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Mate choice in the face of both inbreeding and outbreeding depression in the intertidal copepod *Tigriopus californicus*

Abstract In species vulnerable to both inbreeding and outbreeding depression, individuals might be expected to choose mates at intermediate levels of genetic relatedness. Previous work on the intertidal copepod *Tigriopus californicus* has repeatedly shown that crosses between populations result in either no effect or hybrid vigor in the first generation, and hybrid breakdown in the second generation. Previous work also shows that mating between full siblings results in inbreeding depression. The present study again found inbreeding depression, with full sibling mating causing significant fitness declines in two of the three populations assayed. In the mate choice assays, a single female was combined with two males. Despite the costs of both inbreeding and outbreeding, mate choice showed clear inbreeding avoidance but no clear pattern of outbreeding avoidance. This lack of outbreeding avoidance may be attributed either to the temporary increase in fitness in the F_1 generation or to the absence of selection for premating isolation in wholly allopatric populations with infrequent migration. If this inability to avoid unwise matings is common to other taxa, it may contribute to the problem of outbreeding depression when allopatric populations are mixed together.

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Introduction

A serious consequence of mating amongst close relatives is inbreeding depression – a reduction in mean fitness within inbred lines (Greenwood et al. 1978; Charlesworth and Charlesworth 1987; Lynch 1988, 1991; Waldman 1988). Although most organisms carry many deleterious recessive mutations at low frequency, these mutations typically do not have an effect on individual fitness because they are masked by dominant alleles at the same locus (Lynch 1991; Futuyma 1998). However, because of an increase in homozygosity, inbreeding leads to the expression of these deleterious recessive mutations, reducing fertility, survivorship and growth rates (Waldman 1988; Futuyma 1998).

Conservation programs have relied on two very different strategies to alleviate inbreeding depression. One approach has been to transplant individuals from different geographical locations into small populations in an attempt to inflate the amount of genetic variation and promote rapid growth (Templeton 1986). A second and less commonly used approach has been to intentionally inbreed the population in an attempt to quickly purge it of deleterious recessive mutations (Templeton 1986). Unfortunately, this strategy may rapidly reduce population size and lead to a steep decline in standing genetic variation, and does not take into account that, just by chance, new deleterious mutations will arise in the population.

It is well recognized that crossbreeding between populations can lead to an increase in survival and reproduction (Darwin 1876; Meffe and Vrijenhoek 1988; Leberg 1993). This boost in fitness is generally attributed to a return to the heterozygous condition, where dominant alleles can once again effectively mask deleterious recessives. However, hybridization between populations can also result in a reduction of fitness, referred to as outbreeding depression (Dobzhansky 1948; Templeton 1986; Burton 1990; Lynch 1991). In this case, the reduction of fitness is attributed to the loss of locally

adapted genes (Templeton 1986; Waldman 1988) and/or the breakup of coadapted gene complexes. The disruption of coadaptation may not be realized until the F_2 generation, when recombination can break up the original parental gene combinations, resulting in some individuals who are homozygous for one parent's genes at one locus and the other parent's genes at another locus (Waldman 1988; Burton 1990; Lynch 1991). Because of the potentially detrimental effects of crossbreeding, a more comprehensive understanding of outbreeding depression is critical for the conservation of threatened and endangered populations.

Populations might be expected to evolve mate-choice preferences promoting "optimal outcrossing", which would maximize fitness by avoiding the hazards of both extreme inbreeding and extreme outbreeding (Wilson 1987; Waser and Price 1989; Lynch 1991). The focus of this study was to determine if such an optimal reproductive strategy exists in Tigriopus californicus, a harpacticoid copepod common in supralittoral pools from southeast Alaska to central Baja California. This species is ideal for such a study because it breeds year-round, even under controlled laboratory conditions, and is vulnerable to both inbreeding and outbreeding depression (e.g. Burton 1990; Brown 1991). Although all stages of its life cycle are capable of dispersal, a variety of molecular markers (allozymes, nuclear and mitochondrial DNA sequences) show that gene flow between reproductively compatible populations is extremely limited (Burton and Feldman 1981; Burton 1994, 1997; Burton and Lee 1994; Ganz and Burton 1995; Edmands 1999).

Interpopulation crosses typically show hybrid vigor in the F_1 and hybrid breakdown in the F_2 generation (Burton 1987, 1990; Edmands and Burton 1998; Burton et al. 1999; Edmands 1999) although hybrid breakdown has also been observed in the F_1 generation (Brown 1991). The two pairs of populations used in this study are highly differentiated (> 800 km apart and $> 16\%$ sequence divergence for the mitochondrial COI gene: Edmands 1999). When these populations were hybridized, one pair (populations BCW × NB) showed slight but non-significant fitness increases in the F_1 and mixed results in the F_2 generation, whereas the other pair (BCW × SD) showed significant fitness increases in the F_1 and significant fitness declines in the F_2 generation (Edmands 1999) (see first subsection of "Materials and methods" for locations of Populations BCW, NB, SD). Mating between siblings has also been shown to cause significant fitness reductions in one population of this species (Brown 1991). Effects of inbreeding on the three populations used for mate-choice assays were tested as part of the present study.

Previous studies indicated that individuals in the genus Tigriopus can distinguish species, populations, kin and gender, probably via chemical cues. For example, recognition of females by male T. fulvus is promoted by a sex pheromone, which is species- and even population-specific (Lazzaretto et al. 1990, 1994). Members of the genus may secrete "aggregation pheromones" that allows recolonization of rock pools (Bozic 1975). Chemical cues have also been implicated in the ability of female T. fulvus to recognize and avoid cannibalizing their own offspring (Lazzaretto and Salvato 1992). Finally, surface glycoproteins appear to facilitate sex recognition in T. japonicus (Kelly and Snell 1998).

Despite evidence that Tigriopus spp. are able to recognize relatives, previous behavioral experiments using T. californicus suggest that males and females select mates randomly both within and between populations (Brown 1991; Ganz 1994; Ganz and Burton 1995; Edmands unpublished data). These experiments were done by giving an individual the choice of two different mates and scoring which individuals formed clasped pairs (in this species, males "mate guard" by using their first antennae to clasp onto immature females and retain them for 1 to 7 d until the females are mature enough to breed: Burton 1985). In these past experiments, the copepods were stained with chemical dyes to help the investigator distinguish individuals. Because chemical dyes may interfere with copepod mate-recognition cues, they were not used in this study, and populations that naturally vary in color were used instead. Furthermore, after separating pairs, males and virgin females were isolated for a longer period of time (24 h at 4 °C) in this study than in the earlier studies.

Although both males and females may play a role in mate-choice, our study focused on female choice by giving each female the choice of two males. In these copepods, males mate repeatedly, but females only once (Vittor 1971; Burton 1985). Situations in which male reproductive success varies more than that of the female are expected to promote female choice (Bateman 1948). Although virgin females are smaller than mature males and may appear passive, they may exhibit choice in the extent to which they struggle when a male attempts to clasp them. This type of female choice via protest has been shown in other organisms where males vastly outweigh females, such as elephants (Poole 1989) and elephant seals (Cox 1981; Le Boeuf and Mesnick 1990).

In this study, we used Tigriopus californicus populations that had previously exhibited outbreeding depression when hybridized. We also tested whether these same populations exhibit inbreeding depression when subjected to full-sibling mating. Further, we tested the hypothesis that individuals will maximize their fitness by choosing mates at the optimal level of relatedness.

Materials and methods

Population sampling and culture maintenance

Three populations of Tigriopus californicus were used in this study: (1) Bob Creek Wayside, Oregon (BCW, 44°15′N; 124°07′W, collected in August 1996, January, June and October 1997, and February and April 1998), (2) Natural Bridges State Park, California (NB, 36°57′N; 122°04′W, collected in July and December 1996, July 1997 and Febuary 1998), and (3) San Diego, California

(SD, 32°45′N; 117°15′W, collected in July 1996 and June 1997). Because previous work had revealed high gene flow among different tidepools on the same outcrop (Burton and Swisher 1984) and little change in genetic structure over time periods of up to 18 yr (Burton 1987), samples from the same outcrop collected at different time points should be genetically similar. Our samples from different populations were easily discernible: collections from the two California populations exhibited a bright red/orange exoskeleton, whereas collections from the Oregon population were light red to brown in color.

Cultures were maintained in separate 200 ml beakers under controlled temperature (15 °C), light (12 h light:12h dark cycle) and salinity (≃35 ppt); small amounts of commercial flake-type fish food were added to promote an algal bloom. Replicate beakers from the same location were periodically mixed to promote panmixia. To establish colonies of full-siblings, adult females with egg sacs were isolated in individual beakers containing 50 ml of seawater filtered twice through Whatmann #1 filter paper. These females and their emerging offspring were maintained under similar conditions of temperature, light and salinity and were fed on nutritional yeast. In the wild, copepods incorporate carotenoids from plant material into their exoskeletons, giving them a red/orange color (Vittor 1971). However, when raised on nutritional yeast their bodies are white, because of the inaccessibility of these pigments.

Inbreeding experiments

Populations were maintained in the laboratory for 2 to 8 mo (≃2 to 8 generations) before inbreeding experiments were initiated. All individuals used for fitness assays were maintained in the same walk-in incubator and were cultured in twice-filtered natural seawater, with the addition of 0.02 g yeast per 100 ml seawater: this concentration appeared to provide excess food at all times. Colonies of full-siblings were established in beakers containing 50 ml seawater/yeast as described above. Once copepodids (juvenile copepods) were visible to the naked eye, the female parent was removed. Once siblings formed clasped pairs (mature male + virgin female), the pairs were separated using a fine probe and segregated by sex. To form the inbred crosses, one male and one sibling virgin female were united in a petri dish containing 35 ml seawater/yeast. To form the control crosses, one male and one non-sibling virgin female from the same population were united in a petri dish.

The dishes were monitored daily throughout the inbreeding experiments. When females formed egg sacs, they were moved to a new dish. On the day that nauplii emerged, the number of individuals in the first clutch (hatching no.) was determined using a Pasteur pipette to transfer each nauplius to a new dish. Occasionally, females dropped their egg sacs but no nauplii could be found. In these cases, the females were monitored to see if nauplii were produced in the second or third clutches. If no nauplii were produced in the first three clutches, the "0" for the first clutch was excluded from the data set because the female may never have been fertilized. Fourteen days after hatching, offspring were recounted by transferring individuals into a new dish to determine survivorship no. (number of nauplii plus metamorphosed copepodids) and metamorphosis no. (number of metamorphosed copepodids). The fitness of inbreds and controls was compared by a one-tailed Student's t-test (inbreeding depression was predicted a priori).

Mate-choice experiments

Copepods were maintained in captivity for between 2 wk and 11 mo before the mate-choice assays were conducted. Males of approximately the same size were removed from each population, isolated, and inspected under a dissecting microscope to ensure maturity – determined by size and shape of their clasping antennae. Virgin females (Copepedid Stages II to V) were easily obtained by collecting clasped pairs and carefully separating the male from the female under the microscope using a fine probe. To avoid bias from injury, males separated in this way were not included in the experiment. We isolated virgin females for 24 h (at 4 °C, a temperature low enough to arrest development) to allow them to overcome stress that may have been induced during separation. Following this isolation period, the water temperature was allowed to increase to 15 °C; the females were then placed in a 100×15 mm petri dish containing 20 ml seawater (15 °C) and a choice of two males. Female choice was assessed by three different cross types: the female could select between (1) a male from within her population (same outcrop) versus a male from a distant outcrop, (2) a male from the same outcrop as the female versus a sibling, and (3) two males from the same outcrop as the female that had been raised on different diets (algae vs nutritional yeast). This third set of mate-choice experiments was conducted to test the effects of color/diet on mate choice in the inbreeding-avoidance assays, since the majority of siblings were raised on yeast while all non-siblings were raised on algae.

After introduction of the females, the petri dishes were monitored for clasped pairs over a 2 h period. When a pair joined, the type of male selected (determined by color) was recorded for the individual female. Females that did not select a mate within the 2 h monitoring time were excluded from the data set. Chi-square tests were used to determine if mate selection was significantly greater than would be expected due to random chance. Data were tested for significant preferences within and between populations, and for overall population advantage.

Results

Mating between full siblings caused significant inbreeding depression for two of the three populations of *Tigriopus californicus* assayed (Table 1). Populations NB and SD showed declines in all three fitness components. However, the effect was significant only for survivorship no., which declined by 34% in NB and by 41% in SD. The northernmost population, BCW, showed no significant inbreeding effects.

Results of tests for outbreeding avoidance are shown in Table 2. In tests with populations BCW and NB,

Table 1 *Tigriopus californicus*. Effects of full-sibling mating in three populations. Three components of fitness (*H* hatching number, *S* survivorship number, *M* metamorphosis number) were measured in inbreds and controls, and results were standardized so that control means = 1. Inbreds and controls were compared by unpaired, one-tailed Student's t-tests (*P < 0.05; **P < 0.01) (*BCW* Bob Creek Wayside, Oregon; *NB* Natural Bridges State Park, California; *SD* San Diego, California)

Population	No. clutches assayed			Mean fitness of inbred relative to control		
	Inbred	Control	(*df*)	H (SE, *t*-value)	S (SE, *t*-value)	M (SE, *t*-value)
BCW	43	26	(67)	1.145 (0.084, 1.037)	0.963 (0.114, −0.205)	1.129 (0.188, 0.417)
NB	36	43	(77)	0.898 (0.087, −0.747)	0.662* (0.104, −2.150)	0.525 (0.178, −1.642)
SD	35	31	(64)	0.879 (0.090, −0.947)	0.593** (0.104, −2.461)	0.591 (0.141, −1.618)

Table 2 *Tigriopus californicus.* Results of outbreeding-avoidance tests. Females were given choice of two males: male from same population and male from distant population. Overall choice was determined by summing the number of males from a single population chosen in reciprocal crosses ($df = 1$, $*P < 0.05$; $**P < 0.01$; $***P < 0.001$)

Populations	Female choice, male from:			Female overall choice, male from:		
	same population	distant population	χ^2	Population 1	Population 2	χ^2
BCW (Population 1) vs NB (Population 2)						
BCW female	43	46	(0.101)			
NB female	56	31	(7.184**)			
Sum	99	77	(2.750)	74	102	(4.45*)
BCW (Population 1) vs SD (Population 2)						
BCW female	44	60	(2.462)			
SD female	42	66	(5.333*)			
Sum	86	126	(7.547**)	110	102	(0.302)

BCW females showed no preference, while NB females preferred males from within their own outcrop ($p < 0.01$). Our analysis of "overall" mate choice examines the total number of times males from each population were selected (regardless of cross type). The copepod populations we used differed slightly in the color of their exoskeletons, which could possibly confound mate choice by providing males from one population with an advantage. The overall analysis shows that NB males have an advantage ($P < 0.05$), although this is primarily driven by the preference of NB females. In tests with Populations BCW and SD, BCW females selected SD males 58% of the time. Although males from the distant population (SD) were selected slightly more often than males from the same population (BCW), this difference was not statistically significant. In reciprocal crosses, SD females also preferred males from the opposite population, choosing BCW males 61% of the time (statistically significant at $P < 0.05$). Summation of choice between these two populations reveals a significant preference for males from distant populations ($P < 0.01$). The overall analysis suggests that neither population has an advantage.

Results of tests for inbreeding avoidance are shown in Table 3. BCW and NB females selected non-sibling males from within their population over siblings > 65% of the time, resulting in a highly significant preference for non-siblings in both populations. In contrast, SD females chose siblings exactly 50% of the time. The overall summed analysis between these populations suggests that females select random males from within the population significantly more often than siblings ($P < 0.001$).

Table 3 *Tigriopus californicus.* Tests of inbreeding avoidance. Females were given choice of two males: sibling and non-sibling from the same outcrop ($df = 1$, $***P < 0.001$)

Population	Female choice		(χ^2)
	Male sibling	Male non-sibling from same outcrop	
BCW	22	55	(14.143***)
NB	22	51	(11.521***)
SD	33	33	(0)
Sum	77	139	(17.796***)

The males in the inbreeding experiment were raised on two types of diets, since diet affects exoskeloton color and this variation provided a simple means by which to identify males. Non-sibling males had red exoskeletons from feeding on algae, whereas full-sibling males had white exoskeletons from their diet of nutritional yeast. Because differences in color, or differences in fitness due to diet, are potential confounding factors, we examined the effects of color/diet on mate choice. In this experiment, females were given a choice of two males: (1) a male from the same population as the female that had been raised on yeast, (2) a male from the same population as the female that had been raised on algae. Females showed a slight preference for males raised on algae over males raised on yeast in all three populations (BCW, $\chi^2 = 0.062$, NB, $\chi^2 = 0.472$ and SD, $\chi^2 = 1.653$, sum, $\chi^2 = 1.542$). The trend, however, was not significant in any of the three populations or in the summed analysis.

Discussion

Species such as *Tigriopus californicus* that are susceptible to both inbreeding and outbreeding depression might be expected to evolve mate-choice mechanisms to avoid these two extremes. To test this, we used pairs of populations that had already been shown to experience outbreeding depression when hybridized. We also examined whether these particular populations experienced inbreeding depression, and found that full-sibling mating resulted in significant fitness declines in two of the three populations assayed. Despite the evidence that both extreme inbreeding and outbreeding are harmful, the mate-choice assays revealed that individuals avoided inbreeding but did not clearly avoid outbreeding. In fact, there was some evidence for outbreeding preference.

These results contrast with previous mate-choice studies in this species which showed random mate-choice both within populations (Brown 1991; Edmands unpublished data) and between populations (Brown 1991; Ganz 1994; Ganz and Burton 1995; Edmands unpublished data) In the present study, tests of inbreeding avoidance (Table 3) revealed a highly significant preference for non-siblings over siblings in two populations (BCW and NB), while one population (SD)

showed no preference whatsoever. This inbreeding avoidance was apparently not influenced by variation due to different diets, since females showed no preference for males raised on yeast versus algae. In tests of outbreeding avoidance (Table 2), one pair of populations (BCW and SD) showed an overall preference for the opposite population, while the other pair of populations (BCW and NB) exhibited no overall pattern.

The lack of inbreeding avoidance in Population SD apparently did not arise from an immunity from inbreeding depression in that population (e.g. through purging of deleterious alleles), since Population SD had the highest degree of inbreeding depression of the three populations assayed. Instead, the difference may be attributable to the fact that the SD population was maintained in laboratory culture for up to 11 mo before the mate-choice assays, while the BCW and NB populations were replaced by wild-caught individuals during the experiment. Thus, assuming our samples from SD were highly inbred due to extended periods under laboratory conditions, there may be little genetic difference between siblings and non-siblings. This may also explain the preference of SD females for BCW males (Table 2), since females may prefer males from distant outcrops when their only other option is a male from within their population (a close relative) in order to avoid inbreeding. Therefore, the preference of SD females for BCW males (Table 2) may be driven by inbreeding avoidance instead of an actual preference for crossbreeding. However, this "inbred SD hypothesis" does not explain the non-significant trend of BCW females preferring SD males (Table 2). The overall preference of BCW females for males from a distant population (SD) in this particular pair of populations may be related to the rare-male advantage that has been reported for a variety of taxa (e.g. Sinervo and Lively 1996; Gillespie and Oxford 1998; Singh 1999).

The most probable mechanism enabling *Tigriopus californicus* to recognize close relatives is via chemical signaling. The system may be similar to the mother/offspring chemically-mediated recognition suggested by Lazzaretto and Salvato (1992), whereby it is believed that mothers saturate their offspring with a chemical scent. A less direct method of kin recognition is possible, whereby courtship chemoreceptors are stimulated by novel pheromones (those from a distantly related line) more effectively than by pheromones of a close relative: thus, inbreeding is avoided by default (Averhoff and Richardson 1974).

Interestingly, NB females appear to have a strong preference for NB males (Table 2), implying that a mechanism for outbreeding avoidance may exist. However, the summed analysis between the BCW and NB populations suggests that mate selection is not determined by a preference for outcrossing in these lines but by an overall advantage of the NB males. Variation in body color (whereby BCW is paler than NB and SD) probably did not influence female choice. In fact, when given a choice between a white male (raised on yeast)

and a red male (fed on algae) from within the population, females did not select preferentially. However, individuals from the NB populations tended to be very large and, although every effort was made to provide males of identical size, discrepancies were sometimes unavoidable. To date, the potential advantage of large body size in male copepods is poorly investigated, but future studies in this arena may prove useful.

Throughout this paper we have dubbed mating preferences as "female choice", but male–male competition could also have played a role. Because each mate choice assay involved one female and two males, male–male competition could have had an effect if, for example, males compete more vigorously when the only available female is a non-sibling rather than a sibling.

The evolution of mating preferences discouraging outbreeding in *Tigriopus californicus* may be opposed by the temporary increase in fitness in F_1 hybrids. Alternatively, the lack of premating isolation may be a simple consequence of the absence of selection for such behaviors in wholly allopatric populations. Premating isolation evolves more rapidly in sympatric populations than in allopatric populations (Coyne and Orr 1997). Despite a strong potential for gene flow (*T. californicus* are capable of dispersal at all stages of their life cycle and are sometimes swept out of their pools during storms), genetic data suggest that populations have been isolated for up to millions of years (e.g. Burton and Lee 1994). This isolation may result from high predation in lower tidal zones (Dethier 1980) that prevents copepods from colonizing new sites, or from outbreeding depression when colonists do reach new sites. While our results show no evidence of recognition mechanisms preventing crossbreeding in *T. californicus*, the species does appear to have behavioral mechanisms that limit dispersal: when a wave threatens to remove them from their pools, the copepods dive and cling to the substratum (Vittor 1971). Although such behavior probably did not evolve in response to outbreeding depression, it may nevertheless play a major role in restricting gene flow.

Our results suggest that mating preferences in *Tigriopus californicus* have evolved to avoid the hazards of inbreeding, but not the hazards of outbreeding. If this pattern is common to other taxa, then it may cause problems when formerly allopatric populations are mixed together. While there are few well-documented examples of outbreeding depression in the literature, there is considerable concern over negative effects of crossbreeding that may result from accidental introductions, introgression between cultivated populations and wild populations (Linder et al. 1998), and/or intentional translocation aimed at bolstering population size and combating genetic erosion (Griffith et al. 1989; Avise 1992; Mills and Allendorf 1996). Negative consequences of population mixing could be particularly acute if formerly allopatric populations are unable to avoid outbreeding. These problems would be exacerbated if, as in *T. californicus*, temporary outbreeding enhancement in the F_1 generation results in a dispro-

portionate influx of unfit F_2 and backcross individuals. The fitness consequences of mate choice are rarely known, particularly in marine taxa (e.g. Knowlton and Jackson 1993). Future studies will be needed to determine if the patterns found in *T. californicus* are a common phenomenon.

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