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Genetically based differences in nest characteristics between lake, inlet, and hybrid threespine stickleback from the Misty system, British Columbia, Canada

Joost A.M. Raeymaekers[1,2]*, Lari Delaire[1]* and Andrew P. Hendry[1]

[1] *Redpath Museum and Department of Biology, McGill University, Montreal, Canada and*
[2] *Laboratory of Animal Diversity and Systematics, Katholieke Universiteit Leuven, Leuven, Belgium*

ABSTRACT

Hypotheses: Adaptation to different environments can drive the evolution of mating isolation and thereby contribute to ecological speciation. Adaptive divergence in nest characteristics, which could be one avenue to mating isolation, has received little attention. For it to be important, populations adapted to different environments should show genetic differences in nest characteristics.

Organisms: Second-generation, laboratory-reared stocks of threespine stickleback (*Gasterosteus aculeatus* L.) from the Misty system, British Columbia, Canada. Experimental fish included pure lake fish, pure inlet fish, and lake–inlet hybrids.

Methods: Males of different types (lake, inlet, and hybrid) were paired in aquaria, allowed to build nests, and then exposed to females of all three types (individually and on separate days). The resulting nests were photographed, collected, weighed, and dissected.

Results: Lake nests were bulkier than inlet nests, and less often placed on gravel substrate than sand. Hybrid males tended to build intermediate nests but showed very high variation. Some hybrids built lake-like nests and others built inlet-like nests.

Conclusions: Nest characteristics show genetic differences between lake and inlet stickleback, and might therefore contribute to any mating isolation.

Keywords: adaptive divergence, assortative mating, ecological speciation, hybrids, mate choice, nest architecture, nest building, reproductive isolation, sexual selection.

INTRODUCTION

Adaptation to different ecological environments is thought to drive 'ecological speciation' (Dobzhansky, 1940, 1951; Mayr, 1963; Schluter, 2000; Rundle and Nosil, 2005). Several isolating barriers that can emerge during this process reflect ecologically based selection against immigrants or hybrids. Some of this differential selection can be mediated through differences in viability

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Correspondence: J. Raeymaekers, Laboratory of Animal Diversity and Systematics, Katholieke Universiteit Leuven, Ch. Deberiotstraat 32, B-3000 Leuven, Belgium. e-mail: joost.raeymaekers@bio.kuleuven.be

* These authors contributed equally to this study.

or growth, whereas some can be mediated through sexual selection (Schluter, 2000; Coyne and Orr, 2004; Nosil *et al.*, 2005; Rundle and Nosil, 2005; Nosil, 2007). In this paper we are interested in the latter class of effects. Previous studies of sexual selection against migrants and hybrids have emphasized the importance of morphology, coloration or dynamic mating displays such as behavioural sequences (Miller *et al.*, 1998; Boughman *et al.*, 2005; Podos and Hendry, 2006). Although studied less often, differences in physical structures constructed for the purpose of protecting eggs or juveniles, such as nests, might also trigger sexual selection, and here we begin an examination of this possibility.

Nest characteristics, such as location, size, and quality, can influence reproductive success (Petit *et al.*, 2002; Lukas and Orth, 1995; Canali *et al.*, 1991; Gill and Stutchbury, 2005). Nest characteristics might therefore reflect the builder's genetic quality or potential as a parent (Collias, 1964; Soler *et al.*, 1998a, 1998b). Presumably for these reasons, mate choice within populations sometimes depends on nest characteristics (Jones and Reynolds, 1999; Oliveira *et al.*, 2000; Ostlund-Nilsson, 2000; Tallamy, 2001; Giacomello and Rasotto, 2005). Nest characteristics might also show local adaptation, given the often habitat-specific demands placed on mate attraction, egg care, and juvenile rearing. Adaptive divergence in nest characteristics between environments might then contribute to ecological speciation through mating isolation, particularly if preferences for nests co-evolve with locally adaptive nest characteristics. This possibility has received little attention.

The first step in investigating the role of nest characteristics in ecological speciation might be to document divergence in nest characteristics between populations from different environments. Such studies do indeed often report such differences and suggest that they have an adaptive basis (Jamieson *et al.*, 1992; Olafsdottir *et al.*, 2006). The next step might be to document *genetic* divergence in nest characteristics between conspecific populations adapted to different environments. We are not aware of any studies that have taken this second step – and yet it is critical to considering the nature of mating isolation. For example, genetic differences in nest construction could mean that dispersers build maladaptive nests, which might lead to both natural and sexual selection against migrants (and perhaps any hybrids). Of course, population differences in nest characteristics that do not have a genetic basis could also contribute to mating isolation.

Threespine stickleback

Threespine stickleback (*Gasterosteus aculeatus*) are well known for their dramatic adaptive radiation into different freshwater habitats following the last glaciation (e.g. Reusch *et al.*, 2001; Hendry and Taylor, 2004; Raeymaekers *et al.*, 2007). These populations are reproductively isolated to varying degrees and can be thought of as a large species complex (Bell and Foster, 1994; McKinnon and Rundle, 2002). Part of this reproductive isolation stems from mating isolation, which is known to be influenced by colour, behavioural displays, and morphology (Boughman *et al.*, 2005). Nest characteristics might also be important. Stickleback males build nests in which females lay eggs, and the males then tend the eggs for 7–40 days (Wootton, 1976). Nest characteristics may therefore influence offspring survival and reflect a male's parental ability or genetic quality. For example, Barber *et al.* (2001) hypothesized that nests contain information about male health status and androgen levels, whereas Ostlund-Nilsson and Holmlund (2003) suggested that nest decoration may advertise male paternal skills.

The possibility that nest differences might contribute to mating isolation is supported by several observations. First, nest characteristics are known to influence mate choice within populations (Barber *et al.*, 2001; Ostlund-Nilsson and Holmlund, 2003). Second, nest characteristics are influenced by environmental conditions, such as substrate, vegetation, predators, and male–male interactions (Whoriskey and FitzGerald, 1985; Ridgway and McPhail, 1987; Macdonald *et al.*, 1995). Third, nest characteristics sometimes differ between populations in nature (Olafsdottir *et al.*, 2006; Kitano *et al.*, 2008; Rushbrook *et al.*, 2008; Tsuruta *et al.*, 2008). To date, however, no study has tested for *genetic* differences in nest characteristics between populations. If such differences were to be found, future work might profitably concentrate on their role in mating isolation.

Our research focuses on inlet and lake ecotypes from the Misty watershed, Northern Vancouver Island, British Columbia, Canada. These ecotypes differ dramatically in some aspects of morphology (Hendry *et al.*, 2002; Moore *et al.*, 2007; Sharpe *et al.*, 2008) and breeding behaviour (Delcourt *et al.*, 2008), and show very low gene flow in nature (Hendry *et al.*, 2002; Moore *et al.*, 2007). Genetic incompatibilities are lacking (Lavin and McPhail, 1993), and so the observed reproductive isolation may reflect some combination of partial physical separation (parapatry), reproductive timing differences, ecological selection against migrants and hybrids, and mating isolation. We have tested for some of these potential barriers and, although some effects are present, a 'smoking gun' has not yet been identified (K. Räsänen, J.A.M. Raeymaekers and A.P. Hendry, unpublished data). This uncertainty has led us to consider additional factors that might contribute to reproductive isolation. Given that nest characteristics could be one such factor, we test for genetic differences in these characteristics between laboratory-reared lake, inlet, and hybrid stickleback.

MATERIALS AND METHODS

Study system

In June 2004, mature threespine stickleback were collected from Misty Lake site 1 and Misty Inlet site 4 (Moore and Hendry, 2005). These sites were chosen because they contain lake and inlet ecotypes that are highly divergent with respect to morphology (Moore and Hendry, 2005). We used standard artificial crossing methods (Hatfield and Schluter, 1996) to generate eight pure lake families and four pure inlet families (fewer mature females were available in the inlet at the time of collection). Each family was the product of a unique male–female pair. The fertilized eggs were shipped to our laboratory at McGill University, Montreal, Canada.

The families were raised at similar densities in separate tanks, with several tanks per family, which were placed in different environmental chambers. As very young fry, the fish were fed brine shrimp nauplii (*Artemia* sp.). As juveniles, they were fed a combination of brine shrimp nauplii and frozen blood worms (*Chironomid* sp.). As maturing adults, they were fed frozen blood worms and live black worms (*Lumbriculus* sp.). For the first 9 months, the fish were reared under constant 'summer' conditions (16 h light/8 h dark, 17°C). For the next 6 months, they were reared under constant 'winter' conditions (8 h light/16 h dark, 12°C). In September to October 2005, the climate chambers were switched back to summer conditions, which were maintained until the fish reached sexual maturity. These mature fish were then used for studies of divergence in morphology (Sharpe *et al.*, 2008), male mating behaviour (Delcourt *et al.*, 2008), and mate choice (K. Räsänen and A.P. Hendry, unpublished data).

At maturity, this first generation of laboratory-reared fish was subjected to artificial crossing methods (as above) to produce a new generation of pure lake and pure inlet fish (six families each), as well as lake–inlet hybrids (seven families). Each cross represented a unique male–female pair and siblings were not mated together. These new families were reared using the above protocols, but experienced a shorter period of winter conditions (2–3 months). The fish reached maturity in April to June 2007 and were then used for our experiment (see below). Because these were second-generation, laboratory-reared fish, any differences among male types (lake = L, inlet = I, hybrid = H) were likely to be genetic, rather than plastic or maternal.

The experiment

The experiment was conducted in fifteen 102-litre aquaria (92 cm long, 32 cm wide, 39 cm deep). The bottom of each aquarium was covered with sand that extended for 20 cm from either end towards the centre, and the middle was covered with dark gravel. Strips of black garbage bag tied around handfuls of gravel were used to create plastic 'plants', two per tank, one at each intersection of sand and gravel. The tanks were filled with water to a depth of 20 cm and all sides were covered with brown paper so as to minimize visual disturbances.

Two male stickleback of differing type were placed into each tank (L × I, L × H, or I × H). This allowed us to examine nest characteristics in the presence of interactions between male types, as would occur in nature when individuals dispersed between environments. Nesting material was provided in excess so that any such interactions were not the result of limited supplies, which is unlikely in nature. Three types of material were added: (1) 1600 pieces of 7-cm long white sisal rope separated into individual threads, (2) 400 pieces of 7-cm long black sewing thread, and (3) 21 cm^3 of dead plant material originally collected from Misty Lake and Misty Inlet.

Males were 'stimulated' to build nests by the twice-daily presentation, in the centre of their tank, of a single gravid female. These presentations were started on the day males were introduced into their aquaria, and continued until the day before a trial. The female was contained within a clear glass jar with mesh over the top, which allowed males to see and smell the female but prevented physical interactions and movement of the female towards a nest. Our aim was to encourage males to build nests but not let their behaviour be influenced by female responses to the nest. Stimulus females included all three female types (L, I, H), and were presented sequentially and in random order.

As soon as at least one of the males had constructed and was guarding a nest, we stopped the above presentation of stimulus females and instead started the experimental trials. We sequentially (on separate days) introduced one gravid female of each type (L, I, and H in random order) into the tank. These females were always from a different family than the males. Each interaction was allowed to proceed for one hour, or was stopped earlier if the female entered a male's nest. In the latter case, the female was induced to leave the nest before ovipositing by gently squeezing her tail with a pair of long tweezers. The above procedures ensured that we examined the nests after males had been allowed to interact freely with a single unrelated female of each type, but that none of the males had actually cared for eggs.

Once a male pair had been exposed to all three female types, all fish were measured with a ruler (± 0.1 cm) for standard length (tip of upper jaw to end of hypural plate) and body depth (anterior insertion of first dorsal spine to bottom of the pelvic girdle, perpendicular to the lateral line). Water was then drained from the tank so that the nests could be digitally photographed from above, each alongside a standard ruler. The nests were then transferred to paper plates and frozen for future analysis. The experimental tanks were cleaned and new water and nest materials added. A new male pair was then introduced in the tank and the above procedures were repeated. By the end of the experimental period, we had collected nests from 15 lake, 19 inlet, and 15 hybrid males. All of these nests had been actively guarded by their owners. For each of these nests, we recorded opponent 'performance' – that is, whether or not the male opponent in the tank also had a nest.

For the photograph of each nest, we used Image J (version 1.37) to analyse several parameters, generally following Barber *et al.* (2001). Total area was the entire surface area, including all nesting material visible as part of the nest. Bulk area was the surface area of nest through which the substrate was not visible (i.e. completely covered by nest material). The compactness index was taken as bulk area divided by total area, providing a measure of nest 'density' (Barber *et al.*, 2001). We also recorded the position of each nest in the tank (in a corner, under a plastic plant or in the open), as well as the substrate on which the nest was built (sand or gravel).

The frozen nests were thawed and then dried at 70°C for 12 h. We then measured total dry mass and dissected the nest to measure the dry mass of their individual constituents. These included mass of white rope, mass of black threads, and all materials that did not pass through a 2-mm^2 mesh filter.

Statistical analyses

First, we used multivariate analysis of variance (MANOVA) and analyses of variance (ANOVA) (followed by Tukey tests where applicable) to consider the effect of nest owner type (including or excluding the hybrids) on combined and individual nest characteristics (total dry mass, total area, compactness index, materials >2 mm, mass of black threads, mass of white rope). Before these analyses, total dry mass, materials >2 mm, and mass of black threads were $\log_{10}$-transformed to improve normality. Subsequently, we performed a discriminant analysis to quantify how well the nests of each male type (including the hybrids) could be discriminated from the other male types. Hybrids were then excluded to identify which characteristics discriminate lake and inlet nests. The resulting discriminant functions were then used to classify the hybrid nests as lake-like or inlet-like.

We next used additional multivariate analyses of covariance (MANCOVA) and analyses of covariance (ANCOVA) to compare the effect of nest owner type (L, I, H) with the effect of fish size (standard length and body depth) and the effect of the opponent male on nest characteristics. The effect of the opponent was incorporated either as opponent performance (comparing opponents with or without a nest) or as opponent type (L, I, H). The latter was evaluated after excluding all cases where opponents did not build nests, ensuring that all males in the analysis were real opponents, interested in the females. Non-significant effects were removed from the models using a backward stepwise model-building strategy. Because the effects of nest owner type, fish size, and opponent type may confound each other (fish size differed significantly among nest owner types, and nest owners were never confronted with opponents of the same type), we also tested all effects separately.

Finally, associations between nest owner type, opponent performance, opponent type, and two categorical nest characteristics (substrate and nest position) were analysed with chi-squared tests. All statistical analyses were performed in Statistica (version 8.0).

RESULTS

Multivariate analysis of variance revealed significant differences in nest characteristics among nest owner types (Wilks' $\lambda = 0.450$, $F_{12,78} = 3.195$, $P = 0.0009$). Analysis of variance (Table 1) revealed significant nest owner type effects for total dry mass (L > H > I; Fig. 1A) and the dry mass of material >2 mm^2 (L > H > I; Fig. 1B). These effects also made up the

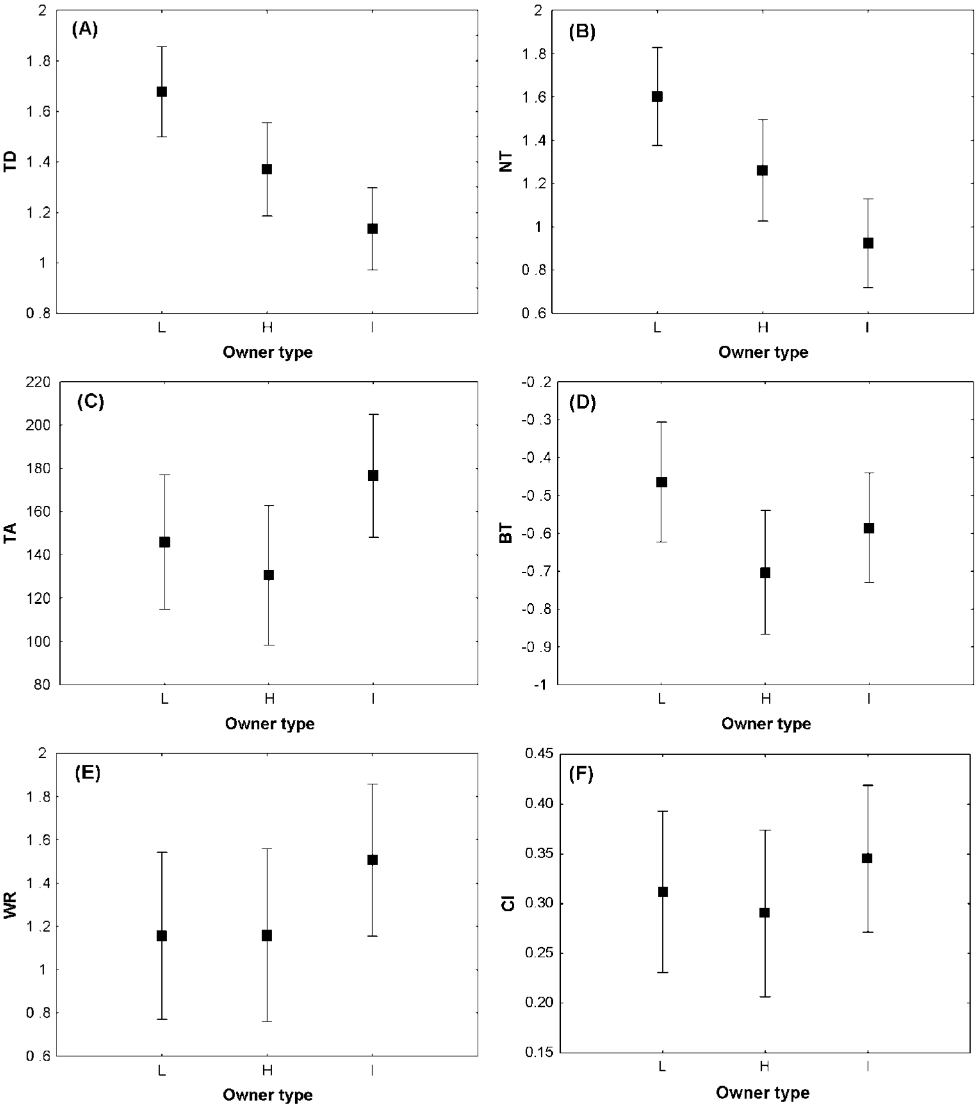

Fig. 1. Nest characteristics by nest owner type (L = lake, H = hybrid, I = inlet). (A) Total dry mass (TD); (B) dry mass of material >2 mm^2 (NT); (C) total area (TA); (D) mass of black threads (BT); (E) mass of white rope (WR); (F) compactness index (CI). Vertical bars denote 95% confidence intervals. Total dry mass, dry mass of material >2 mm^2, and mass of black threads were log$_{10}$-transformed to improve normality.

Table 1. Mean nest characteristics by nest owner type (lake, hybrid, inlet) and results of ANOVA (A) with and (B) without hybrid males included (standard errors in parentheses)

	Nest owner type			(A) With hybrids		(B) Without hybrids	
Trait	Lake	Hybrid	Inlet	$F_{2,46}$	P	$F_{1,32}$	P
Total dry mass (g)	62.60 (14.03)	33.50 (7.21)	16.78 (2.59)	9.724	**0.0003**	22.738	**< 0.0001**
Materials >2 mm (g)	55.11 (13.34)	27.98 (6.38)	12.52 (2.31)	10.133	**0.0002**	20.921	**< 0.0001**
Total area (cm^2)	145.91 (13.39)	130.54 (15.71)	176.59 (15.63)	3.154	0.0520	2.383	0.1325
Mass of black threads (g)	0.43 (0.07)	0.23 (0.03)	0.33 (0.06)	2.8965	0.0653	0.923	0.3439
Mass of white rope (g)	1.16 (0.18)	1.16 (0.23)	1.51 (0.16)	1.874	0.1653	2.988	0.0935
Compactness index	0.31 (0.04)	0.29 (0.04)	0.35 (0.03)	0.725	0.4899	0.232	0.6332

Note: Significant *P*-values are shown in **bold** font.

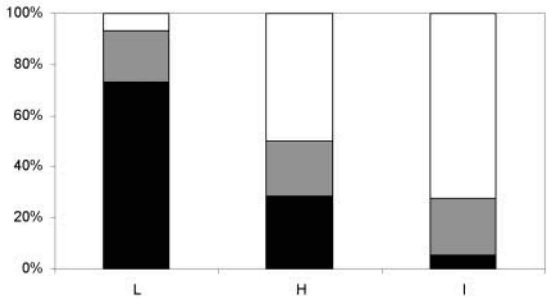

Fig. 2. Variation in nest characteristics among lake (L), hybrid (H), and inlet (I) ecotypes as revealed by a discriminant function based on six traits (total dry mass, dry mass of material >2 mm^2, mass of black threads, mass of white rope, total area, and compactness index). Shown are the proportions of nests of a given male ecotype (x-axis) that were assigned to a given male ecotype (y-axis). Black areas correspond to the proportion assignment to lake ecotype, grey areas to hybrid ecotype, and white areas to inlet ecotype.

main difference between the nests of the pure type males (Table 1). Analysis of variance (Table 1) also revealed possible, although non-significant, effects for total nest area (I > L > H; Fig. 1C) and the mass of black threads (L > I > H; Fig. 1D). Other characteristics (compactness index and mass of white rope) were not significantly different among nest owner types (Table 1; Fig. 1E, F). Tukey tests following these ANOVAs revealed the following differences among particular male types: Lake males had nests with a greater total dry mass ($P = 0.0002$) and greater amounts of material >2 mm ($P = 0.0003$) than inlet males. Hybrid males had nests with less total area than inlet males ($P = 0.0463$), and with marginally less black thread than lake males ($P = 0.0542$).

Discriminant analysis showed that lake and inlet fish were well classified based on their nest characteristics (percent misclassified: L = 26%; I = 28%), whereas 79% of the hybrids were misclassified to either lake ($n = 4$) or inlet ($n = 7$) ecotypes (Fig. 2). When hybrids were excluded from the analysis, the classification of lake and inlet nests improved considerably (percent misclassified: L = 7%; I = 11%). The resulting discriminant function assigned the hybrid nests either to lake or inlet nests with high probability ($0.71 < P < 0.99$). Seven hybrid nests were classified as inlet-like, and seven hybrid nests were classified as lake-like. This classification did not distinguish the reciprocal hybrids (I × L and L × I).

One-way MANOVA showed that neither opponent performance (Wilks' $\lambda = 0.869$, $F_{6,40} = 1.00$, $P = 0.44$) nor opponent male type (Wilks' $\lambda = 0.732$, $F_{12,56} = 0.79$, $P = 0.66$) had a significant effect on nest characteristics. There were also no effects of the opponent on individual nest characteristics (Table 2). Opponent effects could be removed from any model that already included nest owner type. Fish body size, on the other hand, had a significant effect on nest characteristics as indicated by a multivariate regression (standard length: Wilks' $\lambda = 0.712$, $F_{6,40} = 2.70$, $P = 0.0269$; body depth: Wilks' $\lambda = 0.686$, $F_{6,40} = 3.06$, $P = 0.0148$). This was mainly due to a positive correlation between fish body size and nest bulkiness (total dry mass and materials >2 mm; Table 2). However, this relationship was only observed in inlet fish (results not shown), and both measures of body size could be removed from any model that already included nest owner type.

A chi-squared test revealed that inlet males built their nests more often on rocks (31.58%) than did lake (0%) or hybrid males (0%) ($\chi^2 = 12.735$, d.f. = 2, $n = 49$, $P = 0.0017$), but males

Table 2. (A) Analysis of variance of nest characteristics by opponent performance and opponent type. (B) Pearson correlation between nest characteristics and fish body size (standard length, body depth)

(A)	Opponent performance		Opponent type	
Trait	$F_{1,47}$	P	$F_{2,35}$	P
Total dry mass	2.617	0.1124	2.671	0.0832
Materials >2 mm	2.395	0.1286	2.760	0.0775
Total area	3.370	0.0727	0.057	0.9448
Mass of black threads	0.0617	0.8049	0.512	0.6036
Mass of white rope	0.138	0.7124	1.293	0.2877
Compactness index	0.0139	0.9066	1.568	0.2228

(B)	Standard length		Body depth	
Trait	R	P	R	P
Total dry mass	0.438	**0.0021**	0.315	**0.0309**
Materials >2 mm	0.451	**0.0014**	0.372	**0.01**
Total area	−0.186	0.2104	−0.361	**0.0126**
Mass of black threads	0.073	0.6278	0.017	0.9091
Mass of white rope	−0.125	0.4017	−0.148	0.3199
Compactness index	−0.130	0.3825	−0.085	0.5690

Note: Significant *P*-values are shown in **bold** font.

of the different types did not build their nests in different positions in the tank ($\chi^2 = 8.749$, d.f. = 4, $n = 49$, $P = 0.0677$). Owner performance was significantly associated with nest position ($\chi^2 = 10.439$, d.f. = 2, $n = 49$, $P = 0.0054$): nests were more often placed in a corner or under plastic plants when opponents built nests, and more in the open when opponents did not build nests. Opponent type was not associated with substrate type or nest position.

In summary, nest owner type was the strongest determinant of nest characteristics. The nests of lake males were less often placed on rocks and were bulkier than were the nests of inlet males. Hybrid nests were, on average, intermediate (Fig. 3). However, individual hybrid nest characteristics were either more lake-like (total area), more inlet-like (mass of black theads) or highly variable (total dry mass and materials >2 mm). Consequently, some hybrids built lake-type nests, others built inlet-type nests, and few hybrids built nests that were truly intermediate (Fig. 3).

DISCUSSION

When inlet and lake ecotypes from the Misty system were raised for two generations in a common laboratory environment, males showed several differences in their nest characteristics. This result supports the likelihood of genetic divergence in an important component of the mating system. We cannot be certain, however, that the specific aspects of divergence seen in the laboratory would parallel those seen under more natural conditions (i.e. genotype × environment interactions). It would therefore be useful also to sample nests

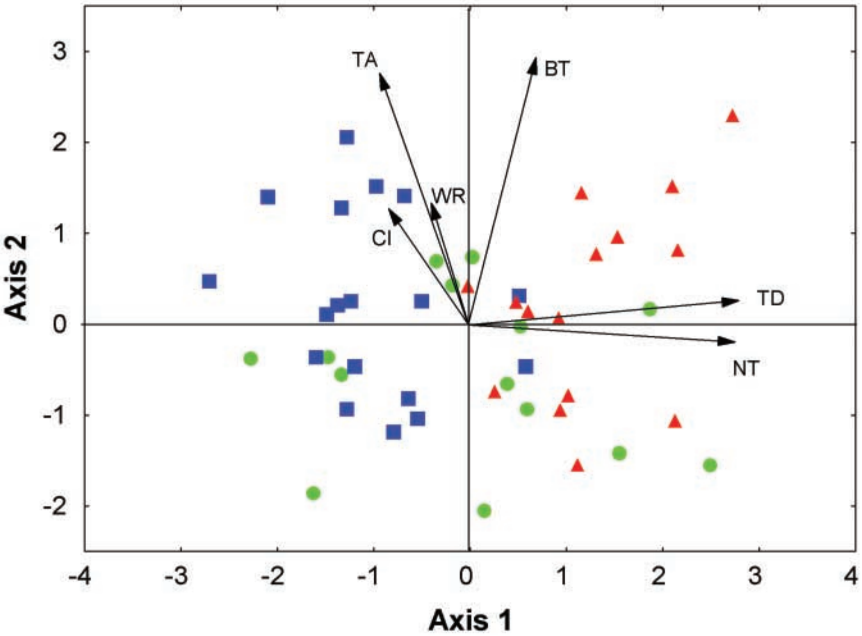

Fig. 3. Discriminant analysis plot showing nests by nest owner type as differentiated by nest characteristics. Triangles, dots, and squares represent lake, hybrid, and inlet nests, respectively. Loadings of the nest characteristics with respect to the discriminant functions are represented as vectors.

from nature – although this is very difficult owing to the very dark (tannic) water in the Misty system. For the moment, we will proceed under the assumption that the differences seen in the laboratory would be similar to those seen in nature – as is the case for morphology (Lavin and McPhail, 1993; Hendry *et al.*, 2002; Sharpe *et al.*, 2008). This allows us to postulate some adaptive hypotheses for the observed differences. At the same time, we acknowledge that the differences might well reflect non-adaptive processes, such as genetic drift or founder effects. In this case, divergence in nest characteristics might still influence mating isolation – but not because of adaptive divergence.

The main observed differences were that the nests of lake males were less often placed on rocks (more often on sand) and were bulkier than those of inlet males. With respect to nest placement, lake males may simply not recognize gravel as a suitable nest site – given that this type of substrate is much less common in Misty Lake than in Misty Inlet (J.S. Moore, unpublished data). Or the choice of location may be adaptive and increase mating success or offspring survival (Kraak *et al.*, 1999; Blais *et al.*, 2004). Here, some possibilities are that gravel substrates may be less suitable for nests in Misty Lake, lake males might be less adapted at building nests on gravel, or the lake females might avoid nests built on gravel. With respect to nest size, lake males may build bulkier nests simply because they are themselves larger (mean in nature = 58 mm) than inlet males (50 mm) (Moore and Hendry, 2005). If so, it would reflect an evolutionary difference, not a proximate constraint, because we found little evidence that individual male size influenced nest bulkiness in our experiment. Alternatively, lake males may build bigger nests to accommodate lake females, which are larger (mean 61 mm) than inlet females (52 mm) in nature (Moore and Hendry, 2005) and in the laboratory (J.A.M. Raeymaekers, unpublished data). We cannot, at present, discriminate among these or other adaptive and non-adaptive explanations for the observed differences in nest characteristics.

Lake and inlet males clearly show a genetic difference in characteristics of the nests they build, and this may have implications for sexual selection against migrants in nature (see below). Examining the nests of lake–inlet hybrids can yield some crude insights into the possible nature of these differences and can also inform the possibility of sexual selection against hybrids. We found that some nest characteristics of hybrids were intermediate, on average, between the two parental forms. This pattern initially suggests an additive genetic basis for nest characteristics. Closer examination, however, points to the possibility of more complicated effects. In particular, some hybrid males built inlet-like nests whereas others built lake-like nests. Only a few individual males built nests that were clearly intermediate between those built by pure lake and pure inlet males (Fig. 3). This variation among individuals suggests the possibility of non-additive effects, such as family-specific dominance for a single nuclear gene. We would be surprised, however, if nest characteristics were determined by a single gene. Alternatively, the pattern may be due to genomic imprinting controlled by a polymorphic nuclear gene and epigenetic modifiers (Sambatti *et al.*, 2008). This possibility is also unlikely, however, because we did not find the expected differences in nest characteristics between the reciprocal hybrids (I × L and L × I) (Sambatti *et al.*, 2008). In short, the specific genetic basis will require more focused breeding experiments.

Although nest characteristics might be a direct effect of an individual's genotype, they might also reflect an indirect genetic effect (Moore *et al.*, 1997) of the opponent's genotype. We examined this possibility by testing whether nest characteristics were influenced by opponent male type. Such effects might occur because different male types show different levels of intra-sexual aggression (Bakker, 1994), thus influencing nest construction of their opponents. Although we have not examined this possibility, inlet and lake males do at least differ in their levels of inter-sexual aggression (Delcourt *et al.*, 2008). Alternatively, different male types may vary in exploitative competition for nest sites and nesting material (Whoriskey and FitzGerald, 1994). Despite these possibilities, we found no effect of opponent male ecotype on nest construction. We nevertheless frequently observed males stealing nest material from each other's nests, while nest raids may have prohibited the construction of some opponent nests. We also found that in the presence of a nesting opponent, males were building nests in more sheltered positions, making them easier to defend (see Jenni, 1972, for a similar finding).

Implications

We have argued that genetic divergence in nest characteristics may contribute to mating isolation by not favouring migrants and perhaps also hybrids. This process may reflect the action of ecological speciation if the original nest differences are the result of adaptive divergence. No previous studies appear to have documented genetic divergence in nest characteristics for any organism – but we here show that such differences exist for the Misty Lake and Misty Inlet populations. Although we cannot be certain that these differences are adaptive, we were able to suggest some adaptive hypotheses and many other traits do seem to differ adaptively between these populations (Hendry *et al.*, 2002; Moore *et al.*, 2007; Delcourt *et al.*, 2008; Sharpe *et al.*, 2008). Regardless of whether the differences are adaptive, they might contribute to mating isolation because inlet males that swim into the lake will likely build a different nest than the resident males. The same will be true for lake males that swim into the inlet.

Hybrids males may also have problems given that they sometimes build lake-type nests and sometimes inlet-type nests.

Genetic divergence in nest characteristics then begs the question of whether such differences are used in mate choice. Although this has yet to be tested explicitly, existing work on sticklebacks is suggestive of this. First, studies within populations show that nest characteristics are important to mate choice (Barber *et al.*, 2001; Ostlund-Nilsson and Holmlund, 2003). Second, differences in nest characteristics have been observed for white versus typical marine stickleback (Blouw and Hagen, 1990; Jamieson *et al.*, 1992) and for Icelandic lava versus nitella stickleback (Olafsdottir *et al.*, 2006). Third, we have shown here that differences in nest characteristics can have a genetic basis and would therefore lead to differences between migrants (and perhaps hybrids) and residents. Similar possibilities are likely in other taxa given the frequent use of nests for incubating eggs or rearing young, the demonstrated use of nest characteristics in mate choice (Jones and Reynolds, 1999; Ostlund-Nilsson, 2000), and the evidence for phenotypic differences in nest characteristics among habitats (Järvi-Laturi *et al.*, 2008).

In summary, we suspect that divergence in nest characteristics could contribute to the development of mating isolation during ecological speciation. Studies of ecological speciation often search for the key reproductive barriers that caused the initial development of isolation between populations (Schluter, 2000; Coyne and Orr, 2004). Our search for such barriers in lake–stream stickleback has revealed several candidate barriers but few appear particularly strong, consistent, and symmetrical (K. Räsänen, J.A.M. Raeymaekers and A.P. Hendry, unpublished data). The present study adds genetically based differences in nest characteristics to the large suite of morphological and behavioural differences that might influence reproductive isolation in this system. Perhaps this is the smoking gun for lake–stream stickleback reproductive isolation – or maybe it is just one of several quivering arrows, all of which are necessary, but not sufficient.

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