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Cite this article: Gosden TP, Waller JT, Svensson EI. 2015 Asymmetric isolating barriers between different microclimatic environments caused by low immigrant survival. *Proc. R. Soc. B* **282**: 20142459.
<http://dx.doi.org/10.1098/rspb.2014.2459>

Received: 7 October 2014

Accepted: 22 December 2014

Subject Areas:

evolution, ecology, behaviour

Keywords:

Enallagma cyathigerum, local adaptation, reciprocal transplant, ecological barriers, microclimatic effects

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Electronic supplementary material is available at <http://dx.doi.org/10.1098/rspb.2014.2459> or via <http://rspb.royalsocietypublishing.org>.

Asymmetric isolating barriers between different microclimatic environments caused by low immigrant survival

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Spatially variable selection has the potential to result in local adaptation unless counteracted by gene flow. Therefore, barriers to gene flow will help facilitate divergence between populations that differ in local selection pressures. We performed spatially and temporally replicated reciprocal field transplant experiments between inland and coastal habitats using males of the common blue damselfly (*Enallagma cyathigerum*) as our study organism. Males from coastal populations had lower local survival rates than resident males at inland sites, whereas we detected no differences between immigrant and resident males at coastal sites, suggesting asymmetric local adaptation in a source–sink system. There were no intrinsic differences in longevity between males from the different environments suggesting that the observed differences in male survival are environment-dependent and probably caused by local adaptation. Furthermore, the coastal environment was found to be warmer and drier than the inland environment, further suggesting local adaptation to microclimatic factors has led to differential survival of resident and immigrant males. Our results suggest that low survival of immigrant males mediates isolation between closely located populations inhabiting different microclimatic environments.

1. Introduction

Population divergence in allele frequencies and phenotypic traits is influenced by the relative strengths of spatially variable selection balanced against local genetic drift and gene flow [1]. Populations that experience high levels of gene flow from other populations are expected to suffer a reduction in fitness through a migration load, which can constrain local adaptation [2]. The migration load can potentially cause populations to become maladapted to their local environment and unable to reach their adaptive peaks [3,4], although density-dependent competition and source–sink dynamics can also modulate the effects of migrants on local adaptation [5,6]. Therefore, the realized population divergence in phenotypic traits is thought to be subjected to a delicate balance between the opposing forces of gene flow and the diversifying effects of spatially variable selection [7–11].

Ecological isolation driven by natural selection has been demonstrated in the form of viability differences between insect populations on different host plants [12–14]. In addition, different predation regimes [15–18] or different food resources in different habitats can increase population isolation [19]. Ecological speciation is thought to arise from reproductive barriers that develop as indirect or correlated responses to natural selection for adaptation towards different ecological niches [13,18,20–22]. Ecologically mediated isolation might therefore be a key component generating isolation between populations, especially if isolation acts early in an organism's life history [23]. Local adaptation caused by ecological factors is expected to reveal itself as a statistical signature of phenotype-by-environment interactions between populations in reciprocal transplant experiments and local phenotypes are expected to outperform immigrant phenotypes in their natal environments [24–26].

The type and magnitude of reproductive isolation between populations or closely related species can provide clues to the mechanisms of speciation, and investigations of different isolating mechanisms can elucidate the relative roles of certain barriers to total isolation [11,23,27]. In addition to isolation caused by classical ecological factors, costs arising from sexual selection against migrants might also contribute to population isolation [27,28]. Sexual interactions between species or incipient species can strengthen reproductive isolation through processes such as reinforcement [29], manifest itself as inter-specific interference aggression between males [30], and/or lead to increased risk of predation or parasitism owing to exaggerated male secondary sexual characters [17,31,32].

Here, we examine potential ecological barriers to immigrants within a common, widespread species of damselfly (Odonata: Zygoptera). We performed a spatially replicated experimental field study (reciprocal transplants) between natural populations of the damselfly *Enallagma cyathigerum*, complemented with laboratory experiments. Our aim is to quantify potential sexual and non-sexual isolating barriers mediated by migration between coastal and inland habitats on a small geographical scale that is of interest to local adaptation [33]. The damselfly genus *Enallagma* is well suited for such an investigation as it inhabits more or less discrete populations consisting of water bodies such as ponds and lakes, combined with sexual selection being typically strong and with the potential to fluctuate among populations in this and other closely related coenagrionid damselfly genera, such as *Ischnura* [34] as well as in North American species of *Enallagma* [35]. Our own previous studies across the same geographical scale in another coenagrionid damselfly (*Ischnura elegans*) have revealed a clinal coastal–inland gradient for male body size and large spatial variation between populations in local sexual selection regimes [34]. Coastal damselfly populations emerge earlier in the season than inland populations (E. I. Svensson 2000–2014, unpublished data), and this earlier phenology of coastal populations is presumably caused by a milder microclimate along the coasts, compared with the inland environment. Therefore, reciprocal transplant experiments between coastal and inland habitats are ecologically interesting, as these two contrasting environments are likely to differ in several microclimatic factors. The results presented here have some general implications beyond the study of local adaptation in insects, because we identify low male immigrant survival in a generalist species as a potential new isolating mechanism between ecologically divergent habitats with different microclimates.

2. Methods

(a) Study species

The common blue damselfly, *E. cyathigerum*, occurs throughout Europe from Spain in the south, to Scandinavia in the north [36]. During the reproductive season, males search out females, and attempt to grab the female's prothorax using claspers on the end of the male abdomen (cerci) [37]. The female can then choose to respond by bringing her abdomen up to join the male's genitals, so mating can take place [37]. Males are non-territorial and engage in scramble competition for females with both sexes mating multiple times with different partners [37]. The main flux of dispersal between populations is thought to typically occur during natal periods [37] and dispersal distances are normally relatively short at less than 1 km [38].

(b) Fieldwork and experiments

We performed replicated (pairwise design) reciprocal transplant experiments involving males from four different populations outside Lund in the province of Skåne (Scania), in southern Sweden. These populations were located either along the strait of Öresund or over 20 km inland from the coast. During June–August of 2006–2008, these reciprocal transplant experiments were conducted using two coastal (Höfterup-Borgeby approx. 10 km apart) and two inland (Krutladan-Genarp approx. 6 km apart) populations (electronic supplementary material, figure S1). All coastal–inland transplant combinations were performed; however, logistical issues limited the number of transplant combinations that could be carried out in any given field season. Our previous studies on molecular population differentiation (AFLP-markers) in the same region, on a closely related and ecologically similar coenagrionid damselfly species (*I. elegans*) showed no evidence of isolation-by-distance in the same geographical area and among similar coastal–inland populations [39].

Immigrant males were caught and transported to the relevant field sites for mark and release. Local males (termed 'residents') were caught, dusted and released simultaneously with the experimental immigrant males during each session. Resident and immigrant males were marked with two different colours of transferable fluorescent tracking dust [40]. We changed the combination of colours used at each site during the field season to control for any potential impacts of a particular colour of dust on the survival of individuals. Males were dusted in two separate places on their body: on the clasp organ located at the end of the abdomen (cerci) and on their secondary genital organs. Reciprocal transplants in each population were intended to be carried out 7 days apart; however, weather conditions and population densities influenced the number of times this goal was achieved during each season. We returned to all of our study populations over the following days after the dusting sessions to recapture resident and immigrant dusted males. To differentiate between ecological and physiological differences in survival, we conducted a separate laboratory experiment where we investigated if there were any intrinsic differences in survival times under benign conditions between coastal and inland males (see the electronic supplementary material, Methods).

The male dusting technique employed in this study can also be used to score female matings in the field [40,41]. By catching single females and females found in copula and checking for the presence of fluorescent dust on the female's genitalia, we were able to determine whether any of the females have mated with released resident or immigrant males, and quantify differences in the fecundity of females mated to the different male classes. We brought all wild-caught females after each catching session back to a laboratory and checked for the presence of fluorescent dust under a dissecting microscope. Females were subsequently set up in oviposition jars and left to lay eggs for 2 days before being released. After 3 days, the eggs were counted.

(c) Statistical analyses

All statistical tests were performed using SAS v. 9.3 (SAS Institute, Cary, NC), unless otherwise stated. Survival for the two groups of males (resident versus immigrant) was binomially distributed, where the probability distribution is the number of individuals caught over the days proceeding a dusting session, with the number of dusted individuals released at the previous dusting session representing n independent Bernoulli trials, and analysed using a generalized linear model approach, run using PROC GENMOD with a binomial probability distribution and logit link function. We ran separate models for coastal and inland environments, with replicate cross (one of two possible population transplant combinations in each model) and year included in the model.

An expected outcome of local adaptation is a significant interaction between the relative fitness of local versus immigrant phenotypes and habitat in a two-way analysis of variance framework [24,26], with native males being expected to have higher fitness in their local habitat compared with immigrant males if local adaptation is present [25,42]. To evaluate this, we used the same generalized linear model approach run as above, with a binomial probability distribution and logit link function for the full dataset including all transplant experiment replicates (a coastal–inland population pair) and including the two-way interaction between environment of origin and release environment.

We also quantified the extent of local adaptation in mating success of immigrant and resident males by recording the number of females caught over a field season with dust on their genitalia. The colour of the dust indicates whether the female mated with either a resident or an immigrant male. We assigned the females with a binomial measure depending on the male she had mated (1, resident; 0, migrant), and we used a generalized linear model approach using PROC GENMOD with a binary probability distribution and a logit link function. The predictor variables were female environment (inland/coastal), population and year, and the latter two variables were nested within environment.

Finally, we quantified differences in female fecundity when mated to immigrant and resident males by counting the number of eggs laid by females mated with different classes of males. Again, we used the colour of the dust as an index of whether mating had taken place with either a resident or an immigrant male. Egg counts between females mated with the two groups of males (resident versus immigrant) were analysed using a generalized linear model approach run using PROC GENMOD with a Poisson probability distribution and log link function. Female environment, male partners environment and the two-way interaction were the independent variables. We also included population (nested within female environment) and year in this model.

To quantify microclimatic differences between the coastal and inland environments, we used the WorldClim dataset [43]. In ARCMAP (ESRI 2011), we enclosed all four of the study populations within a closely bound rectangle. Within this rectangle, we created 258 randomly generated point locations and extracted climate data from these points (see the electronic supplementary material, Methods). For the analysis, we used nine climate measurements from the WorldClim dataset: precipitation for June–August and minimum/maximum temperature for June–August. We used the estimated first principle component of the nine climate measurements and tested for differences between the two environments using a simple *t*-test.

(d) Quantifying the strength of sequential isolation barriers between populations

We calculated three different potential isolating barriers for both environments using the reproductive isolation indices of Sobel & Chen [44]:

$$RI_i = 1 - 2x,$$

where x is the probability for gene flow, and used a bootstrapping with replication approach to estimate 95% confidence intervals (CIs) of the point estimates (see the electronic supplementary material, Methods). The between-population isolation estimates were subsequently combined to calculate the total population isolation using the EXCEL spreadsheet program developed by Ramsey *et al.* [23]. The overall isolation gives an index of total isolation controlling for the various contributions from the different barriers that operate at different stages of intraspecific population interactions [23].

Figure 1. Differences in recapture rates between coastal and inland habitats. Males caught at inland sites (dashed line) had a higher recapture rate than coastal males (solid line) when released at inland sites. However, when inland males were released at coastal sites, they were recaptured with the same frequency as coastal males. Bars denote 95% CIs.

3. Results

We recaptured a total of 1060 (20.08%) of a total of 5278 males that were marked and released across our four replicate populations over 3 years. However, our marking methods mean we are unable to identify instances when the same male was caught over multiple days. These recaptures included 173 recapture days across both coastal and inland environments. Initial analysis found all field caught recapture data to be over-dispersed, and we therefore scaled the dispersion parameter by the deviance, using the option SCALE.

Coastal males released as immigrants in the inland populations had a significantly lower recapture rate than the resident (native) inland males ($\chi^2_1 = 16.80$, $p < 0.001$; electronic supplementary material, table S1). We found significant differences in the number of individuals recaptured across years ($\chi^2_1 = 13.05$, $p < 0.001$; electronic supplementary material, table S1), but no difference between replicate crosses ($\chi^2_2 = 3.41$, $p = 0.188$; electronic supplementary material, table S1).

In striking contrast to the pattern found inland, there was no difference in average recapture rate between residents and immigrant males at coastal sites ($\chi^2_1 = 2.5$, $p = 0.114$; electronic supplementary material, table S2). As with the inland environment, we found significant differences in the proportion of males recaptured between years ($\chi^2_1 = 8.83$, $p = 0.003$; electronic supplementary material, table S2), but there was no significant difference between replicate crosses ($\chi^2_2 = 4.13$, $p = 0.127$; electronic supplementary material, table S2). Finally, results from the model combining data from both coastal and inland environments were consistent with the pattern predicted by local adaptation [24,26]; we found evidence of a significant interaction between site of release (inland/coastal) and male phenotype on the proportion of recaptured males (environment of origin $\times$ release environment: $\chi^2_1 = 14.31$, $p < 0.001$; figure 1 and electronic supplementary material, table S3). We found no difference in survival between males from the different environments under laboratory conditions

Table 1. Components of reproductive isolation and absolute contributions to total isolation for the three reproductive barriers studied, with 95% CIs in brackets estimated from bootstrapping with replication (see the electronic supplementary material, Methods). (Isolation components can vary from negative one (complete immigrant advantage) to one (complete isolation). Zero component values indicate life-history stages with no barrier to gene flow. Isolation components are shown for the two contrasting environments ('coastal' and 'inland').)

	coastal		inland	
	component of isolation	absolute contribution to isolation	component of isolation	absolute contribution to isolation
immigrant inviability	0.109 (−0.065, 0.25)	0.109 (−0.065, 0.25)	0.307 (0.097, 0.507)	0.307 (0.097, 0.507)
sexual isolation	0.064 (−0.074, 0.202)	0.052 (−0.066, 0.188)	0.219 (0.081, 0.348)	0.154 (0.051, 0.26)
fecundity	−0.068 (−0.245, 0.112)	−0.057 (−0.215, 0.096)	0.079 (−0.062, 0.223)	0.043 (−0.034, 0.129)
total isolation		0.103 (−0.185, 0.35)		0.504 (0.29, 0.672)

(log-rank: $\chi^2_1 = 0.665$, $p = 0.415$), suggesting no intrinsic differences under benign conditions in survival times between coastal and inland males.

Over the course of our 3 year study, we captured 342 females from across our four replicate populations that were either caught mating with a dusted male or had dust on their genitalia, indicating a mating with a marked male. Of these 342 females, we caught 58 females that had both colours of dust, indicating that they had mated with both a resident and a migrant male. Our generalized linear model approach to the data indicated no difference between environments in a female's propensity to mate with resident or migrant males ($\chi^2_1 = 0.73$, $p = 0.392$; electronic supplementary material, table S4), and there was neither a significant difference across years ($\chi^2_4 = 1.54$, $p = 0.819$; electronic supplementary material, table S4) or among populations ($\chi^2_2 = 4.30$, $p = 0.117$; electronic supplementary material, table S4). We included females that had mated with both resident and immigrant males, which could introduce a degree of pseudo-replication; however, the results remain the same if they are excluded. We set up 317 females that had mated with a marked male in the field to lay eggs, 51 of which had mated with both immigrant and resident males. As with our survival data, initial analysis found our egg count data to be overdispersed, and we thus scaled the dispersion parameter by the deviance, using the option SCALE. Our generalized linear model approach revealed no significant difference between environments in female fecundity ($\chi^2_1 = 1.08$, $p = 0.298$; electronic supplementary material, table S5), and the interaction between female environment and male status (resident or migrant) suggested that there was no significant post-copulatory or immigrant male disadvantage in terms of female fecundity ($\chi^2_1 = 1.58$, $p = 0.209$; electronic supplementary material, table S5). We also found no significant difference across years ($\chi^2_2 = 1.08$, $p = 0.583$; electronic supplementary material, table S5) or between populations ($\chi^2_2 = 3.55$, $p = 0.17$; electronic supplementary material, table S5) in female fecundity. As with the mating data, the results remain quantitatively the same if females that had mated with both are excluded. We summarize the cumulative effects of all three fitness components (immigrant viability, sexual isolation and female fecundity) on total isolation between the four population pairwise comparisons in table 1 and figure 2. Consistent with the results above, immigrant viability of males was by far the most important component influencing total isolation (table 1 and figure 2). For the coastal environment, the 95% CIs of the isolation indices overlapped zero, suggesting

Figure 2. Absolute contributions of the components of reproductive isolation to total isolation for the three reproductive barriers studied with 95% CIs estimated by bootstrapping with replication (see Methods). Total isolation is the sum of the three components combined, calculated from Ramsey *et al.* [25]. Negative component values indicate life-history stages at which immigrants are favoured. Isolation components are shown for the four study populations (figure 1). 'Borgeby' and 'Hofferup' are the coastal populations, and 'Genarp' and 'Krutladan' are the inland populations. The contrasts between the environments (populations pooled) are shown in table 1.

the isolation indices were not different from zero and that none of our measures of isolation acted as barriers against migrants (table 1). For the inland environment, the 95% CIs for both immigrant inviability and sexual isolation did not overlap zero (table 1), suggesting that both these isolation indices contributed as barriers to total isolation against migrant males. Examination of the replicate inland population level indices suggests that Krutladan drives this result for sexual isolation, as the 95% CIs overlap zero for Genarp (figure 2).

We found that the first principal component (PC1) explained 84% of the variation in the climate values in this region. The eigenvectors of PC1 were loaded positively on minimum and maximum temperature for all three months and negatively on precipitation for all three months, meaning that sites which scored high for PC1 were symptomatic of a warmer, dryer environment than those that scored lower. A test for the equality of variances revealed evidence of unequal variances between our two environments (the

folded F -statistics $F' = 1.85$, with $p = 0.001$). Using the Sat-
terthwaite test for unequal variances, we found a significant
microclimatic difference between the inland and coastal
environments ($t_{215.83} = 13.65$, $p < 0.001$). The coastal environ-
ment had a larger mean value of PC1 (2.987), revealing a
warmer and dryer microclimate than the inland environment
(PC1 = -3.435) in this small geographical region.

4. Discussion

Understanding barriers to gene flow between geographically
close populations is of fundamental interest in studies of local
adaptation [33]. Here, we have shown that immigrant dam-
selfly males from coastal populations survive less well at
inland sites than the native inland males, but there is no evi-
dence of such local adaptation in the coastal environment,
resulting in an asymmetric isolation barrier (figure 1). More-
over, in our laboratory experiments, we found no intrinsic
difference in viability between males from the two contrast-
ing environments, which strongly suggest one or several
extrinsic environmental causes behind the reduced immi-
grant survival at the inland sites. In contrast to male
viability, mating rates of resident and immigrant males did
not differ between the environments in the field, with the
exception of one of the replicate inland sites, Krutladan,
where resident males were found to have mated more fre-
quently (figure 2). Our data did not lend any support for
any post-mating reproductive isolating barriers, and do not
suggest any intrinsic quality differences between females
that mated with either a resident or immigrant male. In com-
bination, these experimental results strongly suggest that
reduced immigrant male survival contributes to most of the
observed isolation between these populations, at least
moving from the coast towards inland (table 1 and figure 2).
The reduced lifespan of immigrant males in the inland
environment might be particularly severe when poor weather
conditions and heavy rains over several days reduce the
window of mating. Such unfavourable weather conditions
are common in the short summers at high latitudes, such as
in Sweden. Because the aim of our study was to mimic natural
gene flow and possible barriers to migrants in natural field
environments, our use of wild-caught individuals means
there are some limitations to the level of inference that can
be made, particularly in our ability to uncover the relative
role of plasticity versus genetic differences. However, because
our finding of higher isolation barriers against immigrants at
inland sites seems to be primarily driven by natural selection,
how plasticity contributes to isolation between environments
will depend on whether it is expressed before or after dispersal
occurs [45]. Such limitations are common in field studies involv-
ing reciprocal transplant experiments in the wild, but are
important for capturing the underlying environmental vari-
ation responsible for the different selection pressures that
probably lead to local adaptation [25].

When a significant interaction exists between habitat and
male category (resident versus immigrant), as in our study,
this is necessary but not sufficient evidence for local adap-
tation [24]. One also needs to demonstrate higher fitness
rank of residents than immigrants in one or both habitats
[24–26]. Here, we found evidence for local adaptation in
the inland habitat, but not in the coastal habitat, revealed as
an asymmetric fitness interaction (figure 1). An implicit

assumption in many previous experimental studies using
reciprocal transplants is that mean population fitness is simi-
lar between environments and a symmetric fitness trade-off
owing to the statistical interaction is expected [but see 46].
In a recent meta-analysis, Hereford [25] divided results
from studies of local adaptation into four different quadrants,
depending on the relative fitness advantage of a population
when native/non-native. Hereford [25] found that 48% of
studies fell into the 'trade-off' quadrant, where populations
had relatively higher fitness when native, and lower relative
fitness when non-native. In contrast, we found no evidence
of a trade-off *per se*; as there was an asymmetric fitness inter-
action and lower population mean fitness (viability) of coastal
populations at inland sites only (figure 1), similar to the
asymmetry seen in a recent reciprocal transplant experiment
on sticklebacks (*Gasterosteus aculeatus*) between inlet versus
lake populations [46]. In the context of the Hereford meta-
analysis, our environments fall along the edge of the quad-
rants, and suggest no trade-off in local adaptation in this
instance [25]. Instead, our results suggest that the coastal
environment might act as a sink and the inland environment
a source. Future studies of local adaptation using reciprocal
transplant experiments would benefit from a more explicit
integration of source–sink perspectives and considera-
tion of population differences in mean fitness in different
environments [5,6].

Population genetic models of the evolution of species
ranges suggest that local adaptation at range margins might
be compromised owing to asymmetric immigration from
larger and well-adapted core populations at the centre of
the range [3,47]; however, density-dependent competition
and source–sink dynamics can modulate such effects [5,6].
Although these models were developed to explain adaptation
over larger spatial scales (e.g. continents) than the scale of our
study, they are relevant as the coastal–inland gradient might
be profound for such a small insect such as *E. cyathigerum*
which is highly dependent on limiting water bodies
(ponds) for survival, mating and reproduction. The asym-
metric survival differences between coastal and inland
immigrants detected in this study (figure 1) can potentially
influence the magnitude and direction of gene flow between
these closely situated populations.

The low survival of coastal immigrant males in the inland
environment will result in lowered lifetime mating opportu-
nities, which may be the underlying cause of the significant
sexual isolation found in the isolation indices for Krutladan
(figure 2). Lower survival rates of immigrant males might
therefore constitute an efficient reproductive barrier through
reduced immigrant viability (table 1 and figure 2). Such
direct selection on reproductively mature immigrants
should play a much stronger role than selection at later life
stages, such as during mating and reproduction, owing to a
reduction in the number of individuals available for selection
to act on [13]. A small note of caution however, with the cur-
rent data we are unable to separate whether this finding
represents females at Krutladden preferring local males, or
simply an effect of the higher survival rates of males at this
locality. Second, as we found this in only one of our inland
replicates, the lack of difference at Genarp (figure 2) could
suggest that immigrant males at this population do better
than local males in finding mates, but this difference is
masked by their lower survival rates. It is likely that the
difference/lack of difference between mating success for

immigrant and resident males at the different replicate inland sites are driven by sample size and higher survival rates of males, but it does make the question of sexual isolation between these sites worthy of further investigation. Asymmetric mating interactions can have consequences for the maintenance of diverging populations, especially after a period of separation. If one population or incipient species would have higher competitive ability and advantages in mating interferences upon secondary contact, that group is more likely to displace the other group through competitive exclusion [48]. One outcome of this could be asymmetric gene flow between populations that could permit inland males to invade coastal environments, but not vice versa (figure 1).

We suggest that native inland males are locally adapted to the colder and more humid microclimates in their natal habitat where they outcompete immigrant coastal males. Note that in the benign laboratory conditions, we found no intrinsic differences between males from inland and coastal sites. This suggests that differences in male viability are only expressed under the more variable environmental conditions in the field or that any intrinsic differences between males from the different environments are only expressed under stressful natural conditions regardless of the specific natural environment [49]. As the average mating rates do not differ between these two environments, it is unlikely that the lower survival rates

in the coastal environment was caused by higher mating activity. Our data do not provide any support for local adaptation to the coastal environment, as immigrants from the inland populations had similar survival to resident coastal males (figure 1). We suggest that asymmetric fitness differences between immigrant and resident males arise from their differential environmental tolerance to microclimatic differences between coastal and inland populations. Competitive asymmetries between immigrant and resident males, and how they influence male survival and local adaptation should be investigated in depth in future studies of local adaptation and isolation between populations.

Data accessibility. All data used in this study has been archived at Dryad: doi:10.5061/dryad.ft8pq.

Acknowledgements. We are grateful to Emma Björk, Emmanuel Pasco-Viel and Lisa Orr for assistance during the field and laboratory work. We are thankful to Andrew Hendry and one anonymous reviewer for constructive comments on earlier versions of this manuscript.

Funding statement. This research was supported by the European Union Seventh Framework Programme ((FP7/2007–2013) (FP7/2007–2011)) under grant agreement no. (AEMMPN 237271) and Helge Ax:son Johnsons Stiftelse to T.P.G. and by research grants from ‘Carl Tryggers Stiftelse’ (CTS), The Crafoord Foundation and the Swedish Research Council (VR) to E.I.S.

Conflict of interest. The authors declare no conflicts of interest.

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