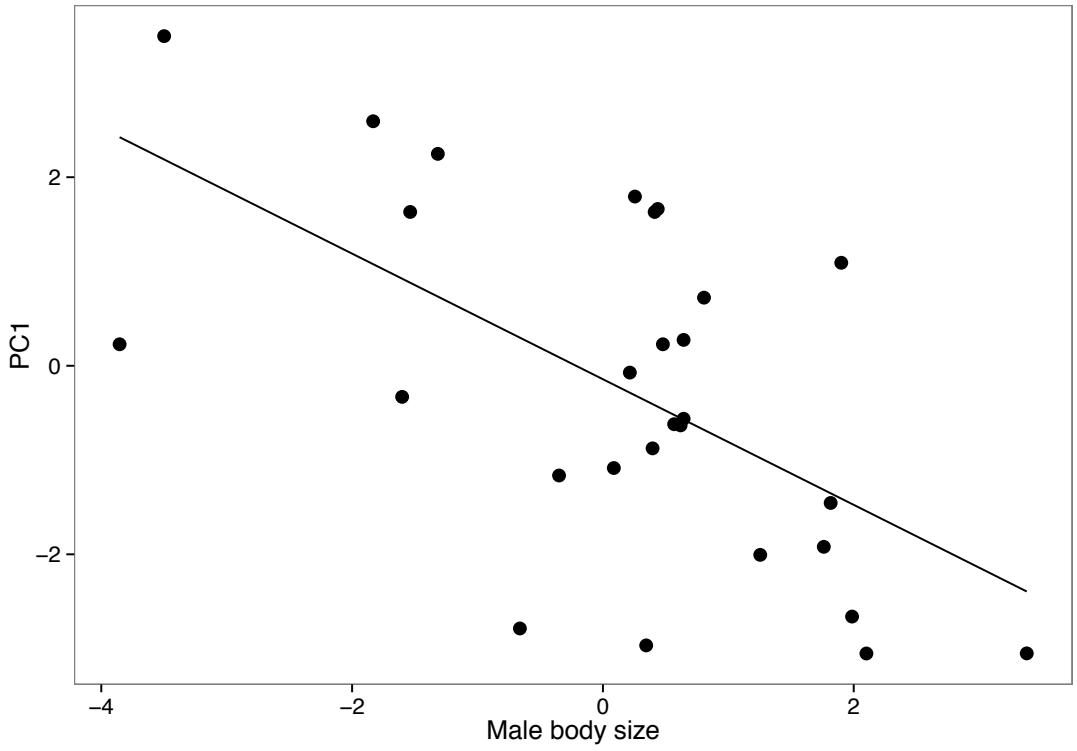


Figure 1: Correlation between male body size and PC1 in recently captured male lizards before they underwent the different basking conditions.

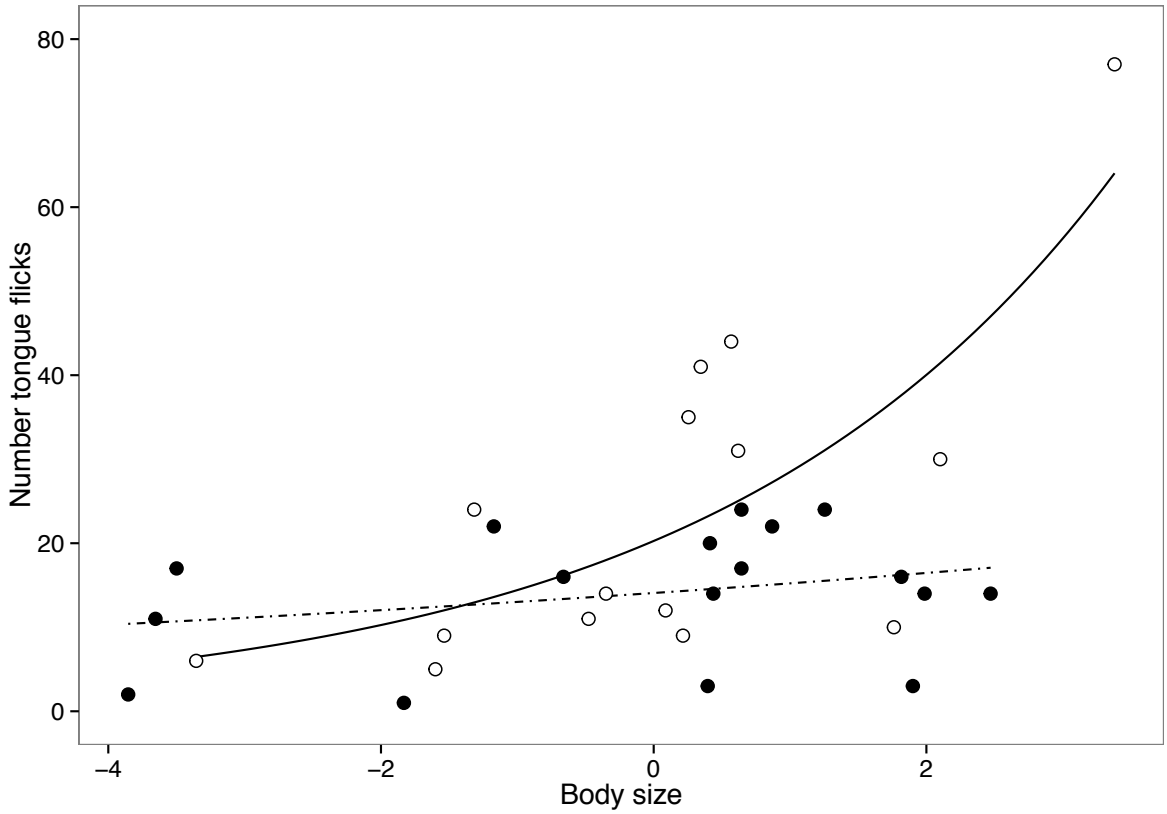


Figure 2: Interaction between a male's body size (based on the first principal component between mass, SVL, head length and head width) and basking treatment in the number of tongue flicks a female directed towards male-scented cotton buds. Males in the high-quality treatment are filled points with the dashed line, and low-quality treatment males are white points with the solid line. Note that one single outlier (male body size=0.81, tongue flicks= 157) has been removed for clarity of presentation.

CHAPTER SIX

GENERAL DISCUSSION

Unpublished manuscript

6.1 Overview

This thesis has addressed how behavioural interactions mediate patterns of hybridisation upon secondary contact in a lizard. In Chapter two, I showed that there are strong asymmetries in male dominance between the Italian and French lineage, which appear to be driven by historical differences in the strength of sexual selection. Specifically, males from the Italian lineage exhibit exaggerated expression of a suite of traits that have been shown previously, and in this thesis, to be associated with male competitive ability. However, the extent to which this dominance extrapolates into gaining access to females was found to depend on the spatial clustering of a key resource: basking sites. Chapter three moved on to address the actual patterns of paternity that arose from these behavioural interactions. I showed that whilst Italian males did father more offspring overall, this was perhaps not as high as expected based on their dominance, and there was in fact a surprisingly high degree of assortativity in terms of courtships and, in particular, parentage in the enclosure-sired offspring. Rather than being due to female choice reducing the extent to which Italian male dominance translates into paternity, I showed that the Italian-biased male dominance actually contributes to assortative paternity. By aggressively defending the largest females, which are of Italian origin, Italian males are reducing the ability of French males to hybridise. Furthermore, it appears that Italian males exercise some degree of discrimination against French females, leaving them more easily accessible to the sub-dominant French males. The enclosure experiment also provided me with an opportunity to address the phenotypic traits that predict hybridisation at the individual level in my fourth chapter. As expected from the previous chapters, hybridisation was particularly high in small Italian males and large French males,

which have relatively low reproductive success and are subordinate relative to large Italian males. Additionally, males with a low dominance status were more likely to sire hybrid offspring, implying that males become less 'choosy' for conspecific females as their competitive ability to gain access to these declines. Importantly, this result also suggests that both lineages of males may show conspecific mate choice independent of female size. Body size also appeared to influence the propensity of females to hybridize, with a trend towards smaller Italian and larger French females to have increased proportion of hybrid offspring, although the actual trait most important in determining this pattern was head size. In my final chapter, I showed that scent marks, a major signal used in general communication in lacertids, were also strongly affected by a male's opportunity to thermoregulate. Whilst females could detect this change, they surprisingly did not appear to prefer males from good basking environments, even though females have been suggested to extensively use scent mark cues in mate choice. Regardless, environmental shifts such as changes in thermal opportunity are unavoidable consequences of secondary contact, which raises questions about how the communication dynamics in ectotherms that invade new habitats might be affected as a result.

6.2 The future of secondary contact in wall lizards

Given the behavioural factors that mediate mating patterns between the two wall lizard lineages, what are the potential evolutionary consequences of this system? This is a realistic prospect to consider since several of the introduced wall lizard colonies in Germany and the UK are already, or have the potential to be, in secondary contact

through either human translocation or range expansion through natural dispersal (see Schulte et al. 2012; Michaelides et al. 2013). Since studies investigating genetic patterns of secondary contact often speculate about the underlying processes that drive them presently or drove them in the past (e.g. Hewitt 1993; Klingenberg et al. 2000; Arntzen et al. 2009; Chatfield et al. 2010), I feel validated in speculating from the opposite end of the spectrum here.

Firstly, when considering this question, we must remember that the results I have reported in Chapters two, three and four relate only to a 'first generation' of secondary contact. If hybrids between these lineages are fertile, the mechanisms that maintain assortativity discussed in Chapter three would rapidly break down as the morphological and behavioural variables become blurred through introgression. If so, and given the strong benefits of being an Italian in direct staged interactions and in the enclosures, I would expect Italian traits to spread rapidly and create an Italian-biased 'hybrid swarm'.

Chapter two suggests that Italian male dominance and asymmetrical mating success, and therefore potential for biased introgression, is enhanced in habitats with clumped basking resources with high local population densities. Interestingly however, this result did not result in increased hybridisation rates in the clumped basking-site resources (Chapter four), possibly because the more dominant males with greater mating success are also able to exercise more choice towards the best females (large Italians). Nevertheless, in some scenarios at the invasion front, where conspecific mates might be rare, this dominance effect is likely to play an important role when there are fewer conspecific females available than a dominant male is able to

monopolise access to. My data suggests that, in these scenarios, dominant Italian males are likely to preferentially court the largest females (at least in regards to head size, which correlates strongly with body size) and therefore most fecund heterospecific females, potentially accelerating introgression into the subordinate lineage. This prediction is consistent with on-going hybridisation in the human-mediated secondary contact range of this species in Germany, where introduced Italian nuclear genes are rapidly introgressing into the native *P. m. muralis* subspecies (similar in appearance and sister lineage to the *P. m. brogniardi* used in this study). However, hybridisation is likely to be particularly extensive when a small number of individuals are introduced into the range of another lineage due to a low relative availability of conspecific mates (Wirtz 1999; Willis 2013; see Chapter one). Whilst the Italian genome will likely be swamped in these scenarios, Italian animals possess a suite of traits that make them strongly dominant in competition with French males. Depending on the underlying genetics of these traits, there is potential that some might be maintained even when small numbers of Italian animals are introduced into French populations. For example, if green dorso-lateral colouration in Italian animals enhances reproductive success, and the genes responsible for its expression show tight linkage, it is possible for this trait to be retained even when the rest of the Italian genome has been swamped. For example, the golden collar in the manakin *Manacus vitellinus* has been shown to spread rapidly through populations of white-collared manakins *Manacus candei*, even whilst the latter seem to have retained the majority of their lineage-specific characteristics (Parsons et al. 1993). Even so, the introductions of small numbers of Italian animals into larger native populations represent an extreme version on the scale of secondary contact, the other being the more readily recognisable consequences when much larger populations expand their

range following the removal of a geographical barrier, where the relative density of conspecifics and heterospecifics can be similar (such as the conditions I simulated in my enclosure experiment) (Haffer 1969; Barton and Hewitt 1985; Barton and Hewitt 1989; Zamudio and Savage 2003). In these cases, we would expect introgression to be slower since Italian males would have a greater availability of conspecific females.

It must be stressed that any speculations on the future of hybrid zones require information on the costs associated with hybridisation. These costs can include anything from marginally reduced survivorship of hybrid offspring (e.g. Lancaster et al. 2007), to absolute reproductive 'failure', where embryos may die prematurely (Tyler et al. 2013), or where the offspring are totally sterile (Haldane 1922; Hewitt et al. 1987). If the costs of hybridisation are high enough, or if the competitively inferior lineage can subsist in less preferred habitats, even strong asymmetries in dominance and interspecific mating success can allow the sustained coexistence of hybrid zones in the wild (Sperling and Spence 1991; Pearson 2000; Pearson and Rohwer 2000; Reudink et al. 2006; Vallin et al. 2012). At the moment, there is only limited data on hybrid performance between the two lineages of wall lizards in this thesis. However, there is no evidence that hybrid offspring show increased hatching failure or reduced growth in captivity (T. Uller & GM While unpublished data), or that females produced more infertile eggs if they had hybridised (Chapter four). Further work that quantifies these costs, and in particular the fertility of male and female hybrids, will be able to shed light on whether they may be sufficient to reduce introgression sufficiently to maintain a sustainable hybrid zone under some circumstances between these lineages.

Assuming the costs of hybridisation are high enough, lineage integrity could possibly be retained long enough for the populations to respond to selection against hybridisation. If this occurred, then the phenotypic patterns I uncovered in Chapter five imply that disruptive selection might exaggerate the current phenotypic differences between the lineages; increasing the size of the Italian animals and decreasing that of the French. However, perhaps a more likely response would be evolution of lineage recognition. The present data suggests that either scenario is perhaps unlikely given the rates of hybridisation and limited evidence for low hybrid fitness. Thus, I conclude by suggesting that of the possible outcomes of hybridisation in this system, overall asymmetrical hybridisation from the Italian into the French lineages is the most likely outcome of secondary contact between the lineages.

6.3 Signalling plasticity and its potential consequences in secondary contact

The scent-mark plasticity I detailed in Chapter five was focused on the influence of basking opportunity on olfactory communication, and it raises some intriguing questions regarding how introduced species may interact following environmental shifts. For example, there is clear evidence that scent mark composition in lizards acts a 'signature mixture' in regards to advertising individual and heterospecific identity and competitive ability (Carazo et al. 2007; Carazo et al. 2008; Font et al. 2012). Since secondary contact often involves a shift in environmental conditions for at least one of the lineages, we should consider how this phenotypic plasticity will influence

inter- and intra-specific communication and competition in a secondary contact zone. One possibility is that there is a clear difference between the Italian and French male femoral pore secretions in respect to the relative composition of several compounds, the most notable of which were heptadecene, hexedecanal and octodecanoic acid; Italian males also secreted α-tocopherol (Chapter three). Interestingly, I found that these same three compounds were plastic in response to basking opportunity; lower heptadecene and hexedecanal and higher octadecenoic acid were found in the high-quality basking treatment. Thus, there is the potential that environmentally-induced changes in scent mark composition could alter the degree to which heterospecifics recognise and appropriately respond to one another (sensu Fisher et al. 2006). Clearly, a major area that needs further exploration is exactly how scent mark composition relates to male-male communication and competition, as female discrimination did not appear to be functional, at least according to the observations of spatial behaviour in the settlement experiments and outdoor enclosures (Chapter 3 and 5). In addition, my results provide support for the idea that females, rather than males, are acting as the signallers in lacertid olfactory-based mate choice, and yet the chemical characterisation and function of female chemical signatures have been almost totally ignored (see Font et al. 2012 for an exception). Clearly, a shift from the 'female mate choice' focus in lizard olfactory studies and a greater focus on the potential role of male mate choice in response to female signals, or male assessment of rivals (sensu (Carazo et al. 2007; Carazo et al. 2008), may prove particularly rewarding.

6.4 The role of male and female choice in driving hybridisation

An interesting theme that has emerged from this thesis is the apparent absence of female choice for male characteristics, whether it is by lineage, size or other indices of 'quality'. It is possible that by being allopatric, and thus naive to any direct selection pressures associated with costly hybrid matings, the lack of conspecific female preference we report here is simply a non-adaptive response. However, female choice does appear to be very rare in lizards (reviewed in Olsson and Madsen 1995; Tokarz 1995), although the olfactory-based discriminatory and preference tests we conducted throughout this thesis have previously been used to demonstrate sophisticated olfactory-based 'choice' abilities in female lacertids (reviewed in (Mason 1992; Mason and Parker 2010a; Martín and López 2011, but see Font et al. 2012); See below for an overview of the methodologies used in lizard mate choice studies). In line with our results, however, other researchers have also recently argued that male choice, rather than female, is the predominant mechanism determining mating patterns and heterospecific mating avoidance in this taxon, with limited chemical mate choice in females (Carazo et al. 2011; Font et al. 2012). Indeed, our results suggest that patterns of hybridisation will largely be driven by males with no, or at least very limited, contribution by female choice. This contrasts with the theoretical expectation that females, by being the sex that invests more per capita of offspring, should be the 'gatekeepers' of hybridisation, as they should experience the costs of hybridisation more intensely compared to males (Wirtz 1999; Randler 2002).

So why are most female lizards so cavalier in their choice of the males that court them? It has been previously argued that because lizards are strongly dependent on

temporally stable and patchy resources such as basking sites and shelters for almost all aspects of biological functioning, the onus on female choice in many species is not so much in relation to male quality but to the best resources (Tokarz 1995). In turn, this facilitates male-male competition for obtaining access to these resources and their associated females, removing the necessity to choose males directly as male quality and territory/resource quality will be correlated (Tokarz 1995). Whilst this provides a relatively convincing argument for why female choice for male quantitative traits may not be highly developed in this taxa, we failed to provide evidence that females use male scent marks as cues about habitat quality. In contrast, male choice appears to be common in lizards, in particular for larger females (Olsson 1993; Whiting and Bateman 1999), something that this thesis has confirmed, and that also has implications for the patterns of hybridisation at the individual level.

There has been a recent surge in interest for how male choice can evolve. Traditionally, male choice has been considered to primarily occur in species where male investment is high, such as through paternal care or material investments such as nuptial gifts or copulatory plugs (Trivers 1972; Gwynne 1991; Shine et al. 2003). Male choice should be particularly pronounced when the number of available females exceeds a males ability to mate with all of them (Trivers 1972; Edward and Chapman 2011), which can involve situations such as female-biased sex ratios due to sex-linked mortality (Kokko and Monaghan 2001), time-consuming courtship investments (Shine et al. 2003), or monogamy (Jones and Hunter 1993; Jones et al. 2001). But how can we explain male choice in a species that enjoys high polygyny and provides no paternal care, such as in the majority of lizard species? One potential suggestion is that high levels of sperm competition can result in correspondingly high ejaculate

volumes, which in themselves can be costly in terms of energetic investment and/or time to replenish (Dewsbury 1982). This has been used to explain why male *Drosophila sp*. show relatively sophisticated choice abilities for more fecund females (Byrne and Rice 2006; Long et al. 2009). Sperm competition is a relatively under-studied area in herpetology (Uller et al. 2010), although the often-extreme degree of multiple paternity in lizards and other reptiles (see Uller and Olsson 2008) suggests that it may be high and thus sperm may be a limiting factor in male reproductive success. In addition, some species exhibit mate guarding to reduce the ability of other males to copulate with females (Olsson 1993; Cooper Jr 1999; Zamudio and Sinervo 2000). Any time spent guarding a female means a male cannot spend time searching for and courting other females, in which case mate choice should be particularly evident through this behaviour, as shown in this thesis and previous studies of sand lizards (*Lacerta agilis*) (Olsson 1993); Chapter three). In summary, male mate choice is a subject that clearly requires more research (Edward and Chapman 2011), and in particular, the results from this thesis suggest that it may be an underappreciated cause of the varying mating patterns that arise in the early stages of secondary contact.

6.5 The use of tongue flicks and other paradigms in lizard mate choice studies

Some of the inferences in this thesis have relied on the use of assessing mate choice in lizards by quantifying tongue flicks in the lab and determining female settlement patterns in naturalistic enclosures or large indoor cages. Tongue flicks are a very

commonly used paradigm for assessing lizard mate choice, which many authors use under the assumption that increased 'preference' for a mate results in increasing tongue flick rates (references in Mason and Parker 2010b). However, the assumption that tongue flicking should correlate with increased mate preference may not always be a valid one (Cooper, 1994; Mason 1992) and the results in this thesis suggest further questions about the use of tongue flicks as indicators of preference. Increased sensory exploration of cues can occur under multiple scenarios, for example such as when objects or items are perceived as novel (Falls 1982; McGregor 1993). As such, additional experiments are often needed to determine the biological interpretation of tongue flicks. My thesis provides clear evidence for this requirement. Whilst female lizards showed increased tongue-flick rates for the scent of small males from the low-quality basking enclosure (Chapter 5), they did not show any preference to spatially associate with males based on either body size or basking treatment in the settlement experiment. Additionally, male Italian lizards only showed a difference in tongue flicking for female lineages when females were small, and French males only showed a difference when females were large (Chapter 3). If this data was interpreted as 'preference', then Italian males should have preferred small Italian females, and French males preferred large French females. Clearly the courtship data I obtained did not support this assertion. In all, the data in this thesis collectively suggests that differential tongue flicks can only be interpreted in terms of discrimination between chemical cues, and further experiments are necessary to corroborate the biological function of any discrimination uncovered.

Throughout all the behavioural observations I obtained during this thesis, there were no instances where females appeared to instigate courtship or mating behaviour with

males. The possibility of female choice in lacertids is therefore most likely via female spatial behaviour. One potentially useful paradigm for assessing the functional significance of tongue-flick tests is therefore the settlement-style experiments I used in Chapters three and five (and used in Carazo et al. 2011). Considering that females are almost always found in close association to males in the wild (Pérez i de Lanuza et al. 2013; R. Heathcote pers. obs), and that males emerge from winter dormancy before females (Kwiat and Gist 1987; Edsman 1990; Olsson and Madsen 1996), some authors have suggested that females likely settle within the territories of males based on certain sensory cues (e.g. Martín and López 2012). I tested this using artificially scent marked basking resources in outdoor enclosures and large indoor cages, and whilst females preferred to settle in proximity to a male when the scale was large enough (i.e. the outdoor enclosures rather than the indoor cages), they did not show a preference for different males. The association with male scent over controls in the more naturalistic setting provides some proof-of-principle that females do make use of scent marks in their decisions on where to settle. However, the lack of discrimination between males that differ substantially in their scent marks is strong evidence against scent marks being used in female choice. A potential reason for this lack of effect might be that females are using additional sensory cues as well as olfactory ones. A potential further experiment would be to test female association preferences based on visual cues as well (e.g. Font et al. 2012). However, there was no evidence that females might assort by lineage in regards to their home ranges, further suggesting female wall lizards exhibit a surprising lack of pre-copulatory choice (Chapter three).

6.6 Outstanding questions

As mentioned above, the inferences that we can make regarding the consequences of any behavioural interactions for patterns of introgression will depend on the extent to which hybridisation is costly. The current data suggests costs are minor, but this will require further studies. From a more mechanistic perspective, one area I have not been able to address are the potential postcopulatory factors that may influence paternity patterns in this system. The staged mating trials that I outlined in Chapter three, whereby a single female was allowed to mate once with a single male from both lineages, were also used to obtain some preliminary data on postcopulatory paternity via genotype parentage (the data for which has only just become available as of 09/10/13). Preliminary findings suggest that paternity is biased towards Italian males, regardless of the female he mated with (80% with Italian females (37/46 juveniles), and 62.5% with French females (15/24 juveniles)). Overall this suggests that the behavioural factors that I have uncovered here are clearly of pivotal importance for the underlying paternity that arose from the enclosure experiment, as paternity was also strongly assortative with the French females.

So what might these postcopulatory mechanisms be? Generally speaking, whilst postcopulatory paternity biases have been well established in some reptile species (e.g. Olsson et al. 1996; Calsbeek and Bonneaud 2008; Keogh et al. 2013), the exact mechanisms that determine them are poorly understood. For example, sperm storage is well established in many lizard species (although poorly so in *P. muralis* (R. Heathcote, T. Uller unpubl. data)), usually via sperm storage tubules in the oviducts

(Fox 1963; Conner and Crews 1980), which allow females to store sperm for many months after mating (e.g. Calsbeek et al. 2007). Sperm storage has been suggested by some authors to provide a means by which females may be able to exercise some control over which male's sperm fertilises her eggs (Passek 2002). The potential role of male sperm competition in driving paternity patterns has received little attention within populations (Olsson et al. 1994), and, as far as I am aware, conspecific sperm precedence has not been addressed in lizards. Until detailed physiological studies are performed on lizards, in a similar manner to birds (e.g. Birkhead and Brillard 2007), the mechanisms that mediate postcopulatory patterns will remain elusive.

6.7 Concluding remarks

This thesis has focussed on the behavioural determinants of mating patterns in the European wall lizard during the early stages of secondary contact. I have shown that divergence in male sexual characters in allopatry is likely to generate biased patterns of introgression, from the more dominant lineage into the less dominant one. However, the extent to which this happens may to some degree depend on the habitat structure that determines the ability of dominant males to monopolise females. In fact, paternity was largely assortative upon first contact, although the potential for reinforcement should still be limited. Female choice was found to be of no importance in generating these patterns. Overall, the effects that are demonstrated here provide a partial explanation for biased, but variable, hybridisation rates found across naturally occurring contact zones, and suggest potential mechanisms by which variable mating

patterns can arise during the early stages of secondary contact when mate choice is non-adaptive.

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Appendix 1

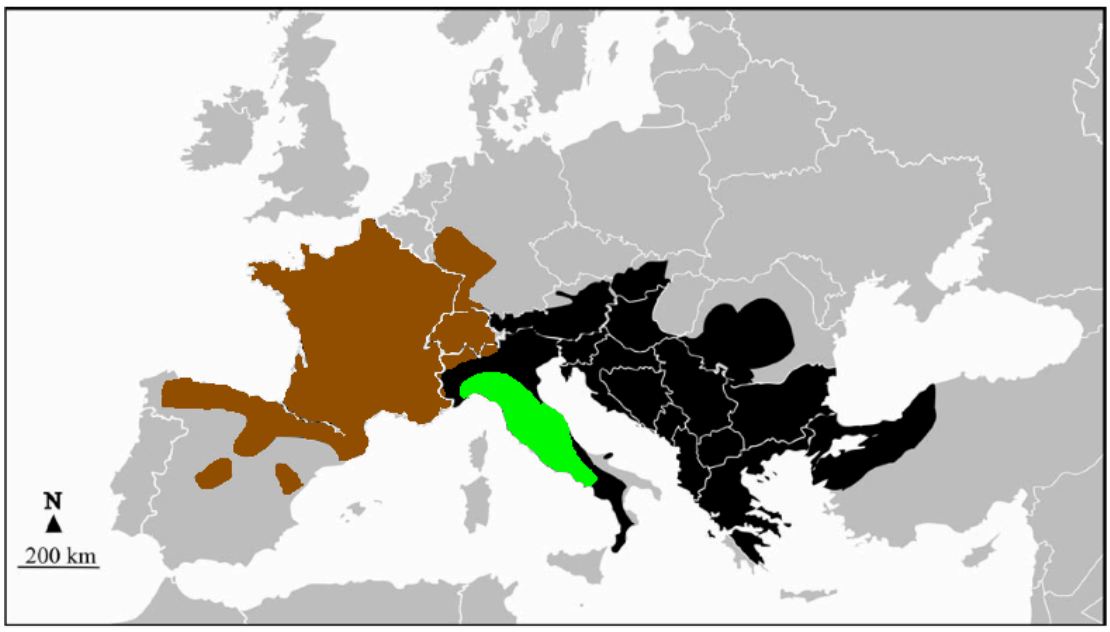


Figure S1) Range map of *Podarcis muralis*, showing the native distribution of the two different subspecies being used in this study. Green areas refer to *P. m. nigriventris* and brown to the *P. m. brogniardii*.

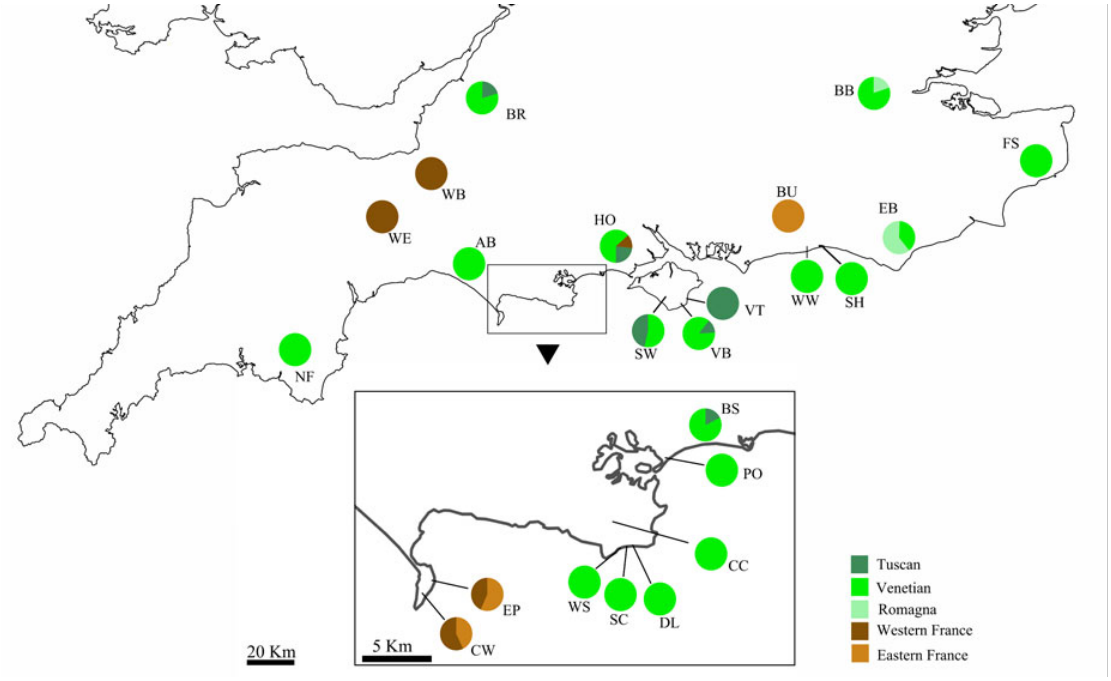


Figure S2) UK distribution of wall lizard populations. Pie charts denote the proportion of haplotypes from either the Italian lineage (green) or French lineage (brown). Taken from Michaelides et al, 2013.

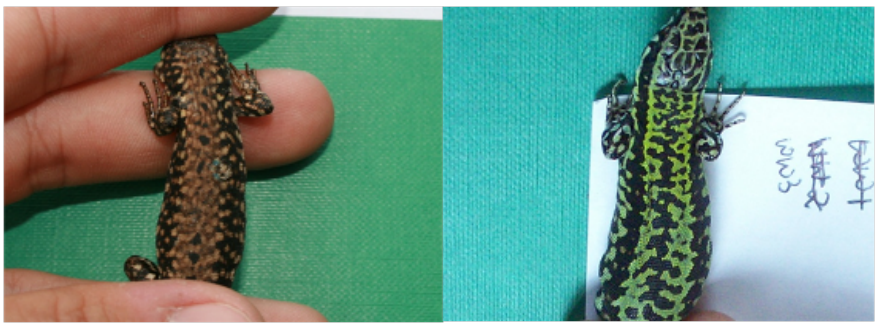


Figure S3) Differences in dorsal colouration between the French (left) (*P. m. brogniardi*) and the Italian (right) (*P. m. nigriventris*) wall lizard subspecies found in the UK.

Table S1) Summary of morphological differences between the two major wall lizard lineages in the UK, based on UK animals caught in 2012. 'Italian' refers to the *P. m. nigriventris*, and 'French' refers to *P. m. brogniardi*. All values are mean +/- standard error.

Lineage	n	SVL	Mass	Head length	Head width
Italian	81	61 +/-0.5	5.6 +/-0.13	13.65 +/-0.178	6.82 +/-0.089
French	87	59 +/-0.4	4.8 +/-0.11	13.45 +/-0.205	6.88 +/-0.111

Appendix 2

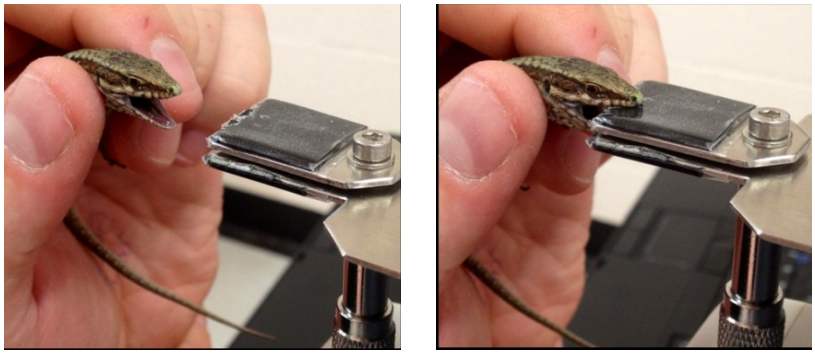


Figure S1) Taking lizard bite force recording with the bite force meter.

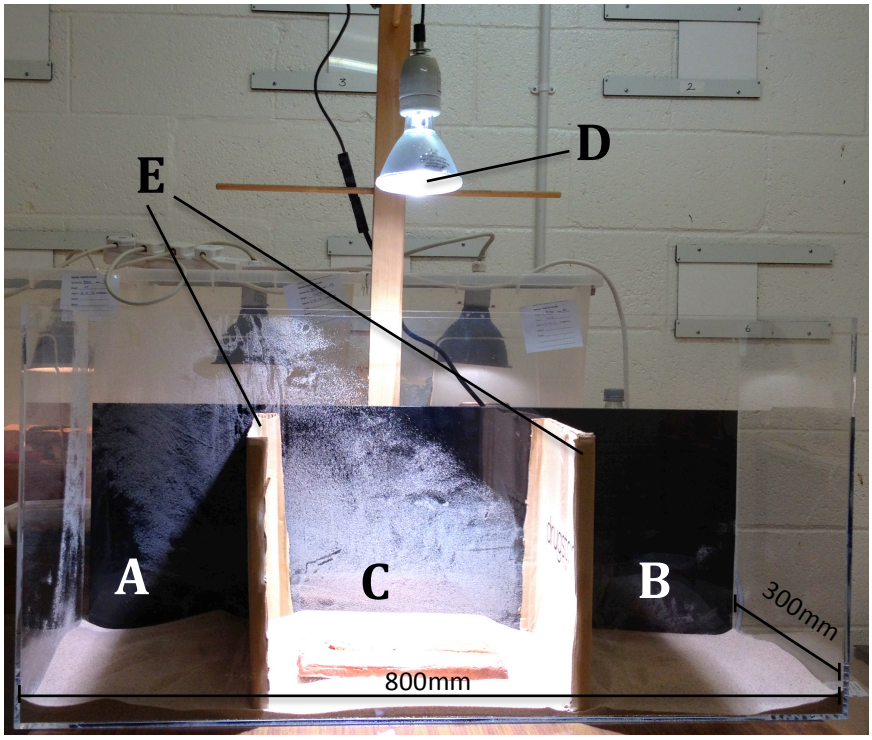


Figure S2) Annotated photograph of indoor enclosure used for the staged territorial interactions between male lizards. Compartments 'A' and 'B' represent the areas that two competing males were habituated in whilst the opaque dividers ('E'), restricting their access to the basking site ('C'), were in place. 'D' shows the full spectrum bulb ('Lucky Reptile Bright Sun') that emits the ultraviolet light visible to most lizards, which was used to create the contested basking area during trials.



Figure S3) Outdoor enclosures, Oxford University Field Station, Wytham, Oxfordshire.

Table S3) Ethogram of behaviours associated with territorial disputes between lizards used in the staged enclosures. Displays can occur concurrently with approaches, touches, charges, and bites. Tail undulations can occur concurrently with foot shakes.

Behaviour	Aggressive/submissive	Score
Approach	Aggressive	1
Display	Aggressive	2
Touch	Aggressive	3
Charge	Aggressive	4
Bite	Aggressive	5
Chase	Aggressive	6
Fight	Aggressive	7
Retreat	Submissive	NA
Foot shake	Submissive	NA
Tail undulation	Submissive	NA

Table S4) Ethogram of behaviours associated with territorial disputes and courtship behaviour between lizards in the staged enclosures.

Category	Behaviour	Description
Maintenance		
	Bask	Exposure to direct sun whilst stationary, often angling body to maximise heat absorption
	Feeding	Pursuing or handling prey
	Patrolling	Active movement along stereotyped pathways characterised by frequent tongue flicking and temporary periods spent stopping. Usually associated with encounters with prey or conspecifics
Male Aggressive		
	Approach	Direct approach towards conspecific at regular patrolling speed
	Display	Stereotypical posturing, involving arching the thorax with the nose pointing down, extending the jowels downwards to expose the throat, lateral flattening to expose the dorso lateral colouration including the blue spots
	Charge	Extremely rapid movement with mouth open towards conspecific male
	Contest	Both lizards displaying towards one another, often facing opposite directions within 1 body length
	Lunge	Quick, rapid movement to strike opponent with mouth open
	Bite	Result of Lunge and Charge where initiator makes contact and bites down on opponent
	Head grasp	Opponents lock jaws during contest
	Chase	Victor rapidly pursues loser as the latter retreats
	Fight	Rapid attempts to bite form both contestants
Courtship		
	Approach	Direct approach towards conspecific at regular patrolling speed
	Display	Stereotypical posturing, involving arching the thorax with the snout pointing down, extending the jowels, lateral flattening to expose dorso lateral colouration including the blue spots
	Tongue flick	Tongue flick the tail of a female.
	Tail grab	Grasp tail in jaws
	Mating	Copulation
	Mate guarding	Male and female pair spends extended periods of time in close proximity (within one body length) of one another >1 hour.
Defensive		
	Retreat	Rapidly retreat from antagonist
	Foot shake	Rapid foot shaking in response to aggressive conspecific, or from unreceptive females towards males
	Tail quiver	Rapid quivering of tail by unreceptive female in response to tail tongue flick from male

Appendix 3

Table S1) Primer sequences of the nine novel microsatellite markers used in this thesis, with allele characteristics based on 526 lizards (including all individuals used in Chapters 3 and 4). H_O/H_E refers to observed/expected heterozygosity.

Locus	Primer sequences (5'-3'; F, forward; R, reverse)	Repeat motif	Size range (bp)	Annealing temperature (°C)	No. alleles	Individuals Amplified	H_O/H_E
C150	F: GTCAGCTTTGCAGCACCTTAG	CA	176-204	57	12	523	0.71/0.88
	R: GCGATTAGAGAAGGCGTTTG						
C168	F: GGTCCGGCTTCAAAGAATAAG	TTTC	225-257	57	14	525	0.81/0.85
	R: CAGAGGACTCGCTCAAGGAC						
C275-278	F: GCTTAAAATTAATGCTGCTATTGTATC	TATC	219-553	57	44	524	0.84/0.95
	R: ATAGGTAGAAAATTTATAAACCCTTGG						
C028	F: TTGCTTCTGAATACGCCTAGC	TATC	228-328	57	16	524	0.74/0.85
	R: AGTGTATTGCGACTGTCAATGG						
C038	F: CAATGTGCAGTGTTGGGTTG	TATC	211-548	57	27	516	0.80/0.93
	R: ATGTGAGCGACTCCTGGATG						
C164	F: ATCGATGAATGAAGGGCAGT	GATA	185-222	57	9	526	0.54/0.80
	R: CCAGGCATTGTCAAACTATCTG						
C103	F: CCAGGTCTTGTGATCGAGTG	GATA	325-480	57	21	519	0.67/0.90
	R: CCCGACCCAAACTAGTGC						
C109	F: AGGAGCCCAGCAGCTGAA	GTA	297-337	57	13	522	0.74/0.88
	R: TTTACATAGACCTGCGGGTATGG						
C356	F: GATCTTCAGATGAAGGGTAGTTAGAT	GTTA	137-171	57	8	524	0.62/0.70
	R: ATGAAGACAAACAGGCTTGG						

Appendix 3

Table S2) Identified compounds from femoral pore secretions of male lizards used in the female-tongue flicking trials, with their characteristic retention time and diagnostic ions.

Retention time (s)	Compound ID	Diagnostic ions
7.88	Heptadecene	55, 41, 69, 57, 43, 83, 97, 70, 56, 71, 238
8.045	Unknown	57, 71, 85, 196, 239
8.85	Hexadecanal (Palmitaldehyde)	43, 57, 82, 41, 55, 96, 68, 83, 69, 71, 240
9.441	2-Heptadecanone	43, 58, 59, 71, 41, 29, 55, 57, 27, 85, 254
9.53	Unknown	57, 71, 85, 224, 267
9.9	n-Hexadecanoic acid	60, 73, 57, 43, 55, 41, 129, 71, 69, 83, 213, 256
11.141	Unknown	57, 71, 85, 252, 295
11.35	Oleic Acid	55, 69, 41, 83, 43, 97, 57, 67, 56, 70, 264, 282
11.53	Octadecanoic acid	43, 73, 60, 57, 55, 41, 129, 69, 71, 83, 185, 241, 284
12.754	Unknown	57, 71, 85, 280, 323
12.86	Unknown	57, 69, 83, 97, 111, 125, 139, 153, 378
13.2	Unknown	59, 72, 126, 281
14.522	Heneicosane, 11-(2,2-dimethylpropyl)-	57, 56, 41, 43, 55, 29, 71, 27, 39, 42, 308, 351, 366
15.13	Unknown	57, 71, 85, 99, 113, 183, 224
17.17	13-Docosenamide	59, 72, 55, 41, 43, 126, 69, 112, 57, 67, 337
17.69	Squalene	69, 81, 41, 95, 68, 121, 137, 136, 93, 67, 410
18.848	Unknown	105, 141, 155, 197, 251, 349, 364
20.09	Unknown	57, 179, 219, 444
21.086	Cholesterol	43, 55, 57, 105, 386, 107, 95, 81, 91, 41, 145, 213, 275, 301, 353, 368
21.12	Vitamin E (α-Tocopherol)	165, 430
21.526	Cholesta-5,7dien-3-ol	351, 384, 43, 55, 325, 145, 143, 41, 57, 69
22.241	Campesterol (Cholest-5-en-24-methyl-3beta-ol)	43, 55, 41, 57, 81, 95, 107, 71, 105, 145
22.699	Ergosta-5,8-dien-3-ol, (3β)-	365, 143, 43, 145, 41, 55, 57, 398, 69, 157
22.894	Cholesta-4,6-dien-3-one	43, 136, 382, 41, 55, 160, 57, 95, 81, 247, 338
23.3	Unknown	81, 107, 165, 215, 233, 401, 416
23.403	Unknown	57, 214, 267, 365, 380, 416
23.6	Unknown	143, 353, 379, 412
30.447	9-Octadecenoic acid (Z)-, tetradecyl ester	43, 55, 57, 41, 264, 69, 83, 97, 71, 98, 478

Table S3) Eigenvector loadings of the different chemical compounds for the seven main principal components.

Compound	PC1	PC2	PC3	PC4	PC5	PC6	PC7
Heptadecene (7.88)	0.210	-0.212	0.479	-0.300	0.109	-0.147	-0.136
Hexadecanal (8.85)	0.207	-0.282	0.487	-0.170	0.050	-0.002	0.095
Unknown (13.20)	-0.052	-0.374	-0.301	-0.022	0.132	0.026	0.688
13-Docosenamide (17.17)	0.071	-0.382	-0.296	0.220	0.200	0.191	-0.209
Squalene (17.69)	-0.259	0.031	-0.208	-0.211	-0.327	-0.661	-0.232
Unknown (18.85)	-0.178	0.208	0.241	0.430	0.551	-0.080	-0.157
Unknown (20.09)	-0.331	-0.278	0.102	-0.086	-0.157	0.153	-0.163
Cholesterol (21.09)	0.334	0.192	-0.275	-0.158	0.164	0.171	-0.285
Pro-vitamin D (21.53)	-0.400	0.097	0.040	0.002	0.243	0.116	-0.135
Campesterol (22.24)	0.387	0.092	-0.208	-0.070	-0.070	0.135	-0.252
Ergosta-5,8-dien-3-ol (22.70)	-0.304	0.279	-0.157	-0.233	0.024	0.225	0.093
Unknown (23.3)	0.342	0.251	-0.029	-0.118	-0.039	0.057	0.165
Unknown (23.40)	0.090	0.422	0.055	-0.097	0.271	-0.333	0.358
Unknown (23.6)	-0.106	0.308	0.306	0.135	-0.486	0.431	0.142
9-Octodecenoic acid (Z)-, tetradecyl ester (30.45)	0.215	-0.017	0.018	0.685	-0.301	-0.252	0.052

Table S4) Summary of Protected ANOVAs conducted on individual principal components from the femoral pore extractions.

	Factor	df	F value	p
PC1	Subspecies	1	82.51	<0.001
	Population	5	1.16	0.343
PC2	Subspecies	1	3.76	0.058
	Population	5	2.54	0.040
PC3	Subspecies	1	0.97	0.329
	Population	5	1.38	0.249
PC4	Subspecies	1	0.45	0.505
	Population	5	2.18	0.072
PC5	Subspecies	1	2.51	0.120
	Population	5	2.84	0.025
PC6	Subspecies	1	1.79	0.190
	Population	5	1.04	0.407
PC7	Subspecies	1	0.01	0.908
	Population	5	1.22	0.313

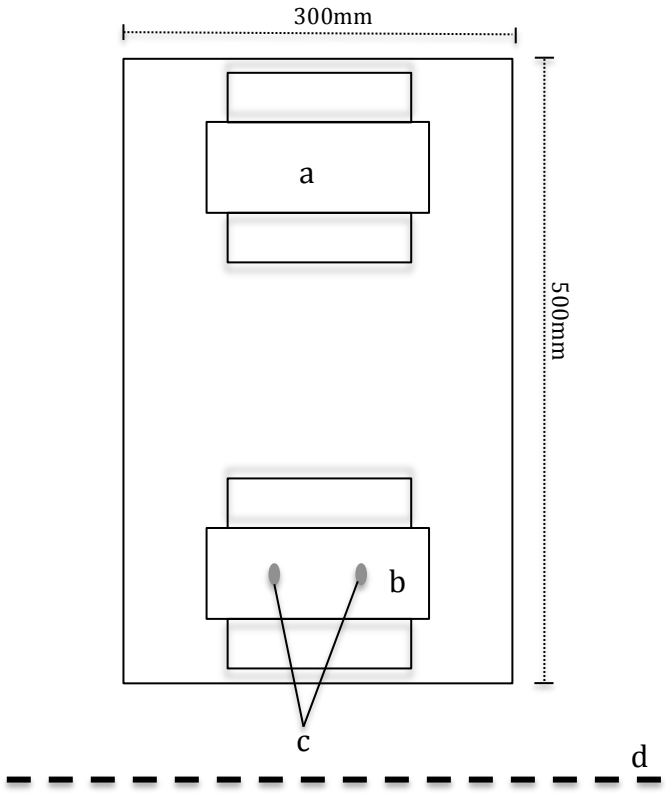


Figure S1) Diagram of indoor enclosures used to house lizards, and the placement of the cotton-buds ('c') during the tongue-flicking trials. 'a' and 'b' represent the two shelters (made from stacked bricks), with the top of 'b' also being the basking spot. The dashed line at 'd' represents the one-way-plastic film that concealed the experimenter.

Table S5) Summary statistics of independent variables that predict the number of tongue flicks a male lizard directs towards a female scented-swab.

Independent variable	df	X^2	p-value
Lineage combination	3	2.75	0.433
Female size	1	6.86	0.009
Lineage combo*Female size	3	10.71	0.003

Table S6) Summary of contrasts between subspecies combinations (male-female, F=French, I=Italian) on how males differ in their number of tongue flicks in response to differently sized females (based on the 1st PC for body size).

Independent variable	Contrast	Estimate	z-value	p-value
French male	F-F vs F-I	3.50	2.44	0.015
Italian male	I-I vs I-F	2.24	-0.98	0.327

Table S7) Summary statistics of GLMMs done on staged mating experiments between both lineages of lizards. Statistics for non-significant results are included at the point of their deletion from the model.

Response	Factor	X^2	p
Court or not	Lineage combo*Female size	6.28	0.099
	Female size	3.89	**0.048**
	Lineage combo	9.83	**0.020**
Mate or not	Lineage combo*Female size	1.08	0.782
	Female size	6.09	**0.048**
	Lineage	6.24	0.100
Mating length (s)	Lineage*Female size	0.68	0.878
	Female size	0.02	0.902
	Lineage	8.31	**0.040**

Table S8) Comparison between lineage combinations on whether a male was observed courting a female or not during the staged mating trials.

Comparison (male-female)	Estimate	SE	Z	p
French-French vs French Italian	-3.88	3.56	-1.09	0.474
Italian-Italian vs Italian-French	12.14	5.48	2.21	0.053

Table S9) Comparison between lineage combinations on the length of mating during the staged mating trials.

Comparison (male-female)	Estimate	SE	Z	p
French-French vs French Italian	3.75	5.70	0.66	0.760
Italian-Italian vs Italian-French	-15.00	6.03	-2.49	0.026

Appendix 4

Table S1) Statistical output of female morphological predictors on hybridisation level. Non-significant term values are included at the point prior to their removal from the model.

Factor	χ^2	p
SVL	0.57	0.449
Mass	0.73	0.392
Head length	0.07	0.794
Head width	0.37	0.543
Lineage*SVL	0.99	0.320
Lineage*Mass	0.01	0.905
Lineage*Head length	**22.48**	**<0.001**
Lineage*Head width	**13.40**	**<0.001**

Table S2) Statistical output of female behavioural predictors on hybridisation level. Non-significant term values are included at the point prior to their removal from the model.

Factor	χ^2	p
Basking site treatment	0.03	0.861
Courtship index	0.06	0.809
Core home range size	0.08	0.777
Ratio male lineage overlap	2.18	0.140
Lineage	2.58	0.101
Multiple paternity	0.01	0.990
Lineage*Basking site treatment	1.84	0.176
Lineage*Courtship index	0.98	0.322
Lineage*Core home range size	1.36	0.243
Lineage*Ratio male lineage overlap	0.07	0.791
Lineage*Multiple paternity	1.15	0.283

Table S3) Statistical output of male morphological predictors on hybridisation level (deviation from expected hybridisation). Non-significant term values are included at the point prior to their removal from the model.

Factor	χ^2	p
SVL	2.82	0.093
Mass	2.76	0.097
Head length	0.01	0.919
Head width	2.54	0.111
Lineage*SVL	**5.60**	**0.018**
Lineage*Mass	**7.77**	**0.005**
Lineage*Head length	0.05	0.823
Lineage*Head width	**10.31**	**0.001**

Table S4) Statistical output of male behavioural predictors on hybridisation level (deviation from expected hybridisation). Non-significant term values are included at the point prior to their removal from the model.

Factor	χ^2	p
Lineage	1.29	0.257
Basking site treatment	0.33	0.569
Dominance	**18.61**	**<0.001**
Courtship index	0.04	0.850
Core home range size (m^2)	2.17	0.141
Ratio same:diff female overlap	2.20	0.116
Lineage*Dominance	0.43	0.512
Lineage*Courtship index.	0.49	0.486
Lineage*Home range	0.09	0.762
Lineage*Ratio overlap home range	0.01	0.990
Lineage*Basking Treatment	0.03	0.874

Appendix 5

Table S1) Compounds identified from lizard femoral pore secretions with their diagnostic retention times and ion peak compositions.

Retention time (s)	Compound ID	Diagnostic ions
7.88	Heptadecene	55, 41, 69, 57, 43, 83, 97, 70, 56, 71, 238
8.045	Unknown	57, 71, 85, 196, 239
8.85	Hexadecanal (Palmitaldehyde)	43, 57, 82, 41, 55, 96, 68, 83, 69, 71, 240
9.441	2-Heptadecanone	43, 58, 59, 71, 41, 29, 55, 57, 27, 85, 254
9.53	Unknown	57, 71, 85, 224, 267
9.9	n-Hexadecanoic acid	60, 73, 57, 43, 55, 41, 129, 71, 69, 83, 213, 256
11.141	Unknown	57, 71, 85, 252, 295
11.35	Oleic Acid	55, 69, 41, 83, 43, 97, 57, 67, 56, 70, 264, 282
11.53	Octadecanoic acid	43, 73, 60, 57, 55, 41, 129, 69, 71, 83, 185, 241, 284
12.754	Unknown	57, 71, 85, 280, 323
12.86	Unknown	57, 69, 83, 97, 111, 125, 139, 153, 378
13.2	Unknown	59, 72, 126, 281
14.522	Heneicosane, 11-(2,2-dimethylpropyl)-	57, 56, 41, 43, 55, 29, 71, 27, 39, 42, 308, 351, 366
15.13	Unknown	57, 71, 85, 99, 113, 183, 224
17.17	13-Docosenamide	59, 72, 55, 41, 43, 126, 69, 112, 57, 67, 337
17.69	Squalene	69, 81, 41, 95, 68, 121, 137, 136, 93, 67, 410
18.848	Unknown	105, 141, 155, 197, 251, 349, 364
20.09	Unknown	57, 179, 219, 444
21.086	Cholesterol	43, 55, 57, 105, 386, 107, 95, 81, 91, 41, 145, 213, 275, 301, 353, 368
21.12	Vitamin E (α-Tocopherol)	165, 430
21.526	Cholesta-5,7dien-3-ol	351, 384, 43, 55, 325, 145, 143, 41, 57, 69
22.241	Campesterol (Cholest-5-en-24-methyl-3beta-ol)	43, 55, 41, 57, 81, 95, 107, 71, 105, 145
22.699	Ergosta-5,8-dien-3-ol, (3β)-	365, 143, 43, 145, 41, 55, 57, 398, 69, 157
22.894	Cholesta-4,6-dien-3-one	43, 136, 382, 41, 55, 160, 57, 95, 81, 247, 338
23.3	Unknown	81, 107, 165, 215, 233, 401, 416
23.403	Unknown	57, 214, 267, 365, 380, 416
23.6	Unknown	143, 353, 379, 412
30.447	9-Octadecenoic acid (Z)-, tetradecyl ester	43, 55, 57, 41, 264, 69, 83, 97, 71, 98, 478

Table S2) Relative eigenvalues for each principal component.

PCs	Eigenvalue	Individual %	Cumulative %
PC1	3.36	21.0	21.0
PC2	2.79	17.4	38.5
PC3	2.03	12.7	51.2
PC4	1.37	8.6	59.8
PC5	1.22	7.6	67.4
PC6	1.05	6.6	73.9

Table S3) Eigenvector loadings of the different chemical compounds for the six main principal components. Highlighted values denote those compounds with particularly high correlations with the principal components.

Compound	PC1	PC2	PC3	PC4	PC5	PC6
Heptadecene (7.88)	0.419	0.007	-0.010	0.028	0.177	0.336
Hexadecanal (8.85)	0.313	-0.043	-0.165	0.136	0.513	0.316
Unknown (13.20)	0.163	0.227	0.147	-0.484	-0.387	-0.061
13-Docosenamide (17.17)	0.314	0.165	-0.186	-0.387	0.025	0.005
Squalene (17.69)	-0.072	-0.276	-0.084	0.399	-0.356	0.250
Unknown (18.85)	-0.212	0.416	-0.009	-0.016	-0.070	0.310
Unknown (20.09)	0.185	-0.393	0.119	-0.074	-0.110	0.063
Cholesterol (21.07)	0.059	0.345	0.034	0.490	0.163	-0.179
Vitamin E (α-Tocopherol) (21.12)	0.129	-0.410	0.052	0.166	-0.246	-0.171
Cholesta-5,7dien-3-ol (Pro-vitamin D) (21.53)	-0.330	-0.076	-0.328	-0.201	0.033	0.128
Campesterol (22.24)	0.308	0.302	0.272	0.146	-0.013	-0.269
Ergosta-5,8-dien-3-ol (22.70)	-0.305	0.002	-0.294	-0.093	0.217	-0.068
Unknown (23.30)	-0.171	-0.049	0.590	-0.018	0.167	-0.060
Unknown (23.40)	-0.199	0.316	0.119	0.216	-0.320	0.418
Unknown (23.65)	-0.361	-0.099	0.316	-0.107	0.371	-0.108
9-Octadecenoic acid (Z)-,tetradecyl ester (30.45)	-0.022	0.141	-0.402	0.188	-0.095	-0.526

Table S4) Summary of Protected ANOVAs conducted on individual principal components from the second femoral-pore extractions

	Factor	df	F value	p
PC1	Basking Treatment	1	18.3	<0.001
	SVL	1	0.38	0.55

	Factor	df	F value	p
PC2	Basking Treatment	1	3.07	0.091
	SVL	1	0.55	0.47

	Factor	df	F value	p
PC3	Basking Treatment	1	0.61	0.44
	SVL	1	0.09	0.76

	Factor	df	F value	p
PC4	Basking Treatment	1	0.06	0.81
	SVL	1	2.53	0.12

	Factor	df	F value	p
PC5	Basking Treatment	1	3.96	0.057
	SVL	1	0.38	0.54

	Factor	df	F value	p
PC6	Basking Treatment	1	14.48	<0.001
	SVL	1	0.20	0.66

Table S5) Statistical results of compounds that had high factor loadings (+/- 0.25) in the principal components that differed between the basking treatments. Each chemical was analysed as the response variable in a separate linear model.

Chemical (retention time)	Percentage difference between basking treatments (high-quality – low-quality males)	
Heptadecene	**-7.5**	**(p=0.017)**
Hexedecanal	**-7.6**	**(p=0.059)**
Squalene	-5.4	(p=0.379)
Unknown (18.85)	-2.3	(p=0.877)
Campesterol	**+9.2**	**(p=0.002)**
Unknown (23.403)	+30.2	(p=0.835)
Octadecenoic acid	**-19.3**	**(p=0.046)**

Table S6) Summary statistics of compounds that correlate with male SVL in the first extracted femoral pore secretions.

Compound	Estimate	t	p
Heptadecene	-0.12	3.81	**<0.001**
Hexedecanal	-0.10	2.72	**0.012**
Docosenamide	-0.15	2.30	**0.030**
Pro-vitamin D	0.15	3.31	**0.003**
Campesterol	-0.08	2.25	**0.033**