

Impact of Nest Substrate Limitations on Patterns of Illegitimacy in the Fathead Minnow, *Pimephales promelas* (Cypriniformes: Cyprinidae)

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The fathead minnow (*Pimephales promelas*) is a nest-building North American cyprinid that spawns multiply and exhibits exclusive male parental care. Here, we examined the effects of nest substrate availability on the genetic mating system in 2 natural populations from the glacial pothole region of eastern South Dakota. The sites differed dramatically with respect to the type and density of available nest substrate. Substrate effects were examined by comparing the percentage and distribution of illegitimate offspring between the 2 sites. A total of 1338 embryos from 42 nests (21 from each site) were genotyped at 4 or 5 microsatellite loci. Bimodally distributed frequencies of illegitimate offspring occurred in the nests of guardian males from both sites and indicate the presence of nest takeovers as well as previously undocumented alternative reproductive tactics. As predicted, the rate of usurped nests, a previously documented behavior, was higher in the nest substrate-limited population; furthermore, males were significantly larger here and acquired more mates, suggesting a difference in sexual selection regime. Although these results are based on a single comparison between 2 sites, the results are intriguing and provide a foundation for future studies.

Sexual selection results from differential mating pressures between males and females and is a prominent force driving the evolution of elaborate sexual dimorphism and extreme variation among animal mating systems (Andersson and Iwasa 1996). The 2 major components of sexual selection, intrasexual competition among the nonlimiting sex and mate choice by the limiting sex, have long been linked to environmental factors (Verner 1964; Selander 1965). Three decades ago, Emlen and Oring (1977) emphasized the importance of considering ecological constraints in predicting patterns of intra- and interspecies mating system variation. Recent studies have continued to draw correlations between environmental variables and the intensity of

sexual selection (Clutton-Brock et al. 1997; Coltman et al. 1999; Gamble et al. 2003). In the present case, we present such a correlation. Although sample size is limited, results spur many interesting directions for future research.

The advent of molecular techniques has led to a better understanding of mating system evolution and allows for empirical assessment of clandestine behaviors that are difficult to observe in the field (Avisé et al. 2002). For instance, alternative reproductive tactics (e.g., sneak fertilization) are now known to be quite common and occur across a wide taxonomic range. These and other relevant behaviors should be under the strong influence of sexual selection (Arnold 1994; Andersson and Iwasa 1996; Sinervo and Lively 1996); however, only a limited number of studies have investigated multiple populations that experience different sexual selection regimes (Kelly et al. 1999; Jones et al. 2001).

The fathead minnow (*Pimephales promelas* Rafinesque, 1820) provides an interesting system for investigation. It is a ubiquitous nest-building North American cyprinid that is sexually dimorphic, spawns multiply, and exhibits male parental care (McMillan and Smith 1974). Mature males construct nests on submergent vegetation, defend an adjacent territory, and court females (Cole and Smith 1987). *Pimephales promelas* is known to exhibit allopaternal care after nest takeovers (Unger and Sargent 1988; Sargent 1989), and it has been suggested that this is due to a female preference for males with nests that already contain eggs (Unger and Sargent 1988; Sargent 1989).

We used microsatellite markers to examine patterns of illegitimacy in 2 natural populations of *P. promelas* that differed dramatically with respect to the type and availability of nest substrate. These markers also allowed us to investigate the possible occurrence of other cuckolding behaviors such as “sneak” and satellite fertilization (Gross 1982, 1991) as well as multiple maternity. Our data did not allow us to distinguish between sneak and satellite fertilizations; therefore, we use the term sneak to encompass

Table 1. Physical comparison of study sites

Site	Maximum perimeter (m ²)	Maximum depth (m)	o.d.s. (m)	NS quantity	NS density	Saturation (estimated)
Substrate limited	1850	3.0	7.5	700	0.05	>0.9
Nonlimited	620	1.5	5.0	2900	0.94	<0.3

NS, nest substrate; o.d.s., occupied distance from shore; NS density = NS quantity \div (perimeter \times maximum o.d.s.), units = NS/m²; saturation = estimated percentage of available substrate occupied by at least one territorial male during the time of collection.

both possibilities. Our a priori expectation was that nest substrate limitation—that is, a reduced availability of substrate—would elicit 1) increased rate of turnover in nest site ownership in territorial males and 2) increased polygyny due to the combination of heightened intrasexual competition among males and diminished opportunity for mate choice among females. To our knowledge, alternative reproductive tactics have never been investigated in *P. promelas* and have only been suggested by one previous author (DeWitt 1993). Due to aggressive nest guarding and concurrent paternal care by nest-tending males, the potential payoff for a reproductive parasite is high and, because fertilization is external, the opportunity to steal fertilizations is readily available to sexual competitors (Taborsky 2001). Consequently, we expected to find genetic evidence consistent with these behaviors.

Materials and Methods

Site Comparisons

Although *P. promelas* occurs throughout much of North America, it often inhabits turbid waters that are not amenable to snorkeling observation; therefore, 2 study sites were identified in the prairie pothole region of eastern South Dakota, an area characterized by an abundance of shallow, clear wetlands. These sites (site 1, 45°52'27"N, 96°55'00"W, and site 2, 44°24'18"N, 97°04'02"W) were chosen because they differed in the type and density of suitable *P. promelas* nest substrate. Because pothole wetlands are generally shallow, winter hypoxia prevents the establishment of most large piscivorous fishes (Peterka 1989). *Pimephales promelas* is tolerant of hypoxia and is one of only a few local fishes that can survive such conditions (Klinger et al. 1982; Magnuson et al. 1985); thus, if present, it is usually abundant.

Prespawn collections were conducted with seines and minnow traps to compare catch per unit effort (CPUE) values, a proxy for population density (Bannerot and Austin 1983), between the 2 sites. Concurrently, snorkeling transects across both wetlands revealed suitable nest substrate only within 7.5 m of the shoreline at site 1 and 5 m of the shoreline at site 2. These zones were investigated further to identify and quantify available nest substrate (Table 1). Individual *Typha* sp. plants extending at least 20 cm below the surface and every 1-m length of wood substrate were counted as one unit. Hereafter, site one is referred to as

“substrate limited” and site 2 “nonlimited.” These terms are based on the availability of nest substrate (Table 1), not on *P. promelas* density.

Progression of the spawning season was monitored by periodic snorkeling visits until at least 20 nesting males were active at each study site. Nests were then marked via tethered floats so that relative positions could be mapped from shore, and guarding males were monitored via snorkeling for several days. Guardians remained in close proximity to their nests at all times; thus, we were able to collect nest-holding males with a standard 23 \times 28-cm aquarium net with high certainty of guardian/nest association. Nest markers allowed the precise measure of distances between nests with a steel tape. These estimates were used to calculate internest distance (i.d.), the mean distance to the 3 nearest neighbors (Goldschmidt et al. 1992). Twenty-one guardian males were collected (with nests) from each site and preserved in 95% EtOH. Other studies have shown that gonadic mass may vary with reproductive strategy employed (Mackiewicz et al. 2002); therefore, we also measured standard length and calculated gonadosomatic indices (GSI) (Jennings and Philipp 1992) for all males collected.

Additional snorkeling observation revealed that, at the time of collection, the substrate-limited site was highly saturated with virtually every unit of available substrate occupied (Table 1). At the nonlimited site, substrate availability was clearly not a limiting factor as large sections of suitable substrate were unoccupied. If this were simply a phenological effect, we would expect to see the opposite saturation pattern; that is, the (higher latitude) substrate-limited site should lag behind the (lower latitude) nonlimited site.

Seven microsatellite loci previously developed by the authors (Bessert and Ortí 2003) and 2 others (Walker D, Avise JC, unpublished data) were assessed for variation and statistical exclusion power in both study populations. Five were selected for use in this study (Table 3). Genomic DNA was extracted from hypaxial muscle tissue from 50 adults in each population. Each locus was amplified in all adults and tested for Hardy–Weinberg (HW) equilibrium and (pairwise) linkage disequilibrium with GENEPOP 3.4 (Raymond and Rousset 1995). Exclusion probabilities were calculated according to Chakraborty et al. (1988).

In order to determine an appropriate sample size for parentage analyses, we considered paternity and maternity separately. Because sneak events typically result in a smaller proportion of illegitimate embryos, we based our sampling

Table 2. Comparison of substrate type and i.d. for analyzed nests

Site	Total no. of nests analyzed	Wood	Substrate			Mean i.d. (m)	
			Typha stem	Leaf blade	Artificial	Total	Outliers*
Substrate-limited	21	20	1	0	0	6.13	3.22
Nonlimited	21	0	9	9	3	4.91	1.92

"Artificial" refers to 3 nests that cooccurred on a single piece of cardboard. Asterisk indicates a significant difference in mean i.d. when 3 extreme outliers (>10 m i.d.) from each site were excluded.

scheme on the assumption that sneak tactics do occur in the species and that the success rate approximates the mean 10% from 11 previous studies of paternity in fishes reviewed by Avise et al. (2002). If this is so, the binomial probability of failing to encounter at least one illegitimate embryo in a sample of 30 or greater is exceedingly small ($P < 0.001$). In considering maternity, we estimated the number of embryos necessary to detect all dams contributing to a nest with the computer simulation BROOD (DeWoody et al. 2000). Given the degree of variation in the selected markers (Table 2), results from this analysis indicated that if 5 or fewer dams contributed equally to a given nest containing 500 embryos, a sample of 35 embryos would be sufficient to detect all females. If fewer than 5 dams contributed, fewer embryos would be sufficient. We genotyped on average 31.9 embryos per nest.

Molecular Techniques

DNA was isolated from embryos using a modified chelex extraction protocol (Walsh et al. 1991). In the nonlimited population, 5 loci were amplified in 2 multiplexed 10 μ l polymerase chain reactions (PCRs). The first contained 2.0 μ l of 1 mM deoxynucleoside triphosphates, 1.0 μ l of 10 \times PCR buffer, 0.4 μ l of 50 mM MgCl₂, 0.4 μ l of 10 mM *Ppro126F**, 0.4 μ l of 10 mM *Ppro126R*, 0.4 μ l of 10 mM *Ppro132F**, 0.4 μ l of 10 mM *Ppro132R*, 0.5 μ l of 10 mM *Pn1.3F** and 0.5 μ l of 10 mM *Pn1.3R*, 0.1 μ l (0.5 units) of *Taq* DNA polymerase, 2.9 μ l of ddH₂O, and 1.0 μ l of (\approx 100 ng) DNA (* = fluorescently labeled). Reactions were denatured at 94 $^{\circ}$ C for 2 min followed by 30 cycles at 94 $^{\circ}$ C (30 s), 53 $^{\circ}$ C (30 s), and 72 $^{\circ}$ C (30 s) with a final extension of 72 $^{\circ}$ C (2 min). Reagents were the same in the second reaction except for primers (0.4 μ l of 10 mM *Ppro118F**, 0.4 μ l of 10 mM *Ppro118R*, 0.4 μ l of 10 mM *Ppro171F**, and 0.4 μ l of 10 mM *Ppro171R*) and volume of ddH₂O (3.9 μ l). Thermal cycling conditions were 94 $^{\circ}$ C for 1 min, then 30 cycles of 94 $^{\circ}$ C (30 s), 66 $^{\circ}$ C (40 s), 72 $^{\circ}$ C (40 s), and a final extension of 72 $^{\circ}$ C (2 min). Amplifications were conducted similarly for the substrate-limited population except that locus *Ppro126* was omitted and the volume of ddH₂O increased to 3.7 μ l.

Analyses

Multilocus genotypes for all embryos sampled from a given nest were compared with the genotype of the respective guardian male. Illegitimate embryos were deduced when an

embryo failed to share at least one allele at every locus with a guardian male. In order to minimize the effects of possible scoring errors and de novo mutations (Jones and Avise 2001; Dakin and Avise 2004), we took a conservative approach and only considered an embryo illegitimate if (both) alleles differed by more than one repeat unit from the guardian at 2 or more loci. A conservative estimate of the minimum number of dams contributing to a nest was determined by subtracting paternal (guardian male) alleles from the set of genotypes present in the sampled embryos from each nest and then determining the smallest integer value equal to or greater than half the remaining number of (maternal) alleles (Kellogg et al. 1998). Standard analysis of variance (ANOVA) and *t*-tests were performed with SPSS 13.0 for Windows (SPSS Inc., Chicago, IL) to compare id, mean parentage, and guardian physical measures between populations.

Results and Discussion

Marker Screening

In the substrate-limited population, loci *Ppro118*, *Ppro171*, and *Pn1.3* did not deviate from HW equilibrium. Under the assumption that neither parent was known with certainty, the combined exclusion probability was 0.986, sufficiently high for our purposes. In the nonlimited population, one locus (*Ppro171*) departed from HW equilibrium ($P < 0.01$), possibly indicating the presence of null alleles. A second locus (*Ppro118*) exhibited a bimodal allelic distribution with a class of very long alleles (>500 bp). To circumvent these problems, we added a fifth locus (*Ppro126*) to the nonlimited analysis. Information from locus *Ppro171* was not discarded but used only to verify illegitimate candidates revealed by the other markers. Even when *Ppro171* was not considered, the combined exclusion probability was very high (0.984).

Illegitimate Frequencies

Illegitimate offspring were detected in 8 of 21 nests from the substrate-limited site and 6 of 21 nests from the nonlimited site (Figure 1). Within-nest illegitimate frequencies (*if*) exhibited a bimodal distribution. One group ranged from 0.03 to 0.10 and the other from 0.40 to 1.00. This finding is consistent with the occurrence of 2 types of cuckolding behavior, sneak fertilizations and nest takeovers, as previously documented in a wide range of teleost fishes

Table 3. Loci used for paternity analysis in *Pimephales promelas*

Population locus/ GenBank accession number	Substrate-limited								Non-limited							
	<i>n</i>	<i>k</i>	<i>s</i>	H_O	H_E	P_E^2	P_E^1	<i>n</i>	<i>k</i>	<i>s</i>	H_O	H_E	P_E^2	P_E^1		
<i>Ppro118</i> AY254352	50	20	175–339	0.90	0.95	0.79	0.88	52	29	167–595	0.90	0.95	0.79	0.88		
<i>Ppro126</i> AY254353	—	—	—	—	—	—	—	50	9	159–183	0.78	0.80	0.44	0.61		
<i>Ppro132</i> AY254354	50	19	130–180	0.84	0.86	0.55	0.71	52	11	130–186	0.54	0.54	0.18	0.36		
<i>Ppro171</i> AY254357	42	18	228–320	0.90	0.94	0.77	0.87	51	23	224–344	0.88	0.92	0.71	0.83		
<i>Pn1.3</i>	50	5	107–117	0.80	0.82	0.40	0.58	49	5	107–117	0.82	0.75	0.32	0.49		
Combined P_E						0.986	0.998	Combined P_E					0.980	0.997		

n, sample size; *k*, number of alleles; *s*, allele size range (bp); H_O , observed heterozygosity; H_E , expected heterozygosity; P_E^2 , exclusion probability when neither parent is known with certainty; P_E^1 , exclusion probability when one parent is known with certainty.

(Gross 1982; Taborsky 2001; Avise et al. 2002; Sato et al. 2004). It is possible that higher *if* values were the result of highly successful sneak events; however, it is more likely that they were the result of nest takeovers given previous documentation of such behaviors in the species (Unger and Sargent 1988; Sargent 1989). When it was possible to discern multiple clutches within the same nest (via developmental differences or spatial isolation), we employed stratified sampling to ensure equal representation in the analysis. We discovered 3 instances where the guardian male was excluded as the sire for all embryos in one (older) clutch but not excluded as the sire for any embryos in one or more newer clutches.

Clearly, these, and cases of 100% exclusion, reflect nest takeover events. Consequently, each nest was placed into one of 3 categories: no cuckoldry, nest takeover, and sneak (Figure 1).

Site Differences

The sites differed in the type, density, and level of occupation (saturation) of available nest substrate at the time of collection (Tables 1 and 2). In addition, there was a difference in spatial distribution (*id*) of occupied nests when 3 extreme outliers (>10 m i.d.) from each site were excluded from an independent sample *t*-test ($t = 2.586$,

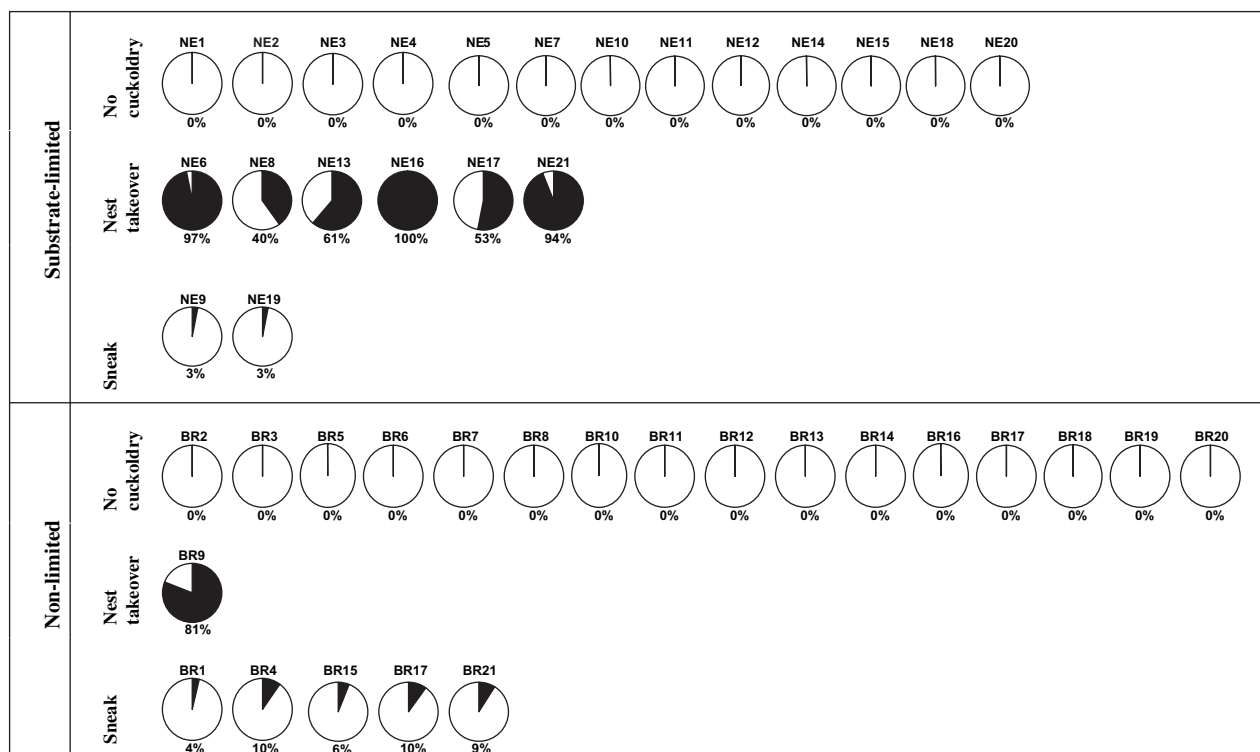


Figure 1. Pie diagrams showing the proportion of illegitimate embryos and parentage category of each nest. Shaded areas represent proportion of illegitimate embryos, and exact percentage is indicated below each nest. Specific nest identity is indicated above each pie diagram.

degrees of freedom [df] = 38, $P = 0.014$; substrate-limited mean $id = 3.22$ m; nonlimited mean $id = 1.81$ m). The substrate-limited site was bordered primarily not only by grasses but also by small patches of *Typha angustifolia* and *Typha latifolia* (Typhales: Typhaceae) and overhanging trees. Throughout the study, most *Typha* (>95%) were above the shoreline at this site. The nonlimited site was bordered by a mixed community of *T. angustifolia* and *T. latifolia* that extended below the shoreline. Based on snorkeling surveys, we estimated at least a 4-fold difference in the quantity of available nest substrate between the low and nonlimited sites (Table 1). Although prespawning collection surveys were limited, it is important to note that the CPUE did not provide any evidence for a difference in *P. promelas* density between the 2 sites ($t = -0.0793$, $df = 4$, $P = 0.941$).

The 2 sites did not differ with respect to number of embryos per nest ($t = 1.20$, $df = 40$, $P = 0.237$), number of embryos genotyped per nest ($t = 1.846$, $df = 40$, $P = 0.072$), or GSI in guardian males ($t = 0.176$, $df = 40$, $P = 0.831$); however, guardian males were larger (standard length: $t = 2.530$, $df = 42$, $P = 0.015$; dry somatic mass [equal variances not assumed]: $t = 4.26$, $df = 35.6$, $P < 0.001$) and acquired more mates (minimum number of dams contributing to a nest: $t = 3.390$, $df = 35$, $P = 0.002$) at the substrate-limited site (substrate-limited mean = 3.63 dams and nonlimited mean = 2.86 dams; see Figure 2). Only one nest takeover occurred in the sample from the nonlimited site (1 of 21 nests), whereas in the substrate-limited site, this number (6 of 21 nests) was significantly higher, albeit narrowly ($P = 0.047$, 1-tailed Fisher's exact test). Usurped nests were examined closely to determine whether any illegitimate embryos could be the product of sneak fertilization by one or more additional males, but we did not detect any such instances. Only 2 nests in the substrate-limited site had been sneaked, whereas 5 had been sneaked in the nonlimited site. These numbers suggest a positive relationship between nest density and sneak incidence, but it is not significant ($P = 0.263$, 1-tailed Fisher's exact test).

In comparing sneaked, usurped, and noncuckolded nests, we found no correlation between substrate type and parentage category ($\chi^2 = 10.608$, $df = 6$, $P > 0.10$). We also found no evidence (via ANOVA) to suggest any differences in i.d. with respect to parentage category (data log transformed: $F_{2,33} = 0.099$, $P = 0.906$). Males guarding usurped nests ($n = 7$) were larger than their counterparts (SL: $t = 2.682$, $df = 40$, $P = 0.011$; log-transformed somatic mass: $t = 3.251$, $df = 40$, $P = 0.002$); however, usurped nests were excluded from minimum number of dam estimates due to difficulties arising from assignment of a nest to more than one sire.

Alternative Tactics

This study indicates that allopaternal care in fathead minnows occurs through both nest takeovers and one or more previously undocumented alternative reproductive tactics—either sneak or satellite behaviors (or both). Although usurping males are known to selectively prune

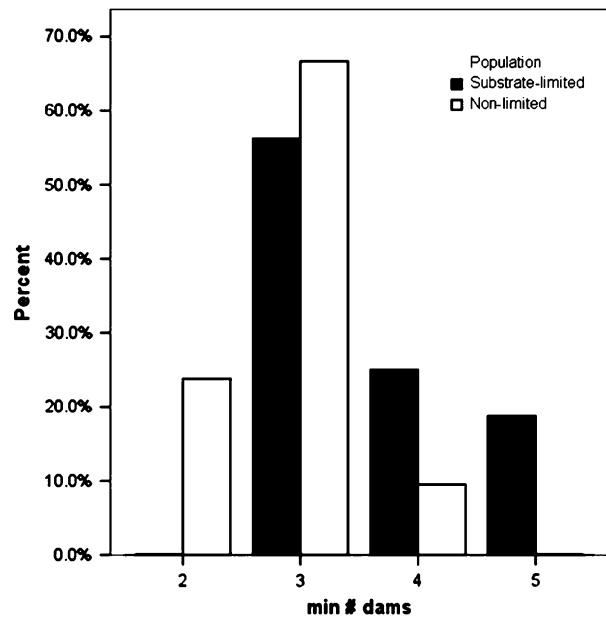


Figure 2. Comparison of mating success for nest-holding males in New Effington (substrate limited) and Brookings (nonlimited) populations. Histograms indicate the minimum number of dams occurring in nests as determined by the minimum method (Kellogg et al. 1998).

away nonsired embryos (Sargent 1989), it is unlikely that this would result in the low frequencies (3–10%) observed in 7 of the nests. The hereditary nature of this strategy is unknown; however, a recent investigation found that smaller male fathead minnows accelerated their reproductive condition and guarded nests only when larger conspecifics were absent (Danylchuk and Tonn 2001). This plasticity suggests that alternative tactics are probably facultative choices rather than a hard-wired, mixed evolutionary stable strategy (Gross 1982).

Substrate Effects

Changes in resource (in this case, nest substrate) availability, type, and density should result in shifts in male–male competition with a corresponding change in the intensity of sexual selection. This, in turn, should manifest itself in predictable empirical differences in mating success among males (Emlen and Oring 1977). There were, indeed, qualitative and quantitative differences between the sites. This is not surprising given that nest substrate was readily available at one site but not the other. The significantly larger size of males at the substrate-limited site is consistent with strong male–male competition. Here, male (and female) intruders swarmed over every nest almost immediately after the guardian was removed. Indeed, nests had to be guarded by hand in order to prevent their destruction prior to collection. At the nonlimited site, no immediate threats of this nature were observed, and nests could be

collected in a relaxed time frame after the guardian was removed.

Although not statistically significant, the data suggest a trend of decreased sneak rates at the substrate-limited site relative to its counterpart. The higher rate of turnover in nest ownership at the substrate-limited site poses some fascinating questions. Here, guarding males may be subject to reduced lifetime reproductive capacity. Further tests are necessary to determine whether this is the case. If so, this mechanism may homogenize, or at least reduce, the overall variance in male reproductive success, thus limiting the potential payoff for reproductive parasites. Larger males at the substrate-limited site may also be better able to defend against sneakers.

At the nonlimited site, nest substrate availability was not a limiting factor as a high proportion of possible nest sites remained unoccupied during the study. At the same time, the analyzed nests were more tightly clustered than their counterparts at the substrate-limited site (Table 2). Because nest sites were in closer proximity to one another, it may be easier for females to assess male quality via visual or chemical cues at this site. This, in combination with longer nest tenure by guarding males, could result in increased variance in male lifetime reproductive potential and increase the potential payoff for sneakers.

Nest density has been shown to impact patterns of cuckoldry as colony nesters are subject to greater rates of sneak tactics in a number of taxa (Jennings and Philipp 1992; Neff 2001; Neff et al. 2004); however, this is usually attributed to cuckoldry by neighboring males in crowded conditions. This pattern was not evident in our study as we were able to attribute paternity of only a single illegitimate embryo to a neighboring male in the substrate-limited population; however, increased sampling may reveal additional cases.

Another factor to consider is predation regime. Kelly et al. (1999) found that guppy populations subject to higher predation pressure exhibited significantly higher incidences of multiple paternity (via multiple insemination). This was attributed to decreased risk of predation by males that employed alternative tactics, but it is unlikely that this factor increased sneak rates at the nonlimited site. In fact, this site probably experienced lower predation pressure than the substrate-limited site. Although local residents claim to have seen *Esox lucius* (Esociformes: Esocidae) here, the only other fish taxon was *Culaea inconstans* (Gasterosteiformes: Gasterosteidae), which is not a piscivore. Other predators may enter during floods but probably would not survive winter hypoxia. At the substrate-limited site, long-term persistence of *Ameiurus melas* (Siluriformes: Ictaluridae), a piscivore, is evident. In early spring snorkeling surveys, large *A. melas* and *Chelydra serpentina* (Testudines: Chelydridae) skeletons could be seen. Predation pressure may indeed be a selective factor for larger size in this population.

Finally, at the nonlimited site, males had little choice but to construct nests on substrates that were highly exposed to potential invaders (e.g., vertical *Typha* stems). Although nest substrate is scarcer in the substrate-limited site, males were

able to construct “typical” *Pimephales* nests—that is, on the undersurface of a submerged object. In fact, several males excavated cavities underneath fallen limbs that rested on the soft wetland substrate. These sites may be more easily defended from sneakers.

Although this study compared only 2 fundamentally different sites, the results are intriguing and pave the way for future investigations with greater replication (e.g., multiple controlled mesocosms to determine with more certainty the effects of substrate limitations on behavior, breeding experiments to determine the hereditary basis of the behavior, etc.). Although genetic surveys are labor intensive, we encourage further efforts. As in this case, we still do not understand the full impact of environmental factors on selection regimes and are not able to predict all outcomes with certainty; yet, the capacity to do so is important as human-mediated environmental factors have already been shown to interfere with sexual selection regimes and cause mating system breakdown (Jarvenpaa and Lindstrom 2004). With molecular tools in place, *P. promelas* and its widespread congeners provide an excellent venue for future studies.

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