

Postzygotic incompatibilities between the pupfishes, *Cyprinodon elegans* and *Cyprinodon variegatus*: hybrid male sterility and sex ratio bias

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Abstract

I examined the intrinsic postzygotic incompatibilities between two pupfishes, *Cyprinodon elegans* and *Cyprinodon variegatus*. Laboratory hybridization experiments revealed evidence of strong postzygotic isolation. Male hybrids have very low fertility, and the survival of backcrosses into *C. elegans* was substantially reduced. In addition, several crosses produced female-biased sex ratios. Crosses involving *C. elegans* females and *C. variegatus* males produced only females, and in backcrosses involving hybrid females and *C. elegans* males, males made up approximately 25% of the offspring. All other crosses produced approximately 50% males. These sex ratios could be explained by genetic incompatibilities that occur, at least in part, on sex chromosomes. Thus, these results provide strong albeit indirect evidence that pupfish have XY chromosomal sex determination. The results of this study provide insight on the evolution of reproductive isolating mechanisms, particularly the role of Haldane's rule and the 'faster-male' theory in taxa lacking well-differentiated sex chromosomes.

Introduction

Understanding the mechanisms by which species are formed and maintained has long been a major goal of evolutionary biology. Speciation involves the formation of reproductive isolating barriers, traits that reduce either the probability (prezygotic barriers) or fitness (postzygotic barriers) of interspecific fertilizations. Considerable progress has been in understanding the most common types of isolating mechanisms, their rates of evolution, and their underlying causes, particularly with regards to postzygotic isolation. For instance, within animals most cases of postzygotic isolation are caused by Dobzhansky-Muller (interlocus) genetic incompatibilities (Bateson, 1909; Dobzhansky, 1933; Muller, 1942; Coyne & Orr, 2004). Other causes of postzygotic isolation, such as polyploidization, chromosomal rearrangements, and cytoplasmic incompatibility play an important role in some

speciation events, but are relatively rare across animal taxa (Coyne & Orr, 2004).

The most striking pattern in the evolution of postzygotic isolation is Haldane's rule (Haldane, 1922), which states that unisexual sterility or inviability almost invariably affects the heterogametic sex of animal hybrids. The primary explanation for this phenomenon is the dominance theory, the idea that recessive incompatibilities that occur on sex chromosomes are masked in the homogametic, but not the heterogametic sex (Muller, 1942; Turelli & Orr, 1995; Coyne & Orr, 2004). Haldane's rule has been overwhelmingly supported in all taxa examined, including Dipterans, Lepidopterans, frogs, mammals and birds (Coyne & Orr, 1989; Wade *et al.*, 1994; Sasa *et al.*, 1998; Presgraves, 2002; Price & Bouvier, 2002; Lijtmaer *et al.*, 2003; Russell, 2003). Notably, most of these studies (frogs are the exception) involve taxa with evolutionarily conserved, well-differentiated sex chromosomes. The dominance theory would predict that Haldane's Rule should operate only in taxa in which the heterogametic sex has hemizygous expression over at least some regions of the sex chromosomes (Turelli & Orr, 1995; Presgraves & Orr, 1998). Thus, it is unclear

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whether Haldane's rule should hold in taxa with homomorphic (cytologically undifferentiated) sex chromosomes. One might predict that strength of Haldane's rule in these taxa would be inversely related to the degree of recombination between the sex chromosomes.

In addition, if Haldane's rule is weakened, the importance of other mechanisms that generate differential postzygotic isolation in each sex may increase. For example, it has been well documented that genes related to male fertility diverge more quickly than those related to female fertility (Tao & Hartl, 2003; Ranz *et al.*, 2004; Zhang *et al.*, 2004; Lima *et al.*, 2004; Jagadeeshan & Singh, 2005; Slotman *et al.*, 2005). This observation forms the basis of the faster-male hypothesis which is that male sterility factors should accumulate between species faster than female sterility factors, thus causing male-biased sterility in hybrids (Wu & Davis, 1993; True *et al.*, 1996). The faster male hypothesis is apparently weaker than Haldane's rule in taxa with well-differentiated sex chromosomes, because examples of male-limited sterility in female-heterogametic taxa are rare (Coyne & Orr, 2004; but see Kobel *et al.*, 1996). It is possible, however, that the faster-male hypothesis will play a greater role in generating patterns of postzygotic isolation in taxa without well-differentiated sex chromosomes.

Only a handful of studies of postzygotic isolation have been performed on species without hemizygous sex chromosomes, but these suggest that (i) male sterility evolves faster than female sterility (perhaps explained by the faster male hypothesis), (ii) male and female inviability evolve at similar rates, and (iii) that the lag time, or time of divergence necessary before postzygotic isolation accumulates, tends to be longer for taxa without hemizygous chromosomes (Kobel *et al.*, 1996; Presgraves & Orr, 1998; Mendelson *et al.*, 2004). Hemizygous expression of recessive incompatibilities may explain why taxa with well-differentiated sex chromosomes tend to have a shorter lag time until postzygotic isolation accumulates (Mendelson *et al.*, 2004). Though these studies have provided valuable insight, more studies of the evolution of postzygotic incompatibilities in taxa lacking well-differentiated sex chromosomes are clearly necessary.

Further work is also needed in understanding how postzygotic isolation influences the hybridization of species in secondary contact. For instance, relatively few studies have examined the absolute and relative contributions of various isolating mechanisms (but see Ramsey *et al.*, 2003) or how the strength of different isolating mechanisms will influence the direction and extent of introgression.

The pupfishes *Cyprinodon elegans* and *Cyprinodon variegatus* form an ideal system for studying these questions. These species diverged in allopatry between 3 and 10 million years ago (Echelle *et al.*, 2005). *Cyprinodon variegatus* is a widely distributed species: its natural range includes estuaries along most of the Atlantic and Gulf

coasts (Lee *et al.*, 1980). *Cyprinodon elegans*, on the other hand, is limited to the springs, irrigation canals, and lake of Balmorhea, TX, USA (Garrett *et al.*, 2002). These fishes came into secondary contact approximately 40 years ago, when *C. variegatus* was unintentionally introduced from the Gulf of Mexico to Lake Balmorhea, TX, USA, a semi-isolated portion of the range of *C. elegans* (Stevenson & Buchanan, 1973). Since that time, two separate studies have found evidence of very limited introgression between these species (Stevenson & Buchanan, 1973; Echelle & Echelle, 1994). Previous work has shown that premating isolation in the form of conspecific mate preference forms a strong, but incomplete barrier to hybridization between these fishes. Females of both species strongly prefer conspecific to heterospecific mates, but show no discrimination against hybrid mates (C. Tech & A. Kodric-Brown, unpublished data). Anecdotal evidence suggests that postzygotic isolation may also occur: hybrids collected from Lake Balmorhea have had female-biased sex ratios and male hybrids may have reduced testicular development (Stevenson & Buchanan, 1973; Echelle & Echelle, 1994).

To date, there is no cytological evidence of sex chromosomes in pupfish; both members of each pair of chromosomes have similar size and shape (Nirchio *et al.*, 2003). Nevertheless, male-limited fitness loss is a common finding in pupfish hybrids (Turner & Liu, 1977; Cokendolpher, 1980). In light of Haldane's rule, this observation of male-limited fitness loss has led to the hypothesis that males are the heterogametic sex in pupfish (Turner & Liu, 1977); however, this hypothesis has not been verified by any other lines of evidence.

In this study, I examine the type and extent of postzygotic isolation between *C. elegans* and *C. variegatus*. Specifically, I address whether, relative to intraspecific crosses, hybrid crosses and backcrosses (i) fail to produce offspring more often, (ii) produce fewer offspring, (iii) produce offspring with reduced survival, or (iv) produce female-biased sex ratios. I then relate these results to the broader questions of the absolute and relative levels of isolation necessary to limit introgression between species and the evolution of postzygotic isolation in taxa without well-differentiated sex chromosomes.

Materials and methods

Breeding stock and husbandry

All fish used in this study are derived from breeding stocks that have been maintained in the laboratory for one to three generations. The *C. variegatus* stock was founded with approximately 200 individuals collected in August 2001 from Lake Balmorhea, TX, USA. The *C. elegans* stock was founded in 2001 with approximately 80 individuals acquired from Uvalde National Fish Hatchery (UNFH), and was supplemented with 60 additional fish from UNFH in 2003. Hatchery stock was used

to avoid depleting wild populations of this federally endangered species. Studies indicate that there has been no significant loss of natural genetic diversity in this hatchery population (Edds & Echelle, 1989).

Fish were bred and reared in 38 L aquaria with gravel and undergravel-filtration. The water was maintained at a salinity of 8 ppt using CoraLife™ (IL, USA) Marine Salt. The aquaria were kept in a greenhouse and exposed to a natural light cycle. Fish were fed once per day with a 70 : 30 mixture of TetraMin™ (Tetra, Blacksburg, VA, USA) flake food and Freeze-Dried™ (UT, USA) brine shrimp, and were provided with as much food as they would consume within 5 min.

Throughout this paper, Ce refers to *C. elegans*, Cv refers to *C. variegatus*, and in hybrids and backcrosses, the maternal parent is listed first. For example (Ce × Cv) × Cv refers to the backcross produced by mating a hybrid female (who was produced by crossing a *C. elegans* female and *C. variegatus* male) with a *C. variegatus* male.

These experiments were conducted in accordance with University of New Mexico main campus institutional animal care and use protocol numbers 20101 and 20416. *Cyprinodon elegans* were acquired, maintained, and tested in accordance with federal permit TE034087-0.

Hybridization experiments

I bred Ce, Cv, and all possible F₁ hybrid crosses and backcrosses using fish of known parentage in no-choice mating trials. In each replicate breeding attempt, one male and two females were placed in a 38 L aquarium for 24 h, after which time all adults were removed to allow the eggs to develop. Females were added to the breeding aquaria 48 h before the males to allow them to acclimate and to ensure mating receptivity. Female sizes varied greatly within a type (minimum SL: 24 mm, maximum SL 45 mm), but were similar across types. All breeding attempts occurred within the natural breeding season of these fishes (April–September, 2003–2004).

The number of offspring produced by each breeding attempt was determined at 3 weeks after fertilization, when all juveniles were removed from the breeding aquaria, counted, and placed into new aquaria for rearing. Up to 30 juveniles, usually from multiple broods, were placed within each rearing aquarium. This maximum number of fish was reduced by five every 3 weeks until 15 weeks after spawning, at which point there was maximum of 15 juveniles within each rearing aquarium. This procedure reduced differences in density between rearing aquaria. At 6, 9, 12, 15, 20, 25, 30 and 35 weeks after fertilization, all fish were removed from rearing tanks and counted to determine whether any mortality had occurred. The gender of each fish was determined by the presence or absence of male secondary sexual characteristics at 35 weeks after spawning. The sample sizes of each cross varied greatly because of differing success in breeding and rearing the fish, and are given in the results section.

Statistical analysis

To determine whether hybrid or backcross breeding attempts failed to produce offspring more often than intraspecific breeding attempts, a multiple comparison contingency table analysis was used (Zar, 1999). To determine whether hybrid or backcross matings produced fewer offspring than intraspecific matings, the number of offspring produced by successful matings was analysed with ANOVA and Bonferroni *post hoc* tests using STATVIEW™ (Cary, NC, USA) software. Prior to analysis, the number of offspring was square-root transformed to normalize the distribution. To determine whether any crosses were female-biased, a chi-squared analysis was used.

To determine whether hybrids and backcrosses have reduced survival relative the parental species, a Dunnett procedure for multiple comparisons among proportions (Zar, 1999) was used to analyse the proportion of offspring surviving to 15 weeks (approximate age of sexual maturity) and to 35 weeks after fertilization. These two measures are not intended to be independent. I decided to use proportion of offspring surviving, rather than formal survival analysis, for the following reasons. Survival is designed both to estimate time to an event and to allow the use of censored data (individuals which enter a study late or leave early, thus providing incomplete information on time to the event). The purpose of this study was not to determine the life span of each cross, but rather to see if there were significant differences between crosses in survival; analysing the proportion surviving to maturity and to the end of the experiment accomplishes this. Moreover, since only a small fraction fish reached the event of death, almost all individuals would be considered censored data; thus, survival analysis has limited ability to provide additional information.

The unit of replication for analyses of proportion of successful breeding attempts and average number of offspring produced is the breeding trio. The unit of replication for analyses of survival and sex ratio data is the individual, as each rearing aquarium typically contained multiple unrelated broods.

Based on previous work by Stevenson & Buchanan (1973) and Echelle & Echelle (1994), I predicted that hybrids and backcrosses would have lower fitness than parental species and that sex ratios would be female-biased; thus, one-tailed *P*-values were used for all statistical tests.

Results

Backcrosses involving hybrid males, Ce × (Ce × Cv) and Cv × (Ce × Cv), produced offspring significantly less often than any other cross ($\chi^2_9 = 47.3$, $P < 0.0001$). In fact, in crosses involving hybrid males, only one breeding attempt produced offspring, a success rate of 0.03%,

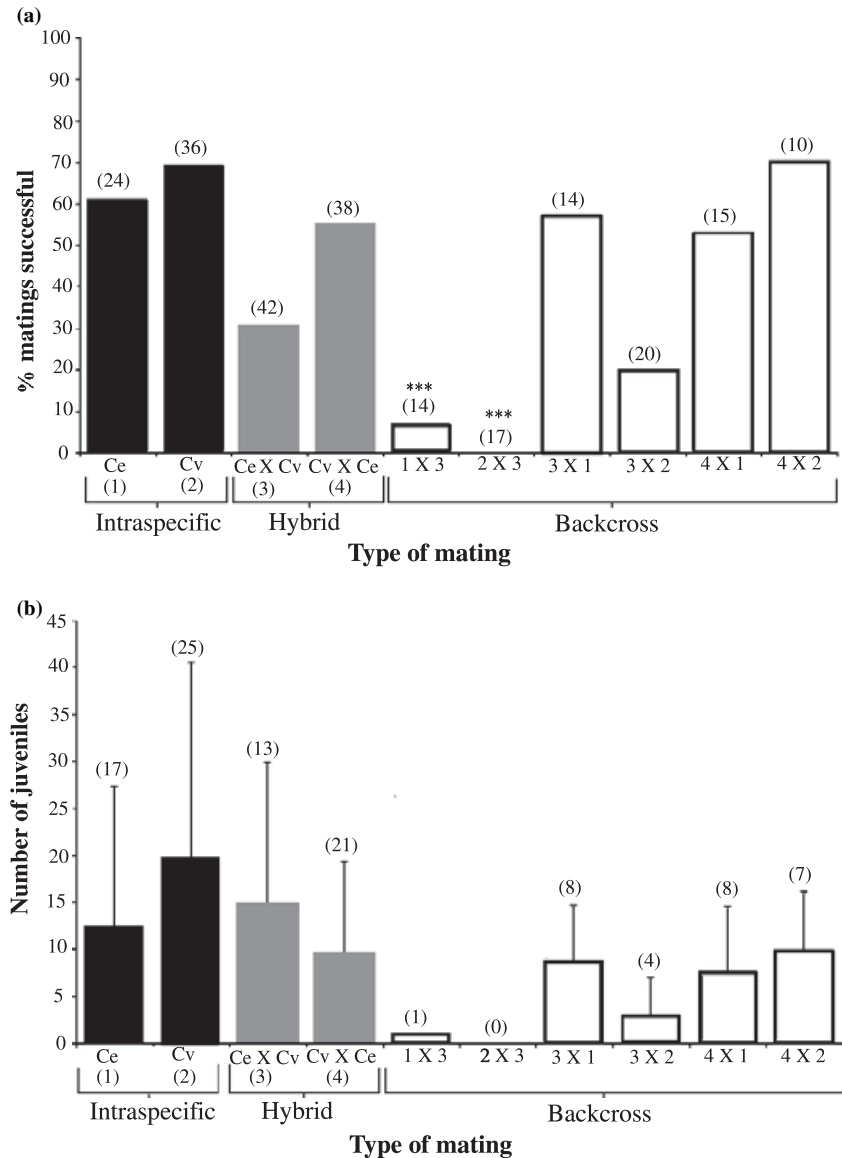


Fig. 1 (a) Percentage of breeding attempts that produced offspring. The sample size (number of breeding attempts) is given above each bar. *** = $P < 0.001$. (b) Average number of offspring produced in successful breeding attempts. The sample size (number of successful breeding attempts) is given above each bar. Error bars represent standard deviations.

compared with a success rate of about 65% for intraspecific crosses (Fig. 1a). Moreover, the single successful breeding attempt with a hybrid male produced only one offspring. Since previous work (Tech and Kodric-Brown, unpublished data) has shown that *C. elegans* and *C. variegatus* females mate readily with hybrid males, the most likely explanation for the low success of matings with hybrid males is that hybrid males have greatly reduced fertility. Excluding backcrosses with hybrid males, the number of offspring produced did not differ between crosses (Fig. 1b, $F_{7,102} = 1.6$, $P = 0.15$).

The two backcrosses into *C. elegans* ($Ce \times Cv$) \times *Ce* and ($Cv \times Ce$) \times *Ce*, had lower survival than any other cross at both the approximate age of sexual maturity, 15 weeks, and at the end of the experiment, 35 weeks

(Fig. 2, Table 1). The cause(s) of mortality are not known; however, these backcrossed fish tended to grow poorly and often appeared starved at death.

Some crosses demonstrated female-biased sex ratios (Table 2). Although most crosses produced nearly 50% males, there were no male $Cv \times Ce$ hybrids and only about 25% males in the two backcrosses with *C. elegans* males ($Ce \times Cv$) \times *Ce* and ($Cv \times Ce$) \times *Ce*. These three crosses had sex ratios significantly less than 50%.

Discussion

These results provide insight on reproductive isolation at many different levels. I focus first on the physiological and evolutionary mechanisms of postzygotic isolation,

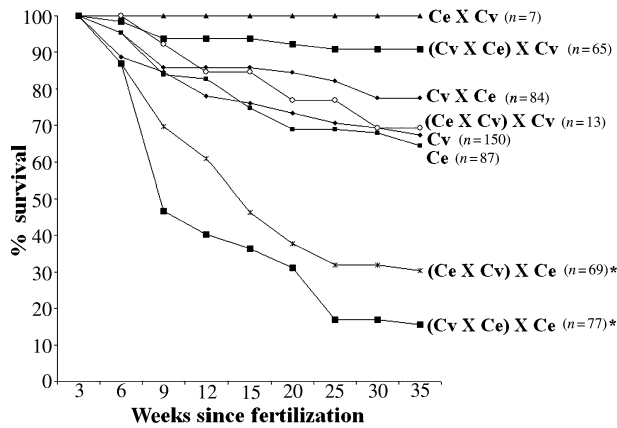


Fig. 2 Proportion of surviving offspring from each cross. For each cross, *n* refers to the total number of offspring reared. * = $P < 0.05$.

Table 1 Statistical results of survival analysis.

Cross	<i>n</i>	Proportion surviving	$q_{(0.05, 1, \infty, 6)}$	<i>P</i> -value
Survival to 15 weeks				
Ce and Cv	237	0.75	N/A	N/A
Ce × Cv	7	1	1.6	>0.05
Cv × Ce	84	0.86	3.2	>0.05
(Ce × Cv) × Ce	69	0.46	2.1	<0.01
(Ce × Cv) × Cv	13	0.85	0.6	>0.05
(Cv × Ce) × Ce	77	0.36	4.7	<0.001
(Cv × Ce) × Cv	65	0.94	6.2	>0.05
Survival to 30 weeks				
Ce and Cv	237	0.64	N/A	N/A
Ce × Cv	7	1	2.2	>0.05
Cv × Ce	84	0.77	3.8	>0.05
(Ce × Cv) × Ce	69	0.30	2.0	<0.01
(Ce × Cv) × Cv	13	0.69	0.2	>0.05
(Cv × Ce) × Ce	77	0.16	5.7	<0.001
(Cv × Ce) × Cv	65	0.91	8.3	>0.05

The proportion of hybrid and backcross offspring surviving to 15 weeks (average age of sexual maturity) and to 35 weeks was compared with offspring of intraspecific crosses using a Dunnett proportion test (Zar, 1999).

Ce, *Cyprinodon elegans*; Cv, *Cyprinodon variegatus*.

then calculate the strength of reproductive isolation between these fishes, and finally discuss how reproductive isolation may influence the direction and extent of introgression.

Mechanisms of sex ratio bias

Hybrids and backcrosses of *C. elegans* and *C. variegatus* exhibit interesting patterns of sex ratio: some crosses, including both parental species, produce approximately 50% males, whereas other crosses produce female-biased sex ratios. Although the cause of the sex ratio bias is not

Table 2 Sex ratio (proportion males) of crosses.

Cross	Observed sex ratio	Sample Size	95% CI
<i>Cyprinodon elegans</i>	0.51	65	0.37–0.64
<i>Cyprinodon variegatus</i>	0.50	103	0.39–0.60
Ce × Cv	0.48	23	0.27–0.69
Cv × Ce	0	110	0–0.03
(Ce × Cv) × Ce	0.27	30	0.12–0.46
(Ce × Cv) × Cv	0.45	11	0.17–0.77
(Ce × Cv) × Ce	0.25	16	0.07–0.52
(Cv × Ce) × Cv	0.48	48	0.33–0.63

In hybrids and backcrosses, the female parent is listed first.

Ce, *Cyprinodon elegans*; Cv, *Cyprinodon variegatus*.

known, the patterns observed in this study are consistent with any combination of two genetic incompatibilities and two phenotypic effects. The possible genetic incompatibilities are an incompatibility between gene(s) on the X chromosome of *C. variegatus* and gene(s) on the Y chromosome of *C. elegans* or incompatibility between dominant gene(s) on an autosomal chromosome of *C. elegans* and recessive gene(s) on the X chromosome of *C. variegatus*. Each genetic incompatibility may produce one of two different phenotypic effects: inviability or the feminization of males. In this study, survival of each cross was monitored beginning 3 weeks after fertilization, thus, inviability of embryos would not have been detected. Similarly, because gender was assigned based on secondary sexual characteristics, not gonadal histology, the feminization of males would also be undetected. Feminization can occur if fish that are genetically male fail to develop the sexual characteristics of males; this may result from any number of developmental problems, including endocrine disruption (e.g. Zillioux *et al.*, 2001; Sumpter, 2005). The phenotypic effect of the incompatibility – feminization or inviability – could be determined in future studies by examining the development of fertilized eggs and the histology of the gonads.

No matter what the phenotypic effect, both of these incompatibilities would explain both the normal sex ratio of the hybrid cross Ce × Cv and the total absence of males in the hybrid cross Cv × Ce. There are small differences, however, in the predicted sex ratio of backcrosses depending on which incompatibility and which phenotypic effect is present (Table 3). The sex ratios observed in this study are in closest agreement with the predicted ratios based upon an incompatibility between gene(s) on the X chromosome of *C. variegatus* and gene(s) on the Y chromosome of *C. elegans* that results in the feminization of males, but the sample sizes are not high enough to provide sufficient statistical power to exclude the other possible mechanisms. The fact that sex chromosomes are involved in each of the proposed mechanisms of sex ratio bias provides

Table 3 Predicted sex ratios (proportion male) based upon four possible mechanisms of sex ratio bias.

Incompatibility Phenotypic effect	Ce 'Y' and Cv 'X'		Ce 'A' and Cv 'X'	
	Feminization	Inviability	Feminization	Inviability
Ce × Cv	0.5	0.5	0.5	0.5
Cv × Ce	0	0	0	0
(Ce × Cv) × Ce	0.25	0.33	0.25	0.33
(Cv × Ce) × Ce	0.25	0.33	0.25	0.33
(Ce × Cv) × Cv	0.5	0.5	0.375	0.5
(Cv × Ce) × Cv	0.5	0.5	0.375	0.5

All four mechanisms produce sex ratios within the 95% confidence intervals of the observed sex ratios in this study.

Ce, *Cyprinodon elegans*; Cv, *Cyprinodon variegatus*.

strong, albeit indirect, support for XY chromosomal sex determination in *Cyprinodon*.

Haldane's rule and faster male evolution

Hybrids and backcrosses of *C. elegans* and *C. variegatus* show male-limited fitness loss consistent with both Haldane's Rule and faster male evolution. Haldane's rule is likely responsible for the female-biased sex ratios of hybrids and backcrosses. Each of the proposed mechanisms for the sex ratio skew directly implicate the dominance theory of Haldane's rule because the greater effect on males than females is related to the expression of incompatibilities occurring on one or both sex chromosomes. Independent, molecular evidence of sex chromosomes in these species is urgently needed. If male heterogametic sex determination is conclusively demonstrated, pupfish will become one of very few good examples of Haldane's rule in fishes and will extend the generality of Haldane's rule to taxa lacking cytologically identifiable sex chromosomes (Russell, 2003).

On the other hand, the faster male hypothesis might play a role in the sterility of male hybrids. The faster male hypothesis states that genes related to male fertility diverge faster than those related to female fertility because of stronger sexual selection on males than on females (Wu & Davis, 1993; True *et al.*, 1996), though other mechanisms, such as the greater vulnerability of spermatogenesis (Kulathinal & Singh, 1998) or the faster substitution of male-specific traits on the X or Y chromosome (Charlesworth *et al.*, 1987), may also play a role. Pupfish males experience very high levels of sexual selection (Itzkowitz, 1978; Kodric-Brown, 1997; Leiser & Itzkowitz, 2003), which makes the faster male hypothesis a likely contributor to the pattern of male-limited sterility in pupfish hybrids. Of course, the faster male hypothesis and the dominance theory of Haldane's rule are not exclusive; both could contribute to hybrid male sterility if (i) genes related to male fertility evolve more quickly than genes related to female fertility and

(ii) some genes related to male fertility are recessive (between species) and occur on sex chromosomes.

Estimation of reproductive isolation

Reproductive isolation is often the result of the multiple mechanisms that reduce gene flow between species, including prezygotic isolating mechanisms such as habitat isolation, temporal isolation, sexual isolation and gametic incompatibility, and postzygotic isolating mechanisms, such as intrinsic hybrid sterility and inviability, ecological inviability, behavioural sterility and fitness loss in backcrosses (Coyne & Orr, 2004). This list is not exhaustive, but shows some of the most common isolating mechanisms. Quantification of the amount of gene flow blocked by each component of reproductive isolation is useful; it permits direct comparisons of different components of reproductive isolation and facilitates predictions about the extent and direction of introgression. Here, I estimate the absolute and proportional contributions of two important components of reproductive isolation – sexual isolation and intrinsic hybrid sterility/inviability – following the calculations by Ramsey *et al.* (2003).

The absolute degree of sexual isolation equals the proportion of matings occurring between conspecifics when mates of both species are available. Since sexual isolation acts prior to hybrid sterility/inviability, the proportional contribution of this barrier equals its absolute strength. In semi-naturalistic mating experiments involving both species, Tech & Kodric-Brown (unpublished data) found that approximately 80% of matings were between conspecifics. Thus, the absolute strength and proportional contribution of sexual isolation is 0.8.

Intrinsic hybrid sterility and inviability are often quantified separately (e.g. Ramsey *et al.*, 2003), but these mechanisms are difficult to separate in this study, as it is unclear whether the lack of Cv × Ce males is because of inviability or feminization (a form of sterility). The results of this study showed that the viability and fertility of female hybrids is similar to that of parental species, but that male hybrids have essentially zero fitness. Thus, the absolute strength of intrinsic hybrid sterility/inviability is 0.5. The proportional contribution refers to contribution of this barrier to total reproductive isolation, and is calculated as the absolute contribution multiplied by one minus the sum of the proportional contributions of all prior-acting barriers. The proportional contribution of intrinsic hybrid sterility/inviability is therefore 0.1.

The combined contribution of sexual isolation and intrinsic hybrid sterility/inviability is 0.9; indicating that 90% of gene flow is blocked by these two mechanisms alone. This is clearly an underestimate of total reproductive isolation, as many other potential isolating mechanisms have not been studied. At the same time, it suggests that the roles of these two isolating mechanisms are extremely important in blocking gene flow between *C. elegans* and *C. variegatus*. The results further suggest

that sexual isolation may be stronger than intrinsic postzygotic isolation, a trend that has been found in other strongly sexually selected taxa (Mendelson, 2003).

Consequences of reproductive isolation

Studies of reproductive isolation can often inform predictions about the degree and extent of genetic introgression between species in nature. For example, the degree of introgression between collared and pied flycatchers is inversely correlated with the fitness of F_1 hybrids (Borge *et al.*, 2005). Similarly, genes found only in the heterogametic sex of species exhibiting Haldane's rule (such as $mtDNA$ in many hybridizing birds) often exhibit lower introgression than autosomal genes (Tegelstrom & Gelter, 1990; Cianchi *et al.*, 2003). The high level of reproductive isolation found between *C. elegans* and *C. variegatus* suggests that introgression should be limited; and, indeed, i.e. what has been found in surveys of the hybrid zone (Stevenson & Buchanan, 1973; Echelle & Echelle, 1994). These results also indicate that there may be differences in both the rates of introgression of different genes and the direction of introgression. The greater fitness loss of male hybrids in *Cyprinodon* suggests that introgression of paternally inherited genes, such as Y-linked genes, will be more severely limited than maternally inherited genes, such as $mtDNA$. Similarly, the higher mortality and greater sex ratio skew of *C. elegans* backcrosses suggests that the introgression of *C. variegatus* genes into the *C. elegans* genome will be more restricted than introgression in the opposite direction, all else being equal. On one hand, this is encouraging news, as it suggests that the rarer *C. elegans* genome may remain relatively pure; on the other hand, the greater fitness loss in *C. elegans* backcrosses may also make this species more vulnerable to extinction through hybridization (Templeton, 1981). These hypotheses could be tested by examining patterns of introgression within the hybrid zone.

Finally, the results of this study may also shed light on the phylogenetic relationship of these fish. Currently, this relationship is in doubt: allozyme analysis indicate that these fishes have a distant relationship (Echelle & Echelle, 1998), whereas analysis of $mtDNA$ sequence (of non-hybridizing populations) indicate a much closer relationship (Echelle *et al.*, 2005). Echelle *et al.* (2005) suggested that a likely explanation for this discrepancy is that *C. elegans* and *C. variegatus* have a distant evolutionary relationship but share similar $mtDNA$ because of recent introgressive hybridization between *C. elegans* and its geographical neighbours, *Cyprinodon bovinus* or *Cyprinodon pecosensis*, both of which are closely related to *C. variegatus*. The high level of postzygotic isolation between *C. elegans* and *C. variegatus*, relative to what is commonly observed within the genus (Turner & Liu, 1977; Cokendolpher, 1980; Rosenfield *et al.*, 2004), supports a distant evolutionary relationship between these fishes.

This study demonstrates that strong postzygotic isolation, with the greatest fitness losses in males, occurs between *C. elegans* and *C. variegatus*. Further study on the mechanisms of sex ratio bias and sex determination in these fishes will greatly increase our understanding of the evolution of postzygotic isolation.

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