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Population divergence in chemical signals and the potential for premating isolation between islet- and mainland populations of the Skyros wall lizard (*Podarcis gaigeae*)

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Abstract

When sexually selected traits diverge because of different local selective environments, premating isolation might arise as a correlated response. However, sexually selected traits might also diverge by stochastic forces. Here, we show that odour-based mate preferences and scent composition have diverged between islet- and mainland populations of Skyros wall lizard, *Podarcis gaigeae*. We quantified the degree of scent-mediated premating isolation between populations. Islet lizards preferred scent from islet lizards, whereas the mainland populations were less discriminatory. The pheromone compositions differed more between islets than between islet- and mainland populations and did not differ significantly between mainland populations. There was a tendency for population divergence in pheromones to be positively correlated with neutral genetic divergence. This might indicate a role for genetic drift in evolutionary change in these signals and partial decoupling between signals and preferences. Our results suggest that chemical signals and associated mate preferences can diverge through stochastic and selective forces and influence premating isolation.

Introduction

The evolution of prezygotic isolation is a central question in the study of population divergence, speciation processes and species differences. Prezygotic isolation mechanisms are often argued to be the most important isolating mechanism for keeping populations or incipient species separate (Jiggins *et al.*, 2001; Kirkpatrick & Ravigne, 2002; Coyne & Orr, 2004). Both prezygotic and post-zygotic isolation between populations are thought to increase with genetic or phylogenetic distance (Coyne & Orr, 2004). However, recent models and data suggest that prezygotic isolation can evolve faster than post-zygotic isolation, the latter which might take several millions of generations to achieve (Coyne & Orr, 1989, 1997; Turner & Burrows, 1995; Gavrilets & Boake, 1998; Boake *et al.*, 2000; Price & Bouvier, 2002).

Prezygotic isolation might often arise as a by-product of divergent natural selection (Schluter, 2000; Rundle & Nosil, 2005; Tobler *et al.*, 2009), divergent sexual selection (Uyeda *et al.*, 2009) or interactions between these different evolutionary forces (Cocroft *et al.*, 2010). Ecological release following colonization of new environments can also fuel population divergence, because of relaxed competition and weakened selection (Schluter, 2000; Thomas *et al.*, 2009). When species colonize islands, novel selective regimes operating on phenotypic traits could indirectly also affect divergence in mate preferences and result in sexual isolation as a correlated response to divergent selection (Rice & Hostert, 1993). Moreover, within islands, disruptive selection might operate in heterogeneous environments with several empty niches and result in either sympatric coexisting ecotypes (Losos *et al.*, 1998) or ecologically driven sexual size dimorphism (Bolnick & Doebeli, 2003; Butler *et al.*, 2007). In support of this, accelerated divergence in sexual size dimorphism on islands has recently been documented in *Anolis* lizards (Thomas *et al.*, 2009).

Ecological differences between more or less isolated populations can also generate differences in sexual

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selection. For instance, sexually selected traits might diverge between populations living in different environments, because of the need to efficiently protrude from the background and selection for increased signal efficiency (Marchetti, 1993; Schluter & Price, 1993; Schluter, 2000; Price, 2007). Geographical isolation of discrete island populations might also facilitate population divergence and prezygotic isolation because of restrained gene flow between populations. Geographical isolation is important, because gene flow will constrain both stochastic population divergence caused by genetic drift and hamper adaptive population divergence driven by selection (Storfer *et al.*, 1999; Langerhans *et al.*, 2003; Hendry & Taylor, 2004; Garant *et al.*, 2007; Räsänen & Hendry, 2008).

However, it is important to underscore that population divergence in sexually selected traits can also arise even without ecological release and even in the absence of ecological differences between populations. Geographical separation and isolation of island populations might allow for mate preferences and sexually selected traits to evolve in different and arbitrary directions due local runaway processes favouring trait diversification in more or less arbitrary directions (Lande, 1981; Day, 2000). Theoretical models predict that genetic drift in mate preferences, in combination with sexual selection, can generate sexual isolation between populations as a by-product (Uyeda *et al.*, 2009). To date, there is little empirical evidence from island–mainland microgeographic systems to evaluate the relative role, if any, for stochastic forces such as genetic drift on signal divergence. There is also very limited knowledge about the possible interactions between natural and sexual selection in the divergence of mate preferences and signals. Some empirical studies have documented patterns of parallelism in traits and sexual isolation in similar environments (Rundle *et al.*, 2000; Schluter, 2000; Johannesson, 2001; Eroukhmanoff *et al.*, 2009). Such parallelism in sexual signals/or preferences for them is the classical and expected signature of ecologically driven divergence and indicates a role for selection, rather than genetic drift (Schluter, 2000).

Historically, there has been considerable scientific controversy about the evolution of mating preferences in subdivided populations, such as those on islands (Barton & Charlesworth, 1984). In the Hawaiian *Drosophila* complex, some populations that have colonized islands have lost some of their courtship elements, presumably owing to founder effects (Kaneshiro, 1980). As a consequence of this, females from these islands have become less discriminatory towards males than the females in the ancestral founding populations (Kaneshiro, 1980). In contrast, recent theoretical models have suggested that island populations would be expected to be more discriminatory in their mate choice (Kirkpatrick & Servedio, 1999). The reason for this is that selection for maintaining locally adapted gene combinations is thought to drive the evolution of reinforced mate preferences on islands (Kirkpatrick & Servedio, 1999). Empirical studies on divergence in courtship elements and associated sexual preferences in multiple and replicated island–mainland systems are needed to distinguish between these alternative evolutionary outcomes.

Chemical cues and olfaction play important roles in mate choice in many organisms, including reptiles such as sea snakes (Shine *et al.*, 2002) and lizards (Cooper & Vitt, 1986; Barbosa *et al.*, 2006; Martín & Lopez, 2006). Tongue-flicking activity is generally considered to reflect sexual interest and has been used in several previous studies of mate preferences and sexual isolation in reptiles (Shine *et al.*, 2002; Barbosa *et al.*, 2006; Martín & Lopez, 2006). These studies indicate that chemical cues do often mediate premating isolation between closely related reptile species, incipient species or populations.

Here, we present a study on olfactory mate preferences and chemical cues in island populations of a lacertiid lizard, the Skyros wall lizard *Podarcis gaigeae*. Our goal was to quantify the degree of scent-mediated premating isolation between populations and investigate whether there are any parallel changes in mate preferences in the islet populations of this endemic lizard species. We investigated male and female mate responses to chemical cues from individuals of the opposite sex. To quantify differences in pheromone composition between populations that might drive scent-mediated sexual isolation, we analysed the chemical composition of secretions from the male femopores (Gabirot *et al.*, 2008). We address the issue of possible parallelism between similar habitats (e.g. islands vs. mainland) in pheromone composition. Low effective population sizes on small islets ($N_e < 100$) in this system, pronounced molecular population divergence between the islets and close mainland populations and low within-population genetic diversity on small islets suggest a potential role for genetic drift in the divergence of these populations (Runemark *et al.*, 2010). Thus, this ecological setting has close similarities to the suggested scenario where Kaneshiro's theory would predict a loss of courtship elements through either founder effects or genetic drift. This geographical and ecological setting also allows us to address the issue of the general evolutionary processes that are likely to drive divergence in mate preferences in island geographical settings.

Materials and methods

Study species and geographical setting

The Skyros wall lizard *P. gaigeae* is a small insectivorous lacertiid lizard inhabiting Greece. It is endemic to the Greek island Skyros and islets in the surrounding archipelago (Poulakakis *et al.*, 2005). In addition, there is also another subspecies, *Podarcis gaigeae weigandi*, which is found further north of Skyros, on the island of Piperi (Arnold & Ovenden, 2002). The Skyros archipelago consists of 36 islands and islets (Fig. 1), which have sequentially become landlocked owing to sea level rise in the Aegean Sea (Lambeck, 1996). On the small islets, predation rates are considerably lower than on the mainland of Skyros (Pafilis *et al.*, 2009a), a common ecological pattern seen on many islands in the Mediterranean (Perez-Mellado *et al.*, 1997). The main reason for the reduced predation rates on lizards on small islets is the absence of raptors and snakes (Pafilis *et al.*, 2009a). The floral community composition on the main island of Skyros and on the surrounding islets also varies considerably, because of variation in the substrate and grazing pressure from goats and sheep. Plant taxa common on the main island of Skyros are absent on some islets, whereas other species reach very high frequencies (Snogerup & Snogerup, 2004). These differences in local plant communities will also have an impact on the invertebrate faunas (e.g. phytophagous insects) and hence the food sources of the lizards. We have recently found pronounced and significant differences between mainland and islet environments in both invertebrate species composition, as well as diets of the lizards, as estimated from stable isotope analyses (A. Runemark, E. I. Svensson, unpublished data). The general differences between mainland- and islets environments are summarized in Table 1. *Podarcis gaigeae* varies considerably in its morphological characters between Skyros and the islets, and there are at least two documented cases of island gigantism (A. Runemark and E. I. Svensson, unpublished data; Pafilis *et al.*, 2009b) on small islets in the Skyros archipelago.

Field work, animal care and maintenance

Lizards were caught on two different islets close to the coast of Skyros and two localities on main island of Skyros (hereafter referred to as ‘mainland populations’), see Fig. 1. The two mainland populations that we

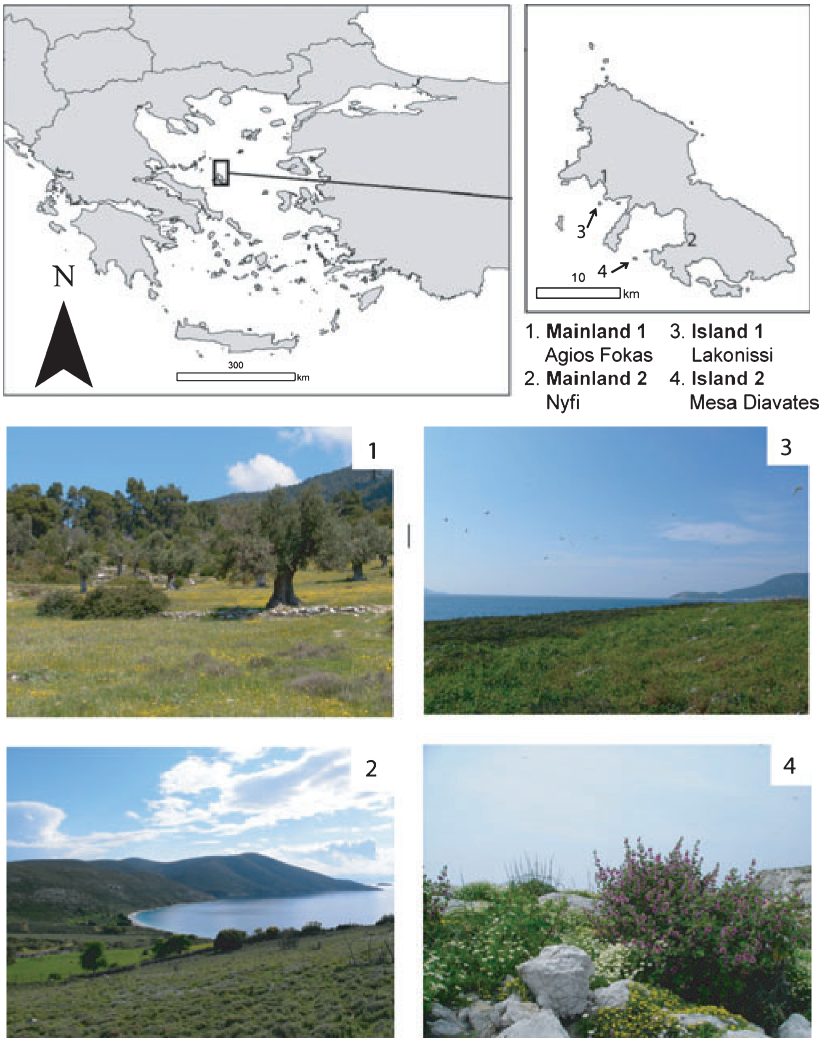
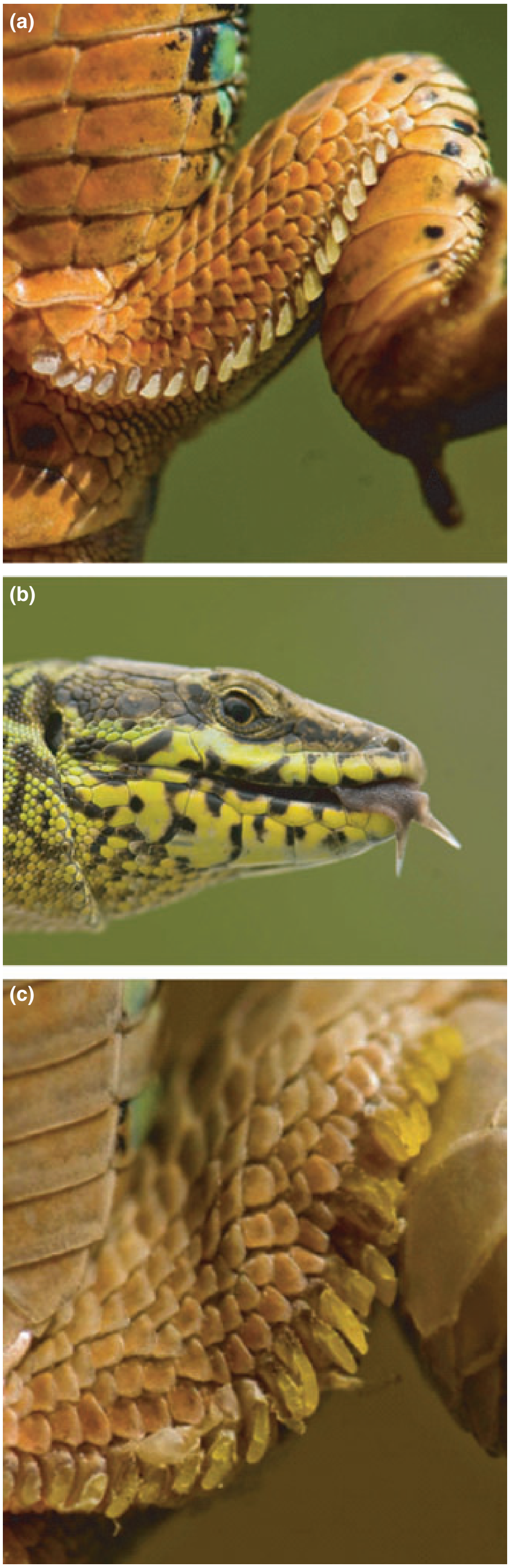


Fig. 1 The island of Skyros in Greece. Marked on the map are the study sites on the main island of Skyros where we caught the *Podarcis gaigeae* that were later used in the experiments. We used lizards from four localities, two islet populations off the coast of Skyros and their two closest and proximate ‘mainland’ populations on Skyros. The environments differ considerably between these sites, because of variation in grazing pressure from goats, percentage woody vegetation and general substrate.



sampled were chosen because they were the geographically the closest to the focal islet populations. The rationale behind this sampling schedule was thus to obtain a parallel experimental design with two independent islet-mainland population pairs (two replicates). We tentatively assume that the two mainland populations are the ancestral populations of the respective islet populations (Fig. 1). This assumption is likely, given isolation-by-distance documented between populations of this species (Runemark *et al.*, 2010) and the fact that islet populations in the Aegean have been subsequently landlocked following a sea level rise (Lambeck, 1996; Hurston *et al.*, 2009), although isolation by distance is not necessarily present in all taxa (Tregenza *et al.*, 2000; Reusch *et al.*, 2001; Makinen *et al.*, 2008; Pineira *et al.*, 2008). All lizards were caught during peak reproductive activity (March–May) in 2007 and 2008.

The sex of the individual lizards was determined through inspection of the femoral pores (Fig. 2a,c) that are more pronounced in males than in females, and the head width in comparison with body size, which is sexually dimorphic in *Podarcis* (Arnold & Ovenden, 2002). All animals used in the experiments were either adult males with well developed femopores or adult females with mating scars. *Podarcis gaigeae* females mate multiple times and have two to three clutches of eggs during the summer (Germanou *et al.*, 2000; Pafilis *et al.*, in press), so the presence of mating scars does not imply that the females are no longer sexually interested. As the size of adult males differs between populations, mean population snout-vent length and standard deviation for the males used for pheromone sampling are provided in Supporting Information, Table S1. Animals were individually marked through toe clipping and kept in 46 × 30 × 17 cm terraria at 25 °C and a 12:12 light/dark regime. Both 40-W incandescent light bulbs for heating and fluorescent lamps providing 2% UVB light were used for illumination. The animals had free access to water and were fed meal worms dusted with vitamins *ad libitum*. All animals had acclimatized to the laboratory environment for at least 2 days before the experimental trials, which implies that no females had been mated during at least 3 days prior to the experimental trial. Remaining scent from previous matings was thus unlikely to confound these results. No animals were kept in captivity for longer than 2 months.

Fig. 2 Image of (a, c) femoral pores with waxy pheromone secretion of *Podarcis gaigeae* males. We sampled the secretions from the femoral pores and analysed the pheromone contents using gas chromatography. (b) A male lizard flicking its tongue to assess the scent of the surrounding air. We assessed sexual interest in the deposited pheromones by quantifying the number of tongue flicks of focal lizards in terraria that contained the scent of smell donor animals (see Methods).

Table 1 Schematic representation of the abiotic and biotic differences between the islet- and mainland habitats of the study populations of *Podarcis gaigeae*.

Factor	Islet 1	Islet 2	Mainland 1	Mainland 2	Source
Geology	A*	A*	B*, A*	B*, A*	Jacobshagen & Ματαράγκας, 1989
Moisture	High	High	Low	Low	Personal observation
Plant community	Mix of herbaceous and woody	Predominately herbaceous	Mix of woody and herbaceous	Predominately woody	Snogerup & Snogerup, 2004; personal observation
Grazing pressure	Moderate	None	High/Low patchy	High	Personal observation
Predation	Low	Low	High	High	Pafilis *et al.*, 2009a,b
Intraspecific competition	High	High	Low	Low	Pafilis *et al.*, 2009b

A* Calcitic-dolomitic marbles.

B* Semi-metamorphosed clastic sedimentary formations.

Premating isolation and sexual interest in scent

We used interest in pheromones from an individual of the opposite sex as a measure of sexual interest. We used this as to quantify the degree of premating isolation between populations. Pheromones are important mating cues in many lizard species, including *Podarcis* lizards (Cooper & Vitt, 1986; Barbosa *et al.*, 2006). The number of tongue flicks (Fig. 2b) during 10 min was used as a measure of sexual interest. This measure should partly reflect the probability of a field encounter between the smell donor and the focal animal in nature because *Podarcis* males have been shown to trace female scent (Gómez *et al.*, 1993). Lizard females can also monitor male dominance status, MHC similarity and health through scent (Lopez *et al.*, 2002, 2009; Olsson *et al.*, 2003), and scent recognition plays a role in species recognition in the *Podarcis* clade (Martín & Lopez, 2006).

Tongue flicking is used in many different lizard behaviours, for example territoriality, predator assessment and sociality. Because male territories encompass several female home ranges (Edsman, 1990; Tsellarius & Tsellarius, 2006) and female lizards are typically not as strongly territorial as males, tongue-flicking activity is unlikely to reflect male territoriality. Although increased tongue-flicking responses to saurophagous lizard scent has been documented (Punzo, 2008), the pheromone composition of the saurophagous *Lacerta trilineata* is chemically very different to that of the *Podarcis* clade (see Martín & Lopez, 2006 and Gabirot *et al.*, 2008). Hence, predator assessment behaviour is unlikely to reflect the patterns of tongue-flicking rates that we found in these trials. In the congeneric relative *Podarcis muralis*, tongue-flicking activity was directed primarily towards scent from the opposite sex, while activity towards individuals of the same sex was not different from that activity directed towards distilled water, i.e. a blank control (Cooper & Perez-Mellado, 2002). Thus, heightened tongue-flicking activities towards the opposite sex are unlikely to primarily reflect territoriality, general sociality or predator detection, at least not in this lizard group, but are highly likely to reflect genuine sexual interest and probability of initiating copulation, if given the chance. In further support of our use of tongue flicks being a good measure of sexual interest, we have found that islet females from the same two islet populations also prefer to associate with islet males in visual trials (*T*-test for dependent samples, $t_{28} = 2.29$, $P = 0.030$), whereas this pattern was weaker and only borderline significant for mainland females ($t_{29} = 1.83$, $P = 0.08$) (Wadlund, 2010). The same experimental islet females also tended to direct more tongue flicks at their own population than another ($t_{110} = 1.83$, $P = 0.07$), whereas mainland females did not discriminate ($t_{126} = -0.17$, $P = 0.87$) (Wadlund, 2010). These consistencies between visual mate choice and number of tongue flicks, the significantly lower number of tongue flicks in terraria where the closely related species *Podarcis taurica* individuals have been housed and the previously documented strong role for olfactory cues in reproductive isolation in the *Podarcis hispanica*-complex strongly suggest that number of tongue flicks does reflect sexual interest in *P. gaigeae*.

We used an experimental set-up that was similar to that described in a recent study on a closely related *Podarcis* species (Barbosa *et al.*, 2006). A smell donor animal of the opposite sex was kept in the experimental terrarium with a filter paper substrate during one night (10–14 h) prior to each experimental session and was removed from the terrarium approximately 5 min before the start of each trial. Because the pheromone production is continuous during the mating season and the deposition of the secretions from the femopores is passive in lizards (Mason, 1992; Alberts, 1993), the nocturnal sampling scheme should not affect the results. The smell donor animals had access to a shelter of crumbled filter paper and water during this period. Before the trial, all visible signs of the smell donor, such as faeces and pieces of shed skin, the paper shelter and the water were removed. The experimental terraria were kept in the same room as the lizards were housed and the light conditions were identical. Trials started by placing a focal lizard in a 46 × 30 × 17 cm terrarium with an incubated filter paper substrate. Thereafter, we recorded the number of tongue flicks on the filter paper the first 10 min after the lizard started moving. Observers were placed behind a blind to avoid disturbing the lizards during the trials. Between trials, the terraria and water bowls were washed with water and alcohol to remove remaining chemical traces and the filter paper was changed. An alternative method where secretions from the femoral pores are sampled on a cotton cue tip (see for example Martín & Lopez, 2006) has also been used to monitor sexual interest in the same populations of *P. gaigeae*. The results from these experiments were largely consistent with those obtained with the method of Barbosa *et al.* (2006) that we applied here (Wadlund, 2010). Thus, our results and conclusions seem to be roboust, independent of the choice of experimental method, and not affected by timing of sampling (diurnal with the cotton cue tip method and nocturnal in the Barbosa *et al.* (2006) method).

In 2007, 13 mainland1, 11 mainland2, 14 islet1 and 9 islet2 focal female lizards and in 2008, 23 mainland1, 20 mainland2, 11 islet1 and 13 islet2 focal female lizards were tested for their interest in male scent. In 2007, 12 mainland1, 14 mainland2, 10 islet1 and 13 islet2 focal male lizards and during 2008, 21 mainland1, 20 mainland2, 16 islet1 and 11 islet2 focal male lizards were tested for interest in female scent. All focal lizards were presented to the olfactory cues from all four populations. All focal lizards also served as donors for lizards from all populations except in a few cases where lizards only donated smell to three of the four populations, because of the larger sample size of their populations. In the populations with lower sample size, some lizards were used as smell donors for the same (larger) population twice. In the cases where a lizard was used more than once, we also tested for the significant effect of identity, to avoid possible problems with pseudoreplication, by treating identity as a random effect. However, such effects were generally weak and nonsignificant, unless otherwise stated.

Behavioural trials were conducted during March–May 2007 and March–April 2008 between 9 AM and 4 PM, the main activity period of the lizards in nature. This schedule was used to ensure that the lizards were active. The temperature in the terraria was kept at 25 °C. In addition to tongue flicks, we also recorded the number of escape attempts during the observation sessions. Trials where the lizards were spending more than half of the experiment time trying to escape or remained inactive for longer than 5 min were excluded from further analysis. A total of 846 trials, 420 where the focal animals were females and 426 where the focal animals were males, were included in the analysis. To evaluate the utility of the number of tongue flicks as a measure of sexual interest, we compared the number of tongue flicks between terraria where *P. gaigeae* had been housed ($N = 846$) to (i) control terraria where no lizard had been present ($N = 40$) and (ii) terraria where the closely related species *P. taurica* had been housed prior to the experimental trial ($N = 8$). We analysed whether there were any differences between these three categories using a one-way ANOVA.

In our analyses of the chemosensory responses, we used a fully factorial three-way ANOVA with the number of tongue flicks as the dependent variable. Habitat origin of the focal lizard (islet or mainland), habitat origin of the smell donor lizard (islet or mainland), sex of the focal lizard and all two-way interactions between these factors and the three-way interaction were included in this original fully factorial model. Initially, we also included the population of the focal lizard and the population of the smell donor as factors. However, the population effects turned out to be nonsignificant and weak, and they were therefore excluded from the models. Nonsignificant terms were sequentially removed from the model starting with the highest level interaction term (i.e. the three-way interaction, followed by the two-way interactions). We also present the reduced models in all the different populations, where we included sex of the focal animal, origin of the smell donor and the interaction between these factors. To more formally test whether the sexual interest differs between different habitat combinations, we performed two general linear models (GLMs), one for male and one for female focal animals, with number of tongue flicks as a dependent variable and habitat combination (island–island, island–mainland, mainland–mainland and mainland–island) as categorical predictor. All of our statistical analyses and models were performed in Statistica (Statsoft Inc., 2004), unless otherwise stated.

Chemical analyses of femoral gland secretions

After 2 weeks of acclimatization to a common garden environment in the laboratory, we extracted femoral gland secretion from males by gently pressing around the femoral pores with forceps and collecting secretions directly in glass vials with Teflon-lined stoppers. Vials were stored at −20 °C until analyses. We used the same procedure on each sampling occasion but without collecting the secretion, to obtain blank control vials that were treated in the same manner to compare with the analysis results from the lizard samples. Before the analyses, we added 250 μL of n-hexane (Sigma-Aldrich, St. Louis, MO, USA, capillary GC grade) to each vial.

We analysed lipophilic compounds in samples by using a Finnigan–ThermoQuest Trace 2000 GC fitted with a poly (5% diphenyl/95% dimethylsiloxane) column (Thermo Fisher Scientific Inc., Waltham, MA, USA, Trace TR-5, 30 m length × 0.25 mm ID, 0.25-mm film thickness) and a Finnigan–ThermoQuest Trace mass spectrometer as detector. Sample injections (2 μL of each sample dissolved in n-hexane) were performed in splitless mode using helium as the carrier gas at 30 cm s^{-1}, with injector temperature at 250 °C. The oven temperature programme was as follows: 50 °C isothermal for 5 min, then increased to 270 °C at a rate of 10 °C/min, isothermal for 1 min, then increased to 315 °C at rate of 15 °C/min and finally isothermal (315 °C) for 10 min. Ionization by electron impact (70 eV) was carried out at 250 °C. Mass spectral fragments below $m/z = 39$ were not recorded. Impurities identified in the solvent and/or the control vial samples are not included in our results and analyses. Initial tentative identification of secretion components was performed by comparison of mass spectra in the NIST/EPA/NIH 1998 computerized mass spectral library. Identifications were confirmed by comparison of spectra and retention times with those of authentic standards from Sigma-Aldrich Chemical Co. (Milwaukee, WI, USA). For unidentified or unconfirmed compounds, we report their characteristic ions, which we used together with retention times and characteristic *m/z* ratios to confirm whether these compounds were present in a given individual.

For the statistical analyses of secretions, the relative amount of each component was determined as the percent of the total ion current (TIC). Then, relative areas of the peaks were transformed following 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). The transformed areas were used as variables in a principal component analysis. The 10 first principal components (PC) were extracted (all with eigenvalues > 2%; collectively explaining 61.62% of total variance) and used as independent variables in a multivariate analysis of variance (MANOVA). The goal of this MANOVA was to determine whether the four populations of male *P. gaigeae* differed in the relative proportions of compounds. A discriminant function analysis (DFA) was used to calculate Mahalanobis distances between populations to see whether they could be separated from each other based on these principal components. We also used the classification function in the DFA to estimate the percentage of correct population classifications of all individuals. We used this test to verify whether chemical compounds in femoral secretions could be used to predict the population of origin of a male lizard and whether the population differences could explain the results from the behavioural trials.

Molecular genetic analyses and correlation between neutral genetic differentiation and pheromone composition

DNA from the tails of 394 lizards (71 mainland1, 113 mainland2, 116 islet1 and 94 islet2 individuals) was extracted using an ammonium acetate extraction protocol (Sambrook *et al.*, 1989) and typed at 20 highly variable microsatellite loci described in Runemark *et al.* (2008) and Wellenreuther *et al.* (2009), respectively. Pairwise F_{ST} values between populations were calculated using F-stat (Goudet, 2001). We estimated the correlation and its significance between the pairwise F_{ST} values between populations and the Mahalanobis distances of the multivariate distances in pheromone composition for the same populations. Because the data points are dependent (pairwise population differences), a resampling procedure implemented in Resampling Stats (Simon, 2000) was implemented to estimate this correlation and its associated *P*-value. We also performed a Mantel's test, a matrix-based test for correlations, implemented in the Isolde application in GENEPOP (version 1.2) (Raymond & Rousset, 1995; Rousset, 2008) to test for such a correlation.

Results

Behavioural assays of scent preferences

Our control trials revealed that *P. gaigeae* lizards differed significantly in their tongue-flicking rates between experimental treatments (ANOVA: $F_{2,892} = 15.89$, $P < 0.001$). Focal animals had significantly higher tongue-flicking rates in terraria with scent of conspecific *P. gaigeae* than in control terraria where no lizards had been housed (Tukey's test: $P < 0.001$) or in terraria with scent of heterospecific *P. taurica* (Tukey's test: $P = 0.03$), but there were no differences between control terraria and those with scent of *P. taurica* ($P = 0.97$). These data suggest that the number of tongue flicks do indeed reflect enhanced sexual interest towards the smell of conspecifics.

We investigated how the number of tongue flicks between population encounters were affected by various factors using a three-way ANOVA involving all study populations (see Methods and Table 2). The three-way interaction [smell donor × origin (e.g. island or mainland) focal animal × sex of focal animal] was not significant ($P = 0.84$) and was therefore removed from the analysis. Subsequently, the following nonsignificant variables were thereafter removed in a sequential manner: origin of focal animal × sex of focal animal ($P = 0.91$), origin of smell donor × sex of focal animal ($P = 0.09$), smell donor population ($P = 0.16$) and focal animal population ($P = 0.07$) (Table 2a).

The remaining variables in the final model that explains the number of tongue flicks were origin of the focal animal ($P = 0.02$), origin of the smell donor ($P < 0.001$), sex of the focal animal ($P < 0.001$) and the interaction between origin of focal animal × origin smell donor ($P < 0.001$) (Table 2b; Fig. 3). The main effect of sex was because of the fact that males had higher overall tongue-flicking rates than females (Fig. 3b). The two-way interaction reveals that the effect of smell donor is contingent upon the origin of the focal animal (Table 2). Two GLMs and their respective Tukeys *post hoc* tests show that in both

Table 2 (a) ANOVAs of factors affecting premating isolation. The number of tongue flicks was the dependent variable, and the origin of smell donor (islet or mainland), the origin of focal animal, sex and all two- and three-way interactions were included in the original model. The model was subsequently reduced by removing nonsignificant variables through stepwise backward selection. (b) The final model including origin of smell donor, origin of focal animal, sex and origin of smell donor × origin of focal animal.

Effect	*Df*	*F*	*P*
(a) Full model			
Origin of smell donor	1	22.36	< 0.001
Origin of focal animal	1	5.42	0.02
Sex focal animal	1	111.17	< 0.001
Origin of smell donor × origin of focal animal	1	14.97	< 0.001
Origin of smell donor × sex focal animal	1	2.98	0.09
Origin of focal animal × sex focal animal	1	0.02	0.90
Origin of smell donor × origin of focal animal × sex focal animal	1	0.041	0.84
(b) Final model			
Origin of smell donor	1	22.63	< 0.001
Origin of focal animal	1	5.74	0.02
Sex focal animal	1	113.30	< 0.001
Origin of smell donor × origin of focal animal	1	14.93	< 0.001

males ($F_{3,427} = 6.89$; $P < 0.001$; Table S2) and females ($F_{3,412} = 9{,}60$; $P < 0.001$; Table S2), islet populations tongue flick significantly more at the scent from islet animals than at scent from mainland animals (males: $P < 0.001$, females: $P < 0.001$ Tukeys *post hoc* tests; Table S2), whereas mainland populations do not show any significant differences in number of tongue flicks directed at mainland- and islet scent (males: $P = 0.73$, females: $P = 0.95$ Tukeys *post hoc* tests; Table S2).

A general and striking finding in both males and females was that focal islet lizards always and clearly preferred the donors from islets and discriminated against mainland lizards (Fig. 3). In contrast, male and female mainland lizards were more indiscriminate and did not show any significant evidence of directed mate preferences, either towards their own population or towards other populations (Fig. 3).

Pheromone analysis

We found 64 lipophilic compounds in femoral glands secretions of males (Table S3). These secretions are a mixture of steroids (79.07% of TIC), several forms of tocopherol (10.87%), 10 waxy esters (5.84%), carboxylic acids ranging between C_{12} and C_{22} and some of their ethyl esters (2.61%), squalene and other similar unidentified terpenoid (0.71%), three alcohols (0.68%) and other minor components (0.23%). On average, the most abundant chemicals were cholesterol (72.38% of TIC), D-α-tocopherol (9.79%), the octadecyl ester of the hexadecanoic acid (3.32%), campesterol (2.38%) and hexadecanoic acid (1.62%) (Table S3). All 64 compounds were present in lizards from the four populations studied, with no compound being exclusive of any population.

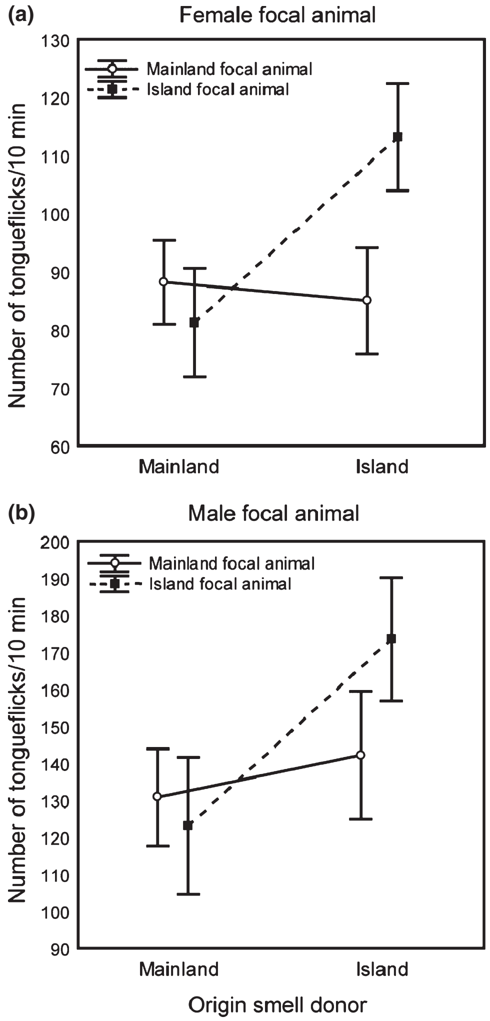


Fig. 3 Olfactory premating isolation between lizard populations, measured as number of the tongue flicks directed towards animals of the opposite sex. There is a significant effect of the origin of the focal animal ($P = 0.02$), the origin of the smell donor ($P < 0.001$), the sex of the focal animal ($P < 0.001$) as well as the interaction term origin focal animal × origin smell donor ($P < 0.001$) (Table 2). (a) Islet females showed enhanced sexual interest, measured as significantly higher tongue-flicking rates, when island males had been present in the terraria, when compared to the responses of mainland females who did not discriminate between male donor categories. (b) Islet males also showed enhanced sexual interests when island females had been present in the terraria, as opposed to mainland males who showed equal interest in the two female categories (Table 2).

A multivariate analysis on the 10 main principal components (PCs) for the 64 shared compounds revealed significant differences between populations (MANOVA, Wilks lambda = 199114, $F_{30,106} = 2.59$, $P < 0.001$; Fig. 4a). A DFA based on these principal components revealed significant differences in chemical composition of the pheromones between the two islet populations (I1–I2 Mahalanobis distance: 7.23; $P = 0.001$, Fig. 4b). Three of four pairwise comparisons between mainland populations and islets were significant and the fourth one close to significant (Mahalanobis distances: M1-I1 8.26, $P = 0.001$; M1-I2 4.99, $P = 0.03$; M2–I1 5.86, $P = 0.01$; M2–I2 3.70, $P = 0.09$; see Fig. 4b). In contrast, the difference between the mainland populations was not significant (M1–M2 Mahalanobis distances: 2.75, $P = 0.35$).

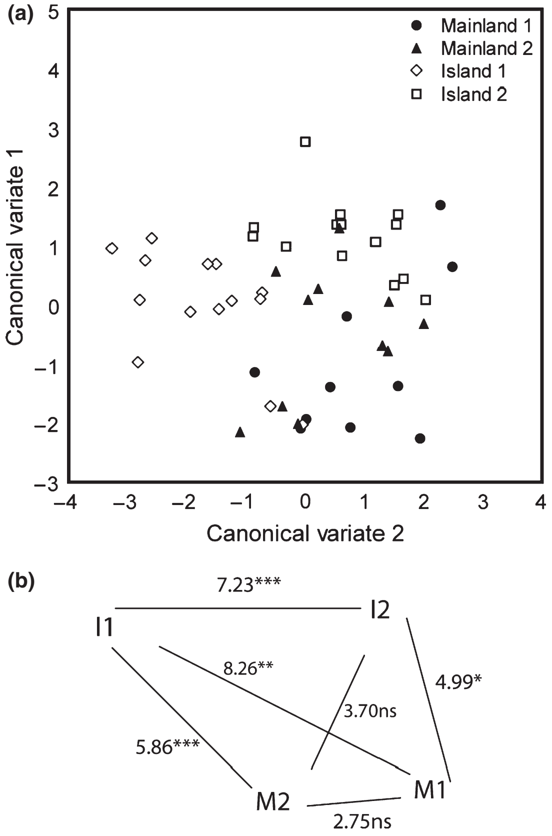


Fig. 4 (a) Pheromone compositions of the secretions from the male femoral pores. The two first canonical variates (CVs) based on 10 principal components are illustrated in the figure. There are significant differences between the male populations in multivariate pheromone space (MANOVA, $F_{27,405} = 2.07$, $P = 0.001$). Post hoc tests revealed that four of six of the between-population comparisons are significant (all $P < 0.05$). The differences within the habitat categories were not significant (i.e. no difference between the two islets and between the mainland populations). (b) Mahalanobis distances of pheromone composition between the different populations. These multivariate distances were obtained from a discriminant function analysis based on the 10 first principal components. Vectors are scaled to the relative distance between populations, and significances are denoted by asterisks ($^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$).

In a classification analysis, 86% of the individuals from islet 1 and 93% from islet 2 were correctly classified into population. The classification percentages were considerably lower for the mainland populations: 50% and 64%, respectively, for mainland population 1 and 2. These analyses indicate that the islet populations have diverged considerably, from both the mainland populations as well as from each other (Fig. 4). Moreover, divergence of the islet populations from the mainland was not parallel, but in different directions (Fig. 4).

Two of the principal components of the pheromone compounds differed significantly between populations after we had applied Bonferroni correction: PC2 (ANOVA $F_{3,45} = 7.13$, $P = 0.005$; Fig. 5a) and PC5 ($F_{3,45} = 4.48$; $P = 0.007$; Fig. 5b). PC2 was negatively correlated with the relative proportions of dodecanone and waxy ester-octadecenoic (factor loadings −0.62 and −0.69, respectively, Table S4). Males from islet 1 had significantly higher PC2 scores than males from mainland locality 2 (Tukey's test: $P = 0.04$) and islet 2 ($P = 0.0003$). PC5 was positively correlated with the proportion of dl-α-tocopherol, the proportion of cholesta-7-en-3-ol, 2, 2dimethyl (3beta, 5alppha) and the proportion of an unidentified steroid (143,253,354,380,395,413) (factor loadings 0.88, 0.68 and 0.62, respectively, Table S4). Males belonging to the mainland populations had lower PC5 scores than males from the islet populations (Fig. 5b). Males from the two islet populations differed significantly in PC5 compared to mainland population 1 (Tukeys *post hoc* tests: island 1 vs. mainland 1: $P = 0.02$; islet 2 vs. mainland 1: $P = 0.04$).

Correlation between molecular divergence and divergence in chemical signals

We calculated pairwise F_{ST} values between populations using 18 neutral molecular microsatellite markers used in Runemark *et al.* (2010) and compared a multivariate measure of pheromone divergence between populations (Mahalanobis distances) to this neutral molecular divergence between the same populations. F_{ST} values ranged from 0.026 (mainland 1 vs. mainland 2) to 0.251 (islet 1 vs. islet 2). F_{ST} values between mainland localities and islets were intermediate; 0.166 (mainland 1 vs. islet 1), 0.111 (mainland 2 vs. islet 2), 0.113 (mainland1 vs. islet 2) and 0.177 (mainland 2 vs. islet 1), see Fig. 6 for a graphical illustration of the F_{ST} values.

Consistent with a scenario where pheromone chemical signals are partly influenced by genetic drift, we found a significant regression between the Mahalanobis distances and molecular genetic distances that was positive and equal to $y = 2.5 + 20.8 \times x$ ($P = 0.038$; Fig. 6). This positive regression reveals that the pattern of population genetic differentiation mirrors to that of differentiation of the pheromone blend, which suggests a role for genetic drift in the divergence of chemical signals. An alternative

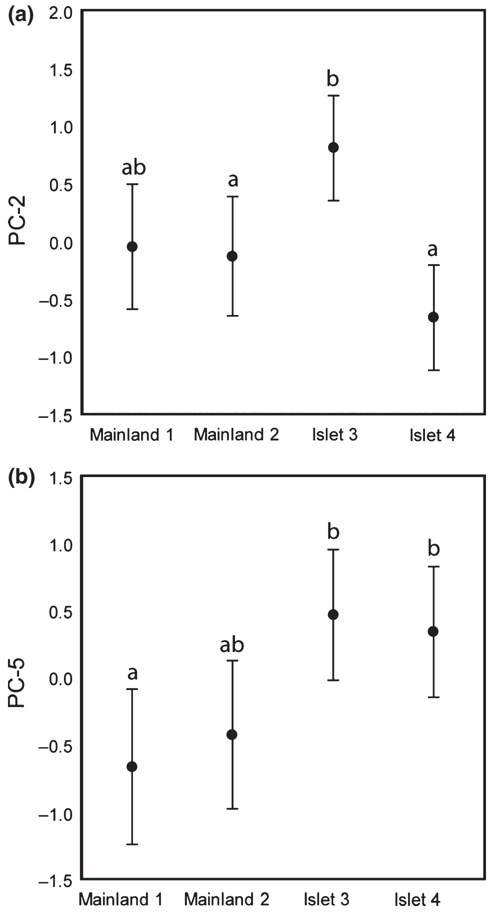


Fig. 5 Population differences in pheromone composition revealed by principal component analysis. (a) PC2 is negatively correlated with the proportion of dodecanone and differs significantly between the four populations after we had applied a Bonferroni correction (ANOVA, $F_{3,45} = 7.13$, $P = 0.005$). Males from islet 1 had higher scores than males from both mainland 2 (Tukey's test: $P = 0.04$) and island 2 ($P = 0.0003$). (b) PC5 is positively correlated with the proportion of dl-a-tocopherol, and the proportion differed significantly between populations ($F_{3,45} = 4.48$; $P = 0.007$). Males from the mainland populations had lower values of PC5 scores than males from the islet populations. *Post hoc* test revealed significant differences between both mainland 1 and islet 1 ($P = 0.02$) as well as between mainland 1 and island 2 ($P = 0.04$).

statistical approach is to use a matrix-based Mantel's test. The Mantel test did not reveal any significant correlation between Mahalanobis distances and molecular genetic distances ($y = -0.01 + 0.028x$, $P = 0.15$; Fig. 6), which might partly be because of the low sample size.

Discussion

Our behavioural trials have revealed a general propensity for islet lizards to prefer the scent of individuals from islet populations, whereas mainland lizards were less discriminatory (Fig. 3). This pattern was similar for both the male and female mate responses (Fig. 3). This indicates asymmetric sexual isolation, i.e. islet populations are more choosy than mainland populations (Fig. 3). Asymmetric sexual isolation of this kind is not uncommon and has been documented in many studies of various taxa (Whiteman & Semlitsch, 2005; Svensson *et al.*, 2007; Nickel & Civetta, 2009; Egger *et al.*, 2010). Although mate discrimination based on pheromone composition has been reported previously between closely related *Podarcis* species (Martín & Lopez, 2006), our results suggest that pheromones could also affect sexual interest at the intraspecific level, between geographically close populations.

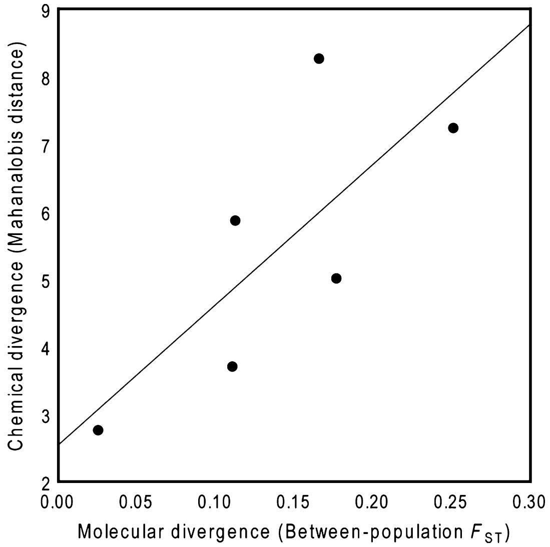


Fig. 6 Relationship between the between-population divergence in chemical composition of male pheromones (from multivariate Mahalanobis distances) and the pairwise neutral genetic divergence between populations (F_{ST} obtained from 18 microsatellite loci). The regression is positive $y = 2.5 + 20.8 \times x$ and statistically significant (correlation resampled with Resampling Stats (Simon, 2000); $P = 0.038$).

The general pattern that islet populations preferred the scent from islet donors could result from parallel divergence in pheromone composition in the islet populations. Interestingly, we found no evidence of any strong parallelism in pheromone composition between the island populations (Fig. 4). Instead, these analyses revealed that the chemical composition of the islet populations had diverged considerably from each other (Fig. 4b) and differed significantly between the two islet populations (Fig. 4b). One possible exception was an increase in PC5 in the islet populations. PC5 is correlated with the proportion of dl-α-tocopherol, cholesta-7-en-3-ol, 2, 2dimethyl (3beta, 5alpha) and an unidentified steroid (143,253,354,380,395,413) (Fig. 5b). An increased preference for these substances in the islet populations could thus possibly partly explain the islet females' preference for islet scent in the behavioural trials (Fig. 3, Table 2). Additional experiments where the different populations' interest in such candidate substances would, however, be needed to more thoroughly test this possibility. Another possibility is that the mate preferences could be plastic, as has recently been demonstrated in a number of different taxa, including insects (Svensson *et al.*, 2010 and references therein). Thus, if the islet populations have been exposed to similar environments, some chemical cues or some previous experiences might have induced similar mate preferences, resulting in the pattern of sexual isolation documented in this study (Fig. 4).

None of the components of the pheromone blend had been lost in any of the islet populations, as would be expected if Kaneshiro (1980) scenario of loss of courtship elements in islet populations because of founder events would be an important factor in this system. Furthermore, the expectation from the Kaneshiro hypothesis that islet individuals should be less choosy in their mate preferences was not supported either (Fig. 3). Our results on mate preference variation and the degree and direction of sexual isolation are therefore more consistent with the model of Kirkpatrick & Servedio (1999). A general prediction from their model is that islet individuals show stronger discrimination against immigrants than mainland individuals. Interestingly, we found no evidence for discrimination between the two islet populations. This is particularly interesting because they are more distant from each other in terms of their phermomone composition than from the corresponding mainland populations (Fig. 4b).

The pronounced divergence in pheromone composition between islet populations mirrors a similar pattern of neutral genetic divergence (Fig. 6). F_{ST} values were higher between islet population pairs than between islet-mainland population pairs. Founder effects and possibly also genetic drift caused by low effective population sizes on the islets are the most likely explanations for the divergence in neutral genetic variation. The correlation between neutral population divergence and pheromone composition divergence suggests a role for genetic drift also at the phenotypic level in these lizards, at least for these sexual signals (Fig. 6), but a larger sample size would be necessary for a thorough evaluation of this hypothesis as indicated by the nonsignificant Mantel's test. It is possible that the differences in pheromone composition between the islets might be influenced by genetic drift, which would result in signal divergence that would go in different and arbitrary directions, on different islands. Such a pattern stands in contrast to the parallel divergence one would expect to find whether selection in similar environments would have favoured similar pheromone composition on the islets owing to similar ecologies.

Although chemical signals might have a genetic basis as a result of natural selection and/or genetic drift, our results do not in any way exclude the possibility that there are also environmental influences on these pheromones. There is an increasing awareness of the role of phenotypic plasticity in both signalling traits and mate preferences (Chaine & Lyon, 2008; Cornwallis & Uller, 2010). Plasticity might lead to more pronounced population divergence, at least initially, than purely genetic mate preferences and signalling traits (Cornwallis & Uller, 2010; Svensson *et al.*, 2010). The role of phenotypic plasticity in sexual signals is increasingly becoming recognized, and recent theoretical and empirical work suggest that plasticity could play a key role in initial signal divergence (Price *et al.*, 2003; West-Eberhard, 2003; Price, 2006; Svensson & Gosden, 2007; Cornwallis & Uller, 2010). In the case of these lizards, it is possible that local diets could influence the local pheromone composition, and local preferences for such plastic and diet-induced signalling traits might subsequently arise in the different populations. Localized selection could then potentially favour individuals with a higher proportion or a more stable production of this pheromone, which might result in genetic assimilation of an originally plastic trait (cf. Price *et al.*, 2003; West-Eberhard, 2003). Differences in local invertebrate diets and other microenvironmental factors could thus potentially and partly reflect genetically assimilated differences in chemical signals.

However, because we collected pheromone samples from males that had been acclimated for 2 weeks to a common garden laboratory environment with a standardized diet, the differences in chemical composition of the pheromones are unlikely to be solely explained by habitat-specific diets. The common garden housing conditions should, at least potentially, have removed some of the effects of local diets and the differences in chemical signals between our populations. Thus, it seems likely that the population differences we have documented are not only reflecting local diets and phenotypic plasticity (Figs 4 and 6). In fact, if differences in local diets have influenced population differences in chemical signals, this will make our statistical tests for population differences more conservative, and hence, the differences in signals might be even larger under natural conditions (Fig. 4).

In this study, we thus found evidence for parallelism in scent preferences, but these parallel mate preferences were not matched by any parallelism in the pheromone compositions of the islet populations. Possibly the islet populations' preferences for the scent of the other islet population is because of stochasticity, or possibly there could be different ways to achieve a pheromone compositions that are attractive to islet lizards. The efficiency of signal transmission in the habitat is clearly important in sexual communication (Marchetti, 1993; Schluter & Price, 1993; Schluter, 2000; Price, 2007), and ecological differences between islet and mainland environments (Table 1) could potentially select for different properties of the pheromone blend. If there would be different ways to achieve a similar property, this might possibly partly explain the general islet population preference for islet scent.

For example, one factor that is likely to differ between islet- and mainland habitats is humidity, because of the water evaporation from the sea and differential proximity to the coast line. Previous studies have shown that differences in composition of chemical compounds in the pheromones are related to the microclimatic conditions of the lizards (Martín & Lopez, 2006). Thus, such local microenvironmental differences might influence the local pheromone compositions, because of selection for different substances in environments that differ in humidity. Tocopherol, a substance that has a high factor loading in PC5 shows some indications of parallelism between islet populations, is a well-known lipophilic antioxidant (Brigelius-Flohe & Traber, 1999; Martínez *et al.*, 2008). The first possible function of increased amounts of tocopherol in secretions of islet lizards might be to inhibit oxidation of other glandular lipid components. Thus, this substance might increase the chemical stability of scent marks in wet conditions of coastal environments (see Alberts, 1992). Trials with experimentally increased tocopherol values to investigate whether islet populations prefer pheromone blends with a higher amount of tocopherol would be an interesting next step.

Differences in pheromone composition and chemosensory recognition have been shown to be one barrier to reproduction between closely related lizard species, such as the *P. hispanica* complex (Martín & Lopez, 2006). Divergence in this sexually selected character in combination with local preferences for the own population in isolated islet populations could thus potentially result in sexual isolation between populations upon secondary contact. We did find that islet populations of *P. gaigeae* can and did discriminate between the scent from different populations, and they clearly showed less interest in the scent from mainland populations. Interestingly, although the two islet populations have diverged more strongly from each other than they have from the mainland populations, there is no discrimination between islet populations (Fig. 3). This parallel development of scent-based discrimination against mainland populations could potentially be adaptive if selection for locally adapted mates favours such discrimination (Kirkpatrick & Servedio, 1999) and both islet populations have developed characteristics that confer fitness benefits in islet environments. In contrast, to such a parallel divergence in scent preferences, the pheromone composition seems to have changed in a more stochastic manner (Fig. 6).

The highly divergent pheromone compositions on the islets might indicate that sexually selected traits and mate preferences can diverge in different and arbitrary directions in small and subdivided populations and that preferences can also be partly decoupled from the signals. It has recently been argued that Lande's (1981) model of sexual selection based on a genetic correlation between traits and preferences should be the expected null model in the absence of selection on preference and trait- or preference–viability correlations (Prum, 2010). Thus, whereas correlations between preferences and traits need no particular explanation, the absence of correlations will need. Plasticity of preferences, and to a lesser extent also the signalling traits, could partly explain such decoupling between the traits and preferences that we have documented in this study. Moreover, genetic drift in combination with sexual selection can generate sexual isolation between populations (Uyeda *et al.*, 2009), which might also play a role in this lizard system, given the low effective population sizes (Runemark *et al.*, 2010) and the additional results we have found in this study. Our results suggest that the links between the actual signals and preferences for them might be more complex than anticipated from simple models of sexual selection.

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Table S1 Mean and standard deviation of snout-vent length for the males used for pheromone sampling.

Table S2 Number of tongue flicks directed at smell donors from different habitats (mainland, island) divided per sex and habitat of origin.

Table S3 Lipophilic compounds found in femoral gland secretions of male lizards *P. gaigeae* from distinct populations.

Table S4 Factor loadings of the different compounds on the 10 first principal components from the principal component analysis on the compositon of the chemical compounds in the secretions from the femopores of male *P. gaigeae*.

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