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Low fertility of wild hybrid male flycatchers despite recent divergence

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Postzygotic isolation may be important for maintaining species boundaries, particularly when premating barriers are incomplete. Little is known about the course of events leading from minor environmental mismatches affecting hybrid fitness to severe genetic incompatibilities causing sterility or inviability. We investigated whether reduced reproductive success of hybrid males was caused by suboptimal sperm traits or by more severe genetic incompatibilities in a hybrid zone of pied (*Ficedula hypoleuca*) and collared flycatchers (*F. albicollis*) on the island of Öland, Sweden. About 4 per cent hybridization is observed in this population and all female hybrids are sterile. We found no sperm in the ejaculates of most sampled hybrid males, and sperm with abnormal morphology in two hybrids. Furthermore, none of the hybrids sired any offspring because of high levels of hatching failure and extra-pair paternity in their nests. These results from a natural hybrid zone suggest that the spermatogenesis of hybrid males may become disrupted despite little genetic divergence between the parental species.

1. Introduction

When premating reproductive barriers are incomplete, postzygotic isolation is a major barrier to gene flow between diverging populations in sympatry. As divergence between two populations increases, the strength of selection against hybrids is likely to increase. Extrinsic sources of postzygotic isolation, when hybrids experience lower fitness for ecological reasons or owing to lower sexual attractiveness, are thought to play an important role at early stages of population divergence [1,2]. At advanced stages of genetic divergence, intrinsic genetic incompatibilities eventually lead to hybrid sterility or inviability. Extrinsic factors affecting hybrid fitness have been studied in the wild [1], while hybrid sterility and inviability have traditionally been studied in artificial crosses, most recently in model organisms, focused on the genetics of intrinsic postzygotic incompatibilities (reviewed in [3]). However, genetic incompatibilities are likely to evolve slowly and accumulate over time [4]. Therefore, hybrids between young sister species may experience minor reductions in fertility caused by combined effects of environmental and physiological mismatches. A better understanding of the gradual build-up of genetic incompatibilities requires that hybrid fertility also be measured in natural environments.

We investigated the fertility of male hybrids between pied and collared flycatchers (*Ficedula hypoleuca* and *albicollis*). The two species diverged less than 2 Ma and came into secondary contact on Öland (Sweden) about 50 years ago [5]. Around 4 per cent of heterospecific pairing is observed; female hybrids are completely sterile, whereas male hybrids have reduced fitness [6]. Sexual selection against the intermediate phenotype of hybrid males has been proposed as the main reason for their reduced fitness [6]. However, 3 per cent of all breeding pairs include a hybrid male, and while hybrids experience higher rates of extra-pair offspring in their nests than pure-species males [6], backcross individuals have been reported [7,8]. The low paternity rate of

hybrid males could result from females actively seeking extra-pair copulations when paired to less attractive males [6]. Furthermore, the sperm of hybrid males may also compete poorly against pure-species sperm, and mechanisms of cryptic female choice may be at play [9]. In addition, hybrid males may produce low quantities of sperm, or the sperm may swim poorly or fail to fuse with eggs because of incompatibilities between reproductive proteins [9].

In this study, we aim to test whether the reduced fitness of hybrid flycatcher males is caused solely by their low sexual attractiveness, as suggested in previous studies [6], or whether they also experience reduced fertility. Moreover, we aim to investigate whether a potential decrease in fertility is caused by suboptimal sperm traits or more severe genetic incompatibilities by comparing ejaculates, hatching failures and rates of paternity among hybrid, pied and collared flycatcher males.

2. Material and methods

Male collared, pied and hybrid flycatchers were caught during three breeding seasons (2010–2012) on the island of Öland, Sweden, in a study area monitored since 2002 [6]. Ejaculates were sampled from 102 individuals by massaging the cloacal protuberance [10]. We collected all unhatched eggs and sampled blood from all breeding individuals (males and females) and nestlings, as part of the population monitoring. Hybrids were identified through their intermediate plumage (broken collar on their neck) and mixed song (incorporating characteristics of both parental species songs) [5]. Hybrid status was genetically confirmed for a subset of six individuals that were genotyped using 42 000 single-nucleotide polymorphisms (H. Ellegren and A. Qvarnström, unpublished data). The ejaculates, preserved in formalin (5%), were scanned under the microscope (Olympus BX41, magnification 400×) and 30 randomly chosen spermatozoa per male were photographed with a digital camera (Nikon digital sight DS-2M v. 2 Megapixels). When possible (i.e. when present and not degraded, $n = 8$), the perivitelline layer of unhatched eggs was dissected and scanned for sperm using fluorescence microscopy [11]. The data were deposited in the Dryad repository: http://dx.doi.org/10.5061/dryad.5d44q [12].

Paternity analyses were performed on all nestlings with known social fathers in 2010, and only on nestlings from the nests of hybrid males in 2011 and 2012 ($N = 443$). Nestlings and their putative parents were compared at 10 microsatellite loci (FhU1, FhU2, FhU3, FhU4, PdoU5, Fhy304, Fhy401, Fhy403, Fhy407 and Fhy454) using the software CERVUS (v. 3.0.3, copyright Tristan Marshall 1998–2007, www.fieldgenetics.com). We simulated data for 10 000 offspring allowing five different candidate fathers, assuming that 70 per cent of the individuals in the population had been sampled. We only included individuals with data from at least four microsatellite loci. The criterion for paternity assignment was a 95% confidence level. Statistical analyses were performed in R [13].

3. Results

We found no spermatozoa in the ejaculates of 11/13 hybrids sampled between 2010 and 2012. Samples from the other two hybrid males contained sperm with only abnormal morphology (figure 1): thick, undeveloped heads; no visible mitochondrial helix; and an overall irregular shape compared with sperm from pure-species males. Sperm were found in 70 per cent of the collared and pied flycatcher ejaculates,

and no abnormal sperm was noted (table 1). The proportion of samples without morphologically normal sperm was significantly higher in hybrid (13/13) than in pure-species males (27/89) (Fisher's exact test, $p < 0.001$).

Eight females paired with hybrids laid full clutches of eggs, which never hatched ($N = 56$ eggs, 6.22 eggs per clutch). Thus, 62 per cent (8/13) of the hybrid males' nests presented complete hatching failure (none of the eggs in the clutch hatched), which is significantly more than in collared (5.5%) and pied flycatchers (10.7%) (Fisher's exact test, $p < 0.001$; table 1). No sperm was detected on the perivitelline membrane of the eight unhatched eggs examined.

Five hybrid males raised young in their nests (36 nestlings in total, six nestlings per clutch). Based on paternity analysis, none of these nestlings was sired by the hybrid social father; they all resulted from extra-pair copulations (table 1). By contrast, significantly more offspring (82%) were sired by the social male among pure-species collared and pied flycatchers (Fisher's exact test, $p < 0.001$; table 1).

4. Discussion

We report strongly reduced fertility of male hybrids between collared and pied flycatchers under natural conditions. The lack of normal sperm and the incapacity to sire offspring suggest that hybrid males experience impaired sperm production, despite a relatively short divergence time between the parental species and the frequent occurrence of heterospecific pairing in this hybrid zone. Reproductive isolation is known to evolve slowly in birds [14] and complete speciation is often observed without any consequence for hybrid sterility or inviability, owing to strong premating barriers [15] (e.g. Darwin finches [14], ducks [16], pigeons and doves [17]). The average divergence time for the production of infertile hybrids in birds is seven million years (estimated for 368 species using taxonomic relationships, phylogenies generated from DNA–DNA hybridization and divergence in mtDNA sequences) [18]. Because pied and collared flycatchers diverged less than 2 Ma [19], we did not expect such strong signs of infertility in hybrid males. Although several factors may contribute to low fitness of hybrid males, the combined results of our study, i.e. the lack of normal sperm in the ejaculates of the hybrid males and the fact that they did not sire any offspring, imply highly reduced fertility.

Our results suggest that the fertility of male hybrid *Ficedula* flycatchers has been over-estimated in the past, possibly because previous studies used either indirect evidence for hybrid fertility (e.g. hatching success [20]) or few genetic markers to confirm paternity and/or hybrid status [7,8,20]. This has important implications for interpreting the dynamics of this recent hybrid zone. Veen *et al.* [21] suggested that hybridizing female collared flycatchers could bias the sex ratio of their offspring towards fertile hybrid males and benefit from better habitat conditions, thus presenting hybridization as adaptive under certain conditions. This is clearly not the case if hybrid males suffer from strongly reduced fertility, as our results suggest.

Sperm is known to evolve very rapidly [9] and divergence in sperm characteristics between the parental species could lead to suboptimal fertilization capacity in hybrids with intermediate sperm traits. The severity of problems affecting hybrid sperm should depend on the degree of divergence
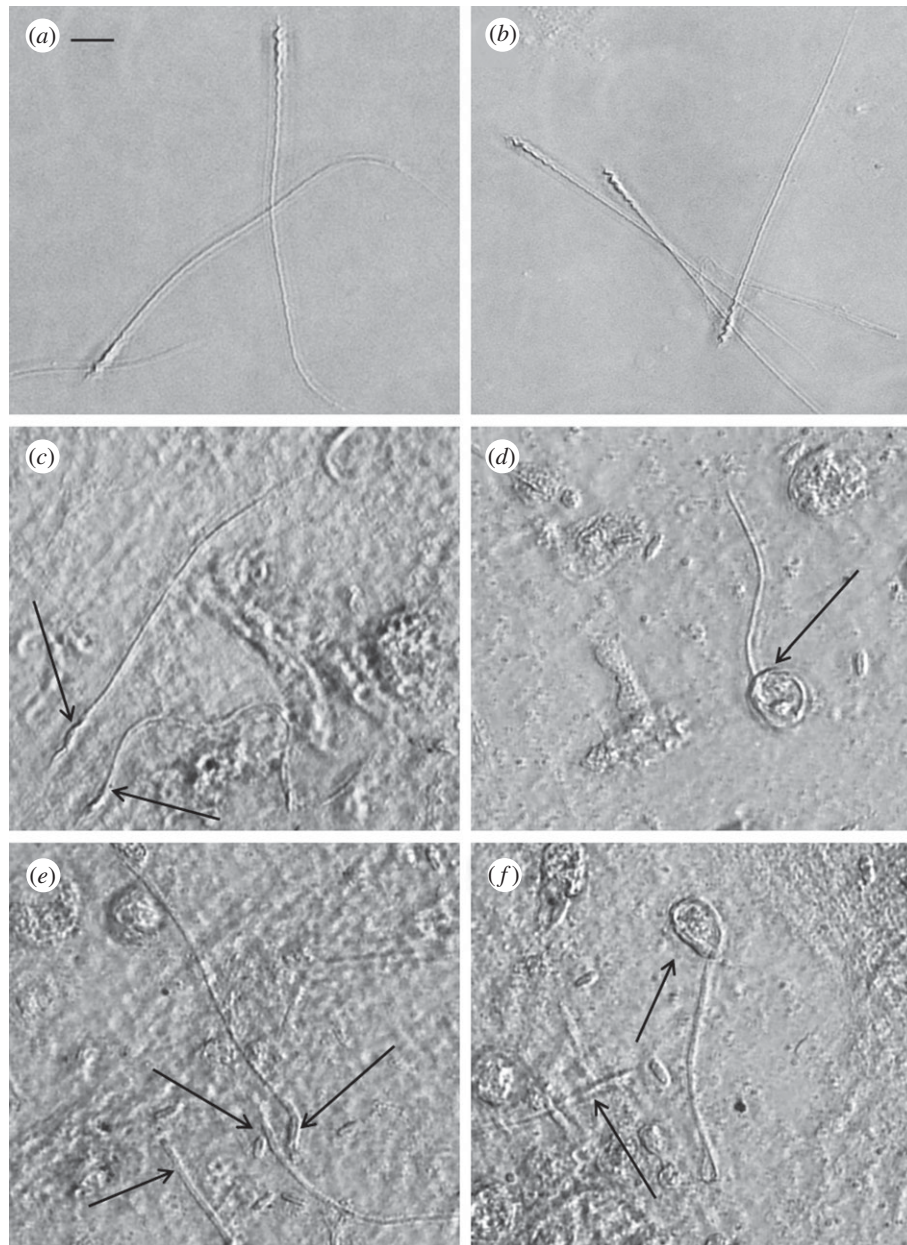
Figure 1. Flycatcher sperm. Scale bar (*a*) = 10 μm, identical for all pictures. (*a*) Typical sperm from a collared flycatcher, (*b*) typical sperm from a pied flycatcher, and (*c*–*f*) abnormal sperm from two hybrid flycatchers, indicated by arrows.

Table 1. Comparison of the occurrence of morphologically normal sperm, abnormal sperm, clutches with complete hatching failure (where none of the eggs in the clutch hatched) and extra-pair nestlings between hybrid, collared and pied flycatchers.

	hybrid	collared	pied
males with normal sperm	0% (*N* = 13)	68% (*N* = 68)	76% (*N* = 21)
occurrence of abnormal sperm	2/13 males	never observed	never observed
clutches with complete hatching failure (*N* = nb of clutches)	62% (*N* = 13)	5.5% (*N* = 109)	10.7% (*N* = 28)
% of extra-pair nestlings (*N* = nb of nestlings)	100% (*N* = 36)	17.2% (*N* = 331)	22.4% (*N* = 76)

between the parental species. The sperm of hybrid *Lepomis* sunfish is functional but outcompeted by the sperm of both parental species [22], while hybrid *Nasonia* wasps produce low sperm quantity [23]. Different degrees of hybrid sterility were observed in different combinations of crosses of *Drosophila* [24] and house mice [25]. However, most studies describe cases of severe spermatogenesis dysfunction, and there is hence no consensus on whether reduced hybrid fertility evolves gradually or not. We know from *Drosophila* that misregulation of genes that play a minor role in normal spermatogenesis can have strong effects on hybrid sterility [26], which could lead to an abrupt loss of fertility in hybrids, rather than a gradual build-up of sterility. The genetics of hybrid sterility have been studied in heterogametic males (XY sex chromosomes, e.g. mammals and insects), but little is known about incompatibilities in homogametic males

(ZZ, e.g. birds and Lepidoptera). The mechanisms are likely to be more complex in this case since males with two copies of the same sex chromosome can compensate for recessive incompatibilities and will only be affected by dominant ones. The pied and the collared flycatchers have very similar nuclear genomes that seem to lack any important chromosomal rearrangements or chromosome inversions [19]. Possible mechanisms behind this relatively fast evolution of hybrid male incompatibilities include mitonuclear interactions and meiotic drive [19].

By combining different methods to investigate reproductive success in a natural hybrid zone, we show that strongly impaired fertility can evolve relatively quickly in hybrids between recently diverged bird species, possibly because of the sensitivity of spermatogenesis to any genetic change. More studies are needed before we can conclude whether hybrid sterility generally evolves in an abrupt fashion where few genetic changes have large phenotypic consequences, or whether reduced fertility may sometimes build up gradually.

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