

Tracking hybrid viability across life stages in a natural avian contact zone

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Abstract

Hybrid inviability is an important post-zygotic reproductive barrier between species, but emerging signs of reduced viability can be difficult to study across the lifespan of natural hybrids. We use a combination of long-term monitoring, extra-pair paternity, and mitochondrial DNA identification in a natural hybrid zone of *Ficedula* flycatchers to detect emerging signs of intrinsic hybrid inviability across their entire lifespan. We evaluate possible evidence of Darwin's corollary to Haldane's rule, predicting asymmetries in inviability between hybrids resulting from reciprocal crosses, due to incompatible genetic factors with sex-specific inheritance patterns. We found higher hatching failure among mixed-species pairs, possibly indicating early developmental impairments associated with specific parental genetic combinations. Adult hybrids had a higher basal mortality rate than both parental species and different age-specific mortality trajectories. There were signs of differences in age-independent mortality rates between the reciprocal hybrid crosses: hybrids with a pied flycatcher mother experienced slightly increased mortality later in life. Using an exceptional dataset with many natural hybrids tracked across life stages, we provide evidence for several emerging signs of reduced hybrid viability. Incompatibilities between alleles located on autosomes and uniparentally inherited factors such as Z-linked and/or mitochondrial genes are strong candidates underlying intrinsic hybrid dysfunction in this system.

Keywords: hybrid viability, genetic incompatibilities, *Ficedula* flycatchers, survival, hybrid zone, long-term monitoring

Introduction

Hybrid intrinsic dysfunction is thought to represent an important reproductive barrier between genetically diverged populations (Coyne & Orr, 2004). The Bateson–Dobzhansky–Muller model has become a keystone concept in speciation research (Coyne & Orr, 2004) by explaining hybrid dysfunction as the result of epistatic incompatibilities between two or more genetic elements that have diverged between the parental populations (Bateson, 1909; Dobzhansky, 1936; Muller et al., 1942). Bateson–Dobzhansky–Muller incompatibilities (BDMIs) are predicted to accumulate increasingly fast, i.e., “snowball,” once they start to arise (Orr, 1995) but should also be highly sensitive to purging at secondary contact (Barton & Bengtsson, 1986; Blanckaert et al., 2020; Xiong & Mallet, 2022). Empirical knowledge of how genetic incompatibilities develop and manifest themselves in nature lags far behind theoretical work on the subject (Reifová et al., 2023). The general understanding of the role that BDMIs play during speciation and/or in hindering speciation reversal, therefore, remains elusive.

BDMIs have been characterized in detail in model systems such as fruit flies, marine copepods, yeasts, and plants (Ellison & Burton, 2008; Lee et al., 2008; Presgraves, 2010b; Sweigart et al., 2006; Tang & Presgraves, 2015) and in very

few vertebrates (Powell et al., 2020; Turner & Harr, 2014). These studies support early observations that when hybrids suffer from incompatibilities, hybrid sterility often evolves before hybrid lethality (e.g., Moyle & Graham, 2005; Presgraves, 2010b; Turissini et al., 2018), although subtle differences in selection against hybrids may often be overlooked (e.g., Delmore & Irwin, 2014). In addition, hybrids of heterogametic sex usually suffer most from lethality and sterility among the interspecific F1 hybrids (i.e., Haldane's rule, Haldane, 1922). These patterns likely arise because hybrid sterility seems to require fewer negatively interacting loci than hybrid lethality (e.g., Bundus et al., 2018) and because recessive X- or Z-linked BDMI alleles are expressed in hemizygous hybrids but masked in the homozygous sex (Muller et al., 1942; Turelli & Orr, 1995). Another common observation is that the degree of sterility or inviability can differ strongly between hybrids resulting from reciprocal crosses, a pattern that is referred to as Darwin's corollary to Haldane's rule (Good et al., 2008; Presgraves, 2002). The latter phenomenon highlights the contribution of genetic factors with sex-specific inheritance patterns, because the parents are of two different species, and sex chromosomes and mitochondria are inherited differently in reciprocal hybrids. Genetic factors with sex-specific inheritance patterns generally diverge quickly

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between populations, for different reasons. For example, genetic conflict over the transmission of sex chromosomes can lead to coevolving meiotic drive systems and rapid divergence of genes related to gametogenesis (Presgraves, 2010a). Furthermore, maternally inherited mitochondria can be incompatible with the paternally inherited genome in hybrids, as there is tight co-adaptation between the nuclear and mitochondrial genomes (Burton et al., 2013; Hill, 2017).

Detailed studies of BDMIs and their effects on hybrid phenotypes in natural systems are limited. Cline analyses provide solid evidence for an important role of divergent or disruptive natural selection driving populations genetically apart (Johannesson et al., 2020; Mullen & Hoekstra, 2008; Stankowski et al., 2017) and studies of natural hybrid zones often reveal evidence of asymmetric patterns of gene flow (Teeter et al., 2008; Wallace et al., 2011). However, the relative effects of various reproductive barriers generally remain unknown. Asymmetric patterns of gene flow do not necessarily result from Darwin's corollary to Haldane's rule but can also be caused by asymmetry in other sources of reproductive isolation. For example, biases in pre-mating barriers (Hochkirch & Lemke, 2011; Takami et al., 2007) or in post-mating, prezygotic isolating mechanisms (Dean & Nachman, 2009; Pease et al., 2016) can alter the initial proportion of produced offspring from reciprocal crosses and thereby consecutive patterns of gene flow. In natural populations, the scenario is further nuanced by demographic dynamics such as migration and asymmetric population sizes, which can override prezygotic isolation mechanisms in biasing hybridization patterns (Qvarnström et al., 2023). To fully understand the build-up of intrinsic postzygotic isolation under natural conditions, we require high-resolution data to account for these factors.

Naturally hybridizing collared (*Ficedula albicollis*) and pied flycatchers (*F. hypoleuca*) are an ideal study system for examining emerging intrinsic incompatibilities causing reduced viability in hybrids, due to fairly high levels of bidirectional mixed-species pairing combined with the possibility to follow the fate of individual birds throughout their lives (Qvarnström et al., 2010). The two species diverged less than one million years ago (Nadachowska-Brzyska et al., 2013), share similar breeding biology and exist in sympatry in Central and Eastern Europe and on the Swedish islands of Öland and Gotland (Alatalo et al., 1990; Qvarnström et al., 2010). Genomic analyses suggest recent asymmetric gene flow from pied to collared flycatchers, occurring after the Last Glacial Maximum (Nadachowska-Brzyska et al., 2013) but monitoring data reveals strong current levels of reproductive isolation. We observe multiple premating barriers between the two species. Habitat isolation arises as the less aggressive pied flycatchers are displaced to poorer breeding habitats (Rybicki et al., 2016). Furthermore, collared flycatchers, more sensitive to starvation, have a stronger response to selection for earlier breeding with climate change induced earlier peaks in food availability, resulting in temporal isolation (Sirkiä et al., 2018). There is also assortative mate choice: females prefer males of their own species based on male song and plumage characteristics (Qvarnström et al., 2006; Saether et al., 2007; Wiley et al., 2005). Moreover, postzygotic isolation is high despite the short divergence time. Hybrid nestlings have an intermediate growth strategy and environment-dependent survival (Vallin et al., 2013), all hybrids appear sterile: females experience complete hatching failure and males suffer from impaired sperm morphology

and lack paternity in their nests (Ålund et al., 2013). In addition, there is evidence of physiological dysfunction expressed as the high metabolic rate in hybrid males, which may result from mito-nuclear incompatibilities disrupting mitochondrial respiration (McFarlane et al., 2016; Van der Heijden et al., 2019). Taken together, pied and collared flycatchers can be considered to have reached an advanced stage in the speciation continuum with many documented sources of prezygotic reproductive barriers, and some postzygotic barriers present (extrinsic and intrinsic affecting fertility). The current study evaluates if the ongoing accumulation of incompatibilities starts to manifest as reduced hybrid viability. An earlier study that reported successful reproductive events of *Ficedula* hybrids detected no evidence of reduced hybrid viability (Veen et al., 2001). However, the lack of molecular resources at that time probably resulted in the misclassification of nonadmixed individuals as hybrids, and thus the false conclusion that later generation hybrids could still be produced between the species, because all hybrids later screened using more extended genomic information have proven to be sterile (Ålund et al., 2013; Segami et al., 2022a). Reproductive isolation is thus complete in this system, and we are focusing here on detecting possible differences in viability between hybrid and nonadmixed individuals. Although BDMIs detected after the stage of complete reproductive isolation may not strictly form part of the speciation process, such BDMIs may nevertheless play an important role by reducing the risk of speciation reversal.

Here we examine F1 hybrid viability across different life stages. Since intrinsic genetic incompatibilities often show asymmetry depending on the pairing combination, we are particularly interested in evaluating such patterns. Previous research on the flycatchers shows a higher level of species divergence on the Z chromosome (Hogner et al., 2012; Nadachowska-Brzyska et al., 2013), and divergence in important, maternally transmitted mitochondrial genes (McFarlane et al., 2016; Van der Heijden et al., 2019). We use a combination of 17 years of long-term monitoring of individual birds in a hybrid zone and molecular information to evaluate possible evidence of Darwin's corollary to Haldane's rule. Extra-pair paternity analyses allow us to distinguish between hybrid nestlings and their conspecific half-siblings (Cramer et al., 2016) and mitochondrial DNA identification reveals the maternal species of immigrant hybrid adults. The detailed monitoring records of both adults and their offspring means that we can control for possible biases in the initial production of hybrid individuals through asymmetries in prezygotic isolation and for possible biases in the immigration of hybrids with respect to their maternal species. Since emerging signs of reduced hybrid viability may be caused by ecological factors selecting against their intermediate phenotype, we consider patterns of consistently reduced hybrid viability compared to nonadmixed individuals across environmental conditions as the most reliable evidence for underlying BDMIs. Our two main expectations are that (a) the most severe emerging genetic incompatibilities affecting hybrid viability (i.e., including nonfixed genetic variants between the two species) should be revealed already during the early developmental stages of the hybrids, and (b) possible differences in survival between hybrids from the reciprocal crosses would signal genetic incompatibilities involving uniparentally inherited factors.

Material and methods

Long-term monitoring

We studied collared and pied flycatchers on the island of Öland, Sweden, where they have occurred in sympatry since the 1960s and are known to hybridize (Cramer et al., 2016; Sirkiä et al., 2018). The population has been monitored during the May and June breeding season every year since 2002, using over 2,000 nestboxes distributed in forest patches across the island (Qvarnström et al., 2010). Each year, all nestboxes are monitored for signs of nest building and the day the first egg is laid is recorded (hereafter laying date). Females lay five to seven eggs incubated for 12 days. All adults are identified by species according to their plumage, alarm call, and song characteristics, marked with unique rings, measured and blood is sampled for DNA analyses. The initial number of nestlings is recorded shortly after hatching, and they are measured at 12 days post hatching (mass and tarsus), individually ringed and small samples of their blood are taken. The proportion of hatched nestlings still alive at 12 days of age is used as “survival in the nest” in our analyses below. Recapture of adult birds over multiple years is used in our analyses of adult survival.

Environmental factors are likely to strongly influence hybrid fitness in the early stages of the evolution of incompatibilities. Food availability differs strongly across the breeding season depending on the tree composition around each nestbox, as different tree species support different amounts of caterpillars, the main and most nutritious food for nestlings (Rybinski et al., 2016; Sirkiä et al., 2018). The strong seasonal variation in food availability is captured by adding laying date and habitat quality as co-variables in all analyses. Habitat quality was estimated for each breeding pair by combining measures of tree species and size in a 150 m perimeter around their nestbox, with estimates of caterpillar availability on each tree species. Caterpillar larvae biomass was estimated through a collection of fecal pellets under the 12 most common tree species in our study areas in 2013, and the habitat quality index is thus computed in mg of caterpillar feces produced per m³ of feeding territory over the nesting phase (see Rybinski et al., 2016 for more details).

Unhatched eggs development

Unhatched eggs were collected from natural nests at least 3 days after the predicted hatching date, either after all other nestlings had hatched in the nest or when the absence of a growing embryo was visually confirmed. Eggs were carefully opened within a few days of collection, and the contents were inspected visually. Any sign of development was noted (e.g., visible blood vessels, nonintact yolk, or visible embryo or fetus) and the content was preserved in ethanol for further investigation. Eggs with completely intact yolks were preserved in formalin. All animal monitoring and handling was in accordance with Swedish laws, following the ethical standards of Linköping's committee of ethical animal research (Linköpings djurförsöksetiska nämnd).

Paternity analyses and hybrid mitochondrial DNA identification

Occurrence of extra-pair copulations is highly elevated in mixed-species pairs (up to 62% of all nestlings [Cramer et al., 2016]), and many of the nestlings born and raised by mixed-species parents are therefore not actually hybrids,

as nearly all extra-pair offspring are sired by an extra-pair male of the same species as the female (Cramer et al., 2016). Paternity analyses were performed on 895 nestlings from 182 nests with known, mixed-species social parents between 2002 and 2018, based upon allele-sharing at 12 polymorphic microsatellite markers (see Supplementary materials for detailed methods). In CERVUS 3.03 (Kalinowski et al., 2007), we simulated data for 10,000 offspring with five candidate fathers, assuming 70% of the population had been sampled and only included individuals that could be compared at six microsatellite loci or more. Offspring were considered extra pair if the confidence level for the pair-wise comparison with their social father was less than 95%. The maternal species identity of immigrant F1 hybrids of unknown parents was determined by amplification of a stretch of mtDNA containing a 32-bp indel as described in (Saetre & Mowm, 2004).

Statistical analyses

All statistical analyses were performed using R (version 4.3.1) (see [Supplementary methods](#) and code for details on the packages used). We report the results of the analysis of deviance with Type III Wald tests in the main text, and linear model coefficients in the tables corresponding to each model. For most models (see details in [Table 1](#)), we fitted the response variable of interest and included the following explanatory variables: a three-way interaction between pairing type (either two collared, two pied, a collared female and a pied male, or a pied female and a collared male), standardized laying date and habitat quality. The year was added as a random effect in all models. To control for nonindependence of nestlings raised in the same nests, and of those born from the same mothers over multiple years, a unique nest identifier, and the mother's identity were added as random effects where applicable. The laying date was standardized around zero for each year, zero being the day when the maximum number of nests were started each given season.

Many analyses of hybrid zones focus solely on the genetic composition of the individuals. Here we instead assessed the survival of hybrids compared to nonadmixed individuals across several life stages. This approach enables us both to detect at what life stages possible signs of reduced hybrid viability are present and to control for possible biases in pre-zygotic isolation. We also investigate patterns of dispersal and immigration, which may have a strong impact on the genetic structure of the monitored mixed-species populations of flycatchers. To achieve these goals, we used nine different linear models described in [Table 1](#) and in the [Supplementary materials](#), complemented by Bayesian analyses of adult survival (see next section). The response variables for the linear models were: (a) hatching success (indicator of survival during development in the egg), (b) nestling survival to the age of 12 days, (c) nestling mass at 12 days (often used as an indicator of general health and correlates with survival probabilities to return and breed as adult [Linden et al., 1992]), (d) relative survival of hybrid versus nonhybrid half-siblings in mixed-species nests, (e) mass of hybrid versus nonhybrid half-siblings in mixed-species nests, (f) recruitment (individuals ringed as nestlings later recruiting to the breeding population as adults), (g) natal dispersal (the distance in meters between the birth nest and the first nest where a recruit was found breeding as an adult), (h) breeding dispersal (the average distance between two nestboxes in which each adult individual bred across all years where they were recorded), and

Table 1. List of the statistical models, variables included, and sample sizes.

Model number	Model name	Model type	Response variable	Type of response variable	Sample size	Explanatory variable	Random effects
1	Hatching success	GLMM	Number of hatched/un-hatched eggs	Binomial logit	3,643 nests	Pairing type * laying date * habitat quality	Year (N = 17) Mother identity (N = 2,332)
2	Nestling survival	GLMM	Alive/dead at 12 days	Binomial	22,586 nestlings	Pairing type * laying date * habitat quality	Nest: N = 3,526 Year: N = 17 Mother identity (N = 2,636)
3	Nestling mass	LMM	Mass at 12 days	Continuous	18,291 nestlings	Pairing type * laying date * habitat quality	Nest (N = 3,144) Year: N = 17 Mother identity (N = 2,374)
4	Half-siblings survival	GLMM	Alive/dead at day 12	Binomial	662 nestlings	Nestling species (collared/pied/hybrid with collared mother/ hybrid with pied mother) * laying date * habitat quality + proportion of extra-pair nestlings	Nest (N = 131) Year (N = 17) Mother identity (N = 122)
5	Half-siblings mass	LMM	Mass at 12 days	Continuous	571 nestlings	(Collared/pied/hybrid with collared mother/hybrid with pied mother) * laying date* Habitat quality + proportion of extra-pair nestlings	Nest (N = 118) Year (N = 17) Mother identity (N = 109)
6	Recruitment	GLMM	Recruited to the population as an adult (yes/no)	Binomial	22,947 nestlings	Nestling species (Collared/ pied/ hybrid) * laying date * habitat quality	Nest (N = 3,530) Year (N = 16) Mother identity (N = 2,629)
7	Natal dispersal	LMM	Distance between birth and first breeding nestbox	Continuous	1,247 recruiting individuals	Species (collared/pied/ hybrid)	Year (N = 17)
8	Adult dispersal	LM	Average distance between 2 breeding nestboxes across all records	Continuous	7,201 adult individuals	Species (collared/ pied/ hybrid with collared mother/ hybrid with pied mother)	-
9	Immigration	GLMM	Number of immigrant/ returning adults	Binomial logit	10,681 adult individuals	Species (collared/ pied/ hybrid with collared mother/ hybrid with pied mother)	Year (N = 15)

(i) immigration from neighboring, nonmonitored areas (the proportion of adult individuals that were not ring-marked as nestlings). Nestlings born in 2018 were excluded from the recruitment analysis because there was no information yet on their recruitment as adults in this dataset. The two first years of our long-term monitoring, 2002 and 2003, were excluded from the immigration analysis since most of the adult birds breeding these years could not have been previously ringed as nestlings.

We ran two types of extra simulations confirming our results are robust to (a) assumption of species status in extra-pair nestlings (i.e., simulation of 10% of these nestlings assigned as hybrids again instead of unadmixed half-siblings) and (b) large differences in sample sizes between collared, pied, and hybrid flycatchers (resampling and permutation tests with equal sample sizes for all pairing types, equal to that of the less abundant pied flycatchers, see Supplementary materials).

The contribution of each barrier in reducing the number of hybrids produced was calculated following Sobel and

Chen (2014), and their equation RI_{454} as described in their [Supplementary Appendix D](#), accounting for the differences in abundance between parental species. This formula compares the number of observed heterospecific matings or hybrid individuals produced to the expected number under random mating or equal survival to unadmixed individuals, taking into account the numbers of both conspecific and heterospecific matings.

Hybrid survival through adult lifespan

To estimate survival differences and mortality patterns across the whole adult lifespan of collared, pied flycatchers and hybrids resulting from the two types of crosses between the parental species, we performed a Bayesian survival trajectory analysis (BaSTA) ([Colchero et al., 2012](#)). This package works with mark-recapture data, and it is especially suitable for our data, as it deals with missing birth and death information ([Colchero et al., 2012](#), detailed methods in [Supplementary materials](#)). We explored the three available mortality functions

(Gompertz, Logistic, and Weibull) together with three different mortality shapes (simple, Makeham, and bathtub), as well as the exponential function with a simple shape, following the advised procedure in (Colchero et al., 2012), and retained the model with the lowest deviance information criterion (DIC, similar to the Akaike information criterion [AIC]), indicating the best fit to our dataset. Trace plots were visually inspected to assure convergence and we controlled that the potential scale reduction for each model was close to 1, and that serial autocorrelations were lower than 0.05. The Kullback–Leiber discrepancy criterion (KLDC) was used to assess parameter differences between the categorical covariates, measuring the overlap between the model parameters' posterior distributions (Burnham & Anderson, 2001).

We first fitted a model using all of our records of birds of at least one year old from 2002 until 2018 with species (collared, $N = 5,805$, pied, $N = 1,314$, or hybrid, $N = 153$) as a categorical covariate using four Markov Chain Monte Carlo (MCMC) parallel simulations with 1,200,000 iterations, 200,000 burn-in period and thinning of 500. The logistic model following a bathtub shape had the lowest DIC (Supplementary Table S1). This model has an initial exponential increase in mortality that later decelerates until it reaches a plateau (Pletcher, 1999) and the bathtub shape allows modeling both the decrease in mortality as animals mature, and a later increase in mortality with senescence.

To explore differences between the two types of hybrid crosses, we used a subset of data with hybrid individuals for which the mother species was known ($N = 79$, 30 with collared mtDNA, 49 with pied mtDNA), and fitted the models using maternal species as a categorical covariate. Because the data set was smaller, we fitted the models using five MCMC parallel simulations with 3,000,000 iterations, 700,000 burn-in period, and thinning of 4,000. The three Gompertz models converged appropriately, but their goodness-of-fit metrics (pD) and by extension their DIC values were calculated at negative infinity, suggesting improper posteriors, and we thus removed them from further consideration. The Weibull model, which allows modeling accelerating/decelerating increases, decreases, and constant mortality rates (Pinder et al., 1978) with a simple shape had the lowest DIC (Supplementary Table S2). However, all retained models showed moderate levels of overdispersion, suggesting either nonindependence between hybrid types or, more likely in our case, higher variance than optimal for our sample size (Amstrup et al., 2010). Note that while hybrid males may be less likely to breed than males of the parental species, they are usually detected via their mixed song and caught each year even if they do not attract a female. Furthermore, we have no a priori reason to expect differential recapture probabilities for one of the hybrid crosses, and thus do not expect the relative difference in mortality between crosses to be affected by sampling probability.

All data and code used for these analyses is available on Dryad (Ålund et al., 2023).

Results

We monitored 3,775 breeding flycatcher pairs in Öland, Sweden, between 2002 and 2018, including 187 mixed-species pairs, most often formed by a pied flycatcher female and a collared flycatcher male (hereafter “mixed pied,” while mixed-species pairs of the opposite cross will be referred to

as “mixed collared,” Figure 1). Confirming previous results based on smaller datasets (Rybinski et al., 2016; Sirkiä et al., 2018), the different types of breeding pairs settled in habitats of significantly different quality ($F_3 = 163.76$, $p < .001$, Supplementary Table S3), differed in the onset of breeding (first egg laid, $F_3 = 50.71$, $p < .001$, Supplementary Table S4), and laid clutches of different sizes ($F_3 = 7.65$, $p < .001$, Supplementary Table S5). Collared flycatchers inhabited territories with higher availability of protein-rich caterpillars and started breeding earlier, while the opposite was true for pied flycatchers. Mixed-species pairs bred in territories of intermediate habitat quality and started breeding later than collared, but earlier than pied flycatchers (see Supplementary material for more details). Conspecifically mated pied flycatcher females on average laid larger clutch sizes (mean 6.60 ± 0.78) than collared flycatchers (6.43 ± 0.78 , $T_{03.95}$, $p < .001$, Supplementary Table S5). Female collared flycatchers produced significantly smaller clutches when paired with a pied flycatcher male (6.16 ± 0.92 , $t = -2.51$, $p = .012$), while the clutch size of heterospecifically paired female pied flycatchers (mean $= 6.47 \pm 0.74$ eggs, $t = 0.52$, $p = .61$) did not significantly differ from female pied flycatchers mated to conspecifics.

Nestling survival during development and growth

Offspring growth during the nestling stage is largely affected by breeding time (earlier laying dates better match the peak in caterpillar larvae emergence [Burger et al., 2012]) and habitat quality (differences in food availability [Rybinski et al., 2016]), included in all models examining nestling survival and growth. We used hatching success (the proportion of laid eggs that successfully hatched) as an estimate of survival in the egg for 3,085 collared, 379 pied flycatchers, and 179 mixed-species nests (55 with a female collared and 124 with a female pied flycatcher). There was a significant interaction between laying date and parental pairing type on hatching success ($\chi^2_3 = 9.67$, $p = .021$, $N = 3,643$, Supplementary Table S6). Hatching success was generally lower among late-breeding birds but the decline in success over the season was steepest for collared flycatchers with extremely late-breeding collared flycatchers having very low hatching success (Figure 2). The average hatching success was lowest for females in mixed-species pairs. Female collared flycatchers on average experienced 89.3% (± 16.7) hatching success when paired with a conspecific male and 88.2% (± 16.3) hatching success when paired with a heterospecific male. Female pied flycatchers on average had 89.3% (± 15.1) hatching success when paired with a conspecific male and 86.3% (± 21) when paired with a heterospecific male. This combination, with a pied flycatcher female and a collared flycatcher male, had the lowest hatching rate overall, significantly different from that of collared flycatchers independent of laying date (post hoc test, $z = 2.12$, $p = .034$, Figure 2, Supplementary Table S6).

To assess whether hatching failure was caused by fertilization failure or by embryo mortality, we examined a subset of 241 unhatched eggs for signs of embryonic development. The majority of them showed visible signs of embryonic development (54% of mixed pied eggs, 64% of mixed collared eggs, and 55% and 97% of conspecific collared and pied eggs, respectively, see Supplementary Table S17). Furthermore, in all but two nests where unhatched eggs were opened, other eggs in the same nests showed signs of development, or even hatched, indicating that the female did mate and receive enough viable sperm to fertilize at least some of her eggs.

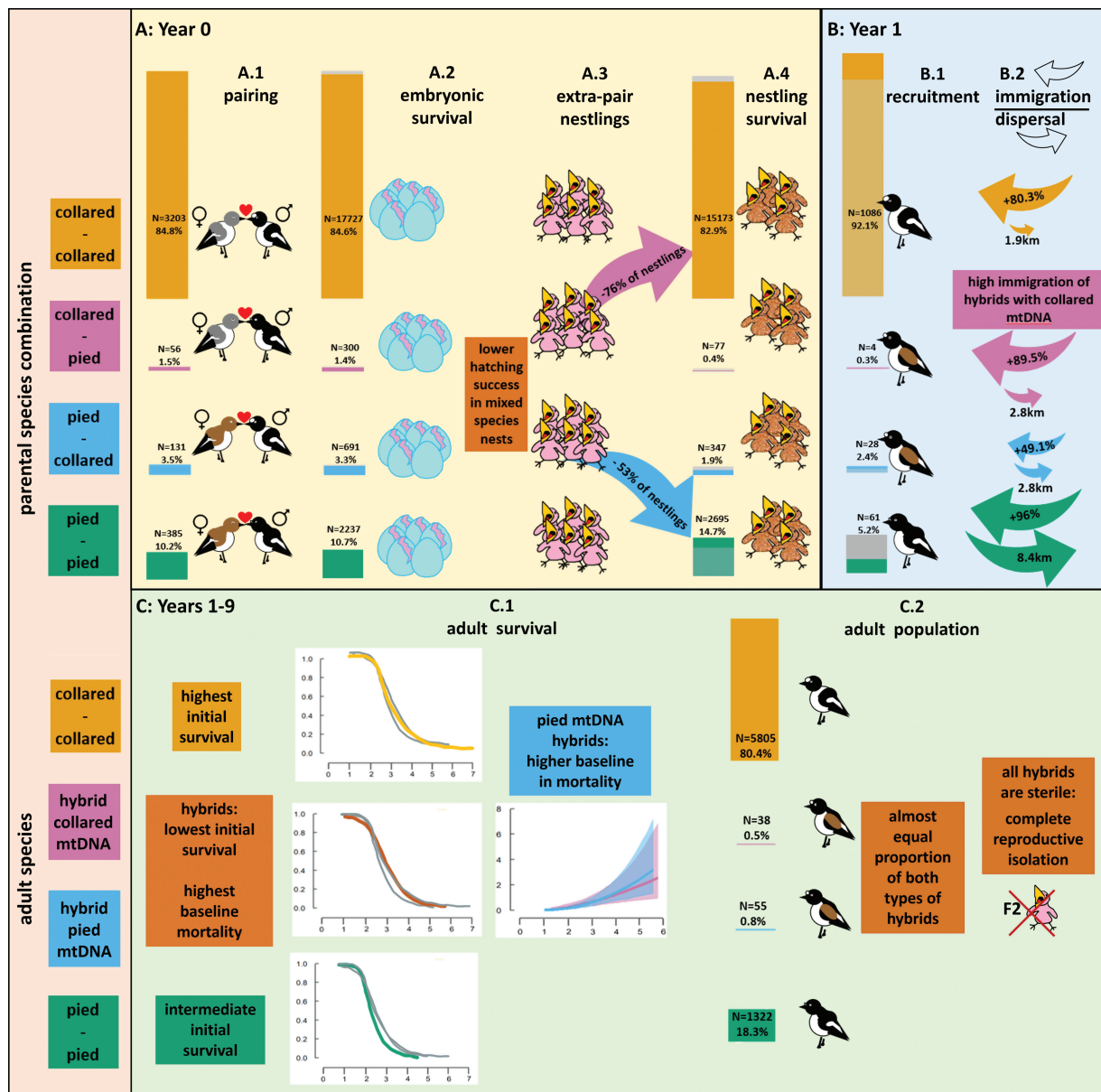


Figure 1. Summary of mixed-population structure and the relative survival of hybrids across the main life stages in a *Ficedula* hybrid zone. Panel A shows stages happening the season hybrids are born (year 0), with strong assortative mating (A.1), lower hatching success in mixed-species nests (A.2), high rates of extra-pair paternity producing nonadmixed nestlings instead of hybrids (A.3), and slightly reduced hybrid nestling survival (A.4), resulting in a bias in initial production of hybrids with pied flycatcher mtDNA. In year 1 (panel B), the initial higher production of hybrids with pied flycatcher mtDNA and their relatively high rate of recruitment (B.1) is counter-acted by a higher rate of immigration of adult hybrids with collared flycatcher mtDNA (B.2). Across their adult life (panel C), hybrids show higher mortality rate than the parental species (C.1), the proportion of adult hybrids (C.2) is lower than what would be expected given pairing patterns (A.1) and the initial bias in hybrids with pied flycatcher mtDNA is nearly absent at this stage. The reduction in number of hybrids compared to what would be expected given random mating, no sperm competition and equal survival rates to that of nonadmixed individuals is shown at the top for years 0 and 1. Note that because all hybrids are sterile, reproductive isolation is complete (C.2). Shaded areas above or on top of the bar graphs in panels A and B indicate the proportion of individuals of each species at the previous stage.

After discarding nests with obvious predation occurring before nestlings were 12 days of age, or missing information on their status at that age, we investigated the survival of 22,586 nestlings. A total of 18,292 were found alive in their nests 12 days after hatching (collared: $N = 15,009/18,625$, mixed collared: $N = 241/303$, mixed pied: $N = 646/787$, and pied: $N = 2,396/2,871$). Laying date significantly affected survival probability such that nestlings born relatively later experienced a lower probability of survival ($\chi^2_1 = 7.53$, $p = .006$,

$N = 22,586$ nestlings from 3,526 nests, [Supplementary Table S7](#)). There was no significant interaction between pairing type and laying date, nor any significant effect of pairing type on nestling survival to day 12.

We also used mass measured 12 days post hatching, just before fledging, as an indicator of nestling health and growth performance, measured for 18,291 nestlings. There was a significant three-way interaction between pairing type, habitat quality, and laying date on nestling mass at 12 days of age

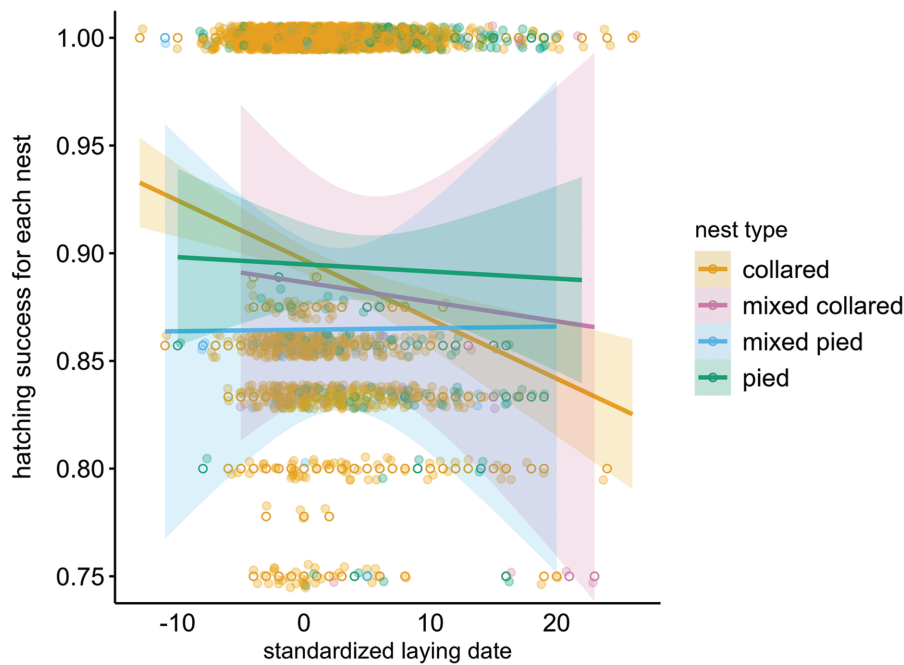


Figure 2. Hatching success plotted as the proportion of eggs that successfully hatched in a given nest depending on the timing of breeding (i.e., laying date standardized around zero being the day for which the majority of breeding pairs started laying each given year). The datapoints are coloured according to the species of the social parents: collared flycatchers nests $N = 3,143$, mixed-species nests with a collared female $N = 56$, mixed-species nests with a pied female $N = 128$, and pied flycatchers $N = 381$. This plot zooms in on the top part of the distribution of hatching rates (between 0.75 and 1) to better visualize the regression lines, and shows a significant interaction between laying date and species combination on hatching success.

($\chi^2_3 = 10.48$, $p = .015$, $N = 18,291$ nestlings from 3,144 nests, Figure 3, Supplementary Table S8). In areas of high habitat quality, collared flycatchers experienced a strong effect of seasonality, with nestlings having the highest mass early, and the lowest mass late in the season. Pied flycatcher nestlings and nestlings raised by mixed-species pairs had lower mass early in the season but there was no decline in average nestling mass over time in these types of nests. In habitats of low quality, most nestlings had higher mass earlier than later in the season, except for nestlings raised in mixed-species nests attended by collared flycatcher females and pied flycatcher males, who did relatively better later in the season.

To control for the strong environmental effects on offspring survival at the nestling stage, we used the natural occurrence of extra-pair paternity in mixed-species nests to compare the performance of hybrid nestlings with their nonhybrid half-siblings (i.e., assuming that extra-pair nestlings are sired by conspecific males in mixed-species nests). There was a significant interaction between species and laying date on the probability of being alive at 12 days of age in the nests of mixed-species parents ($\chi^2_3 = 10.35$, $p = .016$, $N = 803$ nestlings from 131 nests, Figure 4A, Supplementary Table S9). Hybrids with a pied flycatcher mother did relatively worse than their nonhybrid half-siblings earlier in the season, when food is more abundant, but had a slight survival advantage in very late nests. When looking at the mass of hybrids compared to their nonhybrid half-siblings (sired by an extra-pair father) growing in the same nest, we found a significant three-way interaction between species, laying date, and habitat quality ($\chi^2_3 = 9.12$, $p = .028$, $N = 682$ nestlings from 118 nests, Figure 4B, Supplementary Table S10). Differences between hybrids and nonhybrid half-siblings with a pied flycatcher mother were stronger in relatively worse quality habitats, while the opposite was true for mixed-species nests of the other cross:

hybrids with a collared mother did better than their half-siblings early, and worse late in the season in habitats of relatively higher quality. These results hold to a simulated rate of 10% hybrids among extra-pair nestlings (i.e., in rare cases where females would have extra-pair matings with another heterospecific male, see Supplementary material for more details). Thus, patterns of relative survival of hatched hybrids up to the age of 12 days depended on environmental conditions, confirming earlier findings of context-dependent relative hybrid nestling growth at this stage (Vallin et al., 2013).

Probability to return and breed in the population

Out of 22,947 nestlings with known parental species born in our areas from 2002 to 2017, 1,179 returned to breed as adults (Figure 1). There was a significant difference between species in the probability of a nestling later returning to the population to breed ($\chi^2_2 = 36.24$, $N = 22,947$, $p < .001$, Figure 5A, Supplementary Table S11). Collared flycatchers were most likely to recruit as adults (5.8% of the nestlings returned as adults), followed by hybrids (3.7%, posthoc test: $z = -1.94$, $p = .053$) and pied flycatchers with the lowest recruitment rate, significantly different from that of collared flycatchers (1.8%, posthoc test: $z = -5.77$, $p < .001$). The sample size of recruiting hybrids was too low to test for a difference in recruitment rate between hybrids depending on their maternal species.

Immigration of adult individuals from neighboring areas

Of the 10,576 adult individuals caught in our study plots, 5,550 were not ring-marked as nestlings and hence did not hatch in our monitored nest boxes. There were significant differences between species in the proportion of immigrant adult individuals that had dispersed into our study

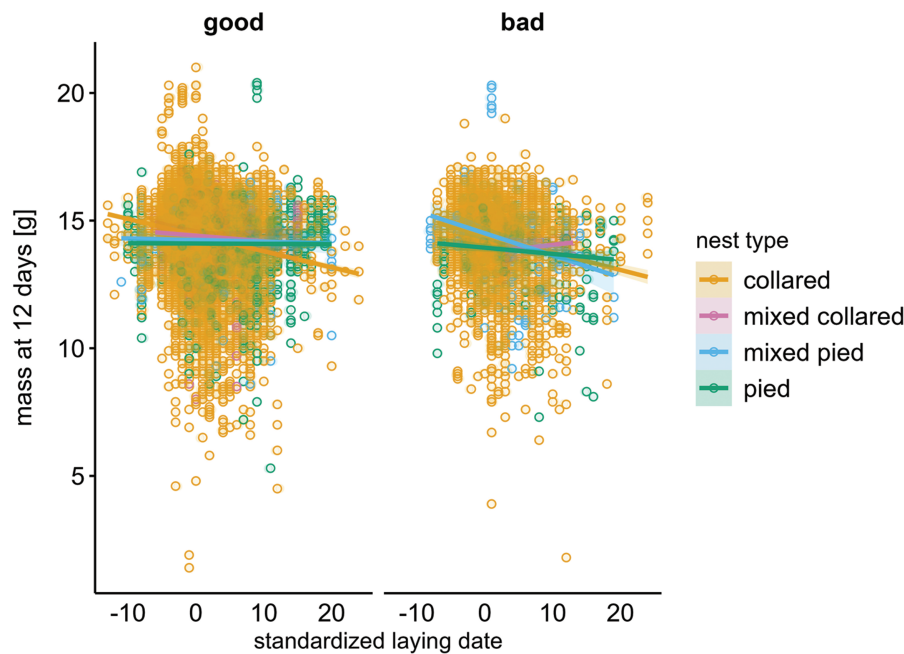


Figure 3. Nestling mass 12 days post hatching, plotted against standardized laying date and separated in two categories according to habitat quality: bad and good. The datapoints are coloured according to the species of the social parents (two collared flycatchers, $N = 15,009$ nestlings, a mixed-species pair with a collared female, $N = 240$, a mixed-species pair with a pied female, $N = 646$, or two pied flycatchers, $N = 2,396$). There was a significant three-way interaction between pairing type, habitat quality and laying date on nestling mass at 12 days of age. In high-quality habitats, collared nestlings are heavier early, lighter late, while pied and mixed-species nestlings are lighter earlier in the season but do not decline in mass with later breeding dates. In bad habitats, all but mixed pairs between a collared female and a pied male show a decline in nestling mass with advancing season.

plots from other places ($\chi^2_3 = 204.55$, $p < .001$, Figure 5B, Supplementary Table S12). Overall, hybrids with a collared flycatcher mother represented the highest proportion of immigrants into our population from other areas in relation to the number of such birds born in our areas (69% of all adult hybrids with a collared flycatcher mother were immigrants), followed by pied flycatchers (67%), hybrids with a pied flycatcher mother (50%), and collared flycatchers (49%).

Dispersal

There was a significant difference in the distance traveled by recruiting adults from their birth nests to the nest where they bred for the first time (i.e., natal dispersal, $\chi^2_2 = 53.56$, $p < .001$, $N = 1,247$, Figure 6A, Supplementary Table S13). Pied flycatchers showed the longest natal dispersal (mean = 8.40 km), followed by hybrids (mean = 2.78 km) and collared flycatchers (mean = 1.90 km).

The average distance between nests from one breeding year to the other (i.e., breeding dispersal) followed a similar pattern (Figure 6B). For this analysis, there were enough individuals to also test for a possible difference between the two types of hybrids ($F_3 = 4.14$, $p = .006$, $N = 7,201$, Supplementary Table S14). Pied flycatchers dispersed the longest distance between nesting sites (mean between-year dispersal: 1.63 km, significantly different from collared, $t = 3.5$, $p < .001$) followed by collared flycatchers (mean between-year dispersal: 0.64 km), hybrids with pied mothers (mean between-year dispersal: 0.47 km), and hybrids with collared mothers (mean between-year dispersal: 0.31 km). However, there was no significant difference between collared flycatchers and the two types of hybrids.

Adult survival

There were substantial differences in mortality rates between the species (see the nonoverlapping prior distributions of parameters a_0 , b_0 , b_1 , and b_2 , Figure 7A). A logistic model with a bathtub shape provides six different parameters describing how mortality and survival rates change across the birds' lifetime. The two alpha parameters (a_0 and a_1) describe the exponential decline in survival that can be observed soon after capture (i.e., between the first and second time an animal is captured). The alpha parameter a_0 showed some difference in apparent mortality after initial capture, which was lowest in collared flycatchers, intermediate in pied flycatchers, and highest in hybrids (KLDC between 0.94 and 1, Figure 7A, Supplementary Table S15). There was no effect of age on the probability of surviving between the first and second capture (a_1). Three beta parameters are also produced, which describe the baseline mortality rate (b_0 , i.e., overall mortality rate across age classes), the initial logistic increase in mortality rate with age (b_1), and the deceleration in an increase in mortality rate before reaching a plateau (b_2). Hybrids showed the highest baseline mortality (b_0) across age classes, characterized by both higher mortality between 1 and 2 years of age (Figure 7C) and a slow deceleration in the increase of mortality rate with age (b_2), meaning that their mortality rate kept increasing later in life (i.e., at 3 and 4 years of age), when collared and pied flycatchers had already reached a plateau in mortality rate around 2 years of age. Collared flycatchers showed an opposite pattern with the lowest baseline mortality, but both a steep increase and quick deceleration in the increase in mortality rate. Pied flycatchers were intermediate in all three parameters. Pairwise KLDC between hybrids and collared flycatchers, hybrids and

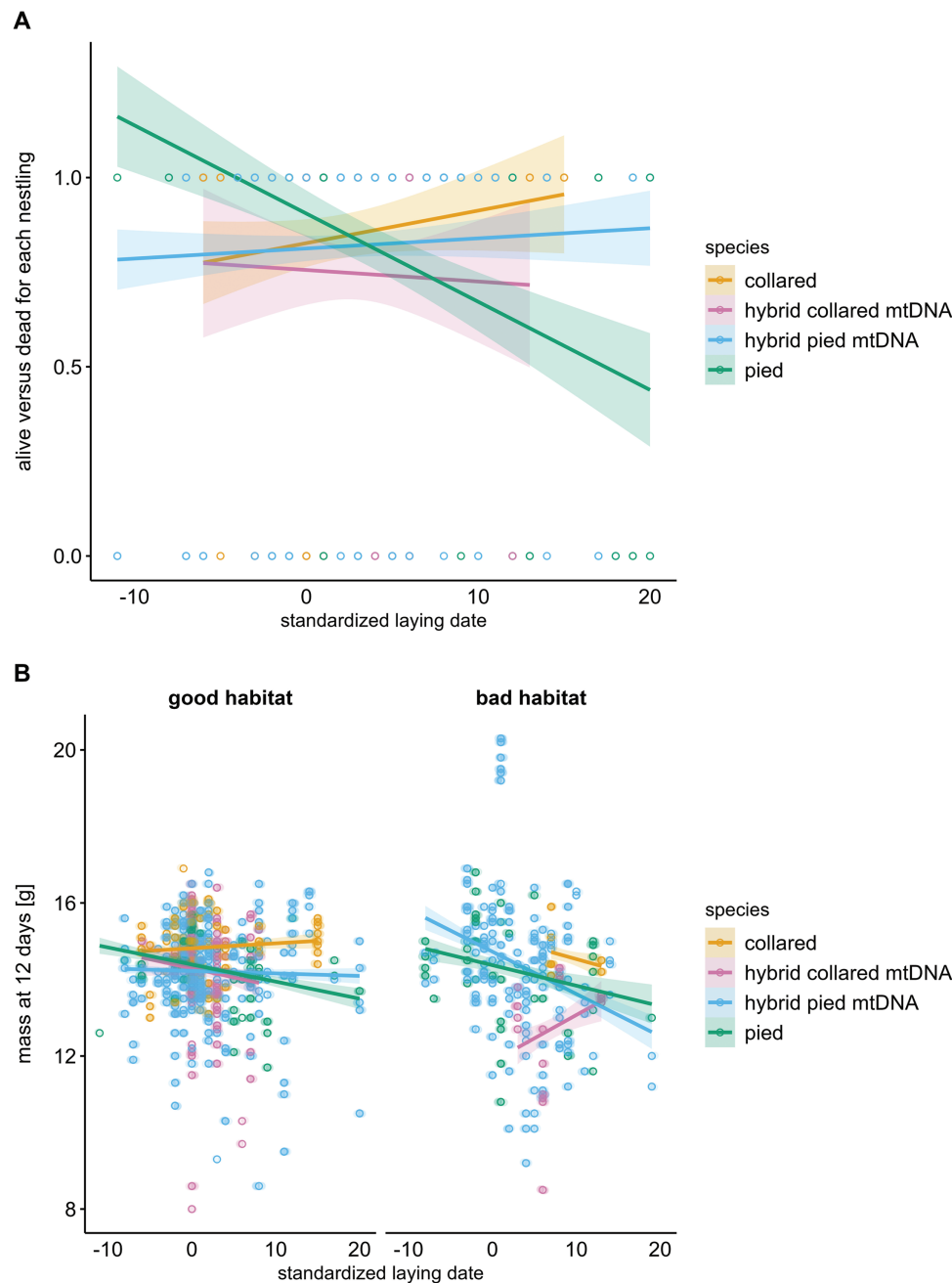


Figure 4. Hybrids and nonhybrid half-siblings at the age of 12 days. The datapoints are coloured according to species: within-pair nestlings with a collared flycatcher mother and a pied flycatcher father (hybrid collared mtDNA) in pink represent the bottom lines of both graphs, extra-pair nestlings in those nests, assigned the species collared flycatchers in yellow (top line in both graphs), within-pair nestlings with a pied flycatcher mother and a collared flycatcher father (hybrid pied mtDNA) in blue (the middle line that is almost horizontal on the left, and starts highest on the right graph), and extra-pair nestlings in these nests, assigned the species pied flycatcher in green (the regression line spanning the longest time period, from left to right, in both graphs). (A) Survival of hybrid versus nonhybrid half-siblings to day 12, plotted as a binomial variable (alive or dead) for each nestling depending on the timing of breeding (i.e., laying date standardized around zero being the day for which the majority of breeding pairs started laying each given year) and species (hybrid collared mtDNA, $N = 91$, collared, $N = 149$, hybrid pied mtDNA, $N = 403$, pied, $N = 160$). This plot shows a significant interaction between laying date and species on nestling survival to day 12 in mixed-species nests. (B) Mass at day 12 of hybrid versus nonhybrid half-siblings plotted against standardized laying date and separated in two categories according to habitat quality: bad and good, collared by species (hybrid collared mtDNA, $N = 77$, collared, $N = 126$, hybrid pied mtDNA, $N = 347$, and pied, $N = 132$). There was a significant three-way interaction between species, habitat quality, and laying date on nestling mass at 12 days of age in mixed-species nests.

pied flycatchers, and collared and pied flycatchers were all maximal for these three parameters (b_0 , b_1 , and b_2 all equal 1, Figure 7, Supplementary Table S15). The recapture probabilities were 0.585, 0.562, and 0.522 for collared, pied, and hybrid flycatchers, respectively.

There were low levels of differentiation in mortality trajectories between hybrids resulting from the two different crosses. A Weibull-simple model, the best fit for this dataset, provides two posterior parameters: b_0 , describing time-independent survival and b_1 , indicating the mortality rate at

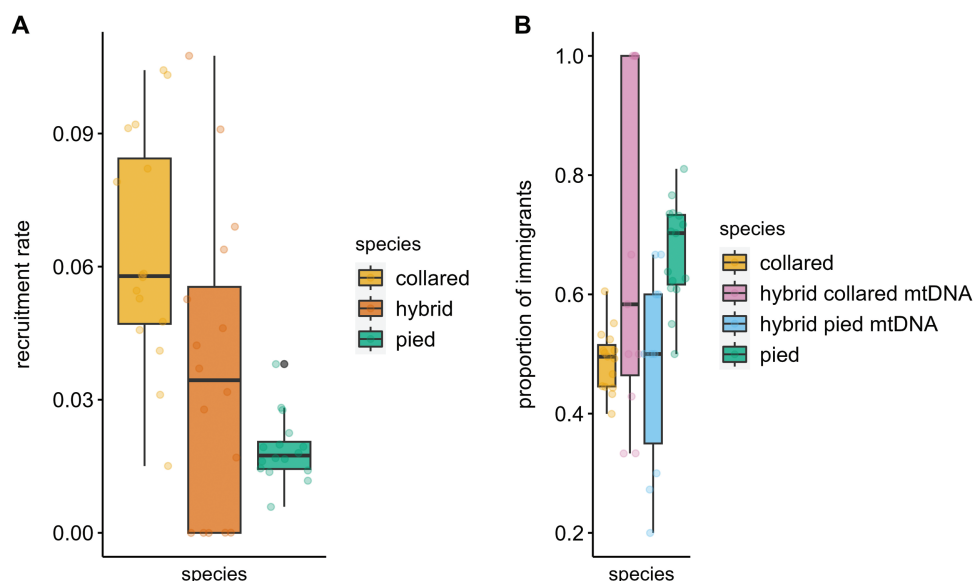


Figure 5. (A) Proportion of individual collared, pied, and hybrid flycatchers that were born in our areas and return as breeding adults. Recruitment rate represented here with one datapoint per year and species for visualization purposes. (B) Proportion of adult birds that immigrated to our monitored areas as adults, grouped by species and species mitochondrial DNA for hybrid individuals.

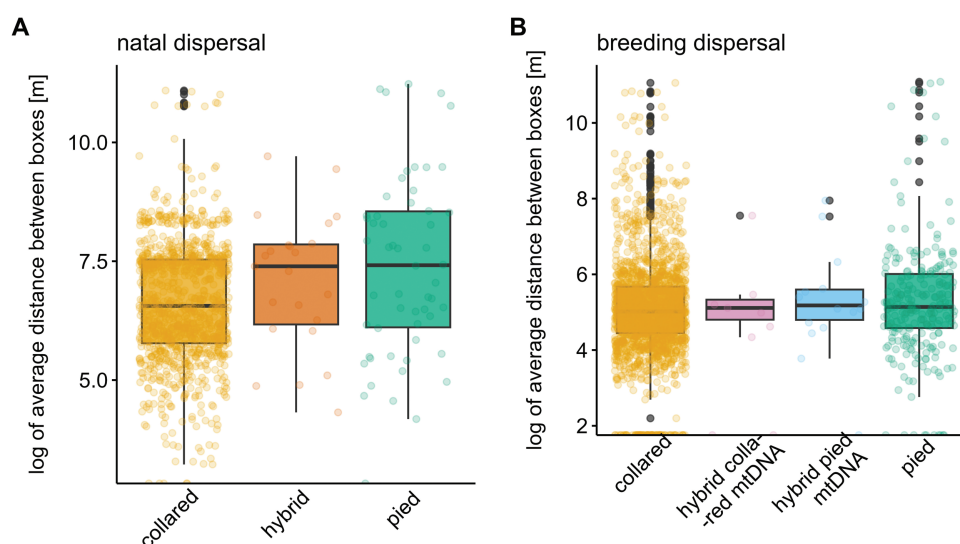


Figure 6. (A) Natal dispersal: the distance between the nest where a bird was hatched and the nest where it was first recorded breeding as an adult. (B) Breeding dispersal: mean distance between nestboxes where adults are recorded breeding from one year to the other. The hybrids are separated depending on their mother's species (hybrid CF have a collared mother, hybrid PF a pied mother). Note that both distances are represented on a log-scale to better visualize differences between species.

each age category. Both hybrid types showed an accelerating increase in mortality, but hybrids with pied mitochondrial DNA had slightly higher levels of baseline, time-independent mortality (Figure 8, b0, b0 HY_PF = 2.98, b0 HY_CF = 2.57, KLDC = 0.66, Supplementary Table S16), whereas the two crosses displayed no substantial difference in age-dependent mortality (Figure 8, b1, KLDC = 0.60, Supplementary Table S16).

Discussion

We investigated signs of reduced viability of hybrids resulting from crosses between pied and collared flycatchers at different life stages under natural conditions and found evidence

consistent with the presence of intrinsic incompatibilities (Figure 1). First, mixed-species pairs experienced higher hatching failure than conspecific pairs of pied and collared flycatchers at the monitored hybrid zone in Öland, Sweden (Figure 2, Supplementary Table S6). This increased rate of hatching failure in mixed-species pairs could be due to early developmental problems associated with specific parental genetic combinations. Moreover, there were differences in growth and survival patterns of hybrid nestlings and their half-siblings (fathered by an extra-pair male) raised in the same nest by the same social parents. Because environmental factors are largely shared within each nest and because parents are only expected to discriminate between offspring of different perceived “quality” when resources are limited (Lee & Chung,

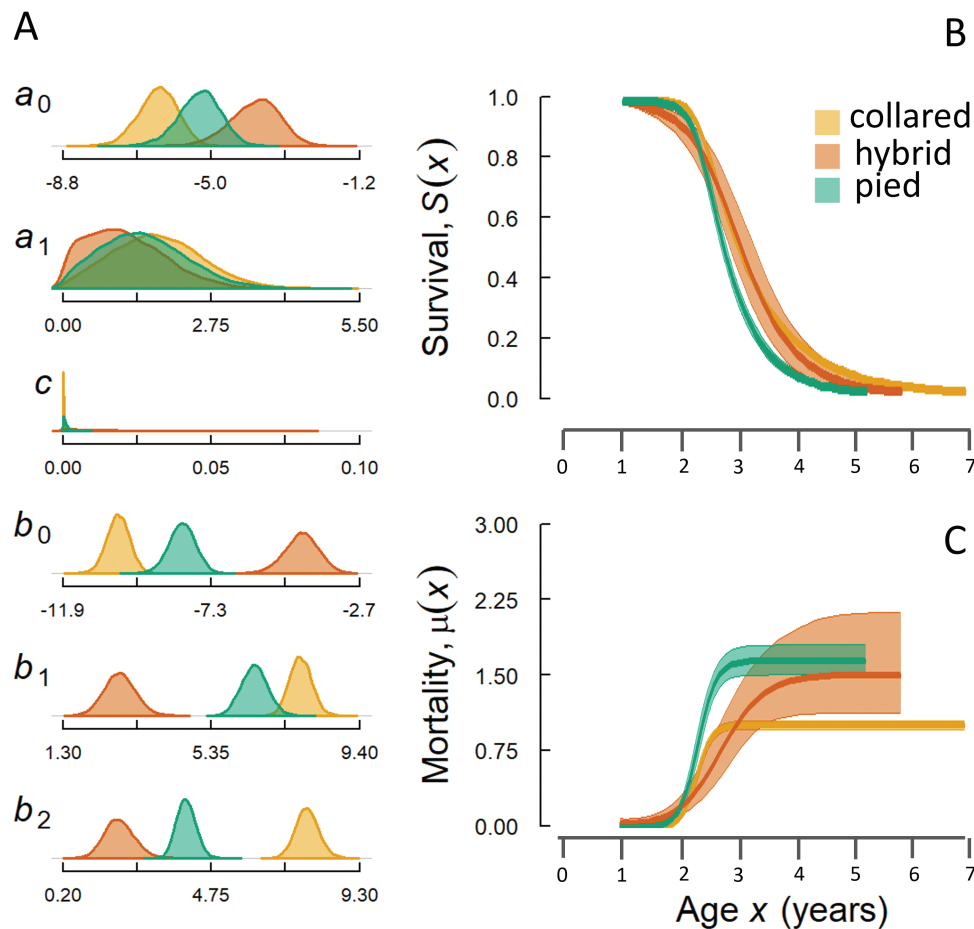


Figure 7. Results of adult survival analyses including collared, pied and hybrid flycatchers. (A) Posterior distributions for the six model parameters, (B) survival, and (C) mortality curves are shown for the Logistic-bathtub mortality model, and 95% confidence intervals represented. Hybrids experience the highest baseline mortality.

2020), this result implies genetic differences in the viability of hybrids also during the nestling growth stage. At the adult life stage, hybrids showed higher baseline mortality rates than the parental species (Figure 7, parameter b_0 , Supplementary Table S15), which kept increasing until later in life compared to the mortality rate of collared and pied flycatchers (Figure 7, parameter b_2). There were no strong asymmetries in adult viability depending on the direction of the parental cross, but hybrids with a pied flycatcher mother showed signs of higher baseline mortality as compared to hybrids with a collared flycatcher mother (Figure 8, b_0 parameter). Such asymmetries are consistent with Darwin's corollary to Haldane's rule and imply that genetic factors with sex-specific inheritance patterns likely contribute to genetic incompatibilities. Below we discuss the implication of these findings in relation to speciation.

A snapshot into the evolution of hybrid inviability

Collared and pied flycatchers are at a late stage of speciation. Complete reproductive isolation between these two species results from hybrid sterility (Ålund et al., 2013), likely caused by disruption of meiosis during gamete production (Segami et al., 2022a). Incompatibilities are expected to accumulate in a snowballing fashion once they arise but may have cryptic or,

at least, diminishing effects on the phenotypic level (Guerrero et al., 2017; Kalirad & Azevedo, 2017). Nevertheless, the build-up of BDMIs beyond the stage of complete reproductive isolation has important consequences for the future fate of young species, because mutations may restore hybrid dysfunction and incompatibilities are highly sensitive to purging at secondary contact (Barton & Bengtsson, 1986; Blanckaert et al., 2020; Xiong & Mallet, 2022). There are only 303 genes with fixed nonsynonymous differences between collared and pied flycatchers across their whole genomes (Segami et al., 2022b) and only a subset of these are expected to be causing negative epistatic interactions with severe effects on hybrid phenotypes. In line with this expectation, many important traits such as nestling growth, dispersal, and recruitment patterns show intermediate values in hybrids compared to the two parental species, which is consistent with additive inheritance of these traits. Nevertheless, we also find evidence of slightly reduced viability of hybrids during both the embryonic and adult life stages as compared to both parental species (Figure 1). Complete hybrid inviability often evolves more slowly than complete hybrid sterility (Moyle & Graham, 2005; Presgraves, 2010b; Turissini et al., 2018) and is therefore expected to have a more complex genetic basis. Because we do not observe complete hybrid inviability in our system, the arising BDMIs that affect hybrid survival probabilities are likely either caused by allelic variants that are not

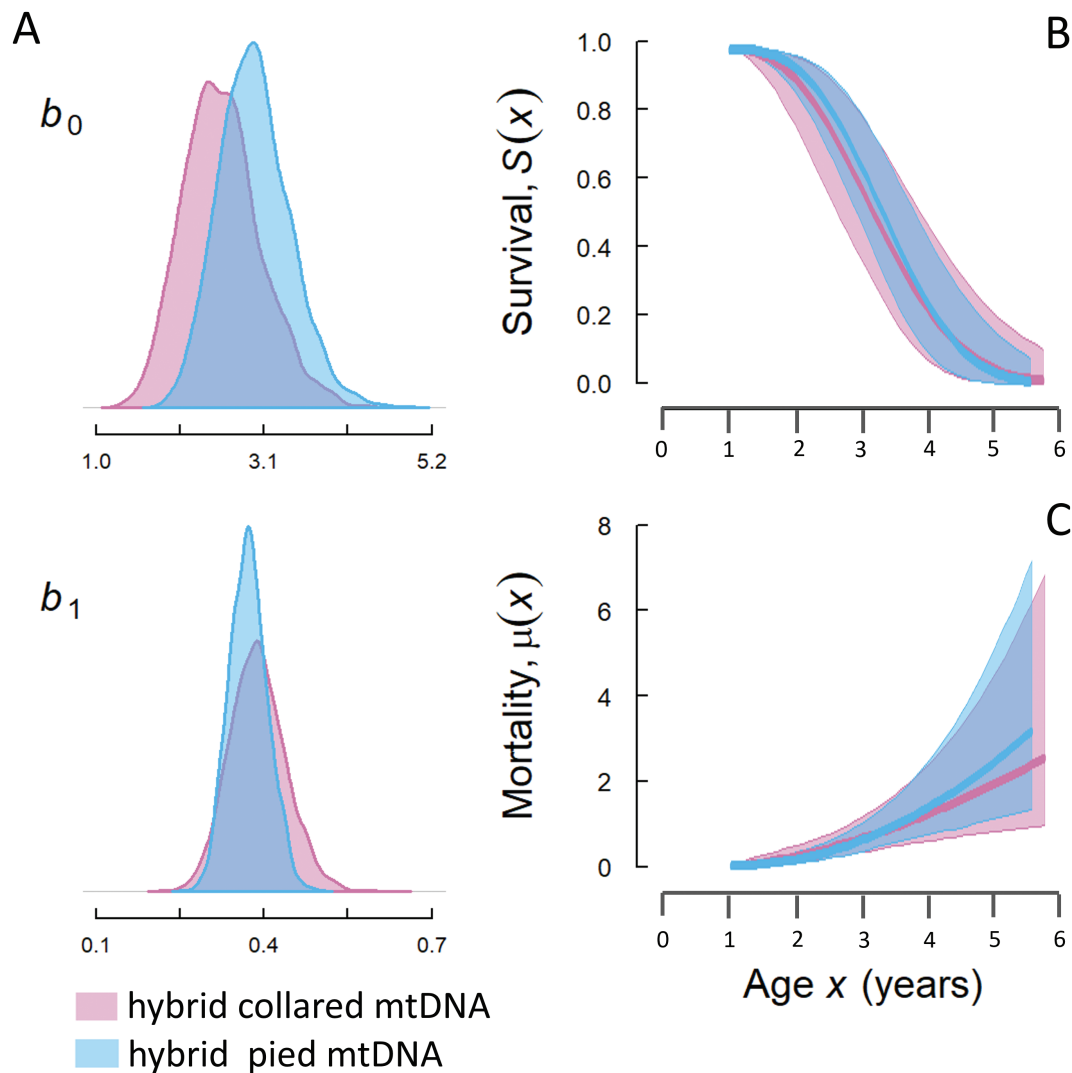


Figure 8. Results of adult survival analyses including only hybrids of the two reciprocal hybrid crosses, with collared flycatcher mitochondrial DNA in pink (left curve in A b_0 , lower line in B, lower peak in A b_1 and C) and with pied flycatcher mitochondrial DNA in light blue (right curve in A b_0 , upper line in B, highest peaks in A b_1 and C). (A) Posterior distributions for the two model parameters, (B) survival, and (C) mortality curves are shown for the Weibull-simple mortality model, and 95% confidence intervals are represented.

fixed between the two species (Coughlan & Matute, 2020; Cutter, 2012) or by fixed allelic differences that only mildly reduce hybrid survival. Any fixed difference causing complete hybrid inviability would result in the complete lack of hybrids between these species.

We furthermore expect individuals carrying the allelic variants causing the most serious BDMIs (i.e., disrupting basic physiological functions) to die already at an early life stage, meaning that observed survival differences between hybrids and nonadmixed individuals should decline across each life stage. In line with this expectation, we found a significant reduction in hatching success of mixed-species pairs and higher baseline mortality for adult hybrids. Since we rely on natural pairing patterns, there are some possible confounding factors, including the possibility of lower fertilization rates in interspecific crosses (see [Supplementary materials](#)) but the fact that hatching failure in hybrids does not co-vary with external factors (i.e., it is independent of laying date and habitat quality), as opposed to what is observed in the parental species, suggests that incompatibilities are present. The speciation field generally has a poor understanding of the stages

of transition between hybrids mostly suffering from extrinsic incompatibilities (due to intermediate phenotypes), and full hybrid lethality (due to BDMIs), which is often observed between long-diverged species. Studying subtle interactions between extrinsic and intrinsic factors affecting hybrids requires in-depth knowledge of the study system in their natural habitat, combined with physiological measurements and genomic analyses.

The application of mark-recapture models allowed us to estimate mortality distributions despite missing information on the exact moment of death for most birds. These powerful models require many years of monitoring of numerous individuals of each category that must be captured multiple times over several different years, in order to accurately estimate survival trajectories. Our analyses reveal that hybrid individuals have a higher baseline mortality than both collared and pied flycatchers across their lifespans. This could be due to a generally less efficient metabolism (McFarlane et al., 2016), or caused by suboptimal migration patterns (e.g., Delmore & Irwin, 2014), or both. Hybrids with a pied flycatcher mother show a slightly higher baseline mortality than the other cross.

Several more years of monitoring and measurements will allow us to confirm any bias in the survival of hybrids of one cross over the other, which again is consistent with emerging incompatibilities between alleles of minor effects, accumulating in the population. Although few studies have investigated the reproductive output of mixed-species pairing under natural conditions, increased rates of hybrid embryonic mortality have been reported from a wide range of taxa including birds (Sellier et al., 2005), fish (reviewed by [Bolnick & Near, 2005]), and insects (Tyler et al., 2013). Even fewer studies follow naturally occurring hybrids through multiple life stages. Intermediate rates of survival and longevity or similar trajectories to one or both of the parental species have been reported in plants (Kenzo et al., 2016), amphibians (Scroggie, 2012), rodents (Hunter et al., 2017), and birds (Neubauer et al., 2014), when no distinction was done between the reciprocal hybrid crosses or sexes. Some studies report signs of higher survival in naturally occurring hybrids (Fitzpatrick & Bradley Shaffer, 2007; Grant & Grant, 1996; Griebel et al., 2015; Weeks et al., 2017) or, strong negative effects on hybrid viability (Saulsberry et al., 2017). More subtle effects on survival, as detected in our study, are difficult to reveal under natural conditions because following individuals from birth to death is often not feasible, especially in animals. However, we need to increase our knowledge of how accumulating genetic incompatibilities manifest themselves under natural conditions (Reifová et al., 2023). Although the genetic incompatibilities causing the observed decline in flycatcher hybrid viability remain unknown, we can make some important conclusions. Since only a small subset of hybrid nestlings fail to hatch, these severe genetic incompatibilities should be caused by nonfixed variants segregating in the parental species (Cutter, 2012). There may indeed be some rare combinations of recessive allelic variants that are causing severe developmental failures within each parental population as well, but would be mostly masked in heterozygous individuals, and could be exposed in hybrids because of frequency differences in these alleles between the two species (Moran et al., 2021). By contrast, the milder effects detected later in life could be caused by fixed differences, as such incompatibilities are not exposed to purging in the two species and can further build up in the hybrid zone because hybrids are already sterile (Coughlan & Matute, 2020).

Darwin's corollary to Haldane's rule

We were particularly interested in possible asymmetries in hybrid survival depending on the maternal species in the mixed-species crosses. There were no strong differences between the hybrid crosses in specific reproductive traits and behavior, but some trends were observed. Hybrids with a pied flycatcher mother were slightly less likely to hatch and tended to experience higher baseline mortality across adult age classes as compared to hybrids resulting from the other cross (Figure 8, Supplementary Table S16). These patterns are consistent with Darwin's corollary to Haldane's rule and imply that genetic factors with sex-specific inheritance patterns, such as sex chromosomes and mitochondria, are involved in BDMIs. Sex chromosomes have been experimentally shown to be associated with hybrid sterility and inviability in artificial crosses (mostly in fruit flies [Coyne & Orr, 2004]), and harbor a higher proportion of highly divergent loci between species than autosomes do, in both X-Y and Z-W systems

(Payseur & Rieseberg, 2016). Previous research has shown elevated divergence between pied and collared flycatchers on the Z chromosome compared to autosomes (Ellegren et al., 2012; Hogner et al., 2012), which is also where the genes coding both for male plumage characteristics and female preference are found (Saether et al., 2007). In addition, there is genetic divergence in mitonuclear genes underlying the OXPHOS pathway responsible for cell respiration between collared and pied flycatchers (Van der Heijden et al., 2019), differences in plasticity of resting metabolic rate were detected between nestlings of the two parental species (McFarlane et al., 2018) and adult hybrid males suffer from suboptimal, high resting metabolic rate (McFarlane et al., 2016). All fitness traits rely on efficient respiration, which necessitates a good match between nuclear and mitochondrial genes (Wolff et al., 2014). Birds have high aerobic demands related to their ability to fly, and there should thus be a strong selection for optimal respiratory functions (Lane, 2011). Metabolic functions are sensitive to the environment, and coevolution of mitochondrial and nuclear genes may evolve quickly under strong climate-related selection, as observed in hybridizing warblers (Wang et al., 2021). Emerging inefficiencies in metabolism may be particularly relevant during periods of exponential growth, meaning that even small deficiencies may influence nestling conditions and survival chances. Poorly optimized metabolism may also cause a free radical leak, which in turn leads to apoptosis and results in increased rates of diseases late in life (Lane, 2011; Pichaud et al., 2019). The best documentation of such asymmetric incompatibilities comes from laboratory experiments, in *Drosophila*, for example, where reduced mitochondrial respiration efficiency is exacerbated with age, due to the accumulation of reactive oxygen species over time (Pichaud et al., 2019). Oxidative damage was also documented in hybrid copepods, even between lines with low levels of divergence, suggesting that these pathways may easily be disrupted (e.g., Barreto & Burton, 2013). Thus, mitonuclear incompatibilities are strong candidates for explaining the higher and slightly biased baseline mortality detected among hybrid flycatchers, given the previous differences in metabolism detected in our system. While challenging to study with high phenotypic and genotypic resolution together with reasonable sample sizes in natural systems, further investigation of survival trajectories of hybrids of both sexes with different mitonuclear combinations would be an interesting future avenue of research in the flycatcher system.

Conclusion

In summary, we took advantage of 17 years of long-term monitoring on naturally hybridizing pied and collared flycatchers to investigate potential signs of hybrid inviability at numerous life stages. While hybrid individuals were intermediate in several important fitness traits, mixed-species pairs experienced lower hatching success, which may be the result of early embryonic problems of hybrids resulting from some specific incompatible combinations of the parental genes. Adult hybrids also had a higher basal mortality rate than both parental species, with rates of mortality that kept increasing with age after pied and collared flycatchers had reached a plateau in mortality rate. While asymmetries between the reciprocal hybrid crosses were subtle, some trends emerged from our analyses encouraging future work investigating potential differences between them, and between the sexes, which could

Table 2. Strength of isolating barrier, showing the reduction in hybrids produced at each stage compared to situations of random mating, absence of sperm competition, and equal survival of hybrids compared to collared or pied flycatchers. In each case, hybrids were compared to their maternal species. Note that because all hybrids are sterile, reproductive isolation is complete in this system. Negative numbers indicate cases where more hybrids are found than expected by chance.

Isolating barrier	Collared flycatcher	Pied flycatcher
Mate choice/ assortative mating	0.77	0.49
Extra-pair copulations and conspecific sperm precedence	0.44	0.17
Egg hatchability	0.005	0.018
F1 nestling survival	0.007	0.008
F1 hybrid recruitment to the population as adult	0.40	-0.38
F1 hybrid immigration from outside of the monitored population	-0.06	0.24
F1 hybrid sterility	1	1
Total isolation	1	1

unveil different patterns of incompatibilities between mitochondrial-, nuclear-, and sex-chromosome-linked genes. In animals, hybrid incompatibilities have mainly been explored in laboratory crosses between species that are often not naturally hybridizing, while studies of natural hybrid zones instead frequently involve populations and species at early stages of speciation, where few signs of intrinsic hybrid inviability are detected. Hybrids between collared and pied flycatchers show advanced signs of intrinsic dysfunction in terms of sterility of both sexes despite a relatively short divergence time between the parental species. Here we report subtle viability differences between *Ficedula* hybrids and parental species that open possibilities to study physiological and genetic mechanisms underlying the evolution of hybrid dysfunction in the wild.

Studying the relative contribution of all the different parameters included in this study provides important insights into the complexity of interactions between extrinsic and intrinsic factors affecting hybrid fitness, and how these may change over time as species move along the speciation continuum. Table 2 illustrates the contribution of each life stage studied here to a reduction in the number of hybrid individuals produced compared to expectations of random mating and equal survival of hybrids to unadmixed individuals. Reproductive isolation is total in our system, due to complete hybrid sterility. Assortative mating and postmating prezygotic isolation are also very high, but strongly biased between the reciprocal crosses. Figure 1 illustrates that while we start with a bias in pairing and extra-pair copulation patterns, leading to a higher production of hybrids with a pied flycatcher mother, this bias is mitigated by a higher immigration of adult hybrid individuals of the other cross, such that differences in the proportion of adult hybrids of both types are minimal in our population. The detected reductions in survival at the different life stages contribute little to the observed number of F1 hybrids with different maternal species compared to these demographic factors. Nevertheless, such knowledge is important in the context of understanding how genetic incompatibilities manifest themselves at the phenotypic level. We show here that combining long-term monitoring documenting subtle phenotypic

differences between hybrids and nonadmixed individuals, coupled with physiological measurements and genetic analyses, allows for different conclusions about sources of extrinsic and intrinsic postzygotic isolation than would have been possible by solely looking at one point of the complex stages in this hybrid zone.

Supplementary material

Supplementary material is available online at *Evolution*.

Data availability

All data and code are available on Dryad: <https://doi.org/10.5061/dryad.dz08kps42>

Author contributions

M.Å. and A.Q. designed the study with contributions from J.C.S.M. and W.J., M.Å., W.J., J.C.S.M., and A.Q. collected data. M.Å., J.C.S.M., and W.J. performed data analyses. P.N.K.M. performed molecular analyses with support from J.C.S.M., M.Å., Y.Z., and A.Q. wrote the manuscript with input from J.C.S.M., W.J., and P.N.K.M.

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Conflict of interest: The authors declare no conflict of interest.

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