Marine Biology Research, 2007; 3: 182–187

ORIGINAL ARTICLE

Environment-specific heterozygote deficiency and developmental instability in hybrid *Mytilus*

STEVAN A. SPRINGER[1] & DANIEL D. HEATH[2]

[1]*Department of Biology, University of Washington, Seattle, WA 98195, USA,* [2]*Department of Biological Sciences and Great Lakes Institute for Environmental Research, University of Windsor, Windsor, Ontario N9B 3P4, Canada*

Abstract

The multiple discrete hybrid zones that characterize *Mytilus* blue mussels allow a novel, non-manipulative, examination of the selective pressures that create and maintain species. If endogenous genetic incompatibility is solely responsible for post-zygotic isolation, then individuals of a specified hybrid genotype are expected to show similar average fitness across environments. However, if hybrid fitness differs across environments, then exogenous selection is implicated, either via ecological selection or environment-specific expression of intrinsic genetic incompatibilities. Correspondence between developmental instability of hybrids and heterozygote deficiency, estimated in two *M. trossulus* × *M. galloprovincialis* hybrid zones on the coast of North America, indicates that environment-dependent selection against hybrids may contribute to reproductive isolation among Pacific *Mytilus* species.

Key words: *Developmental stability, environment-dependent selection, heterozygote deficiency, Mytilus*

Introduction

Scenarios of the origin and maintenance of species implicitly assume that exogenous or endogenous selective forces determine hybrid fitness, and the two most common classes of hybrid zone models reflect this dichotomy (Barton & Hewitt 1985; Moore & Price 1993). In exogenous (ecological) selection models, the coherence of taxa is maintained by reduced hybrid fitness due to ecological circumstance (Moore & Price 1993; Kruuk et al. 1999). In contrast, endogenous (genetic) selection models are built without environmental variables; hybrid dysfunction is caused by the fusion of incompatible genetic systems and hybrid fitness is reduced relative to parental types in all environments (Barton & Hewitt 1985; Kruuk et al. 1999). A third alternative is that genetic incompatibilities are conditional, and cause reduced hybrid fitness in an environment-dependent manner (Kondrashov & Houle 1994; Rundle & Whitlock 2001). The importance of conditional genetic incompatibility in speciation is perhaps underestimated, as most tests of reproductive isolation occur in artificial conditions. All three models predict heterozygote deficiency, relative to Hardy–Weinberg equilibrium, when selection acts to reduce hybrid fitness. However, the effects of strict genetic incompatibility are expected to be relatively stable across environments (Rundle & Whitlock 2001). Examining the fitness of natural hybrids of the same parental taxa in different environments can therefore implicate ecological selection in species formation.

Decreased hybrid fitness manifest as increased developmental stress in hybrid individuals is consistent with both exogenous and endogenous hybrid zone models. However, the cause of developmental stress differs in each case. Exogenous speciation involves divergent adaptation to different environments. Thus, hybrid individuals suffer increased developmental stress as a result of reduced competitive ability in parental environments or increased competition among hybrid individuals (Shykoff & Møller 1999). The degree of exogenous developmental stress experienced by a hybrid individual depends on local environmental conditions and hybrid zones resulting from the same two parental

---

Correspondence: S. A. Springer, Department of Biology, University of Washington, Seattle, WA 98195, USA. E-mail: stevan@u.washington.edu

Published in collaboration with the University of Bergen and the Institute of Marine Research, Norway, and the Marine Biological Laboratory, University of Copenhagen, Denmark

(Accepted 26 February 2007; Printed 11 June 2007)

ISSN 1745-1000 print/ISSN 1745-1019 online
DOI: 10.1080/17451000701320564

taxa are not necessarily expected to have the same average hybrid developmental stress, or fitness. Endogenous selection models involve hybrid dysfunction caused by the breakdown of co-adapted gene complexes; developmental instability in hybrid individuals results from intrinsic genetic incompatibility (Graham 1992), and hybrid individuals are expected to suffer reduced developmental stability independent of ecological circumstance.

This study used fluctuating asymmetry, as a proxy of developmental stability (Palmer 1994), to estimate the effect of hybridization on the developmental stability of natural *Mytilus* hybrids. Polymerase chain reaction (PCR)-based nuclear species markers identified *Mytilus* species and hybrids from two previously characterized *M. trossulus* × *M. galloprovincialis* hybrid zones; Chemainus (Heath et al. 1995) and San Francisco (Rawson et al. 1999). The comparison of two hybrid zones involving the same parental taxa in different environments allowed a novel analysis of the selective pressures that maintain hybrid zones and species. If endogenous genetic incompatibility primarily maintains species, hybrid zones of the same two parental species should exhibit the same pattern of developmental stability and heterozygote deficiency regardless of ecological circumstance. If differences in hybrid developmental stability and genetic disequilibrium occur among environments, then some form of exogenous selective force is implied.

Materials and methods

Sampling

In total, 279 individuals (17–122 mm shell length) were collected from four sites on the Pacific coast of North America (Figure 1). Two samples from areas where *M. trossulus* and *M. galloprovincialis* are known to co-occur and hybridize (Heath et al. 1995; Rawson et al. 1999); Chemainus British Columbia (CH: 48°91′N 123°69′W; 1995, n = 124) and San Francisco California (SF: 38°02′N 122°50′W; 1997, n = 95), and two samples from areas known to contain only a single *Mytilus* species, Newport Oregon (NP: 43°74′N 124°13′W; 1999, n = 30) and San Diego California (SD: 32°79′N 117°23′W; 1999, n = 30). Samples were collected from low wave-action sites on the sub-tidal flotation units of docks; mantle tissue was stored at −20°C in 95% ethanol, and the left and right shell valves of each individual were retained for morphometric analysis.

Species identification

PCR-based nuclear species markers are available to identify, with varying degrees of resolution, the

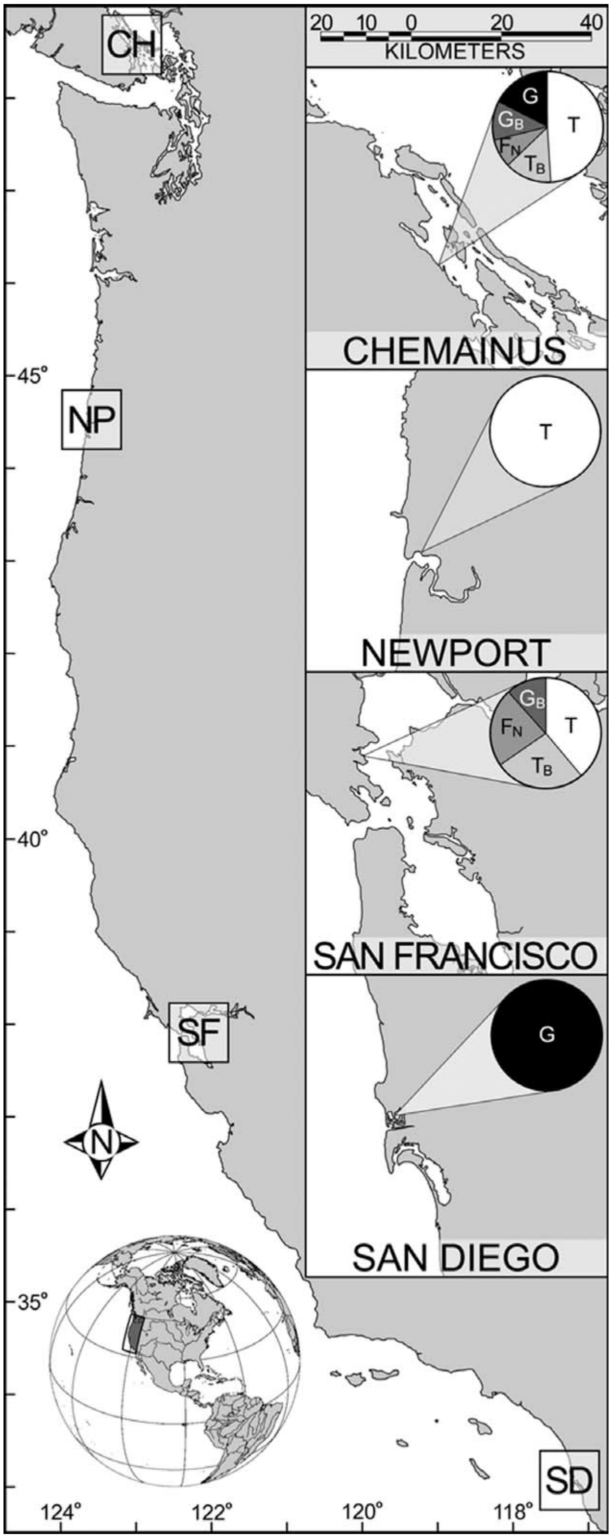

Figure 1. Locations of the four sampling sites: Chemainus British Columbia (CH), Newport Oregon (NP), San Francisco California (SF) and San Diego California (SD). Pie charts indicate the approximate sampling location and relative frequency of pure *Mytilus trossulus* (T), backcross *M. trossulus* (T_B), hybrid (F_N), backcross *M. galloprovincialis* (G_B) and pure *M. galloprovincialis* (G) individuals in each sample. Frequencies are the consensus of three diagnostic species markers, ITS, PLII and GLU (Table I).

members of the *Mytilus* species complex. Three nuclear loci were used to assign species status to *Mytilus* individuals: ITS and PLII, developed by Heath et al. (1995), and GLU, developed by Rawson et al. (1996).

ITS is a co-dominant species marker based on the internal transcribed spacer (ITS) regions between the 18S and 28S nuclear rDNA coding regions (Heath et al. 1995). The ITS marker can distinguish *M. trossulus* from other members of the *Mytilus* species complex, as well as *M. trossulus* ×*M. edulis* and *M. trossulus* ×*M. galloprovincialis* hybrids. PCR amplifications of the ITS locus produce a single band (1250 bp) and diagnostic banding patterns when cut with restriction endonuclease *Hha I* (Heath et al. 1995). Multiple copies of ITS exist in the *Mytilus* genome, but analysis of experimental backcrosses indicates that ITS exhibits Mendelian inheritance (Heath et al. 1995).

PLII is a dominant marker based on a protamine-like sperm-specific protein locus (Heath et al. 1995). PCR amplifications of the PLII locus produce a single band (475 bp) and, when cut with restriction endonuclease *HinfI*, diagnostic banding patterns capable of distinguishing *M. galloprovincialis* or *M. edulis* individuals from other members of the *Mytilus* species complex (Heath et al. 1995). The PLII marker cannot distinguish *M. trossulus* ×*M. edulis* and *M. trossulus* ×*M. galloprovincialis* hybrids from pure *M. trossulus*.

GLU is a co-dominant marker based on a polyphenolic adhesive protein used in the attachment of byssal threads to the substrate (Rawson et al. 1996). Size differences of amplified PCR products diagnostically identify *M. trossulus*, *M. edulis*, *M. galloprovincialis* and hybrids between any of the three species (Rawson et al. 1996).

All PCR amplifications were performed using approximately 100 ng of template DNA, 50 ng of each primer, 0.2 mM of each dNTP, and 1.0 U Gibco/BRL *Taq* DNA polymerase. 1.5 µl of PCR buffer, consisting of 50 mM KCL, 10 mM Tris-HCL (pH 9.0), 0.1% Triton X-100, 1.5 µl of 25 mM $MgCl_2$, and double-distilled water composed the balance of the 25 µl reaction volume. The thermal cycler protocol for ITS and PLII was 30 cycles of 45 s at 94°C, 45 s at 51°C and 90 at 72°C; GLU amplifications consisted of 30 cycles of 30 s at 94°C, 30 s at 54°C and 30 s at 72°C. PCR reactions were performed on an MJ Research PTC-100 thermal cycler. GLU amplifications were modified from Rawson et al. (1996) to include a cyanine dye-labelled reverse primer and one of two forward primers designed to amplify fragments averaging 100 and 220 base pairs. GLU amplifications were multiplexed (2 samples × 2 dyes) and visualized using a Visible Genetics MicroGene sequencer. Exact U-tests of Hardy–Weinberg expectations and F_{IS} estimates were calculated using Genepop 3.2a (Raymond & Rousset 1995).

Genotype analysis

Individuals were assigned a genotype based on the consensus of the three diagnostic markers. Pure types (T and G) were identified as parental at all three loci. Putative first-generation, hybrid (F_N) individuals were heterozygous at the co-dominant ITS and GLU markers and homozygous dominant at the PLII marker. Backcross and higher-order hybrid crosses were identified whenever two markers diagnostically disagreed, the direction of the hybrid cross (T_B or G_B) determined by the homozygous locus (loci). The presence of a recessive PLII restriction pattern was used to place individuals into an appropriate hybrid cross class. Ambiguities resulting from the dominance of PLII were resolved by placing the individual into an appropriate parental or F_N hybrid class. Using only three diagnostic markers, error in scoring hybridization history by genotype is inevitable (Boecklen & Howard 1997); the genotype assignments described here are therefore made conservatively with respect to the test implemented in this paper (Rundle & Whitlock 2001). Positive confirmation of hybrid cross status was required to place an individual into the T_B or G_B class. Advanced introgression, beyond the first-generation backcross, rarely occurs in Pacific *Mytilus* (Rawson et al. 1999) and the T, G and F_N classes can be conservatively regarded as composites of primarily pure type or F_1 individuals and the occasional advanced hybrid cross, remaining misidentifications would reduce differences among mean genotypic fluctuating asymmetry estimates.

Fluctuating asymmetry

Five continuous traits of the left and right shell valves were measured and combined into an index of fluctuating asymmetry for each individual (Figure 2). Each trait was measured twice for every individual; the same observer conducted blind repeat measurements within a week of the original measurement. The magnitude of non-directional asymmetry relative to measurement error was assessed with replicated trait measurements and a mixed-model two-way ANOVA (fixed effect = left versus right; random effect = replicates; Palmer 1994). To estimate total asymmetry, studentized residuals from a linear regression of trait size and absolute difference between left and right trait measurements were used to estimate fluctuating asymmetry independently for

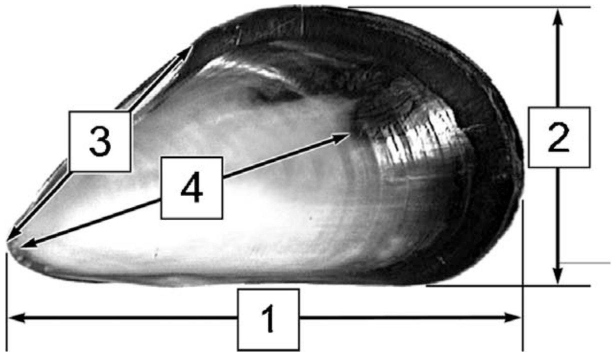

Figure 2. Traits used to estimate fluctuating asymmetry for each individual. (1) Length, measured from the exterior of the umbo to the most distant point on the valve. (2) Height, maximum distance measured perpendicular to the ventral shell margin. (3) Hinge plate, measured from the exterior of the umbo to the end of the hinge plate. (4) Umbo to adductor, measured from the interior apex of the umbo to the posterior adductor scar. (5) Mass, the dry mass of each shell valve, is not depicted. Mass was measured to the nearest 0.001g; all other traits were measured to the nearest 0.01 mm.

each of the five traits and summed to yield a total asymmetry estimate for each individual. Bonferroni-adjusted one-way ANOVA was used to test for differences in total asymmetry estimate between genotypes.

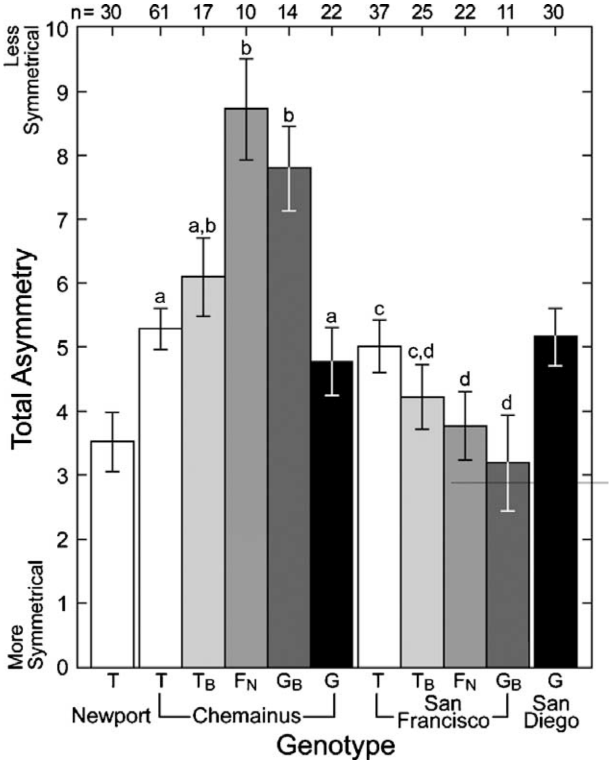

Figure 3. Total asymmetry estimate (mean ± 95% confidence interval) for each genotype from each sample (T, *Mytilus trossulus*; T_B, *M. trossulus* hybrid cross; F_N, unspecified hybrid; G_B, *M. galloprovincialis* hybrid cross; G, *M. galloprovincialis*). Separate analyses were conducted for Chemainus and San Francisco samples. F_N and G_B individuals from Chemainus are less symmetrical than *M. trossulus* or *M. galloprovincialis* individuals. F_N and G_B individuals from San Francisco are significantly more symmetrical than San Francisco *M. trossulus*. a, b and c, d indicate significantly different means after sequential Bonferroni adjustment.

Results

The Chemainus population showed significant heterozygote deficiency (Table I) consistent with the results of Heath et al. (1995); the analysis of total asymmetry ($F_{4,123} = 4.028$, $P = 0.004$; Figure 3) indicated that F_N and G_B individuals were significantly less symmetrical than pure T or G individuals. The San Francisco population exhibited a slight excess of hybrid individuals. Survey of the ITS locus showed a non-significant heterozygote deficiency. However, the GLU locus recorded a significant heterozygote excess (Table I). F_N hybrid and G_B hybrid cross individuals were significantly more symmetrical than pure T individuals in the San

Table I. Observed frequencies of *Mytilus trossulus* (T) and *M. galloprovincialis* (G) genotypes for the ITS, PLII and GLU species marker loci. Single-locus exact U-tests of heterozygote deficiency (†), or excess (‡) and Weir and Cockerham F_{IS} estimates were conducted in Genepop 3.2a (Raymond & Rousset 1995). Statistically significant departures from Hardy–Weinberg expectations are in bold.

Population	Locus	N	T/T	T/G	G/G	F_{IS}	Exact U
Chemainus (CH)	ITS	124	76 (61.3%)	15 (12.1%)	31 (26.6%)	**0.7269**	**(p < 0.001)**†
	PLII	124	90 (72.6%)	–	34 (27.4%)	–	–
	GLU	124	70 (56.5%)	25 (20.1%)	29 (23.4%)	**0.6065**	**(p < 0.001)**†
Newport (NP)	ITS	30	30 (100%)	0 (0.0%)	0 (0.0%)	0	(p = 1.000)†
	PLII	30	30 (100%)	–	0 (0.0%)	–	–
	GLU	30	30 (100%)	0 (0.0%)	0 (0.0%)	0	(p = 1.000)†
San Francisco (SF)	ITS	95	57 (60.0%)	31 (32.6%)	7 (7.4%)	0.1026	(p = 0.232)†
	PLII	95	87 (91.6%)	–	8 (8.4%)	–	–
	GLU	95	43 (45.3%)	52 (54.7%)	0 (0.0%)	**−0.3623**	**(p < 0.001)**‡
San Diego (SD)	ITS	30	0 (0.0%)	0 (0.0%)	30 (100%)	0	(p = 1.000)†
	PLII	30	0 (0.0%)	–	30 (100%)	–	–
	GLU	30	0 (0.0%)	0 (0.0%)	30 (100%)	0	(p = 1.000)†

Francisco sample (total asymmetry: $F_{3,94} = 6.722$, $P < 0.001$; Figure 3). In all traits, asymmetry was significantly larger than measurement error ($P < 0.001$, left versus right × replicate; Palmer 1994). No directionality was evident in any of the five traits; any departures from normality were leptokurtic, consistent with expectations of an fluctuating asymmetry signal (Palmer 1994). Intra-individual correlations among traits were weak, but mean genotypic fluctuating asymmetry values showed high correspondence across all five traits.

Discussion

Recent work on the role of ecology in speciation has renewed interest in the selective pressures that create and maintain species, and govern the fitness of hybrid individuals (Moore & Price 1993; Kruuk et al. 1999). Correspondence between environment-specific developmental stability of hybrids and heterozygote deficiency in hybrid zones between the same two parental taxa indicates that environment-dependent selection against hybrids probably contributes to reproductive isolation between Pacific *Mytilus* species. In Chemainus, G_B hybrid cross and F_N hybrid genotypes suffer reduced developmental stability relative to parental genotypes; the effect reverses in the San Francisco environment, indicating that simple intrinsic genetic incompatibility cannot be operating. Intrinsic genetic incompatibility may exist in San Francisco populations, but remain unexpressed because of the availability of a benign intermediate habitat where hybrid fitness is unaffected.

Three additional scenarios could explain the observed patterns of Hardy–Weinberg disequilibrium. (1) Distinct colonization sources or history may have created genetic differences between the sampled Pacific *M. galloprovincialis* populations. Both Northern and Southern *M. galloprovincialis* are constantly re-introduced and homogenized by ballast water transport of larvae, but southern *M. galloprovincialis* are also independently maintained by existing introduced populations (Geller et al. 1994). The lack of heterozygote deficiency and hybrid developmental instability in San Francisco may therefore result from past selection for hybrid performance, or a genetically distinct source for *M. galloprovincialis* populations in southern California. (2) Stochastic dispersal and recruitment patterns have been shown to result in temporal genetic patchiness and could drive Wahlund effects (Watts et al. 1990) The genetic structure of Pacific *Mytilus* hybrid zones is dynamic (Braby & Somero 2006), but scenarios invoking population structure do not predict the observed correspondence between developmental stability and relative hybrid abundance. (3) Non-random mating, mediated by gamete interactions, has been observed in experimental *Mytilus* crosses (Bierne et al. 2002) and could produce heterozygote deficiency. It is possible that the extreme heterozygote deficiency observed in Chemainus is a composite of non-random mating reinforced by environment-dependent selection against hybrid individuals. These scenarios are consistent with and may contribute to the observed patterns of heterozygote deficiency. However, the recent and ongoing nature of *M. galloprovincialis* introductions to and among Pacific populations limits the potential for historical differentiation and suggests that the observed correspondence between reduced hybrid developmental stability and heterozygote deficiency is best explained by variable patterns of current ecological selection.

Non-manipulative studies of environment-dependent selection are attractive for a number of reasons: the findings are immediately relevant to the populations studied; experimentally induced artefacts, enclosure effects, and artificial selection are absent, and the difficulties of rearing several hybrid generations in the laboratory are avoided. Natural tests also provide an opportunity to examine the importance of environment-dependent intrinsic genetic incompatibilities that are often unexpressed in laboratory conditions (Kondrashov & Houle 1994). Evidence that deviations from Hardy–Weinberg correspond with exogenous selection suggests directions for non-manipulative tests of ecological speciation. For example, heterozygote deficiency driven by exogenous selection should be most pronounced in environmental circumstances where divergent selective pressures are most extreme; geographical surveys of Hardy–Weinberg equilibrium across environments, particularly at the edges of hybrid zones and species' ranges, will shed light on the ecological selective pressures that maintain species. Understanding the full importance of ecology in speciation requires a multi-faceted approach that should ideally include non-manipulative studies of reproductive isolating barriers operating in nature. The presence of multiple discrete hybrid zones between the same *Mytilus* species allows an examination of the ecological maintenance of species in natural conditions; indicating that environment-dependent selection may contribute to reproductive isolation in *Mytilus*.

Acknowledgements

We thank J. Heath, M. Heath, T. Heath, V. Heath, C. Langdon and R. Burton, for collecting mussels. L. Rankin and S. Henry assisted in laboratory analysis. Fieldwork was supported by Yellow Island Aquaculture Limited, laboratory analysis by a Natural Sciences and Engineering Research Council of Canada (NSERC) research grant to DDH.

References

Barton NH, Hewitt GM. 1985. Analysis of hybrid zones. Annual Review of Ecology and Systematics 16:113–48.

Bierne N, David P, Boudry P, Bonhomme F. 2002. Assortative fertilization and selection at the larval stage in the mussels *Mytilus edulis* and *M. galloprovincialis*. Evolution 56:292–8.

Boecklen WJ, Howard DJ. 1997. Genetic analysis of hybrid zones: numbers of markers and power of resolution. Ecology 78:2611–6.

Braby CE, Somero GN. 2006. Ecological gradients and relative abundance of native (*Mytilus trossulus*) and invasive (*Mytilus galloprovincialis*) blue mussels in the California hybrid zone. Marine Biology 148:1249–62.

Geller JB, Carlton JT, Powers DA. 1994. PCR-based detection of mtDNA haplotypes of native and invading mussels on the northeastern Pacific coast: latitudinal pattern of invasion. Marine Biology 119:243–9.

Graham JH. 1992. Genomic coadaptation and developmental stability in hybrid zones. Acta Zoologica Fennica 191:121–31.

Heath DD, Rawson PD, Hilbish TJ. 1995. PCR-based nuclear markers identify alien blue mussel (*Mytilus* spp.) genotypes on the west coast of Canada. Canadian Journal of Fisheries and Aquatic Science 52:2621–7.

Kondrashov AS, Houle D. 1994. Genotype–environment interactions and the estimation of the genomic mutation rate in *Drosophila melanogaster*. Proceedings of the Royal Society of London B 258:221–7.

Kruuk LEB, Baird SJE, Gale KS, Barton NH. 1999. A comparison of multilocus clines maintained by environmental adaptation or by selection against hybrids. Genetics 153:1959–71.

Moore WS, Price JT. 1993. Nature of selection in the northern flicker hybrid zone and its implications for speciation theory. In: Harrison RG, editor. Hybrid Zones and the Evolutionary Process. Oxford: Oxford University Press. p 196–225.

Palmer AR. 1994. Fluctuating asymmetry analyses: a primer. In: Markow TA, editor. Developmental Stability: its Origins and Evolutionary Implications. Netherlands: Kluwer Academic Publishers. p 335–64.

Rawson PD, Agrawal V, Hilbish TJ. 1999. Hybridization between the blue *mussels Mytilus galloprovincialis and M. trossulus* along the Pacific coast of North America: evidence for limited introgression. Marine Biology 134:201–11.

Rawson PD, Joyner K, Hilbish TJ. 1996. Evidence for intragenic recombination within a novel genetic marker that distinguishes mussels in the *Mytilus edulis* species complex. Heredity 77:599–607.

Raymond M, Rousset F. 1995. GENEPOP (version 1.2): population genetics software for exact tests and ecumenicism. Journal of Heredity 86:248–9.

Rundle HD, Whitlock MC. 2001. A genetic interpretation of ecologically dependent isolation. Evolution 55:198–201.

Shykoff JA, Møller AP. 1999. Fitness and asymmetry under different environmental conditions in the barn swallow. Oikos 86:152–8.

Watts RJ, Johnson MS, Black R. 1990. Effects of recruitment on genetic patchiness in the urchin *Echinometra mathaei* in Western Australia. Marine Biology 105:145–51.

Editorial responsibility: Christoffer Schander