Female reproductive investment in response to male phenotype in wall lizards and its implications for introgression

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The likelihood that females will breed or how much they invest in reproduction can depend on the characters of their male partners. Such differential allocation may enhance or limit gene flow between hybridizing lineages, in particular when the lineages have diverged in sexually selected characters. Populations of the common wall lizard (*Podarcis muralis*) in some regions of Italy exhibit striking exaggeration of coloration, morphology and behaviour compared to populations in France. The two forms hybridize in north-western Italy. Gene flow is directional and in concordance with the prediction that the exaggerated sexual characters of lizards of Italian origin give them a mating advantage. To evaluate if differential allocation contributes to asymmetric introgression, we tested experimentally if female reproductive investment is affected by male origin and male secondary sexual characters. Despite the large genetic and phenotypic divergence between males of Italian and French origin, females did not invest more when paired with males of the same origin, nor when paired with males with highly expressed sexual characters. Combined, these results suggest that female responses to male phenotypes in wall lizards are unimportant for explaining the directional pattern of gene flow in regions of secondary contact.

ADDITIONAL KEYWORDS: hybridization – introgression – reproductive allocation – sexual selection – wall lizard.

INTRODUCTION

The value of a reproductive event to an individual often depends on their partner's characters. Theoretical models suggest that, under most conditions, females should increase their investment when partnered with males who exhibit exaggerated sexual characters (Harris & Uller, 2009; Ratikainen & Kokko, 2010). Such differential allocation has received substantial interest in terms of its adaptive value and its effect on variance in fitness within populations (reviewed in Sheldon, 2000; Horváthová, Nakagawa & Uller, 2012).

One context in which differential allocation in response to male traits and its consequences for offspring fitness may be evolutionarily important is during hybridization (Nilsson, Fricke & Arnqvist, 2002; Paczolt *et al.*, 2015). This is particularly likely when hybridizing lineages have diverged phenotypically under sexual selection. On the one hand, phenotypic divergence may make hetero-specific males less effective at stimulating females to ovulate or invest in offspring, thereby limiting gene flow between lineages (e.g. Nilsson *et al.*, 2002). On the other hand, the evolution of exaggerated sexual characters in one lineage could make females of the other lineage allocate more to reproduction when paired with these males, which should promote asymmetric introgression of genotypes and phenotypes. Despite this, there has been little work exploring the potential for differential allocation to contribute negatively or positively to gene flow in regions of secondary contact (e.g. Fricke, Arnqvist & Amaro, 2006); thus, our understanding of its role as a mediator of the evolutionary outcomes of hybridization is limited.

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Common wall lizards (*Podarcis muralis*) exhibit genetic and phenotypic divergence across Europe (Böhme, 1986; Giovannotti, Nisi-Cerioni & Caputo, 2010; Schulte *et al.*, 2012). Specifically, wall lizards in and around Tuscany exhibit striking exaggeration of several characters that typically promote male reproductive success via male–male competition in lizards, including coloration, body size and shape, bite force and aggression (While *et al.*, 2015). We have previously demonstrated that several of these characters predict male reproductive success in free-ranging lizards of Italian origin, including when in competition with lizards from the lineage found in Western Europe, including France (Heathcote *et al.*, 2016; MacGregor *et al.*, 2017). In contrast, common wall lizards from France have not been subject to equally strong sexual selection in the past and generally exhibit weaker correlations between trait values and reproductive success (Heathcote *et al.*, 2016; MacGregor *et al.*, 2017). Importantly, this character divergence makes males of Italian origin outcompete males of French origin in areas of secondary contact. This results in directional hybridization and introgression of genotypes and phenotypes across hybrid regions in the native and non-native range of the species (While *et al.*, 2015). Specifically, we observe strong directional introgression of Italian nuclear genes into French populations and even stronger introgression of the Italian phenotypic traits that function in male–male competition (e.g. body coloration and head size) (While *et al.*, 2015).

Our understanding of the mechanism responsible for this directional introgression in wall lizards has focussed primarily on male–male competition (While *et al.*, 2015). One of the reasons for this is that females do not appear to discriminate between males of the two origins (Heathcote *et al.*, 2016), suggesting that female choice plays a relatively limited role in mediating patterns of gene flow during secondary contact. However, it is possible that female discrimination is manifested in more cryptic ways, such as the likelihood that females ovulate or how much she invests into reproduction. As detailed above such differential allocation may have fundamental implications for the strength and direction of gene flow. To test the extent to which differential allocation contributes negatively or positively to gene flow within this system, we experimentally paired females with a male of either the same or the opposite lineage in a fully factorial design and recorded their reproductive investment in terms of their likelihood to reproduce, their likelihood to mate and their resource allocation to eggs (in terms of both egg size and number).

MATERIAL AND METHODS

We captured 162 males and 105 gravid females from ten non-native populations in the UK (Cheyne Weare, Dancing Ledge, East Portland, Shoreham, Shorewell, Ventnor Botanics, Ventnor Town, Wellington, West Worthing and Winspit; see Michaelides *et al.*, 2015 for full details) in 2 years (2011 and 2012). These populations have been confirmed to be either pure Italian or pure French in origin (Michaelides *et al.*, 2013, 2015). Sexually selected characters do not differ between native and non-native males (MacGregor, While & Uller, in press) and our previous work has not revealed any differences in the causes or patterns of hybridization compared to native animals (While *et al.*, 2015; Heathcote *et al.*, 2016; MacGregor *et al.*, 2017). Specifically, asymmetric introgression occurs both in native hybrid zones and in an introduced population in England (While *et al.*, 2015), which was established by animals from some of the same target populations used in the present study (Michaelides *et al.*, 2015).

All animals were measured for a number of sexual traits and housed individually according to standard procedures described in detail elsewhere (While *et al.*, 2015; MacGregor *et al.*, 2017). Lizards were fed mealworms and crickets daily, which resulted in effectively ad libitum conditions. We measured four morphological traits related to body size [snout-vent length (SVL), mass, head width and head length] and three coloration traits (dorsal greenness, ventral blackness and blue-spot size). For dorsal greenness, the two authors gave each lizard a greenness score (hereafter referred to as 'greenness') from 1 to 10 based on the intensity of the green (1 being pure brown, 10 being pure green; correlation between observer scores = 0.98). These scores have been shown to be highly correlated with values for green chroma extracted using spectrophotometry (see While *et al.*, 2015 for details). For ventral blackness and blue-spot size, we photographed all individuals on their ventral and lateral sides using a Canon EOS 350D digital camera. From these photographs, we quantified the proportion of black to non-black pixels on each lizard's chest as a measurement of ventral blackness (hereafter referred to as 'blackness') as well as the area of the outer ventral scales (OVS) with blue coloration (hereafter referred to as 'OVS blue area').

All animals were captured in early spring when females were gravid with their first clutch of the season. These clutches were laid in the laboratory. Three days after a female had laid a clutch, she was weighed and transferred to a male cage of either the same or different origin. She was left for three days with that male after which she was weighed again and returned to her cage. We did not attempt to observe

behavioural interactions, but the timing of transfer and time allowed for male–female interactions was based on our knowledge of the receptive period of females under these conditions from extensive breeding of the species and previous mating trials (While *et al.*, 2015; Heathcote *et al.*, 2016). Female cages were checked twice daily for eggs. Upon laying, we collected the eggs and scored female reproductive investment in terms of clutch size and mean egg mass, the latter excluding any infertile eggs (easily assessed by visual inspection; Olsson & Shine, 1997). Eggs were then incubated. Fertile eggs in clutches with several infertile eggs (>1 egg) also had embryos that died early. Dead embryos were sampled for DNA using standard molecular techniques (see While *et al.* 2015; Heathcote *et al.*, 2016; MacGregor *et al.*, 2017 for further details). Paternity was assigned using nine microsatellites (see Heathcote, Dawson & Uller, 2015) in CERVUS v 3.0 (Marshall *et al.*, 1998) based on the trio (mother, father and offspring) LOD score and a strict confidence level of 95%. Paternity analyses of dead embryos (in the 2012 sample) revealed that these clutches were sired by stored sperm (7/7 clutches with a dead embryo for which we could test for paternity were not sired by any of the potential males) and hence that females had not re-mated (no surviving embryos were sired by stored sperm). This allowed us to create two levels of female reproductive status: (1) whether females produced eggs (including all females) and (2) whether females who produced eggs re-mated (excluding those with eggs sired by stored sperm).

Our analyses of female reproductive output proceeded in two steps. We first ran models to examine the effect of male and female origin on female reproductive output. This included the female mass loss during mating, the probability that females reproduced, the probability that females re-mated as well as reproductive investment into clutch size and egg mass. These models included male and female origin (French vs. Italian lineage) and their interaction as fixed effects and female mass as a covariate. Because of the lack of effect of male origin on reproductive investment (see Results), for our second step we pooled the data and fitted models to examine the extent to which individual male phenotypic characters influenced egg number and egg mass. For this we targeted a number of male characters including body size (the first principal component of the standardized variables SVL, body mass, head size and head length which explained 81% of the variation; see Table 1), blackness and OVS blue area. These traits are sexually dimorphic in animals of both origins (While *et al.*, 2015; MacGregor *et al.*, 2017). Body size and coloration intensity correlate positively with dominance in free-ranging males and in staged competition trials, and are positively associated with high male reproductive success when mating with females

Table 1. Factor loading and proportion of explained variance for PC1–PC4 from a principal component analyses performed on the male body size characteristics

Trait	PC1	PC2	PC3	PC4
Snout-vent length (mm)	**0.49**	**−0.40**	**0.73**	**−0.25**
Mass (g)	**0.55**	**−0.25**	**−0.66**	**−0.44**
Head length (mm)	**0.53**	−0.04	−0.09	**0.83**
Head width (mm)	**0.41**	**0.87**	0.14	**−0.20**
PC standard deviation	1.81	0.69	0.43	0.31
Proportion of variance	0.81	0.12	0.04	0.02
Cumulative proportion	0.81	0.93	0.97	1.00

Bold values indicate factor loadings considered strong (> | 0.20 |).

of the same origin. To test the effect of secondary sexual characteristics on female reproductive investment, we ran models including clutch size and egg mass as dependent variables, female origin as a fixed effect, and female body mass, male body size (PC1), male blackness and male OVS blue area as covariates. We did not include dorsal greenness in this model because French lizards rarely exhibited variation in dorsal coloration. Because the relationship between male mating success and male traits is stronger in animals of Italian origin (While *et al.*, 2015; MacGregor *et al.*, 2017), we re-ran the above models for offspring sired by Italian males only. All data were analysed using general linear models or generalized linear models in R version 3.2.0 (R Development Core Team, 2015).

RESULTS

There was no difference in mass loss between French and Italian females irrespective of whether they were held with males of their own or the other lineage (Table 2). Only about half (48%) of the French females produced a second clutch, whereas 80% of Italian females did. However, male origin and the interaction between male and female origin did not affect the likelihood that females reproduced (Table 2). For females who did produce a second clutch, female origin was a significant predictor of whether females re-mated (Table 2). Only 55% of Italian females re-mated compared to 71% of French females. However, this was dependent on male origin, with a decrease in the proportion of Italian females that mated if they were paired with a French male (38% re-mated) compared to an Italian male (83%) but no difference for French females (77% and 62% of females mated when paired with a French and Italian male, respectively) (Table 2).

Among the females that mated and reproduced, there was no difference in clutch size between Italian and French females or between females mated to Italian or French males (Table 2). There was a difference in

Table 2. Results of linear models examining the effect of female origin, male origin and their interaction on female reproductive output

	Female origin	Male origin	Male × female origin	Covariate
Mass loss (g)	$F_{1,100} = 1.83, P = 0.18$	$F_{1,100} = 0.63, P = 0.43$	$F_{1,99} = 2.87, P = 0.11$	Laying status: $F_{1,100} = 0.38, P = 0.53$
Laying status (yes/no)	$\chi^2 = 10.28, P < 0.01$	$\chi^2 = 0.08, P = 0.78$	$\chi^2 = 0.74, P = 0.38$	
Mating status (yes/no)	$\chi^2 = 4.80, P = 0.03$	$\chi^2 = 0.47, P = 0.49$	$\chi^2 = 5.05, P = 0.02$	
Clutch size	$F_{1,36} = 0.01, P = 0.98$	$F_{1,36} = 0.05, P = 0.83$	$F_{1,35} = 0.34, P = 0.56$	**Female mass: $F_{1,36} = 5.16, P = 0.03$**
Mean egg mass (g)	$F_{1,36} = 9.25, P < 0.01$	$F_{1,36} = 1.17, P = 0.28$	$F_{1,35} = 0.01, P = 0.94$	Female mass: $F_{1,36} = 1.43, P = 0.24$

Significant effects are in bold.

Table 3. Results of linear models examining the effect of female origin and the phenotypic traits (body size, ventral blackness and blue-spot size) of the male she was mated with on female reproductive output

	Female traits		Male traits		
	Origin	Female mass	Body size PC	Blackness	Blue-spot size
Mean egg Mass (g)	$F_{1,32} = 6.81, P = 0.01$	$F_{1,32} = 0.86, P = 0.36$	$F_{1,32} = 0.25, P = 0.62$	$F_{1,32} = 0.22, P = 0.63$	$F_{1,32} = 2.67, P = 0.11$
Clutch size	$F_{1,31} = 0.26, P = 0.61$	$F_{1,31} = 10.91, P < 0.01$	$F_{1,31} = 3.46, P = 0.07$	$F_{1,31} = 2.80, P = 0.10$	$F_{1,31} = 0.01, P = 0.94$

Significant effects are in bold.

average egg mass between Italian and French females, with Italian females producing, on average, larger eggs than French females, but no difference between females mated to Italian or French males (Table 2). Females did not modify their reproductive investment in terms of their egg mass or clutch size according to the variation among males in any of the key phenotypic traits [e.g. body size (PC1) or coloration (blackness, blue-spot size); Table 3]. These results were consistent when we re-ran models on only clutches that were sired by Italian males (Table S1).

DISCUSSION

The ability of females to bias reproductive investment in response to male characters not only affects variation in reproductive success and offspring survival within populations but also causes variation in hybrid fitness (Nilsson *et al.*, 2002). Differential female allocation, therefore, has the potential to promote gene flow from lineages with exaggerated sexual characters, enhancing effective asymmetric introgression of genotypes and phenotypes. Our mating experiments show that this is unlikely to be the case in wall lizards. First, while we found some evidence that male origin influenced female re-mating, our previous research suggests that this is likely to be the result of pre-copulatory male, as opposed to female, mate choice (Heathcote *et al.*, 2016). Second, we found no evidence that females adjust their reproductive investment in response to variation in male sexually

dimorphic characters. Combined, this suggests that male–male competition and male mate choice alone are responsible for the observed asymmetric gene flow between lizards of French and Italian origins (While *et al.*, 2015).

We found differences between Italian and French females in their likelihood of re-mating and these differences were dependent on male lineage. Specifically, Italian females paired to French males were less likely to re-mate than those paired to Italian males; however, this was not the case for French females. This has the potential to contribute to the patterns of gene flow between French and Italian populations by increasing the likelihood that introgression will occur from Italian males into French females rather than vice versa (While *et al.*, 2015). However, the mechanism underpinning these results is unclear. These patterns could represent evidence of female choice, with Italian females rejecting copulations from French males but not from Italian males. However, several lines of evidence suggest this is unlikely to be the case. First, we have previously failed to find any evidence for female choice in this system; females do not appear to discriminate between males based on olfactory cues in the laboratory nor do they exhibit consistent preference to settle in territories of Italian vs. French males in semi-natural enclosures (Heathcote *et al.*, 2016). In contrast, male wall lizards of both lineages have been shown to distinguish between females of the different lineages (Heathcote *et al.*, 2016). Male wall lizards also initiate courtship and preferentially court same-lineage females (Heathcote *et al.*, 2016; MacGregor *et al.*, 2017). Our data here

suggest that the extent of male preference may be lineage specific, with Italian males more likely to mate with females of both origins compared to French males, who avoid mating with Italian females. This is consistent with the fact that almost all hybrid offspring come from French female clutches (MacGregor *et al.*, 2017) and suggests a potential role for male mate choice, in addition to male–male competition, in mediating patterns of gene flow within this system.

We also found that females are unlikely to influence patterns of introgression through more subtle forms of mate choice, for example via differential allocation. Indeed, we found no evidence that female wall lizards adjust their investment to the large difference in what are, at least for Italian females (MacGregor *et al.*, 2017), reliable indicators of male competitive ability. This may seem surprising in the context of sexual selection theory (Harris & Uller, 2009; Ratikainen & Kokko, 2010). Empirical evidence for differential allocation in females in response to male phenotypic characteristics has been found in several other vertebrates, in particular birds (Møller & Thornhill, 1998; Cunningham & Russell, 2000; Gil *et al.*, 2004; Limbourg *et al.*, 2004; Horváthová *et al.*, 2012). However, despite sexual selection being strong in many lizards (Stamps, 1977; Olsson & Madsen, 1998), evidence for female choice based on male quantitative characters is surprisingly limited (although post-copulatory choice remains poorly investigated) (Olsson & Madsen, 1995; Uller & Olsson, 2008). In addition, body size and other sexually selected characters are often strongly age-dependent in many animals (e.g. Delhey & Kempenaers, 2006; Freeman-Gallant *et al.*, 2010; Evans & Sheldon, 2013), including lizards (e.g. Martin *et al.*, 2013; Weiss, 2016). This may imply that these characters do not provide much information about an animal's breeding value for fitness. As a result, differential allocation may not often be positively selected in lizards. To the best of our knowledge, the only evidence for differential reproductive investment in response to specific male phenotypes comes from free-ranging sand lizards (*Lacerta agilis*) (Anderholm *et al.*, 2004). Interestingly, genetic effects on offspring fitness appear unusually strong in this population, partly because of a high variance in genetic complementarity among potential male partners. In contrast, a study of captive brown anoles (*Anolis sagrei*), a species likely to have high rates of multiple mating and high genetic diversity of offspring (Calsbeek, Smith & Bardeleben, 2007), did not find an effect of male age or size on female egg investment (Warner, Kelly & Lovern, 2013).

There are several additional mechanisms by which males of different origins could influence female reproductive investment. First, male harassment has been shown to affect female reproductive output in common lizards (Le Galliard *et al.*, 2005). We may therefore predict that the greater aggression and courtship intensity of males of Italian origin may result in females housed with those males suffering a loss of body conditions or reduced reproductive output. However, this was not the case. Second, differences in male courtship behaviour could influence female reproductive output if it acts as a stimulus for female reproduction (e.g. Crews, Grassman & Lindzey, 1986; DeNardo & Autumn, 2001; Mathies, Franklin & Miller, 2004). As detailed above, males preferentially court females of their own origin (Heathcote *et al.*, 2016), results that appear to be supported by this study, but this does not appear to affect the likelihood that females will reproduce. This is in line with previous research showing that female wall lizards kept separated from males will ovulate spontaneously (Pellitteri-Rosa *et al.*, 2012; T. Uller, personal observation). A more systematic study of another Lacertid lizard (*Zootoca vivipara*) also failed to find an effect of male presence on clutch size (Bleu *et al.*, 2011). This is likely because seasonality provides environmental triggers for reproduction and mating opportunities are rarely limited in the wild, making male presence irrelevant as a cue or a limiting resource.

Combined, these results suggest that female reproductive investment is unlikely to limit gene flow or contribute to the asymmetric genetic and phenotypic introgression we see in wall lizards. This supports previous work which also provides no evidence for female choice and asymmetric hybrid viability as alternative explanations (While *et al.*, 2015). Instead our results suggest that the asymmetric gene flow between lizards of French and Italian origins is caused largely by male–male competition facilitated, to some degree, by differences in the strength of male preference for same-origin females (Heathcote *et al.*, 2016; MacGregor *et al.*, 2017). Further work is required to rule out the potential for sperm competition and sperm discrimination to contribute to these patterns, but our current evidence suggests these are likely to be of minor importance. This system, thus, perhaps represents the clearest example that male–male competition and male mate choice alone can have large effects on the rate and direction of introgression following secondary contact.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's website:

Table S1. Results of linear models examining the effect of female origin and the phenotypic traits (body size, ventral blackness and blue-spot size) of male she was mated with on female reproductive output. data restricted to only those females who mated with italian males.