

Multiple paternity and competition in sympatric congeneric reef fishes, *Embiotoca jacksoni* and *E. lateralis*

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Abstract

The black surfperch *Embiotoca jacksoni* and the striped surfperch *E. lateralis* (Embiotocidae, Perciformes) are livebearing temperate reef fishes that live sympatrically over a large portion of their distribution range, where they exhibit strong ecological competition. In order to assess whether mating strategies reflect competition, we investigated multiple paternity in these two species in an area of sympatry. We sampled 24 pregnant females (12 for each species) in Monterey Bay, California, used microsatellite analysis and assessed paternity with the COLONY software. While broods are relatively small (12 to 36 offspring), they were always sired by multiple fathers (2 to 9), with no correlation between the size of a brood and the number of fathers. The number of sires for each brood was not significantly different between the two species (approximately 3.5 sires per brood). We tested the deviation from stochasticity of fathered offspring for each father in one brood. Results showed a significant deviation for both *E. jacksoni* and *E. lateralis*. However, this deviation was not found to be significant between species. The striking similarity in the dynamics of multiple paternity in these species, when sampled in sympatry, may result from several alternative scenarios, including phylogenetic inertia, reproductive behaviour, and ecological competition.

Keywords: *Embiotoca*, microsatellites, multiple paternity, phylogenetic inertia, surfperches

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Introduction

Multiple paternity in natural populations is observed when polyandrous females successfully mate with different males during a single period of reproduction (e.g. Hanken & Sherman 1981; Travis *et al.* 1990; Davies 1991; Klemme *et al.* 2008). Usually, male fitness is determined by the number of successful matings. For live-bearing females, limited by the number of offspring they can bear, fitness is a function of the rate of successful zygote production, thus classically leading to the view that female fitness does not significantly increase with multiple mating (Bateman 1948), or that fitness of the offspring may be affected (Evans & Kelley 2008). The tenets of such sexual conflict were challenged with the application of molecular markers, particularly microsatellites (Avisé 2004), when the observation of multiple paternity became increasingly common (e.g. Birkhead 2000; Simmons 2005). Female multiple matings were then seen as ways to offset

potential costs of monogamy. These include bet-hedging, in order to diversify the potential for finding good genes (Jennions & Petrie 2000; Makinen *et al.* 2007; Hyde *et al.* 2008); and, in the case of intra-locus sexual conflict, where a single locus affects the fitness of both males and females, multiple successful matings allow females to mitigate the conflict by selecting different fathers for producing sons and daughters without lowering their relative fitness (Chippindale *et al.* 2001; Calsbeek & Bonneaud 2008).

The reproductive success of an individual can be measured by the number and size of offspring produced and the genetic diversity of the brood. In this study, we focus on two congeneric live bearing species of surfperches (Embiotocidae) which are in strong ecological competition. For live-bearing species, in which the number and the size of offspring are strictly limited, fitness can be increased with multiple matings during a single period of reproduction. Usually, ecological competition translates in a divergence of some life history trait (e.g. benthic and limnetic sticklebacks, Schluter 2000). Using different food resources should change total energetic intake, growth rates, predation risk and life span, yet none

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of these characteristics seem to apply to our focal species. This paper is thus assessing whether these similarities extend to reproductive behaviour as well.

Surfperches are viviparous teleost fishes which occur in the north Pacific from Japan to Mexico (Tarp 1952; DeMartini 1969; Bernardi & Bucciarelli 1999). Nineteen of 23 species of surfperches occur along the California coast (Miller & Lea 1972) and are considered northern, temperate species (cold affinity; Holbrook *et al.* 1997; Horn *et al.* 2006). They are found in a variety of marine habitats, including sandy and rocky reefs, kelp beds, bays, estuaries and eel grass beds, except the tule perch, *Hysterothorax traskii*, which is found inland California fresh waters (Allen & Pondella 2006; Stephens *et al.* 2006).

Our study focuses on two congeneric sister species, and the sole representatives of the genus *Embiotoca*, the black surfperch *E. jacksoni* and the striped surfperch *E. lateralis*. Black surfperch and striped surfperch live sympatrically in northern and central California, USA, as well as in northern Baja California, Mexico (Bernardi 2000, 2005). These two species are in strong competition for food resources (Hixon 1980, 1981; Holbrook & Schmitt 1986, 1995; DeMartini 1988; Schmitt & Holbrook 1990). While the two species overlap completely in the range of depth occupied (0 to 15 m), their populations are not similarly distributed among depth-related habitats. Striped surfperch are commonly seen in the shallowest microhabitats, where they feed on organisms associated with foliose algae such as *Gelidium robustum*, whereas black surfperch, somewhat more evenly distributed among depths, are more common in deep habitats (Hixon 1980). This distribution pattern might be the result of intense competition between these species for the shallowest habitats, which led to a differentiation in foraging behaviour (such as the capability of winnowing, sorting food within the mouth cavity, in black surfperch, Laur & Ebeling 1983; Bernardi 2005).

E. jacksoni and *E. lateralis* are similar in maximum size (approximately 375 mm in total length), and longevity (seven years) (Baltz 1984). Maximum size and longevity are also identical between males and females (Warner & Harlan 1982). Fecundity is similar, with an average of 30.7 and 32.0 offsprings for seven-year-old *E. jacksoni* and *E. lateralis* females, respectively (Baltz 1984). Both species are reported to be sexually mature between one and two years of age (Isaacson & Isaacson 1966; Behrens 1977; Baltz 1984). Surfperches were observed courting from July through November, with a peak in male gonosomatic index in October (Froeschke *et al.* 2007). However, gravid females were first recorded at the end of December, suggesting that they store sperm for a few weeks and up to six months (Hubbs 1921; Wiebe 1968; Froeschke *et al.* 2007). Dissection of different stages of dwarf surfperch, *Micrometrus minimus*, indicate that vast amounts of sperm thickly line the uterus, and apparently sperm is completely mixed at this stage (Darling *et al.*

1980; Warner & Harlan 1982). During this period of sperm storage, phagocytosis results in a decline of sperm (Warner & Harlan 1982). The storage, mixing and decline of sperm thus provide an opportunity for sperm competition (Darling *et al.* 1980; Warner & Harlan 1982; Phelps *et al.* 1995). As the eggs mature, they are fertilized and the additional sperm is later found in the gut of the babies, possibly used as nutrient (Dobbs 1975; Warner & Harlan 1982). Prenatal mortality was estimated to be approximately 11% (Behrens 1977); these data, however, lack statistical power, and it is difficult to know if prenatal mortality is indeed occurring. While the offspring grow within the uterus, a complex structure made of six interconnected folds, they are fed passively via their expanded dorsal, anal and caudal fins that line the uterine membrane (Behrens 1977). When still small enough to move, offspring frequently change position inside the uterus (Behrens 1977). Finally, offspring are released, in June and July, as free-swimming juveniles, 50 mm to 55 mm in length, which resemble young adults (Isaacson & Isaacson 1966).

Considering the unique mode of reproduction of surfperches, the potential for sperm competition and their relative small size broods, interest arose in determining levels of multiple paternity, if present. Two studies using allozyme assays were performed on the shiner surfperch, *Cymatogaster aggregata*, and the freshwater tule perch *Hysterothorax traskii* (Darling *et al.* 1980; Phelps *et al.* 1995). In both cases, allozymes showed very limited variability, resulting in reduced power to fully assess the levels of multiple paternity; yet in both cases it was shown that the potential for multiple paternity could not be excluded from a subset of the tested broods.

The goal of this study was to assess multiple paternity in two congeneric surfperches, *E. jacksoni* and *E. lateralis*, which are in strong ecological competition. Specifically, we used microsatellite analyses to determine: (i) how many males contribute to a female's brood; (ii) if there is evidence of sexual selection on males; and (iii) if there is evidence of differences in the level of multiple paternity between species.

Materials and methods

Collection and DNA samples

Fish were searched over a 0.2-km² area in Monterey, California (36°37'41.58 N, 121°55'17.90 W), and all encountered individuals were collected while free- or scuba diving, with pole spears, without specifically choosing particular or random individuals except for avoiding non-mature individuals (young of the year). Collections were performed between February and June 2007, thus covering most of the gestation period (which is typically from January to June). For each pregnant female, caudal fin and ovary were taken, put in 95% ethanol and stored at ambient temperature in the field, and then at 4 °C in the lab. Ovaries were later dissected, and offspring were counted and measured to the nearest

Locus	Primers	Repeat	Amp. size	nEJA	nELA	Acc. No.
EJ_A2	F-AGCAAAGGTCAAAGGTCAA R-TTGTGGCTGTTGTTTATGG	(CA) ₂₀	235	6	14	EU781556
EJ_A5	F-AACCGCTGAGTAAGTAAACATC R-TCATCCCACATATTTATAGC	(CA) ₃₀	275	15	12	EU781556
EJ_A11	F-ACTTCCATGACAACAAAGTAGG R-CAAATAAGCCAAGTGTGATG	(CA) ₂₄	283	7	18	EU781559
EJ_A12	F-GAAAGAAGCTCAATGCAATCAC R-AGCAGCTCTCAGATCAGAGGTA	(CA) ₂₄	232	5	N/A	EU781560
EJ_B1	F-ACTCGGACAGTAAAGCTGAGG R-AAAATGTCTCCTTGCAAGGATC	(GATG) ₁₄	180	N/A	5	EU781561
EJ_B5	F-CCACCTGGGGCTAAACTG R-CACGGCAGACAGAGCAAC	(CATC) ₁₅	112	8	N/A	EU781563
EJ_B8	F-GGTCGTATTTTGCAGTATGC	(GATG) ₃₀	266	7	N/A	EU781564

Table 1 Microsatellite loci characteristics. Columns correspond to: microsatellite name (Locus), (F) forward and (R) reverse primer sequence, repeat motif, amplification size of original clone (Amp. size), number of alleles per locus for *Embiotoca jacksoni* and *E. lateralis* (*nEJA*, *nELA*), and GenBank Accession Nos (Acc. No.)

millimetre. Total genomic DNA was prepared from 75–100 mg of tissue using a standard Proteinase K, chloroform extraction procedure (Sambrook *et al.* 1989).

Polymerase chain reaction amplification

In this study, we used seven (out of 21 tested) highly variable microsatellite loci derived from a genomic library based on *Embiotoca jacksoni* DNA and previously analysed (Bernardi 2008). Six of those were used for *E. jacksoni* and four for *E. lateralis* (Table 1). Amplification of 5–50 ng of DNA followed standard reaction protocols, with cycling profiles of 45 s at 94 °C and 1 min at 54 °C, 45 s at 72 °C for 35 cycles, followed by 3 min at 72 °C.

Microsatellite scoring and paternity analysis

Microsatellites were run on an automated sequencer ABI 310 and scored using the software Gene Mapper version 3.7 (Applied Biosystem). Raw scores were then binned and tabulated using ConGene, a MatLab program written by Yann Tremblay (personal communication; available upon request). The potential presence of null alleles was controlled with Microchecker (van Oosterhout *et al.* 2006). In order to analyse paternity, we initially used GERUD 2.0, a program that determines the most likely match (each of which consists of the unique maternal genotype and one or more paternal genotypes), based on patterns of Mendelian segregation and expected genotype frequencies in the population (Jones 2005); however, because our data sets exceeded the maximum number of fathers acceptable by this program (six fathers), we repeated the analysis using Colony (Wang 2004), a likelihood-based program that provides the most likely paternity configuration and does not limit the number of fathers. As for other studies (e.g. Makinen *et al.* 2007; Sefc *et al.* 2008), when comparable datasets were used (less than six fathers), differences between the programs were minimal.

Sperm competition and species differences

Statistical tests were performed to assess the possibility of sperm competition and differences between *E. jacksoni* and *E. lateralis*. We tested for a correlation between brood size and the number of fathers. Differences in number of fathers, per brood, in each species were tested using *t*-tests. Considering that sperm is randomly mixed in the ovary, the degree of paternity for an individual male depends on several factors, including the proportion of his sperm compared to the total amount present, differential sperm motility and chemical interactions (Warner & Harlan 1982; Taborsky 1998; Simmons 2005). We addressed one such variable by determining the deviation in fathered offspring from a stochastic distribution based on a binomial test where each critical *z*-value was compared with the deviation of our data (the null hypothesis being that the proportion of fathered eggs would be the same for each father). In addition, we tested whether the level of deviation from stochasticity was different in *E. jacksoni* and *E. lateralis*, using a *t*-test. Since we wanted to compare the mean of the deviation from the expected of our samples, in *E. jacksoni* and *E. lateralis*, the formula for the *t*-test was a ratio. The ratio compared the difference between the two means to a measure of the variability or dispersion of the scores (the standard error of the difference).

Results

Population results

A total of 51 fish were collected which included 27 mature males and 24 pregnant females (12 *Embiotoca jacksoni* and 12 *E. lateralis*). Within a single pregnant female, offspring were not significantly different in size but varied depending on the gestation stage. There was also no significant difference in offspring size between the two species at a given collection date, indicating that the two species follow approximately the same timing and trajectory in the growth of their offspring

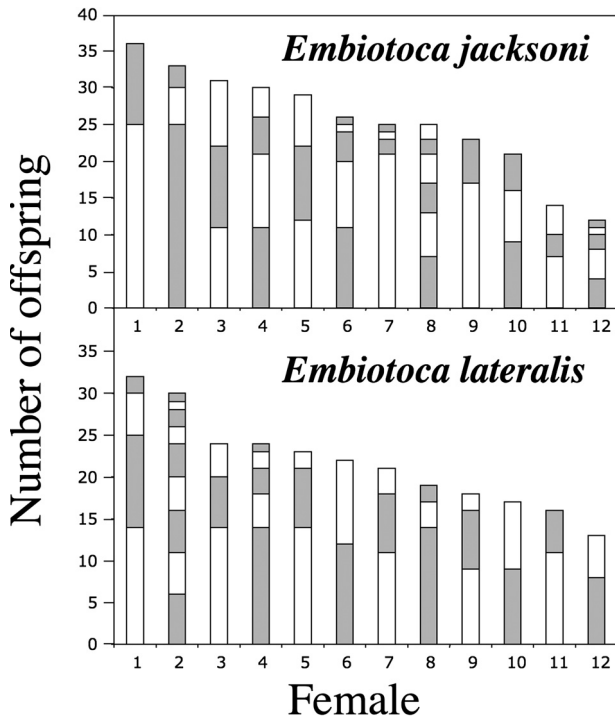


Fig. 1 The most likely division of offspring into full-sib families estimated by the program Colony. Each bar represents the offspring of one female. The bars are divided according to the number of offspring attributed to each male.

and gestation timing. Early in the season (February), the smallest brood had an average size of 8.2 mm, while late in the season (June), the largest brood had an average size of 50 mm. While prenatal mortality has been suggested in the past (Behrens 1977), we did not find a significant relationship between the number of offspring in a brood and the collection date (t -test, $P = 0.38$, for *E. jacksoni* and $P = 0.73$ for *E. lateralis*).

All individuals, including the 24 females and all of their 584 offspring (305 *E. jacksoni*, 279 *E. lateralis*) were successfully amplified and scored. Characteristics for each species and microsatellite locus are described in Table 1. Each locus appeared to segregate normally within progeny arrays and deviation from Hardy–Weinberg equilibrium was not detected in the sample of adult individuals (Bernardi 2008). Microchecker analysis did not reveal the presence of null alleles or possible mutations; indeed, each embryo fitted the maternal genotype for each locus.

Paternity test

Maternal and offspring genotypes were scored, and each potential paternal genotype was assessed using Colony (Fig. 1). Embryos were then attributed to each potential father. Multiple paternities were observed for all females of both species. The number of fathers per brood ranged from two (one *E. jacksoni* and four *E. lateralis* females) to a maximum

of nine fathers (one *E. lateralis* female, Fig. 1). For the *E. lateralis* female who successfully mated with at least nine fathers, both female and brood were re-tested several times independently to ascertain that there were no experimental errors; results were identical in all trials.

Considering that some offspring might disappear over the course of development due to prenatal selection, one might expect higher prevalence of multiple paternity in the early stages of gestation than close to parturition. However, we did not find a correlation between sampling date and either the number of offspring in a brood (as mentioned above), or the level of multiple paternity. In addition, we did not find a correlation between the number of offspring in a brood and the number of fathers for that brood for either *E. jacksoni* ($r = 0.108$, $r^2 = 0.01$) or *E. lateralis* ($r = 0.582$, $r^2 = 0.34$). In order to assess some aspects of sperm competition, we used a binomial test to uncover a possible deviation from a stochastic fertilization of eggs. Results show that five of 12 *E. jacksoni* broods, and also five of 12 *E. lateralis* broods differed from stochastic expectations at the $P < 0.05$ level.

We did not find any putative father that would have been involved in more than one mating event, and we did not collect any male that matched any of the putative fathers, indicating that the sampled population was large enough for us not to sample a small group of interbreeding individuals. On average, *E. jacksoni* broods were fathered by 3.58 males (range 2–6) and *E. lateralis* broods were fathered by 3.50 males (range 2–9). Indeed, the number of fathers per brood was not found to be statistically different between the two species (t -test = -0.3023 , $P = 0.76$). In order to determine if there were differences between deviation from stochastic distribution between species, or if there was greater deviation in one species than the other, we performed a t -test. No significant differences were found between *E. jacksoni* and *E. lateralis* ($P = 0.89$) in that respect.

The ovaries of female surfperches are made of a complex tissue made of six connected chambers. While we carefully dissected fishes and removed the offspring sequentially, the spatial conformation of the uterus is extremely difficult to reconstruct, precluding us from determining a relationship between mating order and mating success, if at all present. However, as mentioned above, offspring frequently move within the uterus during the early phases of the gestation.

Discussion

Small broods and multiple paternity

A variety of reproductive styles have been observed among teleost fishes (Wootton 1990). Males of internally fertilizing species usually make no contribution to the offspring production, except for sperm (Constantz 1984). Sexual selection predicts that such males devote their reproductive efforts to mating, resulting in intense male competition over mates

(Davies 1991; Andersson 1994). Because males may therefore face sperm competition, they may allocate a great part of their reproductive efforts to sperm production to ensure paternity (Andersson 1994; Taborsky 1998; Simmons 2005). While the surfperch broods are relatively small (in our case from 12 to 36 offspring), results demonstrated that all broods were fathered by at least two to nine males.

We examined the data for the presence of a particularly successful male, as evidenced by offspring from different females sharing the same father. Our failure to find such a male suggests that the target population was large enough to preclude us from sampling females that were fertilized by the same male. Yet, it also raises the possibility of a mating strategy where females copulate with several males, while males only mate with a single female.

Distribution of fathered eggs and sperm competition

In addition to multiple paternity, our results highlighted an unequal distribution in the fathered eggs ratio between the different fathers. Previous studies demonstrated that *Embiotoca jacksoni* and *E. lateralis* females are able to store randomly mixed sperm for one to several months (Froeschke *et al.* 2007). Thus, sperm competition may occur inside females, resulting in a post-copulation sexual selection of males. Considering that, in some other fishes, sperm storage can occur for several months to several years (Sogard *et al.* 2007; Thunken *et al.* 2007) it would be important to know the actual duration of sperm storage in surfperches, but also the rate of sperm degradation through time. Sperm competition can result in changes in testes size, sperm production and sperm form and function (Firman & Simmons 2008). Our results are consistent with sperm competition; yet, in the absence of knowledge regarding the relative amount of contributed sperm and other biological and chemical factors, it is difficult to rule out other alternatives (Taborsky 1998). For example, sperm competition can result in genital plugs, toxic semen and dilution factors that have been thoroughly studied in insects (Rice 1998; Simmons 2005). In addition, some mechanical factors have been described where the shape of the penis may help in the removal of previously deposited sperm (Cooper *et al.* 1996; Avise 2004). This is an interesting hypothesis to test in surfperches because, while all surfperches are internal fertilizers, basal genera *Hyperprosopon* and *Amphistichus* lack a penis, while the presence of a penis (called flask organ in surfperches) appears in derived genera, including *Embiotoca* (Tarp 1952; Bernardi & Bucciarelli 1999).

Mating strategy and competition

Our results show that the levels of multiple paternity in *E. jacksoni* and *E. lateralis* are strikingly similar. Such similarity may result from different, and testable, scenarios. The simplest explanation is that the level of multiple paternity evolved

in the ancestor of these two closely related species and was maintained unchanged by 'phylogenetic inertia'. Indeed, in some cases such as honeybees, female reproductive behaviour was shown not to be as precisely fine-tuned as to allow control of the number of eventual sires (Tarp & Page 2000). If this were the case, one would expect other closely related species, such as the rainbow surfperch, *Hypsurus caryi*, to potentially show a similar level of multiple paternity. Another potential scenario concerns the intense ecological competition that exists between sympatric striped and black surfperch. A possibility seldom tested is that the level of multiple paternity itself may provide fitness differences between competing species. Thus, in competing species, multiple paternity levels may reflect the outcome of genetic competition. If this were the case, it is predicted that multiple paternity levels should be identical in competing species, an outcome of 'genetic diversity competition'. Indeed, two possible scenarios are expected. In one case, it is advantageous to have a specific strategy, with females selecting for very high genetic diversity. In this case, we may expect one species to show much higher levels of multiple paternity than the other. In contrast, competition may result in species 'tracking' each other, with diversity and mating strategies being essentially identical.

In the case of *E.* and *E. lateralis*, ecological competition has been thoroughly investigated, and here we show that expectations of one scenario are met, since levels of multiple paternity are virtually identical (*t*-test, $P = 0.32$). While *E. jacksoni* and *E. lateralis* are found in sympatry in Monterey Bay where our study was performed, these two species are found allopatrically in southern California (where only *E. jacksoni* is present) and in Oregon and Washington (where only *E. lateralis* is present) (Bernardi 2005).

The 'genetic diversity' hypothesis may seem far-fetched, and is indeed less likely than the much simpler phylogenetic inertia hypothesis, but it is possible to test it by comparing the levels of multiple paternity in sympatric and allopatric situations. In allopatric populations that are released from competition, we predict different patterns of multiple paternity. In addition, work on sperm viability inside females, egg and sperm recognition, and semen protein competition, would all provide important clues as to the intrinsic mechanisms of sperm competition.

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