Reduced sperm performance in backcross hybrids between species pairs of whitefish (*Coregonus clupeaformis*)

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Abstract: Previous work has demonstrated that genomic incompatibilities work together with ecologically divergent selection to promote and maintain reproductive isolation between incipient species (dwarf and normal) of lake whitefish (*Coregonus clupeaformis* (Mitchill, 1818)). Whitefish spawn in groups with external fertilization, which creates conditions for strong sperm competition. In this study, we asked whether reduced sperm performance in hybrids from whitefish species-pair matings might contribute to postzygotic isolating mechanisms between these taxa. We examined two sperm traits, sperm swimming speed and flagellum length, in pure dwarf and normal whitefish and in their F1 and backcross hybrids. We observed significantly reduced sperm swimming speed in backcross but not in F1 hybrids. Sperm flagellum length was not significantly correlated with sperm swimming speed. These results demonstrate that F1 hybrids formed in nature should be capable of the same fertilization success as the parental species during sperm competition, everything else being equal. However, reduced sperm performance in the backcross generation is consistent with other evidence suggesting that genomic incompatibilities create a range of negative fitness effects in post-F1 whitefish hybrids and provides evidence for an additional postzygotic isolation mechanism involved in the incipient speciation of sympatric dwarf and normal whitefish.

Résumé : Des travaux antérieurs ont démontré que les incompatibilités génétiques agissent de concert avec la sélection divergente en fonction de l'écologie pour favoriser et maintenir l'isolement génétique entre les espèces en émergence (naines et normales) chez les grands corégones (*Coregonus clupeaformis* (Mitchill, 1818)). Les corégones fraient en groupes et ont une fécondation externe, ce qui crée des conditions de forte compétition spermatique. Dans notre étude, nous cherchons à savoir si la performance réduite des spermatozoïdes des hybrides provenant de l'accouplement de paires d'espèces de corégones peut contribuer aux mécanismes d'isolement post-zygotique entre ces taxons. Nous examinons deux caractéristiques des spermatozoïdes, la vitesse de nage et la longueur du flagelle des spermatozoïdes, chez des corégones nains et normaux purs et leurs hybrides de F1 et de rétrocroisement. Il y a une réduction significative de la vitesse de nage des spermatozoïdes chez les hybrides de rétrocroisement, mais non ceux de F1. Il n'existe pas de corrélation significative entre la longueur du flagelle et la vitesse de nage des spermatozoïdes. Ces résultats démontrent que les hybrides de F1 formés en nature devraient pouvoir obtenir le même succès de fécondation que les espèces parentales durant la compétition spermatique, toutes autres choses étant égales. Cependant, la performance réduite des spermatozoïdes de la génération de rétrocroisement est compatible avec d'autres indications qui laissent croire que les incompatibilités génétiques créent une gamme d'effets négatifs de fitness chez les hybrides post F1 de corégones; elle fournit aussi des preuves de l'implication d'un mécanisme additionnel d'isolement post-zygotique dans la spéciation en émergence des corégones sympatriques nains et normaux.

[Traduit par la Rédaction]

Introduction

Our understanding of the process of speciation has increased dramatically in recent decades (Schluter 2000; Coyne and Orr 2004). Many studies have focused on the ecological processes of speciation, by which barriers to gene flow evolve between populations owing to divergent selection resulting from different ecologies (Rundle and Nosil 2005). One major source of evidence for such ecological speciation has come from studies of fish in northern temperate lakes (Hatfield and Schluter 1999; Rundle 2002; Bernatchez 2004; Gow et al. 2007; Landry et al. 2007). Ecological speciation has been the primary focus in these systems because incipient sister species pairs from temperate lakes often differ in phenotypic traits associated with the occupation of divergent trophic niches (Lu and Bernatchez 1999; Schluter 2000; Bernatchez 2004). Nonetheless, recent work suggests that genomic incompatibilities and divergent ecological selection may work together to promote and maintain reproductive isolation in sympatric north temperate fish populations of actual or incipient species pairs (Lu and Bernatchez 1998; Rogers and Bernatchez 2006, 2007).

As with other types of reduced performance in fitness-

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related traits associated with hybridization (Schluter 2000), genomic incompatibilities could contribute to reproductive isolation during the speciation process if sperm performance is reduced in hybrids. Because sperm swimming speed is an important determinant of fertilization success in a wide variety of taxa (Birkhead et al. 1999; Levitan 2000; Froman et al. 2002; Gage et al. 2004; Malo et al. 2005; Casselman et al. 2006), genomic incompatibilities that impair this aspect of sperm performance might be expected to have fitness consequences for males, especially under the competitive conditions found in externally fertilizing fishes (Fitzpatrick et al. 2007). Moreover, because sperm morphometry, and in particular sperm tail length, is expected to contribute to swimming speed (Montgomerie and Fitzpatrick 2009), any genomic incompatibilities that influence sperm morphometry might also be expected to impair male reproductive success.

Recent work on incipient species pairs of whitefish (genus *Coregonus* L., 1758), both of which are currently designated taxonomically as lake whitefish (*Coregonus clupeaformis* (Mitchill, 1818)), from northeastern North America has revealed evidence for both extrinsic, ecologically based mechanisms (Bernatchez 2004; Landry et al. 2007) and intrinsic genomic incompatibilities contributing to reproductive isolation (Lu and Bernatchez 1998; Rogers and Bernatchez 2006). Geographic isolation during the Pleistocene led to genetic divergence between whitefish populations inhabiting different glacial refugia (Bernatchez and Dodson 1990; Pigeon et al. 1997; Lu et al. 2001). Secondary contact of these evolutionary lineages subsequently occurred around 12 000 years before present within at least six lakes. Following contact, both ecological opportunity and character displacement have contributed to the evolution of a limnetic dwarf species that has diverged in sympatry from the "normal" ancestral benthic species (Bernatchez 2004; Landry et al. 2007). These young species still exchange genes where they occur in sympatry (Campbell and Bernatchez 2004), and overall genetic differentiation between dwarf and normal whitefish varies from high to low, apparently depending upon ecological selection pressures imposed in different environments (Lu and Bernatchez 1999). Adaptive trait differences between dwarf and normal whitefish are supported by genetically based phenotype–environment interactions that influence behavior (Rogers et al. 2002), growth (Trudel et al. 2001; Rogers and Bernatchez 2005), morphology (Lu and Bernatchez 1999; Bernatchez 2004), and gene expression (Derome et al. 2006; St-Cyr et al. 2008). Postzygotic genomic incompatibilities are revealed by reduced embryonic survival of both F1 and backcross hybrids in controlled crosses, and significant locus-specific deviations from Mendelian segregation (segregation distortion) in backcross progeny (Lu and Bernatchez 1998; Rogers and Bernatchez 2006).

Whitefish spawn in groups with external fertilization (Fabricius and Lindroth 1954; Rudolfsen et al. 2008), which creates conditions for strong sperm competition and thus large fitness consequences of poor sperm performance (Gage et al. 2004; Stoltz and Neff 2006). We asked whether reduced sperm performance in hybrids between whitefish species pairs could contribute to reproductive isolating mechanisms between these taxa. Even though sperm performance is a trait related to competition that occurs among sperm prior to zygote formation, in general, prezygotic isolating mechanisms apparently do not prevent hybrid formation between incipient whitefish species pairs. If sperm performance is reduced in hybrid whitefish, we consider this an example of a postzygotic isolating factor because it is likely due to genomic incompatibilities that occur within hybrid animals.

The objective of this study was to elucidate the effects of hybridization between an incipient species pair (dwarf and normal) of whitefish on the sperm traits of hybrids. We tested for differences between dwarf and normal whitefish in two sperm traits — swimming speed and tail length — in common-garden-reared individuals from four cross types that have previously revealed evidence for postzygotic genomic incompatibilities (Lu and Bernatchez 1998; Rogers and Bernatchez 2006). We tested for differences in sperm traits between the two pure species (dwarf and normal), F1 hybrids, and backcross hybrids, with the prediction that genomic incompatibilities would lead to reduced sperm quality in hybrids.

Materials and methods

Experimental crosses

In 1996, dwarf whitefish from Témiscouata Lake, Quebec (47°36′N, 68°45′W; Acadian glacial lineage), and normal whitefish from Aylmer Lake, Quebec (45°50′N, 71°26′W; Atlantic glacial lineage), were used to create pure dwarf crosses ($D_♀ \times D_♂$), pure normal crosses ($N_♀ \times N_♂$), and both reciprocal F1 hybrid crosses ($N_♀ \times D_♂$ and $D_♀ \times N_♂$) (for details see Lu and Bernatchez 1998). These fish were maintained until sexually mature under constant environmental conditions in the Laboratoire Régional des Sciences Aquatiques (LARSA) animal facility at Université Laval. In 1999, first generation (F1) captive fish were used to create a second captive generation (F2) that consisted of pure dwarf ($F1D_♀ \times F1D_♂$) and pure normal ($F1N_♀ \times F1N_♂$) crosses, as well as second-generation reciprocal F1 hybrid crosses ($F1N_♀ \times F1D_♂$ and $F1D_♀ \times F1N_♂$) and backcrosses (F1 hybrid$_♀$ ($N_♀ \times D_♂$) $\times F1D_♂$). Note that the second-generation F1 crosses were created between pure F1 dwarf × pure F1 normal individuals. Although they are second-generation individuals held in the laboratory, offspring from these crosses represent "F1 hybrid" individuals that contain equal genetic contribution from pure dwarf and normal whitefish. All fish from this F2 generation were maintained under the same environmental conditions as above until milt (semen containing inactive spermatozoa) was collected from sexually mature males.

Milt collection and transport

In November 2005, males were anaesthetized with eugenol before measurements were made of fork length and body mass and their milt was sampled. One to 5 mL of milt were collected from each of 13 pure normal ($F1N_♀ \times F1N_♂$) and 13 pure dwarf ($F1D_♀ \times F1D_♂$) males, 6 of each type of second-generation reciprocal F1 hybrids ($F1N_♀ \times F1D_♂$ and $F1D_♀ \times F1N_♂$; total $N = 12$), and 15 hybrid backcross (F1 hybrid$_♀$ ($N_♀ \times D_♂$) $\times F1D_♂$) males (hereafter N, D, F1 and BC, respectively). Between 1000 and 1300 (EST), milt was

collected directly from each male's anal pore using disposable plastic pipettes, then placed in a 10 mL "breathable" polystyrene tube with its lid slightly loosened to allow oxygen to enter. Tubes were then wrapped in two layers of damp cloth towels before being placed on ice for shipment to Queen's University (Kingston, Ontario) for sperm analysis. Milt samples were shipped in 40 cm × 40 cm styrofoam containers with 5 cm thick walls containing ice packs and damp sponges to keep them from drying out. Upon arrival at the laboratory (approximately 1100 EST on the day after sampling), all stock tubes were checked for leakage and then refrigerated at 4 °C while sperm measurements were taken.

Sperm swimming speed measurements

We analyzed two samples from each male's milt. To measure sperm velocity, we placed approximately 1 μL of milt (containing inactive sperm) on a 2X-CEL Disposable Sperm Analysis Chamber (Hamilton Thorne, Inc., Beverly, Massachusetts), added a cover slip, and placed the slide on a microscope stage whose temperature was held at approximately 7 °C using a Peltier plate. Spermatozoa were activated (time, $t = 0$) by flooding the slide chamber from one side with a few drops of distilled water at 7 °C, the water temperature of the fish-holding tanks in the laboratory. We recorded all sperm activity on videotape using a high-resolution monochrome CCD camera (model DCR-TRV17 NTSC; Sony, Tokyo, Japan) mounted on a negative phase contrast CH30 microscope (Olympus, Tokyo, Japan) at 100× magnification.

We used a CEROS (version 12) video sperm analysis system (Hamilton Thorne, Inc., Beverly, Massachusetts) to measure sperm behavior. We quantified the swimming paths of spermatozoa in each milt sample for 0.5 s at both 10 and 20 s postactivation because recent studies have shown that most fertilization in externally fertilizing freshwater fishes takes place within 10–20 s of sperm release and activation (Hoysak and Liley 2001). Only those sperm whose discrete paths were wholly captured within the 0.5 s time frame were used to calculate mean sperm swimming speed (VAP; mean smooth path velocity) for each milt sample. We were unable to obtain measurements for one dwarf male's sperm at 20 s postactivation.

Sperm morphometry

Sperm tail length was measured by diluting a 5 μL sample from each male's milt in 750 μL of fixative (3% gluteraldehyde in 0.1 mol/L cacodylate buffer, pH 7.0), then spreading it thinly across a microscope slide and allowing it to air-dry. We then photographed 10 clearly visible sperm on each slide under 400× magnification using a microscope-mounted camera (microscope: Leica PMLS; camera: Panasonic TV camera model WV-1500). We captured and digitized images using BTV Pro (version 5.4) software before taking measurements. We measured the length of the flagellum on 10 sperm from each male to the nearest 0.1 μm using ImageJ (version 1.33 available from http://rsb.info.nih.gov/ij/) software and an Intuos graphic tablet (Wacom Co. Ltd., Saitama, Japan).

Data analysis

We checked all data and analyses to ensure that statistical assumptions were met and particularly that the distributions of residuals were normal. We log-transformed body mass and fork length in all analyses involving both of these variables to linearize the relation between them. When statistical assumptions could not be met owing to outliers,we performed analyses with and without those outliers to check the validity of our conclusions. JMP (version 7.01; SAS Institute Inc., Cary, North Carolina) statistical software was used for all analyses.

Results

Body mass varied significantly among the four types of crosses, with D males weighing significantly less, on average, than males of the F1 crosses (Table 1), even though variation in body length among cross types was not significant (Table 1). Six males (1 D, 4 F1, 1 N) were very heavy for their length, and two BC males were very light, all eight falling well outside the 99% prediction limits of the log mass on log fork length regression for the remainder of the males. To ensure that these outliers did not unduly influence our results, we performed all analyses with and without these males. Both procedures resulted in the same conclusions.

Mean sperm tail length was not significantly correlated with either male body length ($r = 0.16$, $P = 0.25$, $N = 51$) or body mass ($r = 0.18$, $P = 0.20$, $N = 51$) with data pooled for all four cross types. The mean sperm tail length of males did, however, differ among cross types with sperm tail length of D males significantly shorter than that of the other three cross types, each of which did not differ significantly from one another (Table 1).

Sperm swimming speed was not significantly correlated with sperm tail length at either 10 s ($r = 0.08$, $P = 0.59$, $N = 51$) or 20 s ($r = 0.07$, $P = 0.65$, $N = 50$) postactivation with data pooled for all four cross types, nor within any of the cross types (results not shown). For this reason, we do not include sperm tail length in subsequent models to predict sperm swimming speed.

To compare sperm swimming speeds among the four cross types, we constructed an ANCOVA model with log(body mass) as a covariate, because body mass differed among cross types. We also included log(fork length) as an independent variable in these models to control for variation in body size, and thus to assess the effect of residual body mass as a measure of condition (Sutton et al. 2000).

Sperm swimming speed at both 10 and 20 s postactivation varied significantly among cross types, with sperm from BC males significantly slower than sperm from D and F1 males (Table 1, Fig. 1). Mean sperm swimming speed for F1 hybrids was higher than for the other three groups, though this trend was not statistically significant ($P > 0.05$). Sperm swimming speed was uniformly lower at 20 s postactivation than at 10 s postactivation (Table 1), as is typical in externally fertilizing fishes. The decline in swimming speed between 10 and 20 s postactivation was largest for BC males, but the magnitude of that decline, when used as a dependent variable in a separate analysis, did not differ significantly among cross types (ANCOVA, effect of cross type, $F_{[3,46]} = 0.7$, $P = 0.57$). Sperm swimming speeds at both times postactivation were significantly related to body mass control-

Table 1. Mean (SE) body size and sperm traits for the four groups of experimental crosses of whitefish (*Coregonus clupeaformis*) used in this study.

		Fish size		Sperm swimming speed (μm/s)		
	N	Body mass (g)	Fork length (cm)	10 s postactivation	20 s postactivation*	Sperm tail length (μm)†
Cross type						
Normal (N)	12	505.9 (34.2) ab	32.0 (1.1)	45.7 (3.0) ab	37.3 (2.2) a	39.7 (0.6) b
Dwarf (D)	12	378.0 (32.9) a	30.3 (0.9)	49.9 (2.5) a	39.8 (1.7) a	36.5 (0.5) a
F1‡	12	511.8 (35.1) b	30.1 (1.2)	51.9 (1.6) a	42.4 (1.5) a	40.0 (0.7) b
Backcross (BC)	15	427.0 (32.8) ab	32.7 (1.4)	42.2 (1.1) b	30.3 (0.6) b	38.9 (0.3) b
Statistic§						
F		3.5	1.3	5.7	17.0	8.0
df		3, 47	3, 47	3, 42	3, 41	3, 47
P		0.02	0.30	0.002	<0.0001	0.0002

Note: Groups that are not significantly different (Tukey HSD post hoc tests; $P > 0.05$) following significant ANOVA or ANCOVAs have the same letters.

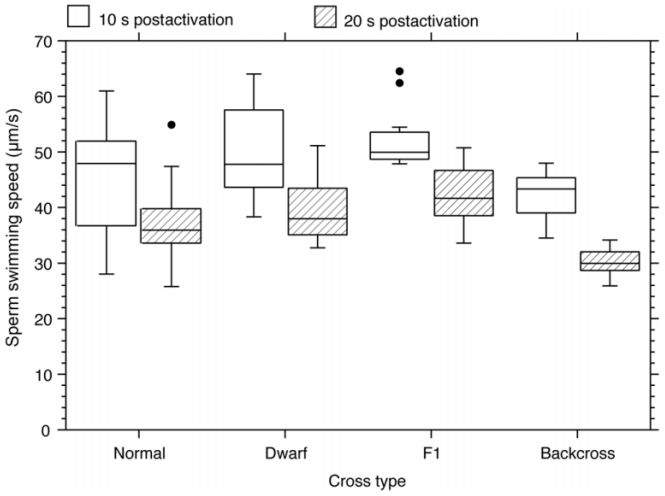
*$N = 11$ dwarf males.

†Analysis of mean values from 10 spermatozoa for each male.

‡Six F1 males from each of two reciprocal crosses: $F1N_{♀} \times F1D_{♂}$ and $F1D_{♀} \times F1N_{♂}$.

§Statistics are from ANOVAs for the effect of cross type on body mass, fork length, and sperm length, and ANCOVAs for sperm swimming speed (controlled for log(body mass), log(fork length), and the interaction between cross type and log(body mass)).

Fig. 1. Sperm swimming speed in hybrid (F1 and BC) and pure (N and D) whitefish (*Coregonus clupeaformis*) species pairs at 10 and 20 s postactivation, illustrated with Tukey box plots. Sperm swimming speed was measured as VAP, the mean smooth path velocity. Black dots are observations that lie outside the median ± 1.5(interquartile range).



ling for fork length (ANCOVA, effect of body mass controlling for fork length: at 10 s, $F_{[1,42]} = 6.0$, $P = 0.02$; at 20 s, $F_{[1,41]} = 10.50$, $P = 0.002$), suggesting that males in better condition had faster swimming sperm.

Discussion

The work presented here demonstrates that backcross whitefish hybrids have reduced sperm swimming speeds compared with pure dwarf and normal whitefish or first-generation hybrids. Sperm swimming speed is likely to have large fitness consequences in group spawning fishes such as Alpine whitefish (*Coregonus zugensis* Nüsslin, 1882) (Rudolfsen et al. 2008). In the closely related Atlantic salmon (*Salmo salar* L., 1758), males with faster swimming sperm fertilized a greater proportion of eggs in competitive trials and relative sperm swimming speed explained approximately half of the variation in male fertilization success (Gage et al. 2004). We therefore suggest that reduced sperm swimming speed would cause reduced fertilization success of later generation (post-F1) hybrids between dwarf and normal whitefish during sperm competition, and consequently could contribute to postzygotic reproductive isolation.

Reduced sperm swimming speed in both F1 and backcross hybrids would have been consistent with hybrid incompatibilities (e.g., Dobzhansky–Muller incompatibilities; Brideau et al. 2006). However, we did not observe slower sperm swimming speed in F1 hybrids; indeed, sperm swimming speed was greatest in F1 hybrids, although this difference was not statistically significant. Therefore, fertilization success should not be impaired and might actually be greater in F1s relative to pure whitefish. Instead of supporting the hybrid incompatibility hypothesis, these observations are consistent with previous data showing whitefish species pairs have arisen recently, still exchange genes to a varying extent in natural populations, and have not evolved well-developed isolating mechanisms (Lu and Bernatchez 1999; Bernatchez 2004; Campbell and Bernatchez 2004). In the absence of other ecological isolating mechanisms (e.g., differences in spawning date; Bernatchez 2004) in a particular lake, F1 fish may be produced and these first-generation hybrids should be physiologically capable of achieving high fertilization success.

On the other hand, backcross males had significantly slower sperm and therefore are likely to have impaired fertilization success relative to pure dwarf and normal whitefish or first-generation hybrids. Together with previous data showing that backcross hybrids have lower embryonic survival, a wider range of hatching times, and exhibit segregation distortion (Lu and Bernatchez 1998; Rogers and Bernatchez 2006), these results provide further evidence for fitness reduction in post-F1 whitefish hybrids. Indeed, slower sperm swimming speed and the concomitant likelihood of reduced fertilization success in backcross hybrids is consistent with outbreeding depression, defined as reduced fitness of offspring from matings between genetically diver-

gent individuals (in this case between whitefish species pairs), either owing to loss of locally adapted gene complexes or disruption of epistasis (Tallmon et al. 2004). Our results thus corroborate the general observation that the effects of outbreeding depression usually appears in post-F1 hybrids (Armbruster et al. 1999; Edmands 1999; Gharrett et al. 1999; Fenster and Galloway 2000; Marshall and Spalton 2000; Marr et al. 2002).

The most likely potential causes of reduced sperm swimming speed in backcross male whitefish are either reduced flagellum length (Gomendio and Roldan 1991; Ball and Parker 1996) or disrupted metabolic function (Cardullo and Baltz 1991; Anderson and Dixson 2002; Burness et al. 2004; Gemmell et al. 2004; Montgomerie and Fitzpatrick 2009). Sperm swimming speed and flagellum length were not correlated in our analysis and therefore a change in flagellum length does not appear to be responsible for our results. A lack of correlation between sperm swimming speed and flagellum length also appears to be the usual intraspecific pattern in fishes (Fitzpatrick et al. 2009). We hypothesize that, instead, disruption of metabolic function upon recombination in F1 males — particularly with respect to sperm energetics — is the mechanism responsible for our observation of reduced sperm swimming speed in backcross males. Interestingly, Fitzpatrick et al. (2009) have identified a change in sperm energetics to be the most likely initial response to selection resulting from sperm competition in cichlid fishes, followed by later selection on sperm length.

In previous controlled crosses of whitefish species pairs, fertilization success was not lower in F1 or backcrosses relative to same generation pure crosses (Lu and Bernatchez 1998; Rogers and Bernatchez 2006), although such an effect might have been predicted from the results presented here on sperm swimming speed. However, those previous in vitro experiments did not aim to rigorously compare sperm performance in a competitive setting, such that the sperm of one male was mixed in direct contact with the ova from one female in a manner designed to maximize fertilization success. Under competitive group-spawning conditions in the wild (Rudolfsen et al. 2008), it is much more likely that a male's reduced sperm performance would result in lower fertilization success.

Sperm of dwarf whitefish raised in a common environment had significantly shorter tail lengths than those of other crosses. This suggests that sperm tail length has a genetic basis, as has been reported in studies of other taxa (e.g., Birkhead et al. 2005). Given the lack of correlation between sperm tail length and sperm swimming speed, our results do not support the hypothesis that differences in sperm tail lengths between dwarf and normal whitefish are adaptive.

In conclusion, our observation of a significant reduction in sperm swimming speed in backcross hybrids provides further support for a role of postzygotic isolating mechanisms in divergence between dwarf and normal whitefish. This research adds to previous studies on postzygotic isolating factors in whitefish species pairs (Lu and Bernatchez 1998; Rogers and Bernatchez 2006, 2007) and suggests that a variety of intrinsic and extrinsic postzygotic factors can reduce genetic exchange during the time course of adaptive diversification in this taxon. Admittedly, sperm competition experiments will be necessary to more explicitly quantify the fitness consequences of reduced swimming speed in post-F1 hybrids. Nevertheless, we propose that our results for reduced sperm quality in post-F1 hybrids may be generally important and could contribute to reproductive isolation in any animal, but especially in species where sperm competition is intense. Such gametic isolation includes all reproductive barriers acting between copulation and fertilization (Coyne and Orr 2004) and has gained increasing recognition for its role in speciation (Ludlow and Magurran 2006).

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