An experimental test of frequency-dependent selection on male mating strategy in the field

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We provide field-based experimental evidence for the frequency-dependent nature of the fitness of alternative mating strategies. We manipulated the frequency of genetically determined phenotypic strategies in six wild populations of the side-blotched lizard, *Uta stansburiana*. The within-population pattern of mating was assessed using nine microsatellite loci to assign paternity. Within populations of the side-blotched lizard exist three colour morphs (orange, blue and yellow) associated with male mating strategy. The frequency of these morphs has previously been found to oscillate over a 4- to 5-year period. We found, as predicted, that the common phenotype lost fitness to its antagonist. The mating patterns of all six populations adhered to *a priori* predictions that were derived from previous empirical and theoretical observations on this system. We found that the frequency-dependent nature of male fitness could be accounted for by the composition of their competitors at a small local population level, driven by associations within a focal female's social neighbourhood.

Keywords: frequency-dependent selection; assortative mating; spatial associations; sexual conflict; side-blotched lizard

1. INTRODUCTION

Frequency-dependent selection (FDS) has long been recognized as a major force in population dynamics and is often cited as an explanation for the maintenance of alternative male mating strategies. However, despite much empirical evidence for its role in maintaining behavioural polymorphisms (Adams *et al.* 1979; Chao & Levin 1981; Gross 1991; Shuster & Wade 1992; Belovsky *et al.* 1996; Sinervo & Lively 1996; Hunt & Simmons 2000; Sinervo *et al.* 2000, 2001), little experimental evidence, particularly from the field, exists (Sinervo & Calsbeek 2006). Previously, the frequency-dependent nature of alternative strategies in bluegill sunfish has been extrapolated from experimental manipulations of cuckolder numbers (Gross 1991). However, it was impossible to separate the interactive effect of density and frequency on male morph fitness.

If alternative strategies are maintained by FDS (Chao & Levin 1981; Gross 1991; Shuster & Wade 1992; Sinervo & Lively 1996), variation in an individual's fitness will fluctuate largely in response to the social environment that it experiences. Studies of FDS have centred upon mean field responses to population-level variation in the frequency of strategies. However, if alternative male mating tactics are the outcome of male–male competition, then we may expect FDS to occur at the level of male social neighbourhoods. In this context, FDS is the summation of didactic interactions within the social neighbourhood of individuals.

The most powerful tool for investigating the existence of FDS of alternative male mating strategies is the experimental manipulation of the frequencies of competing strategies (Brockmann 2001, 2002; Sinervo *et al.* 2001; Clarke 2004). If strategy fitness fluctuates as a function of local population-level processes, then fluctuations in fitness will be correlated with variations in the distributions of competitors, independent of variations in resource distribution (Chao & Levin 1981; Sinervo & Lively 1996).

In addition to frequency-dependent optimum behaviours, density can affect the pay-off to a particular male mating strategy by affecting the level of intraspecific male–male competition. Thus, a complete test of the control of FDS entails a manipulation of both male frequency and population density to determine whether population density has synergistic effects on the success of the male strategies. The ability of a male to defend a female, or resource that she is spatially associated with, is dependent upon their economic defendability, and the economic defendability of a resource is dependent upon the density of competitors (Emlen & Oring 1977). Density may also influence mate choice rules (Van Gossum *et al.* 2001) by affecting the context of direct or indirect effects of mating (Alonzo & Sinervo 2001; Qvarnstrom 2001).

Inherent in populations of the side-blotched lizard (*Uta stansburiana*) are three genetically determined throat colour polymorphisms: orange; blue; and yellow. Each throat colour is linked to a different male mating strategy. Orange males are territorial usurpers; blue are monogamous mate guarders; and yellow males adopt a sneaker strategy. Sinervo & Lively (1996) reported both empirically and theoretically based data to explain a cycle in morph frequency in the side-blotched lizard. Here, we have tested those predictions using data from experimentally manipulated field populations and randomization statistics. Observational data on the variation in throat colour polymorphism

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over a 15-year period suggested a frequency-dependent cycle in male morph (Sinervo & Lively 1996). An empirically based game theoretic model using territorial associations between males and females to infer a male's potential to monopolize a female also predicted this cycle (Sinervo & Lively 1996). Both pieces of evidence pointed to male throat colour being under the control of negative FDS. Predictions from the above work were that (i) orange males usurp paternity from blue males and so their fitness increases with the frequency of blue males with which they compete, (ii) yellow males sneak paternity from orange males and so their fitness increases with orange male frequency, and (iii) blue males guard their paternity against yellow males and so their fitness increases with yellow male frequency. In order to determine whether the fitness of alternative strategies of male side-blotched lizards in the field was frequency dependent, as a function of the social environment, we manipulated the frequency of genetically determined phenotypic strategies in six wild populations and followed their mating patterns.

2. MATERIAL AND METHODS

(a) *Experimental design and study system*

We were interested in the effects of the social environment, as described by both the density and the frequency of throat colour alleles, on patterns of mating in the side-blotched lizard. In order to investigate this, we seeded a number of outcrops with populations of progeny from known parentage (laboratory crosses) at field sites adjacent to our long-term study plots at Los Baños Grandes, California. Both the density of progeny and their frequency of throat colour alleles were manipulated to create six treatments that represented a two-factor design of high and low density, and high and low orange, blue and yellow throat colour allele frequencies. Sex ratio was kept at approximately 1 : 1. Parental throat colour score is highly correlated with offspring throat colour (Sinervo & Zamudio 2001; Sinervo *et al.* 2001) and so can be used as an indicator of hatchling phenotype and putative genotype. The throat colour score was based on a three allele, single-locus model and was used to predict putative hatchling allele contributions to any subsequent, assigned treatment. Both gene mapping studies (Sinervo *et al.* 2006) and laboratory breeding experiments (Sinervo *et al.* 2001) suggest a single genetic factor of major effect controlling male throat colour polymorphism.

Outcrops for the experiment were chosen for their analogous characteristics, all south facing, with a similar area (2505.14 m^2 ±518; range 1836.9–3156.8) of rocky outcrops. Each treatment lay contiguously along a valley, separated from other populations by at least 500 m of grassland. Outcrops were then further subdivided into 35 plots (five high orange, five low orange, six high blue, five low blue, nine high yellow and five low yellow). The average area of habitable rock for each plot was 444.84 m^2 ±213.03 (range 128.2–1273.4).

We removed all adults from our six rocky outcrops, prior to the onset of the breeding season in March 2000. Upon capture, these adults were given a putative score for throat colour (Sinervo *et al.* 2001) and a unique toe clip and brought into the laboratory for participation in a controlled breeding programme. Animals were housed in 38 × 24 cm thermally controlled terrariums with natural light–dark cycles. Food was provided ad libitum (Sinervo & Licht 1991; Sinervo & Denardo 1996). Each cage contained between one and three females and

one male (Sinervo *et al.* 2001). In all, 65 female (34 orange and 31 yellow) and 34 male (11 orange, 6 yellow and 17 blue) lizards were used in the breeding programme. All pairwise combinations of dam and sire throat morph were present in the design; however, pairings were selected on the basis of maximizing the number of the yellow phenotype as these were at their nadir in the morph frequency cycle in 2000. Therefore, matings were biased towards pairing putative yellow homozygotes so that we would generate enough yellow offspring to allow us to control yellow allele frequency.

Hatchlings produced from the controlled breeding programme were toe-clipped for permanent identification, sexed (based on presence or absence of post-anal scales), and their weight and snout-vent length (SVL) were noted. In total, 234 hatchlings were released to the field during May–July of 2000, 1–3 days after hatching, to 33 plots (five high orange, six low orange, five high blue, five low blue, seven high yellow and five low yellow) in the area that had previously been cleared of the adults used in the breeding programme. An average of 2.33 ± 1.65 hatchlings per 100 m^2 were released on high-density treatments and 0.68 ± 0.43 per 100 m^2 on low-density treatments to create variance in hatchling density. Hatchling release was randomized with respect to maternal territory and sibship (Sinervo & Lively 1996) in the sense that hatchlings were not released onto the plot where their mother was caught and no hatchling was released on the same plot as its sibling.

In the following breeding season, from 1 March to 30 June 2001, our experimental generation was surveyed, composed of the individuals released the previous season that were generated from the controlled breeding programme and individuals that immigrated onto the outcrops from surrounding areas. We conducted daily censuses of each outcrop or treatment by mark–recapture; in addition, we also carried out field observations of conspicuously marked individuals to gather data on individual territory use. A 500 m perimeter around each treatment was also searched to maximize the recapture rate of the population census. Upon capture, each individual was sexed, phenotyped, given a putative genotypic score, their location noted and a tissue sample was taken from the tip of their tail. If they were novel individuals, they were also given an individual toe clip. At ovulation, females were brought into the laboratory to lay their eggs, the average duration of their stay was 16 ± 1.21 days, and subsequently released at their point of capture. The offspring from these female's clutches were individually marked, and a tissue sample was taken along with weight and SVL measurements. These hatchlings were then released 1–3 days after emerging.

(b) *Spatial analysis*

To determine nearest neighbours, centroids of convex polygons were extracted from territorial data of our experimental generation using MacTurf (Sinervo 1996) and these were then converted into Gabriel-connected maps (Gabriel & Sokal 1969). Nearest neighbours were calculated for male and female neighbours independently. It was then possible to extract data on the frequency, by sex, of phenotypic associations for each individual. These parameters were then used to define an individual's social neighbourhood. Using data from the Gabriel-connected nearest neighbour analysis, we constructed a randomization test to determine whether the different male and female morphs were spatially associated themselves with a particular phenotype (Zamudio & Sinervo 2000). For each treatment, by sex and morph, 1000 random populations of dyadic

associations were generated, in keeping with the number of observed associations. We could then ask either of these two questions: (i) how many times does the expected number of dyadic associations equal or exceed the number observed, to give us a statistical probability of a positive association between morphs? (ii) How many times is the expected number of associations less than or equal to the number observed, to give us the probability of a negative association between morphs? The alpha level was Bonferroni corrected ($\alpha = 0.00138$) as 36 tests were simultaneously carried out on each randomly generated dataset.

(c) *Population mating patterns*
The pattern of mating and siring success of the experimental generation was determined by microsatellite analysis, using the tissue samples collected at time of capture and from the hatchlings of the experimental generation. We employed the same protocol as in Zamudio & Sinervo (2000). We used nine polymorphic loci (Zamudio & Sinervo 2000) to assign paternity to the hatchlings from 2001 with KINSHIP v. 1.3.1 (Goodnight & Queller 1999). Two separate analyses for each clutch were carried out in order to take into account the increased probability of a hatchling being sired by a male in close spatial association with its mother (Zamudio & Sinervo 2000). Initially, only males identified as nearest neighbours were included. On a second run, all males from the treatment outcrop of the female were included. The highest likelihood ratio from both runs was then used to assign paternity to a particular sire. In total, 430 hatchlings from 75 dams of the experimental generation were hatched in the laboratory using standard oviposition and incubation protocols (Sinervo *et al.* 2000). Of these, 75.5% were successfully assigned a sire (where $\alpha < 0.05$, $\beta < 0.2$), using the likelihood and exclusionary criterion of the program KINSHIP (Zamudio & Sinervo 2000). These values are comparable to paternity assignment in all previous microsatellite studies (Sinervo & Clobert 2003; Sinervo *et al.* 2006).

3. RESULTS
(a) *Treatments*
The difference in hatchling density (individuals per 100 m^2) between high and low experimental plots, that was generated in 2000, did not persist in the following year (d.f. $= 30$, $t = 0.746$, $p > 0.05$), although it did result in considerable variation in adult density between plots (mean of 1.22 ± 0.715 adults 100 m^{-2}, range 0.25–2.8 adults 100 m^{-2}). Frequency treatments proved to be more consistent with the original experimental design. Both the overall gene and phenotype frequencies and the frequency of male gene and phenotype frequencies varied according to their original treatment. Sex ratio across treatments was slightly female biased in surviving adults (mean $= 0.65 \pm 0.25$), deviating from the original hatchling release sex ratio (mean $= 0.51 \pm 0.309$), which is typical for this population of lizard (B. Sinervo 1989–2007, unpublished data).

(b) *Assortative mating*
In the experimental generation, the proportion of orange females with which a male mated (1.00 being all orange females and 0.00 being all yellow females) was positively correlated with the frequency of orange females in a male's neighbourhood ($F_{1,73} = 10.77$, $p < 0.005$) and negatively correlated with the frequency of yellow females ($F_{1,73} = 4.779$, $p < 0.05$). We used residuals of the proportion of

orange females (with which a male mated) derived from a regression of the frequency of orange females in a hierarchical analysis to determine whether there was an additional effect of male morph. A one-way ANOVA with male phenotype found no significant association of male morph with the residuals for the proportion of orange females mated ($F_{1,72} = 0.22$, $p > 0.05$). Thus, males were not found to mate preferentially with a particular female phenotype. Instead, the likelihood of a male mating with a female morph was entirely dependent upon the frequency in which they encounter them in their local neighbourhood. We also tested for assortative (and disassortative mating) by regressing the phenotype of a hatchlings sire and dam. No significant relationship was found between the phenotype of a hatchling's dam and of the hatchling's sire, using dam's identification number as a random variance effect to avoid pseudo-replication from the use of sibs ($\chi^2_2 = 3.42$, $p > 0.05$). In addition, no relationship between female morph and the proportion of hatchlings sired by a particular phenotype within a clutch was found. In conclusion, we found no evidence of assortative mating (or disassortative mating).

(c) *Spatial associations*
We found no evidence that males of any phenotype were spatially associated with a particular female morph. There was some evidence that orange females were non-randomly distributed. Orange females were more likely to have orange females as their nearest neighbour in the high-orange treatment ($p < 0.001$) and were less likely to associate with yellow males in the low-orange treatment ($p < 0.001$). Blue males were the only males to distribute themselves non-randomly, exhibiting a positive association with other blue males ($p < 0.001$) in the low-yellow treatment. No effect was uniform within a treatment for all sexes or morph and no effect was consistent across all treatment for a particular morph.

(d) *Frequency dependence*
We found effects of FDS on the proportional contribution by different male phenotypes to a female's first clutch, dependent on the frequency of male morph in a female's neighbourhood (figure 1). Regression analysis revealed that as predicted by the male intrasexual competition game (Sinervo & Lively 1996), orange males have higher fitness in a high-blue frequency environment and do badly in a high-yellow frequency environment; blue males have an advantage at high-yellow frequencies and a disadvantage in high-orange frequencies (although this last effect was non-significant in the present study); yellow males have higher fitness at high-orange male frequencies and lower fitness in high-blue frequency environments (figure 1). Therefore, the probability that a hatchling was sired by a particular morph was dependent upon the male component of a female's neighbourhood. However, the frequency of colour morphs accounted for between 6.5 and 13.1% of the variation in male morph fitness. Up to 93.5% of the remaining variation was not explained. Therefore, although the contribution of FDS to morph fitness is real, there are other factors that influence morph fitness. A population-level analysis using nominal logistic regression of sire phenotype against density and frequency treatment found a significant effect of frequency treatment on the probability of a hatchling being sired by a particular
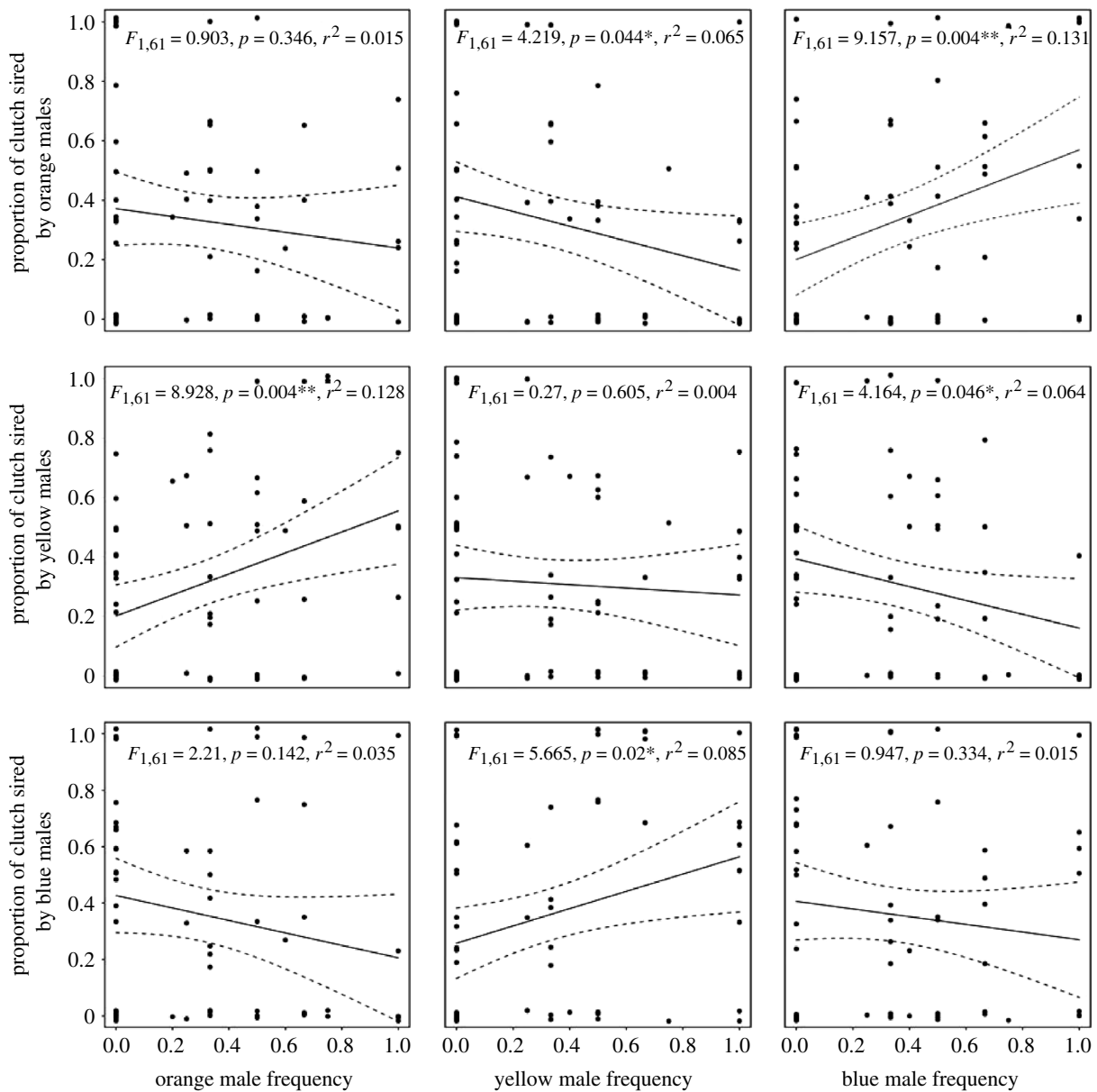
Figure 1. The percentage of a female's clutch sired by the different male morphs as a function of male morph frequency within a female's social neighbourhood. The *F*- and r^2 statistics and *p*-value for each regression analysis are reported in the right-hand top corner. Solid lines represent the line of best fit and dashed lines correspond to 95% CIs; * refers to $p < 0.05$, ** to $p < 0.005$.

morph (Wald $\chi_2^2 = 26.96$, $p < 0.0001$). Again, the contribution of male phenotypes to the next generation was consistent with the predicted pattern of FDS acting on the different male morphs according to the rock–paper–scissors game (figure 2). Density treatment, in keeping with the lack of distinction in density between the treatments reported above, had no effect.

Individual male fitness, dependent upon their phenotype, did respond significantly with respect to frequency treatment (whole model: $F_{8,120} = 5.55$, $p < 0.0001$) at the population level. The interaction between the morph of an individual male and its frequency treatment and its effect on the number of females he mated with was significant ($F_{4,120} = 6.45$, $p = 0.0001$). The relationship with frequency treatment was consistent with our predictions of frequency-dependent male mating success (figure 3). The exception to this rule was the higher mating success of orange males in the high-yellow frequency environment. However, only yellow male mating success exhibited a significant correlation with frequencies of male morphs in their social neighbourhoods.
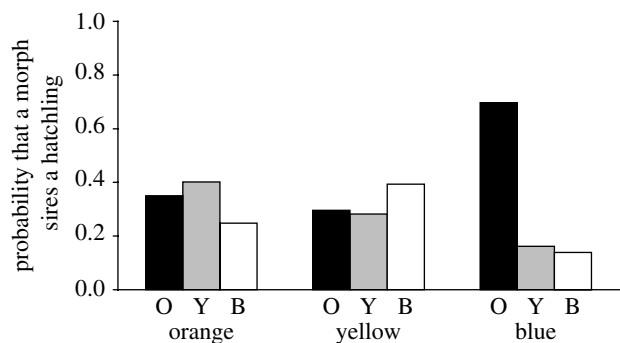

Figure 2. The mean probability of a hatchling being sired by a particular morph as a function of frequency (O, orange; Y, yellow; B, blue) treatment. Where black bars indicate orange, grey bars yellow and white bars blue male morphs.

The number of females a yellow male mated with significantly increased (slope $b = 2.10$) with an increase in orange male frequencies ($F_{1,38} = 6.53$, $p < 0.01$) and decreased (slope $b = -1.70$) with an increase in blue male
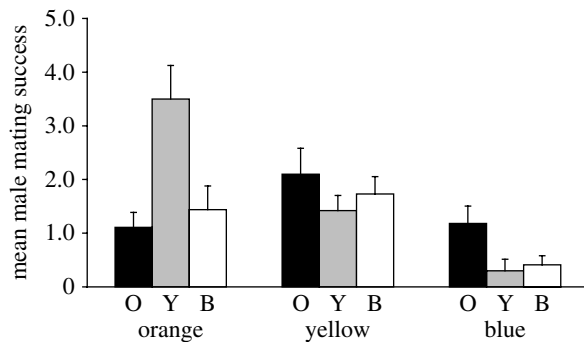
Figure 3. Male morph mating success (O, orange; Y, yellow; B, blue) as a function of frequency treatment (orange, yellow and blue). Mating success was determined by the number of females with which a male sired offspring. Error bars are s.e.m.

frequency ($F_{1,38} = 4.75$, $p < 0.05$). Blue and orange males showed no response to the frequencies of the different male morphs in their social neighbourhoods.

4. DISCUSSION

Male fitness was found to be influenced by the frequency of competing phenotypes and was significantly related to frequency treatment, to the extent that a phenotype at low frequency gained a higher mating success in populations of its antagonistic morph (i.e. yellow phenotypes had high fitness in a high-orange frequency environment). Importantly, this negative FDS was intrinsically driven in that male fitness was associated with the frequencies of the different male morphs in the population. This effect, although apparent at the population level (figures 2 and 3), was subject to negative FDS operating at the level of female social neighbourhoods (figure 1). Only a limited effect of male social neighbourhood on male fitness was found. This is intuitive as females are the resource for which males compete. Therefore, the success of a male's strategy is dependent directly on the prevention of mating success by males that are territorially associated with any given female. Selection is the result of interactions at the level of female social neighbourhoods. Our data suggest that when looking for the presence of frequency-dependent male mating strategies, we should look at the composition of males about a single resource, in small social neighbourhoods, rather than at the population level. In populations where social neighbourhoods are small, as in the side-blotched lizard, we may expect variation between the social neighbourhoods of individuals to be large. This course-grained social environment should act to enhance the strength of FDS (Sinervo 2001).

FDS is a classic paradigm to explain both the presence of phenotypic polymorphisms and the dynamics of polymorphic populations. For example, the maintenance of mimetic species is driven by the relationship between the frequency of the mimic, the model and the predator–prey recognition behaviour. Too many mimics to models and the benefits of the mimetic strategy will decrease due to the reduction in signal efficacy. However, such frequency-dependent systems are driven by the frequency of predator search images in the predator population. This apostatic FDS will therefore be manifest at a local scale, focused upon the behaviour of single predator's behaviour and the frequency of polymorphisms within the territory of that predator. In the damoiselle fly *Ischnura elegans* FDS

acts on female colour polymorphism (Svensson *et al.* 2005). Again FDS operates at a local population level that is centred upon a focal determinant of morph fitness, in this case the ability of morphs to avoid the costs of male harassment (Van Gossum *et al.* 2001) and so the search image of single males and the frequency of morphs within a single males territory. FDS in the case of the side-blotched lizard and also in the trimorphic system of the marine isopod *Paracerceis sculpta* (Shuster & Wade 1992) are also in some respect driven by apostatic selection (Sinervo & Calsbeek 2006) in that a component of the competitive cycle between male morphs is dependent upon the ability of the more dominant strategies to detect female mimics/sneaker male strategies. And here is the rub, in interactions between morphs, FDS is derived from interactions at a number of local scales. FDS operates at the level of the resource that is being competed for, but also at the level of the competing morphs. Orange and blue males of the side-blotched lizard may compete more directly for access to females, but competition between blue and yellow males or orange and yellow males is dependent upon the search image employed by blue and orange males to detect yellow males. This will be dependent upon the frequency of yellow males within the territory of these two morphs and any genetic predisposition to identify yellow males. The manifestation of FDS at the population level is therefore the mean field response of the population (Sinervo & Lively 1996) to hierarchical levels of FDS selection operating around the different interactors (figure 1; Sinervo *et al.* 2006) that determine the selection regime operating on the individual (figures 2 and 3).

Given the size of the effects of morph frequency on male morph fitness (figure 1), it can be concluded that FDS is not the only influence on morph fitness. The size of rock piles, or the quality of individual territories (Calsbeek & Sinervo 2002), along with variation in female preference functions (Bleay & Sinervo 2007) can influence the viability of eggs and the attractiveness of males. In manipulations of territory quality (addition and removal of rock piles used for basking), females were found to preferentially associate with males in the quality-enhanced habitats (Calsbeek & Sinervo 2002). Such behaviour resulted in earlier egg-laying dates and the production of larger eggs. These maternal effects may have influenced the probability of successful hatching that would, in turn, influence our estimate of morph fitness.

Despite previous evidence of an assortative mating rule in this species from laboratory-based mate choice trials (Bleay & Sinervo 2007), no evidence from the pattern of mating or from spatial associations in the wild supports this view. If present, a significant incidence of mating between orange females and males or yellow females and males would be expected (Bleay & Sinervo 2007). However, it is difficult to uncouple female affiliations with male morphs from the evident influence of male–male competition. In addition, it is possible that female side-blotched lizards may operate mate-choice preference functions that are context dependent, and so may be choosing males on the basis of the frequency of male morphs in their social neighbourhood.

The density and frequency of morphs that females experience may have important implications for the costs and benefits of mating with a particular male. In *I. elegans*,

a species with female polymorphisms and male mate choice, a frequency-dependent (Fincke 1994; Van Gossum *et al.* 2001) and density- and sex ratio-sensitive (Van Gossum *et al.* 2001) mate choice rule has been found. It would be reasonable to suspect that such context-dependent mate choice rules may exist in *the side-blotched lizard* (Alonzo & Sinervo 2001), over-riding any mate choice rules found in laboratory mate choice trials (Bleay & Sinervo 2007). The question would then become, is FDS in the side-blotched lizard driven by context-dependent female preference, or is context-dependent mate choice driven by FDS on male–male competition strategies (Alonzo & Sinervo 2001)?

Alternatively, strong intra-sexual competition may swamp the opportunity for females to discriminate between males (Parker & Partridge 1998; Gage *et al.* 2002). Sih *et al.* (2002) found that patterns of mating in water striders were determined in a large part by male–male competition. Large males had a higher mating frequency despite strong female choice for smaller males (Sih *et al.* 2002). Sexual conflict is likely to occur where the outcome of male and female optimal reproductive strategies differ (Johnson & Marzluff 1990; Moore & Moore 1999), i.e. males and females have different optimal mates. In the cockroach, *Nauphoeta cinerea*, sexual conflict can result in disruptive selection on the composition of male social pheromones (Moore & Moore 1999). The constituents of social pheromone that are most attractive to females are different from that which is beneficial in determining the outcome of male–male competition; sexual selection results in disruptive selection or stabilizing selection dependent upon the level of subdivision in cockroach populations (Moore & Moore 1999). Sexual conflict may therefore be involved in the formation and maintenance of the side-blotched lizard. In the side-blotched lizard, females do have a preference for a particular male morph (Bleay & Sinervo 2007), yet they cannot exercise that choice due to competitive exclusion of other males by the dominant male in their social neighbourhood. An observational study of male behaviour and mating success linked to paternity would be the next step in elucidating the driving mechanism for the FDS cycle in this species.

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