VARIATION IN REPRODUCTIVE STRATEGIES OF EMBIOTOCIDS: A COMPARATIVE APPROACH

A Thesis submitted to the faculty of
San Francisco State University
In partial fulfillment of
the requirements for
the Degree

Master of Science

In

Biology: Marine Biology

by

Michael S Izumiyama

San Francisco, California

May 2019

CERTIFICATION OF APPROVAL

I certify that I have read Variation in reproductive strategies in Embiotocids: a comparative approach by Michael S Izumiyama, and that in my opinion this work meets the criteria for approving a thesis submitted in partial fulfillment of the requirement for the degree Master of Science in Biology: Marine Biology at San Francisco State University.

Karen Crow, Ph.D.
Professor of Biology

Eric Routman, Ph.D.
Professor of Biology

Andrew Zink, Ph.D.
Associate Professor of Biology

VARIATION IN REPRODUCTIVE STRATEGIES OF EMBIOTOCIDS: A COMPARATIVE APPROACH

Michael S Izumiyama
San Francisco, California
2019

Reproductive strategies of marine fishes are diverse, surfperches (Embiotocidae) represent a unique family of viviparous fishes that exhibit internal fertilization, gestation to the sub-adult stage, and live-birth. Maternal investment is extremely high in surfperches, which invokes the expectation that broodsize is limited, and females should be selective and choose few mates, yet polyandry occurs in all six species examined to date. Surfperches are found in the northern Pacific in temperate waters with the majority of species residing along the coast of the eastern Pacific. Our study characterized the reproductive life history of calico surfperch (*Amphisticus koelzi*) which is the only species of surfperch missing from previous literature, as well as assigned paternity with the utilization of RADseq. All calico surfperch broods examined were sired by multiple fathers, and the number of fathers ranged from four to eight.

Additionally, we compared the reproductive strategy of Western Pacific surfperches (including calico surfperches) to an Eastern Pacific species of surfperch *Ditrema temminckii* by assigning paternity. We discovered that the prevalence of multiple paternity in Eastern Pacific surfperches ranges from 92-100%, however, the prevalence of multiple paternity is noticeably reduced in *D. temminckii* with only 60% of broods being sired by multiple fathers. In addition, the total number of sires per brood is lower compared to the Eastern Pacific surfperches, suggesting a different mating strategy. The

average number of sires was 1.86 per brood, and paternity was skewed, indicating the majority of paternity was allocated to a single male. The number of fathers is an accurate approximation for the number of mates in *D. temminckii*, which was examined by genotyping spermatozoa within the uterine sacs of females (sperm donors) and sires within broods (paternal contributors). The contrast in the frequency of multiple paternity in *D. temminckii* with eastern Pacific surfperches suggests variation in the mating strategy that may be associated with a stronger female choice on males.

I certify that the Abstract is a correct representation of the content of this thesis.

Karen Gaw — 5-22-19

Chair, Thesis Committee — Date

Acknowledgments

I would like to thank the following agencies for providing funding for this thesis work; Dr. Earl H. Myers and Ethel M. Myers Oceanographic and Marine Biology Trust, San Francisco State University Instructional Related Research Award, National Science Foundation East Asia and Pacific Summer Institutes Fellowship, and Japan Society for the Promotion of Science Summer program fellowship.

I would like to thank Michael Westphal from the Bureau of Land Management for his insight on surfperches as well as many inspiring and motivating conversation throughout the years collecting surfperches. Kristine Lesyna and Ken Oda from the California Department of Fish and Wildlife for helping collect samples. Satoshi Awata from Osaka City University who mentored during my fellowship in Japan and brought me into his family. Faculty and staff of Sado Marine Biological Station at Niigata University who graciously hosted me during my fellowship.

To my lab mates Kayla Hall and John Swenson, you have always been there for me and helped motivate, inspire me through my academic journey without your support I would not be the person I am today.

I am grateful to my family and friends who have supported and believed in me through my academic journey.

Finally, I would like to thank my advisor Karen Crow. When I first took your class as an undergraduate, I never thought I would continue onto graduate school. You have provided so many opportunities and have opened many doors for me. What I am most thankful of is that you believed in me and pushed me to develop as a scientist. Your passion and drive for science is something that I will never forget and will be what I strive to emulate as I continue in my academic journey.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

Figures Page

Introduction

Sexual selection is one of the primary forces driving evolution and has been a subject of intense interest in evolutionary biology. Sexually selected traits arise as a result of variation in reproductive success among individuals. Regardless of whether this variation occurs through intrasexual competition or mate preferences, some individuals exhibit higher reproductive success (fitness) than others. This competition for reproductive fitness has interested biologist for centuries and is associated with the evolution of sexual dimorphism and morphological diversity.

A direct measurement of fitness is defined by the number of offspring that survive to reproduce. While the primary result of sexual selection is increased access to mates for some individuals (Darwin 1871; Andersson 1994), access to mates can be associated with increased fitness, as is the case for male Drosophila (Bateman 1948). The first quantitative analysis of sexual selection was evaluated by Bateman (1948) in a landmark paper that compared differences between the sexes with respect to the relationship between the number of mates (mating success) and the number of offspring (reproductive success). Bateman (1948) assigned parentage in Drosophila using visible genetic markers to infer the number of offspring and quantified the correlation between mating success and reproductive success. He observed that males exhibit increased variation in reproductive success, and a positive correlation between mating success and reproductive success compared to females, indicating that the ability to obtain mates is a limiting factor for males, while the limiting factor in females is maximum egg production.

Bateman concluded that the differences between the sexes were the cause of sexual selection, leading to the notion that females should be choosy due to limited broodsize and the lack of benefit associated with an increased number of mates, resulting in sexual selection on males. The positive relationship between reproductive success and mating success is referred to a sexual selection gradient or Bateman gradient, and a positive Bateman gradient reflects the sum contribution of all sexually selected traits to increased fitness (Bateman 1948; Arnold and Duvall 1994; Andersson and Iwasa 1996).

Many studies have demonstrated support for positive Bateman gradients in males (and females in sex-role reversed systems like seahorses and pipefishes) (Bateman 1948; Jones et al. 2000; Jones et al. 2002; Munroe and Koprowski 2011; Levine et al. 2015). However, relatively few studies have addressed the fitness benefit associated with polyandry and positive Bateman gradients in females, even though polyandry is common in nature (Jennions and Petrie 2000; Zeh and Zeh 2001). Positive female Bateman gradients have been detected in several animal taxa (see Gerlach *et al.* 2012) and in four freshwater fishes (Evans and Magurran 2000; Garant et al. 2001; Becher and Magurran 2004; Dierkes et al. 2008; Neff et al. 2008; Serbezov et al. 2010; Gerlach et al. 2012). However, to our knowledge, there are no examples of positive female Bateman gradients in marine fishes.

Reproductive strategies of marine fishes are diverse, from flexible gender systems, variation in lifetime breeding opportunities, and internal vs. external fertilization (Johannes 1978; Murua and Saborido-Rey 2003). While most fishes are broadcast

spawners, some give birth to live young (viviparity). Viviparity occurs in less than 3% of ray-finned fishes and has arisen independently in multiple lineages (Wourms 1981). Maternal investment is extremely high in viviparous species, which invokes the expectation that broodsize is limited, yet polyandry occurs in many species (Evans and Magurran 2000; Soucy and Travis 2003; Feldheim et al. 2004; Takagi et al. 2008a; Reisser et al. 2009; Ala-Honkola et al. 2011; Liu and Avise 2011; Liu et al. 2013; LaBrecque et al. 2014). There are many challenges associated with parentage analysis in marine fishes from natural populations, such as intractability of sampling all offspring in a brood or observing all mating encounters. Most marine fishes release hundreds of thousands of gametes into the water column making sampling all offspring impossible.

Here we propose to investigate a unique group of viviparous fishes that exhibit internal fertilization, gestation to the sub-adult stage and live-birth the surfperches (Embiotocidae). Surfperches represent a tractable system because females can be collected with their entire brood, allowing for parentage assignment with known maternity among whole or half-siblings. Multiple paternity has been observed in all six species of surfperches examined to date (Takagi et al. 2008a; Reisser et al. 2009; Liu and Avise 2011; Liu et al. 2013; LaBrecque et al. 2014). Therefore, surfperches represent an ideal system for evaluating female Bateman gradients because this mode of reproduction allows for the number of fathers within a brood to serve as an approximation for the number of mates the female has interacted.

A true female Bateman gradient would be compelling in species where broodsize is flexible, allowing for the possibility for fitness gain associated with increased number of mates. LaBrecque *et al.* (2014) have suggested that two species of surfperches exhibit positive female Bateman gradients. However, in one of the species, *Hyperprosopon anale*, lacked sufficient sample size to detect a statistically significant Bateman gradient, and the microsatellite loci used were not developed for that species which could have contributed to a lower estimate of the number of sires (see LaBrecque *et al.* 2014).

Studies of parentage analysis have traditionally been conducted using microsatellite DNA markers that are highly variable within populations. However, one drawback of microsatellites is that when analyzing a new species, specific markers may need to be developed, which is time-consuming and costly. Restriction site associated DNA sequencing (RADSeq) has the ability to detect genome-wide variation as single nucleotide polymorphisms (SNPs), also known as RADTags. RADSeq will generate thousands of SNPs per individual and has the potential to increase the accuracy of parentage assignment compared to microsatellite studies (Hauser et al. 2011).

The mating system of surfperches also has the potential to address a criticism of Bateman gradient studies, that the number of sires within a brood may not be representative of the number of mates due to the inability to detect mates that did not result in fertilization. The assumption that the number of fathers is equal to the number of mates neglects the possibility of postcopulatory selection in females and will shift the Bateman gradient towards a lower number of mates with no estimate of variation in

extra-paternal mating (Birkhead 2010; Gerlach et al. 2012; Parker and Birkhead 2013). Surfperches have multiple mates, sperm storage, pregnancy, live birth, and give birth to only one brood per year. The annual mating season can be characterized by a gonadosomatic index (proportion of gonad mass to body mass (GSI) (Nielsen and Johnson 1983). Mating in surfperches takes place shortly after the male GSI increases (Goldberg and Ticknor 1977; Nakazono et al. 1981; Baltz and Knight 1983). By collecting uterine sacs of female surfperches at the end of the mating season, we can compare the number of alleles in uterine sacs and number of alleles in the broods to determine if the number of sires is a good approximation for the number of mates.

Surfperches exhibit a highly derived reproductive strategy, and species with novel reproductive strategies provide an opportunity to examine how life history and reproductive strategies contribute to species diversity. Therefore, the objectives of this study include the following: 1) Describe the reproductive life history of *Amphisticus koelzi* the only Embiotocid species previously undescribed in the scientific literature 2) Utilize RADseq to infer paternity and evaluate the possibility of a female Bateman gradient. 3) Determine if the number of sires is a good approximation for the number of mates in *Ditrema temminckii* to evaluate an ongoing criticism of Bateman gradient studies.

Objective 1) Describe the reproductive life history of calico surfperch (*Amphisticus koelzi*)

Rationale

The calico surfperch is one of three species in the genus *Amphistichus*, and shares similar size, morphology and feeding habitat with its sympatric congeners, barred surfperch (*Amphistichus argenteus*) and redtail surfperch (*Amphistichus rhodoterus*). Unlike the other two species, which are important commercial and recreational resources, the calico surfperch has received little attention in the scientific literature (see Baltz, 1984). Furthermore, the lack of any known ecological separation from its congeners begs the question of how these three species originally diverged from their common ancestor. Life history and reproductive traits can provide clues to the ecological differentiation of closely related species.

The surfperches (Embiotocidae) is comprised of two major subfamilies which diverged approximately15 mya (Longo and Bernardi 2015). Most of the recent research on the family has focused on the subfamily Embiotocinae, a speciose group of nearshore rocky reef and kelp dwelling species whose ecology is readily observable due to the ease of accessing their environment via diving (Cummings 2004; Bernardi 2005; Cummings 2007) and because many are large, colorful, and conspicuous. Fewer studies have focused on the evolution of the less speciose subfamily, the sandy-beach dwelling Amphistichinae (Westphal et al. 2011), whose association with surf zones makes them more challenging to study.

Within the Amphistichinae, six species representing three genera that radiated relatively recently, (i.e. within the last 5 million years) in nearshore sand/surf environments of the Eastern Pacific (DeMartini 1969; Longo and Bernardi 2015; Longo et al. 2018), Within the genus Amphistichus, phylogenetic relationships and contemporary distributions suggest that redtail surfperch in the north diverged in allopatry from the common ancestor of calico/barred surfperch in the south (Longo and Bernardi 2015). Factors associated with reproductive isolation between the southern forms into calico surfperch and barred surfperch are less clear.

It has been over three decades since Baltz (1984) described the life-history variation of female surfperches in which calico surfperch was omitted due to the lack of data. Our study describes the reproductive life history of calico surfperch, including fecundity and mating season, which has been missing from the literature. We also compared the mating season of calico and barred surfperches to determine if there is reproductive isolation between the two species through reproductive timing. This study is the first to describe calico surfperch reproductive life history and to examine the variation between calico and barred surfperch reproductive strategy.

Methods

Specimens of all size classes of calico surfperch (*Amphisticus koelzi*) and barred surfperch (*Amphisticus argenteus*) were collected from 2010-2017 by hook and line

along the California coast. In total, 333 calico surfperch individuals (187 female and 146 male) and 183 male barred surfperches were collected throughout their range and frozen within hours for later dissection. Ultimately, 52 individuals collected from the northern counties of Humboldt and Mendocino were removed from analysis to minimize population variation that could be associated with latitude. In subsequent analyses, 281 specimens from Central California were evaluated (157 female and 124 male) for standard length (SL, mm), body mass, gonad mass, brood size, and standard length of offspring (mm) when present. Tissue samples for genetic analysis were collected from every individual as well as offspring and stored in 95% ethanol.

Results

Mating Season

The reproductive cycle in surfperches differs from most fishes. With internal fertilization during the mating season followed by pregnancy and a protracted gestation period, the gonadosomatic indices (GSI) of males and females are out of phase. This mode of reproduction contrasts with broadcast spawners where mating season and maximum GSI for both males and females are in register. In surfperches, the mating season is defined by the seasonal peak in male GSI, when enlarged testes and sperm are present in mature individuals in the population. Calico males between 100-131 mm standard length (SL) were sexually immature and did not exhibit enlarged gonads

(Figures 1 and 2). The smallest male collected with enlarged gonads was 133 mm SL with a GSI of 0.58% indicating that sexual maturity occurs at approximately 133mm SL. We defined the mating season by the occurrence of reproductively active males exhibiting GSI values ranging from 0.41-4.43%. We inferred that males with GSI values of 0.41% or less were not reproductively active based on variation in GSI of males that are large enough to be sexually mature that were sampled outside the mating season (Figures 1 and 2). Peak GSI in calico males occurred during October with a sharp increase around October 1 and a short attenuation period during November. Importantly, we sampled several sexually mature but spent calico males (SL> 133mm, GSI<0.41) after December 4, suggesting that the mating season ends in late November-early December in this population. Male GSI remains under 0.41% from January through the end of September (Figures 1 and 2) in calico surfperch.

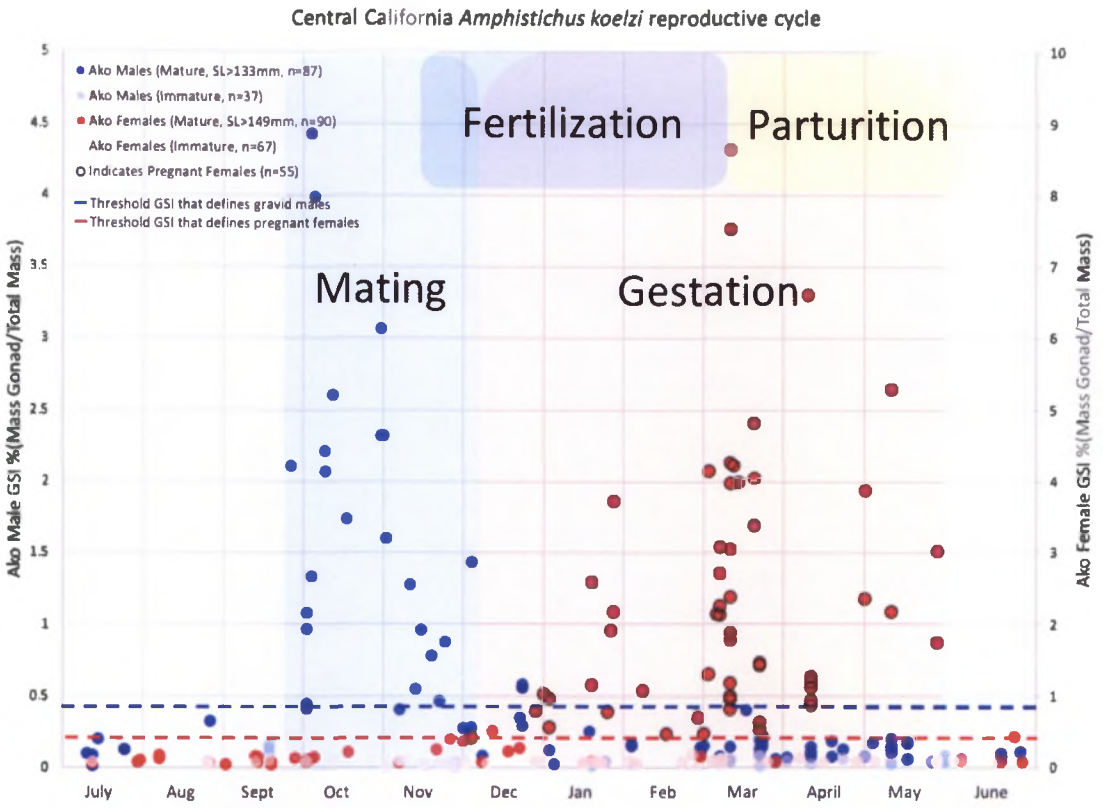

Figure 1. Central California Amphistichus koelzi reproductive cycle characterized by gonadosomatic index (GSI) of males and females. Males are represented by blue and females by red. Immature males and females are represented by a lighter shade. Black outline indicates pregnant females. The dotted line represents the threshold GSI that defines ripe males and pregnant females. Shaded boxes illustrate mating season, gestation period and inferred windows for fertilization and parturition.

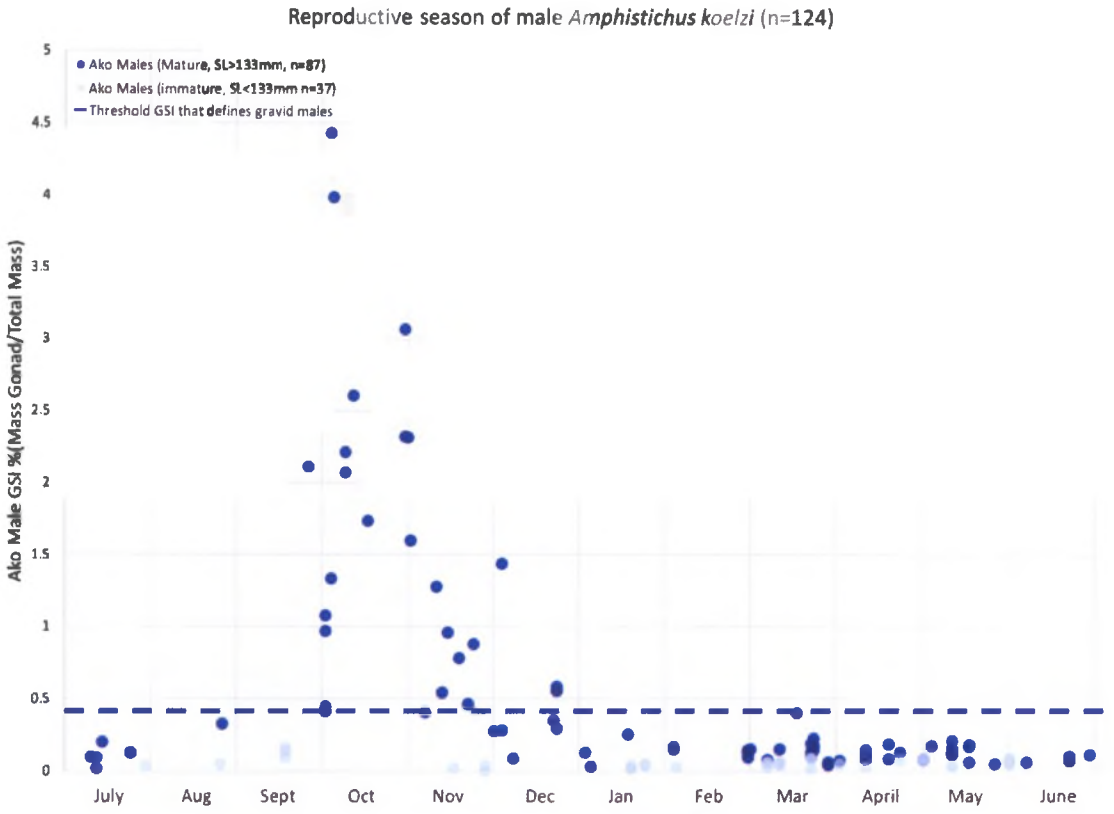

Figure 2. Reproductive season of male *A. koelzi* inferred by annual variation in GSI. Males were considered sexually mature above 133mm SL. The blue dotted line indicates the threshold GSI that defines reproductive males. Presence of sexually mature but non-reproductive males from January to September indicates mating occurs from October to December.

Gestation, Broodsize, and Parturition

In surfperches, pregnancy and gestation are defined by the elevated GSI in females, indicating developing embryos are present in the uterine sac of mature females. In calico surfperches, females between 89-148mm SL were sexually immature and did not exhibit enlarged gonads or fertilized embryos (Figures 1 and 3). The smallest pregnant female collected was 149mm SL with a brood size of 10. We defined the gestation period by the presence of embryos in the ovaries of pregnant females with GSI

values ranging from 0.43%-8.65%. We inferred that females with GSI value of < 0.43% were not gravid based on the GSI values of females large enough to be sexually mature outside of the gestation period (Figure 1 and 3). Pregnant females were observed from December to May with peak GSI values in this population occurring in March. Surprisingly, we did not observe a correlation between increasing GSI and the progression of time throughout the gestation period. Rather, we found that maximum GSI in female calicos was normally distributed, and interestingly, the variation in GSI in any particular month remained high, ranging from 0.5 to the maximum observed. This suggests that fertilization occurs over a protracted time period, and gestation is likely less than five months for any individual female (Figures 3 and 4).

We inferred that parturition occurs before early June based on the observation that all sexually mature females sampled before the end of May were pregnant, and those sampled after the beginning of June were not pregnant (Figure 3). Female GSI remained below 0.43% from June to December (Figure 3).

Broodsize in calico ranges from 7-54 and is correlated with female SL (Figure 5, r^2= 0.744, p<.001). However, we note that small females exhibit a smaller range of GSI compared to medium/large females. The maximum GSI for small females was never greater than 4% with small broodsize ranging from 7-26, while the maximum GSI observed for medium/large females was 8.65%, with broodsize ranging from 16-54 offspring (Figures 3 and 6). We also found a positive correlation between female GSI and SL of embryos (r^2=0.86, p<0.001, Figure 5), suggesting that female GSI is a good

indicator of embryonic development. The SL of embryos from females sampled throughout the gestation period ranged from 5-42mm.

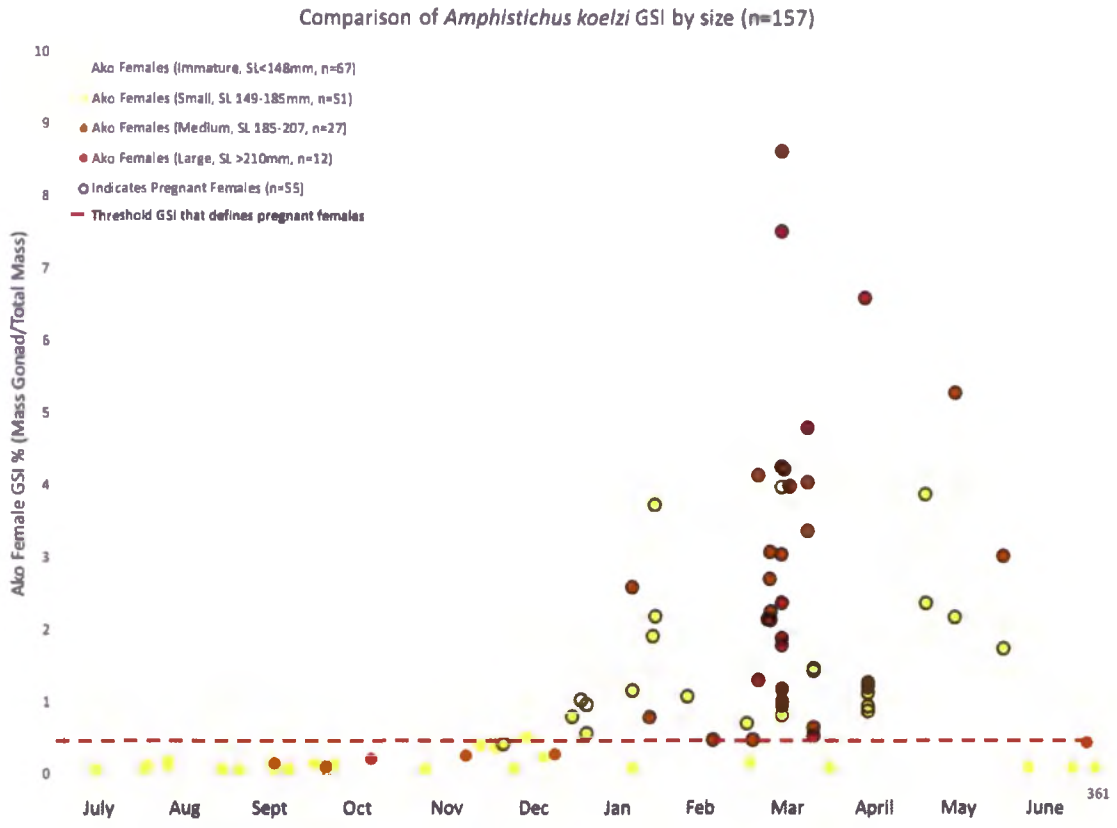

Figure 3. Comparison of A. koelzi GSI by female SL (age class). Outlined data points indicate pregnant females. The red dotted line indicates the threshold GSI that defines pregnant females. Pregnant females were observed from December to May, and reproductive females below the threshold were observed from June to November. Note that pregnant females with varying GSI values were observed throughout the gestation period indicating that timing of fertilization varies among individual females.

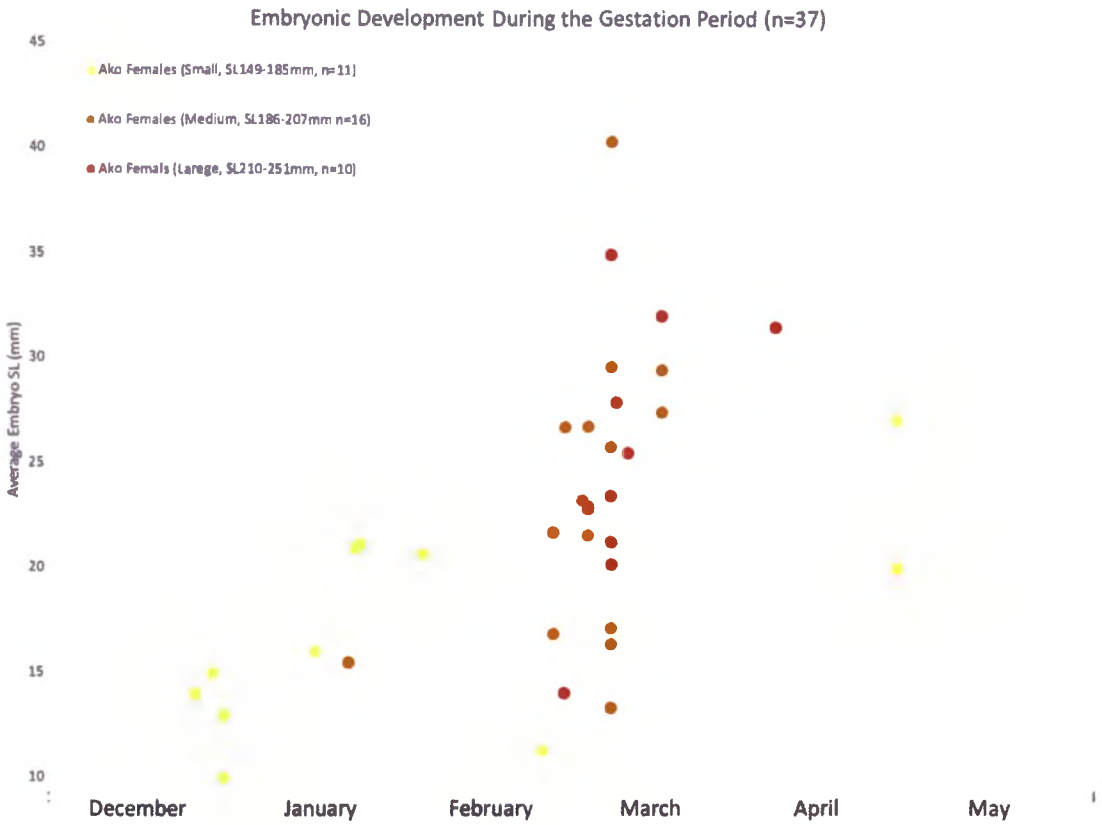

Figure 4. The Average SL (mm) of larvae within a brood is plotted throughout the gestation period in A. koelzi. Yellow indicates small females between 149-185mm SL. Orange indicates medium females between 186-251 mm SL. Red indicates large females between 210-251 mm SL. We did not observe any small females carrying embryos over 27mm SL suggesting that small females give birth to smaller offspring.

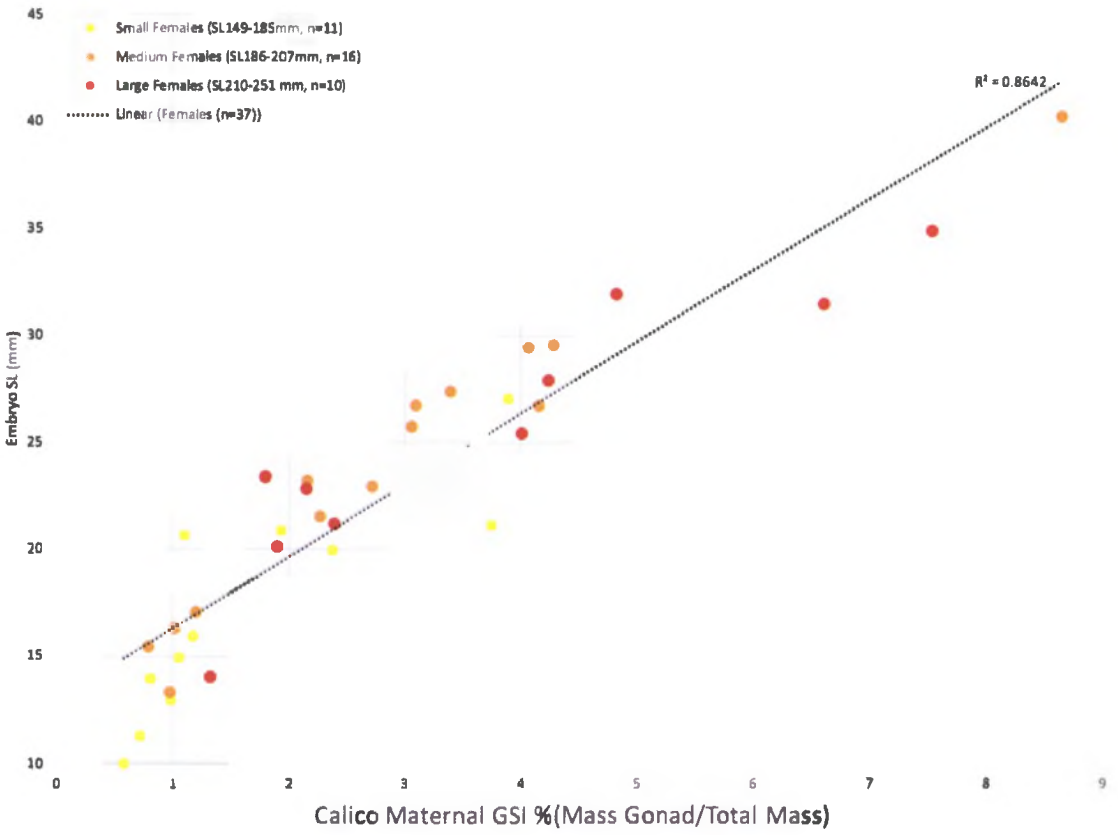

Figure 5. Correlation between female GSI and average embryo SL (r^2=0.86, p<001).

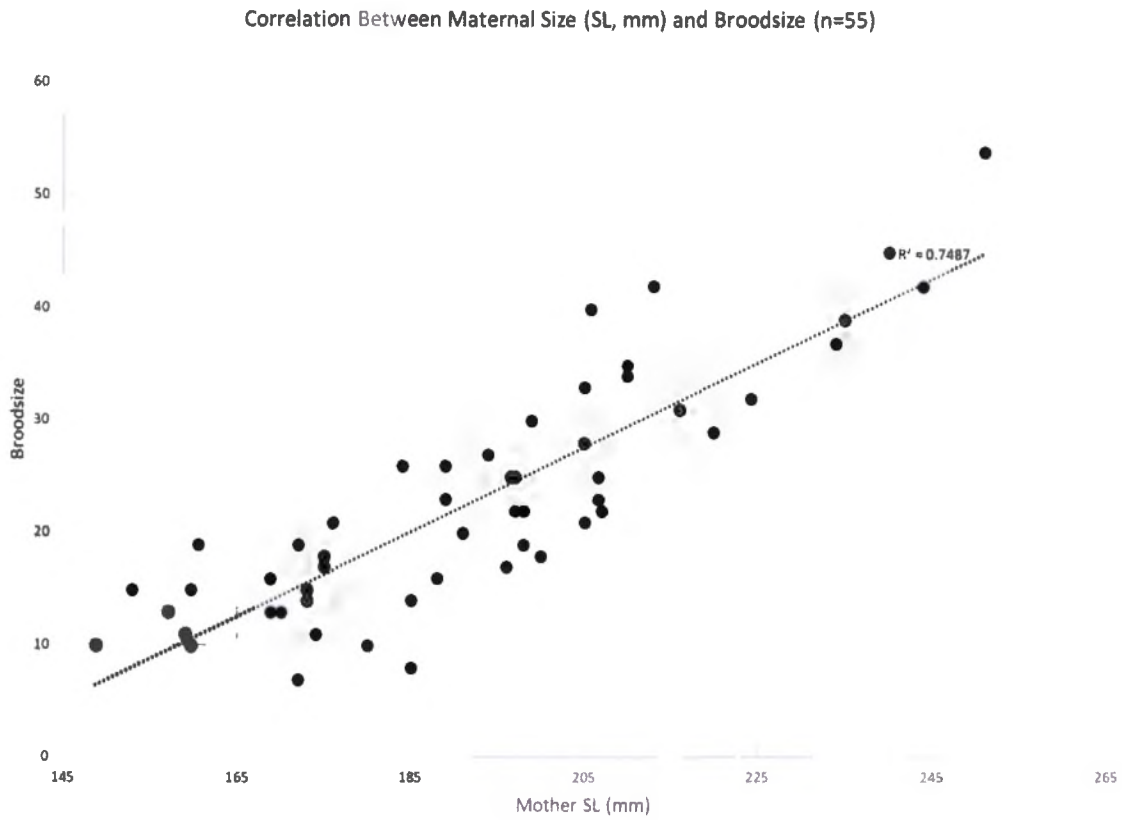

Figure 6. Correlation between female body size and brood size (r^2= 0.744, p<.001).

Fertilization

Interestingly, the SL of embryos within broods was remarkably consistent, with maximum variation less than 6mm (Figure 7), and within 4mm for most broods. Taken together with the observation that surfperches exhibit multiple paternity (Takagi et al. 2008a; Reisser et al. 2009; Liu and Avise 2011; Liu et al. 2013; LaBrecque et al. 2014) and sperm storage (Warner and Harlan 1982; Avise and Liu 2011), we infer that fertilization occurs as a single event in calico females after the mating season and that fertilization within the population occurs approximately from mid-November through

February. This is also supported by the observation that no pregnant females were sampled in October-November, during the mating season.

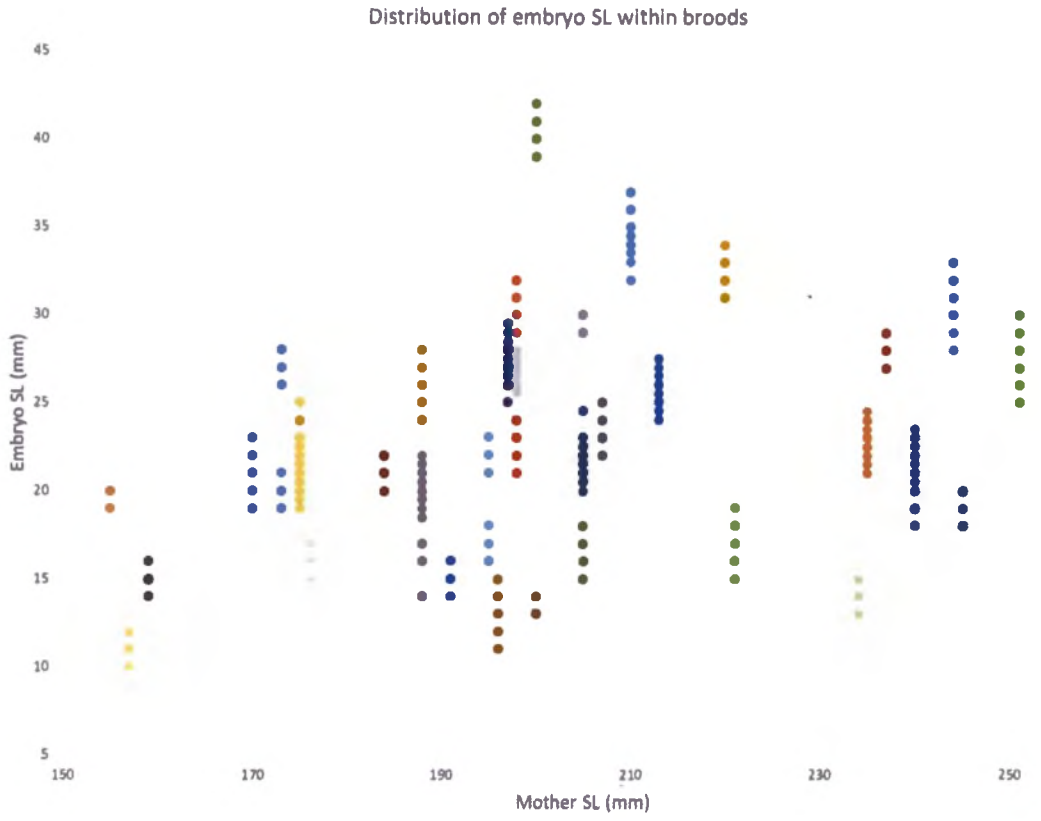

Figure 7. Distribution of embryo SL within broods. Each color represents a different brood. There is little variation among individuals within broods suggesting a single fertilization event for each brood.

Comparison of Amphisticus koelzi and A. argenteus mating season

Amphisticus argenteus (barred surfperch) is the sister taxon of calico surfperch and is found in sympatry throughout Central California (Kells et al. 2016). barred males less than 128 mm SL are immature and do not exhibit enlarged gonads (Carlisle et al. 1960). Mating season was defined by elevated male GSI values ranging from 0.5-4.55. The mating season for barred surfperch is protracted and appears to be at least twice as

long as the calico surfperch. Male GSI for barred surfperch increases sharply at the beginning of October (similar to calico) followed by a longer attenuation period through February (Figure 8).

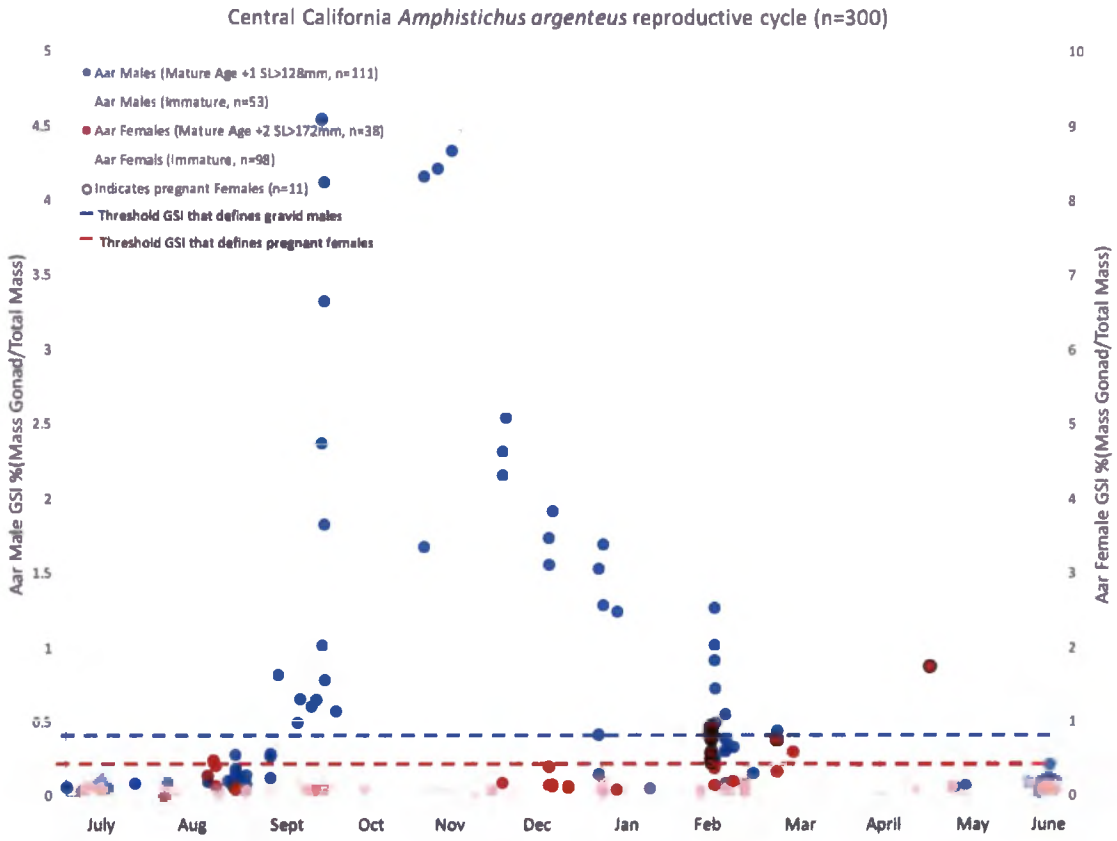

Figure 8. *Amphistichus argenteus* mating season characterized by gonadosomatic index (GSI). Males are represented by blue and females by red, immature males and females are represented by a lighter shade. The dotted line represents the threshold GSI that defines reproductive males. Black outline represents pregnant females, although few females were sampled in this study.

Discussion

Reproductive strategies in Amphisticus koelzi

While the mating season is bounded and occurs within a two-month period in calico surfperch, we found that fertilization and parturition are protracted and occur over

a period of several months based on the following observations. First, there were no pregnant females sampled during the mating season which is consistent with sperm storage and delayed fertilization (Figures 1 and 3). Second, variation in female GSI is maintained throughout the gestation period (Figure 3, indicating that females with recently fertilized broods as well as more developed broods co-occur). Third, all size classes of pregnant females were sampled with broods at various stages of development based on larval SL (Figure 7). Taken together with the observation that female GSI is a good indicator of larval developmental stage (Figure 5), these data suggest that the onset of development varies among females. In other words, while individual females fertilize their entire brood in a single event at some time after cessation of mating, fertilization among females in the population is not synchronized suggesting the possibility of cryptic female choice in paternity, broodsize, and fitness optimization. Following that argument, parturition is likely also variable. However, we did not sample spent females throughout the gestation period which could be associated with our sampling efforts. We focused on surf zones using baited hook and line, suggesting that pregnant females feed during gestation, and that spent females either do not feed/take bait after parturition, or that they move away from turbid surf zones to deeper or protected waters to give birth. Indeed, juveniles of most surfperches are found in microhabitats where cover is present (Baltz 1984). In the redtail surfperch pregnant females move to estuaries where no males or immature females are present (Pruden 2000), suggesting selection for optimal habitats by pregnant females.

It is unclear how long gestation and development lasts in individual females, but there appears to be variation in fitness between size classes of females. Small females are limited in their reproductive capacity, which is supported by the strong correlation between female SL and broodsize (Figure 6, r^2=0.75). Small females also give birth to smaller offspring, based on the observation that we never encountered small females with embryos larger than 27mm. Females larger than 186mm SL had larger broods with larger embryos up to 42mm SL (Figures 5 and 6). Abe (1969) observed this trend in *Ditrema temminckii* a species of Japanese surfperch. If we assume the maximum size of developing pre-birth juveniles is a minimum estimate for the size at parturition, then gestation and developmental time may vary between small and medium/large females. Similarly, in both redtail surfperch (*A. rhodoterus*) and barred surfperch, larger females give birth to larger offspring (Carlisle et al. 1960; Bennett and Wydoski 1977). This is also supported by the observations that small females exhibit a smaller range of GSI and female GSI is correlated with embryonic development and size. In summary, small females in the genus *Amphisticus* have multiple fitness limitations because they have smaller broods and give birth to smaller offspring with increased risk of predation. Given the strong correlation between female SL and broodsize, it is likely that males prefer large females with increased fitness potential. This provides a compelling rationale for delayed onset of sexual maturity in calico surfperches and suggests a selective advantage for increased growth rates.

Shorter mating season in Amphisticus koelzi compared to A. argenteus

The evolutionary split between calico and barred surfperches remains a compelling question due to their similar ecologies (Westphal et al. 2011) they occur in sympatry from Bodega, CA to San Diego, CA (Kells et al. 2016) comprising most of the range of barred surfperch. Calico and barred surfperches diverged about two million years ago (Longo and Bernardi 2015). While red pigmentation in fins is likely the ancestral character state in the Amphistichinae (Westphal et al. 2011), the shift towards larger body size (Carlisle et al. 1960; Baltz 1984) and the loss of red pigmentation in fins of barred surfperch suggests that sexually selected traits may be significant contributors to reproductive isolation and the possibility of divergence in sympatry between calico and barred surfperches. We found that the mating season of barred surfperch is much broader than calico, although with complete overlap. While barred surfperch mate from September through February, the calico mating season is more sharply defined and limited to October and November. It remains unclear if this divergence in the mating season was a contributing factor during the build-up of incipient reproductive isolation. Maximum body size and broodsize are much larger in barred surfperch compared to calico (Table 1). The size at first reproduction is also larger for barred surfperch (172 mm SL) than calico surfperch (150 mm SL) barred surfperches are at least two years old at first reproduction (Table 1). While we did not examine the age of calico surfperches at first reproduction, the assumption that the onset of sexual maturity occurs earlier in calico surfperch aligns with the shorter mating season for calico surfperches. Whereas barred surfperches devote resources to growth and the smaller individual may require more time

to sexually mature and produce gametes. The differences in reproductive strategy may have led to a protracted mating season.

		Max size	At first reproduction			Number of sires		Freq of mult
Subfamily	Species	TL, mm	Age	SL, mm	Fecundity	Mean	Range	paternity
Amphistichinae	*Hypocritichtys analis* [a]	199	1	103	7.8	3.17	2-7	100%
	Amphistichus rhodoterus	406	3	218	9.7			
	Amphistichus argenteus	432	2	172	8			
	Amphistichus koelzi [b,c]	300	Unknown	150	12.75	6.71	5-8	100%
Embiotocinae	*Cymatogaster aggregata* [d]	178	1	82	4.8	4.6	1-8	96%
	Hysterocarpus traskii [e]	171	1	82	17.7	2.5	1-4	92%
	Ditrema temminckii [b]	288	1	123	8.5	1.86	1-5	60%
	Embiotoca jacksoni [f]	390	2	149	5.7	3.58	2-6	100%
	Embiotoca lateralis [f]	381	3	216	9.6	3.5	2-9	100%

Table 1. Age, length fecundity at first reproduction and mean and range of number of fathers within a brood. Age, length and fecundity information was sourced from Baltz (1984), and Miller & Lea (1972). Paternity information was collected from the following sources [a] LaBrecque *et al.* 2014,[b] This study, [c] Liu & Avise, 2011[d] Liu *et al.*, 2013[e] Reisser *et al.*, 2009. * Suisun marsh population of *Hysterocarpus traskii.*

Objective 2: Utilize RADseq to infer paternity and evaluate the possibility of a female Bateman gradient in *Amphisticus koelzi.*

Rationale

Multiple paternity has been observed in the six surfperch species examined to date. However, in the Amphistichinae clade, multiple paternity has only been examined in the ancestral species spotfin surfperch (*Hyperprosopon anale*) (LaBrecque et al. 2014), which was recently renamed to *Hypocritichthys analis* (Longo et al. 2018). To date, no other Amphisticine species has been evaluated and the prevalence of multiple paternity across the Amphistichinae clade is unknown. LaBrecque *et al.* (2014) utilized

microsatellite loci that were not developed for that species which could have contributed to a lower estimate of the number of sires.

The utilization of RADseq for paternity assignment is relatively new (see (Andrews et al. 2018; Thrasher et al. 2018). RADseq incorporates genome-wide SNP variation, and the use of RADTags will circumvent the need to develop new microsatellite markers for parentage analysis. calico surfperches. We utilized RADseq to assign paternity in the calico surfperch if multiple paternity is conserved in the Amphistichinae clade and determined if a Bateman gradient is present in calico surfperch.

Methods

RADseq, SNP filtering, and parentage analysis

We sequenced seven *A. koelzi* females and all offspring within their broods resulting in a total of 165 individuals for RADseq parentage analysis. DNA extraction was accomplished using the DNeasy blood and tissue kit (Qiagen). RADseq libraries were prepared following the protocol from Ali *et al.* (2016) with the following slight modification; pooled DNA libraries were physically sheared using the Covaris S2 sonicator to achieve 400bp fragments. Two libraries containing 96 individually barcoded samples were created and split into two lanes for sequencing on an Illumina HiSeq 2500 at the Vincent J. Coates Genomic Sequencing Laboratory at UC Berkeley with 150bp

single-end reads. SNP locus discovery was accomplished using the software *Stacks* version 1.13 (Catchen et al. 2013). Paternity assignment was performed using COLONY V.2.0.6.5 (Jones and Wang 2010).

De novo locus assembly and parameter optimization in STACKS

We utilized the DENOVO_MAP pipeline in *Stacks* to assemble calico surfperch loci *de novo*. In DENOVO_MAP there are three main parameters: (m) The minimum number of reads required to form a stack within an individual, (M) maximum number of mismatches allowed between stacks to merge them into one locus, (n) the maximum number of mismatches allowed between stacks from different individuals to merge into one locus (Catchen et al. 2013). We utilized the following parameters in DENOVO_MAP m-3, M-3, n-1 following parameter optimization studies of Mastretta-Yanes *et al.* (2015), Andrews *et al.* (2018), and Thrasher *et al.* (2018). We further filtered the output from DENOVO_MAP in the POPULATIONS program in *Stacks* by removing all except one SNP per RAD locus with the parameter -write_single_snp. In the POPULATIONS program we varied the following parameters: (r) minimum percent of individuals within the population to accept the locus from 0.5 to 0.9 and (MAF) retain only loci with minor allele frequency greater than 0.05 (removing minor alleles which are only present in 5% of the population). The application of a stringent filter corresponded to a reduced number of SNPs generated. With the most stringent filter of (r) 0.9 and MAF>0.05, we retained 2234 SNPs. When MAF was not removed, and the (r) was varied the number of SNPs

corresponded to 5790 with an (r) 0.9 and 8114 with an (r) 0.5 with the latter being the most lenient filter allowing for an increased number of SNPs.

Results

Multiple paternity and female Bateman gradient in calico surfperch

Paternity was assigned in COLONY V.2.0.6.5 (Jones and Wang 2010) for all seven calico surfperch broods utilizing the three sets of SNPs generated (2234SNPS r 0.9 MAF>0.05, 5790 SNPs r 0.9, and 8114 SNPs r 0.5) with an error rate of 0.01. All broods examined in all analysis were sired by multiple males. The number of sires per brood ranges from four to eight (2234 SNPs, 5790 SNPs, 8114 SNPs, Figure 9). We did not detect a statistically significant positive correlation between mating success and reproductive success (i.e., female Bateman gradient) for any of the three analysis. (2234 SNPs r^2=0.2982, p=0.2048, 5790 SNPs r^2=0.2201, p=0.3209, 8114 SNPs r^2=0.222, p=0.2883, Figure 9)

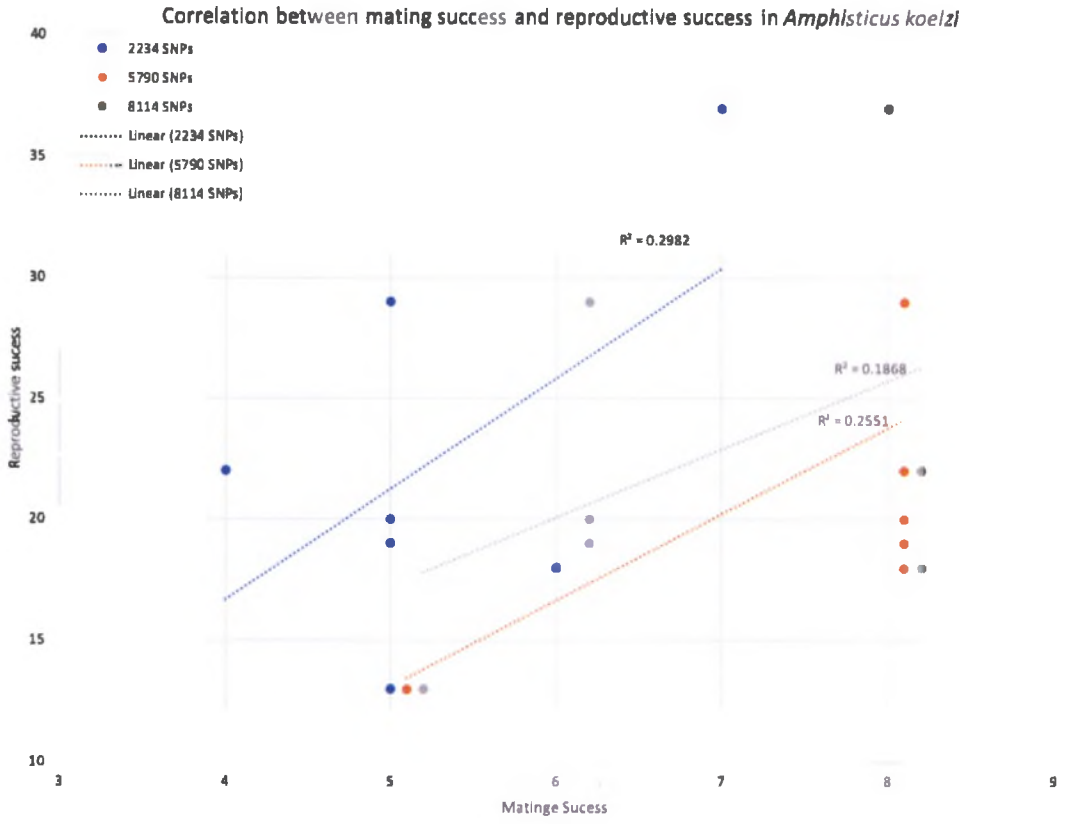

Figure 9. Correlation between mating success and reproductive success in *Amphisticus koelzi* from 7 pregnant females. Each color corresponds to the number on SNPs utilized for the paternity assignments 2234 SNPs r^2=0.2982, p=0.2048, 5790 SNPs r^2=0.2201, p=0.3209, 8114 SNPs r^2=0.222, p=0.2883

Discussions

Paternity assignment with RADseq and SNP filtering

Utilizing RADseq for paternity assignment is relatively new (see (Andrews et al. 2018; Thrasher et al. 2018). The accuracy of paternity assignment utilizing SNPs has been well documented especially for the use in livestock and fisheries management (Anderson and Garza 2006; Jones et al. 2009; Hauser et al. 2011; Fernandez et al. 2013). However, in most cases, a reference genome and specific baits have been developed to

target specific markers that were previously determined to be variable, making paternity assignment reliable. Discovering SNPs *de novo* turned out to be challenging for several reasons.

1) SNPs only have four character states and in most cases are binary, resulting in the need for many more SNPs to accurately assign paternity in surfperches. Further, surfperch broods are relatively large (10-50 for calico surfperch) with little variation among full and half-siblings within broods and the potential for sibling relationships among broods with male polygyny leading to the necessity of more SNP markers.

2) Paternity may not be distributed equally in surfperches leading to the possibility of males only siring a few or single offspring within a brood. Therefore, in our analysis, we did not filter for minor allele frequencies (MAF) due to the possibility of a male siring a single or small proportion of offspring.

3) The variation between SNP signal and noise can be distorted, especially in surfperches due to the potential for varying combination of full and half-siblings, and potential for males that may have only sired a small portion of the brood. The

ability to distinguish signal from a potential unique sire and noise such as null alleles or sequencing errors in SNP data may be distorted.

Multiple paternity in calico surfperch

In calico surfperches, all broods were sired by multiple fathers. Previously, the only species of surfperch in the Amphisticine clade paternity was examined was the ancestral *H. analis,* and all broods in *H. analis* were sired by multiple males (LaBrecque *et al.*, 2014), with multiple paternity observed in all major genera of surfperches it is likely that multiple paternity is a shared reproductive strategy in surfperches.

Objective 3: Determine if the number of sires is a good approximation for the number of mates in *Ditrema temminckii*

Rationale

A common criticism of female Bateman gradient studies is that the number of fathers (or sires) may be an underestimate for the number of mates. Due to the intractability of observing all mating encounters in natural populations, the number of sires is often used as an approximation of the number of mates. However, it is possible that some mates do not receive paternity and would not be detected in the offspring.

Therefore, if mating success is underestimated, the correlation between mating success and reproductive success may be incorrectly inferred (Gerlach et al. 2012).

The reproductive strategy of surfperches represents a system where it is possible to compare the number of mates and sires because females store sperm from mates for up to three months before fertilization occurs. Sperm storage has been documented in three species *Cymatogaster aggregata* (Gardiner 1978), *Micrometrus minimus (Warner and Harlan 1982),* and *Ditrema temminckii* (Nakazono et al. 1981). Broad trends in *D. temminckii* reproduction have been documented (Mizue 1961; Nakazono et al. 1981; Tamura et al. 1981; Lee and Lee 1989; Lee et al. 1996; Lee and Lee 1996), however fine-scale resolution of several aspects of the mating system, such as the correlation between female gonadosomatic index (GSI) and larval development or total number of sires contributing to individual broods, remain unexplored.

For example, Takagi et al. (2008a) used hypervariable microsatellite loci to determine that approximately half of *D. temminkii* broods were sired by more than one father in a population off southern Japan, but broods were subsampled (i.e. not all offspring were evaluated) and the actual number of sires was not estimated. Mizue (1961) and Nakazono *et al.* (1981) measured male GSI to characterize the mating season in *D. temminckii*, which occurred from August through November and peaked in October in a population off Kyushu Japan. Nakazono et al. (1981) and Lee and Lee (1989) measured female GSI to characterize gestation, and found that elevated GSI and the presence of

developing embryos were observed from early January through June, followed by parturition in late June.

In female *D. temminckii* sperm storage occurs based on the observation of sperm in the uterine sacs during the mating season and before fertilization which occurs near the end of December (Nakazono et al. 1981; Tamura et al. 1981; Lee et al. 1996). Nakazono et al. (1981) observed various stages of oocyte maturity within the ovarian folds as well as the presence of spermatozoa in the uterine sacs throughout the mating season, once fertilization occurred in December the presence of oocytes and spermatozoa disappeared and embryos were observed in early January.

The lack of embryos during the mating season indicates that fertilization takes place after an established mating season, and not sequentially after each mating event. With documentation of multiple paternity and sperm storage within the uterine sacs of females, *D. temminckii* represents a system where it is possible to (1) estimate the total number of fathers within broods, and (2) evaluate whether the number of sires is a good estimate for the number of mates by comparing the number of alleles from hypervariable microsatellite markers in uterine sacs of females before fertilization, and within broods after fertilization.

Methods

A total of 241 *D. temminckii* adults were collected from Sado Island, Niigata, Japan by spear, hook and line, or purchased from local fish markets. Of those samples, 214 adults were collected from October 2014 to May 2015 to characterize the reproductive cycle from mid-mating season through mid-gestation. Mating season was characterized by elevated male GSI. The presence of developing larvae and elevated GSI was an indication that fertilization had taken place and females were pregnant. Of the 241 samples, 27 females were sampled near the end of mating but before fertilization in November 2017 to compare the number of sperm donors (mates) based on the number of alleles present in the uterine sac. From the 241 adults collected for this study 15 gravid females sampled after fertilization and onset of larval development, from February to May 2015, were utilized to determine the number of alleles present (for direct comparison with sperm donors), and later the number of fathers that sired each brood. Standard length (SL, mm), total mass, mass of gonad, brood size, and standard length of offspring (mm, when present) was measured for each individual collected. GSI was computed for all individuals as mass of gonad/total mass.

Tissue samples for genetic analysis were collected from every individual and offspring and stored in 95% ethanol. Uterine sacs from November 2017 females were removed immediately after euthanasia and stored in -20 °C freezer until DNA extraction. The uterine sac tissue was opened and allowed to lyse for several hours, excess uterine sac tissue was then removed and DNA was extracted from the lysis buffer using the Gentra Puregene Kit (Qiagen). Seven highly variable microsatellite loci, developed for *D.*

temminckii by Takagi et al. (2008b), were amplified with dye labeled primers using (6-FAM, VIC, NED or PET, Applied Biosystems) using the Type-it Microsatellite PCR kits (Qiagen) for parentage analysis following Blacket et al. (2012). Thermocycling conditions were as follows: 95°C for 5 min; 35 cycles of 95 °C for 30 s, 60 °C for 90 s, and 72 °C for 30 s; and a final extension at 60 °C for 30 min. Microsatellite amplicons were run on a 3130 and 3730 Genetic Analyzer (Applied Biosystems) with a GeneScan 500 Liz size standard ladder (Applied Biosystems) and scored using the software Geneious V:11.0.4 (https://www.geneious.com). Population allele frequency analysis was conducted in GenALEx (Peakall and Smouse 2006; Peakall and Smouse 2012) and Genepop (Rousset 2008). Error rates associated with microsatellite allele calls and null alleles were estimated using the program Pedant (Johnson and Haydon 2007). Paternity assignment was performed using COLONY V.2.0.6.5 (Jones and Wang 2010).

Results

Ditrema temminkii microsatellite diversity

The microsatellite diversity was examined from a total of 185 adults from the population at seven loci. The number of alleles ranged from 13 to 18 with an average of 15.14 alleles. Of the seven microsatellite loci six were in Hardy Weinberg equilibrium $p<0.001$, with the exception of Dte-11 which was removed from further analyses.

Determining the mating season of D. temminckii in Sado island.

The mating season for the *D. temminckii* population off Sado Island appears to peak in early October and continues through November (Figure 10), which aligns with the mating season in a southern population off Kyushu (Mizue 1961; Nakazono et al. 1981), suggesting little temporal or latitudinal variation in this species.

We observed motile sperm (in a saline solution) in the uterine sacs of females collected in November and the presence of developing embryos in the uterine sacs of females in early January (Figure 10). We also noticed that male mating display behaviors had diminished by late November. Taken together, these observations suggest that females sampled from mid to late November would be an ideal timeframe to collect females that have mated but not yet fertilized their oocytes.

The smallest pregnant female collected was 145mm SL suggesting a minimum SL for sexual maturity in this population. All females above 145mm SL sampled from March through May were pregnant with developing broods present (n=19), and we evaluated multiple paternity and the number of sires contributing to each brood from 15 of these females. Brood sizes ranged from12-45, and we evaluated paternity for every offspring within broods, based on the characterization of six microsatellite loci from 15 broods, 13 mothers (tissue from two mothers were missing) and 416 offspring.

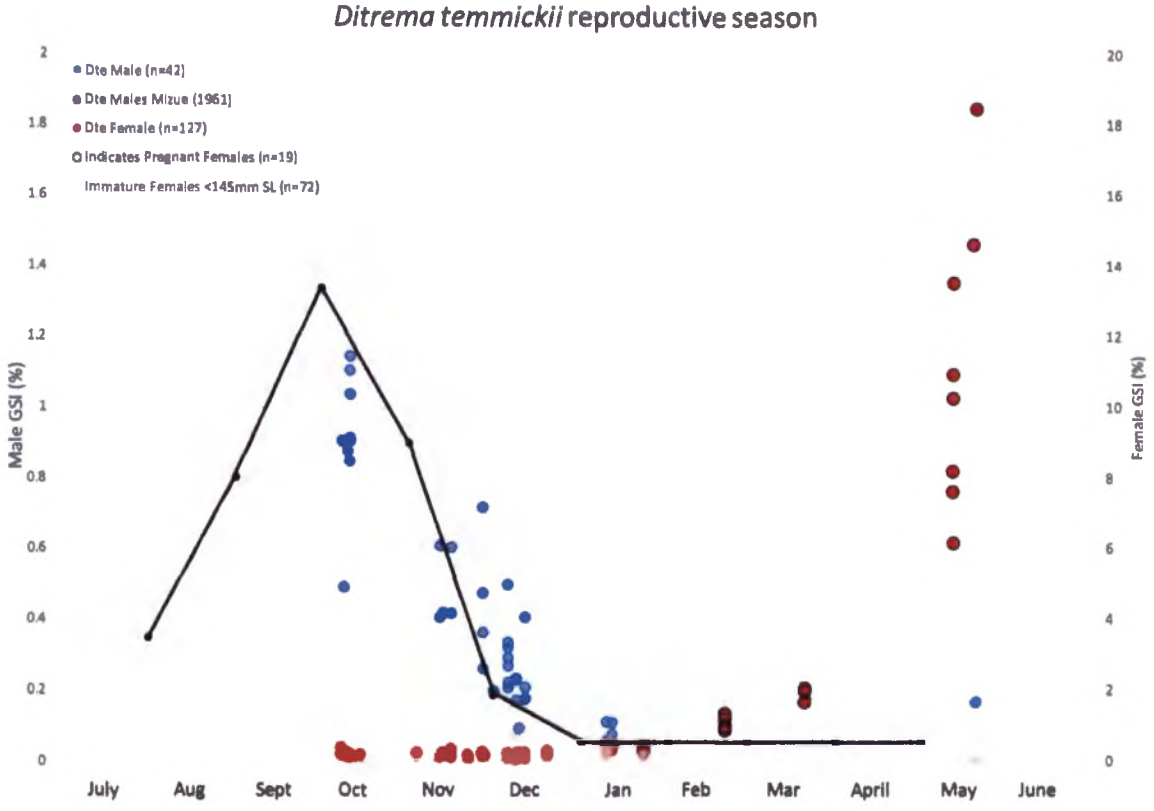

Figure 10. *Ditrema temminckii* reproductive cycle characterized by gonadosomatic index (GSI) of males and females off Sado Island, Niigata. Males are represented by blue (n=42) and females by red (n=127). Black outline indicates pregnant females (n=19). Immature females <145mm SL are represented by pink (n=72). The black line indicates the average male GSI measured from Mizue (1961) from a southern population in Kyushu. The mating season for the *D. temminckii* population off Sado Island appears to continue though November which aligns with the mating season in a southern population off Kyushu from Mizue (1961)

Estimating the number of sires that contribute to broods in D. temminckii

The number of sires within *D. temminckii* broods ranged from one to five, with 60% of broods (nine of 15) exhibiting multiple paternity (i.e. more than one sire detected). Surprisingly, the majority of broods had only one or two sires, with an average of 1.86 sires per brood. In addition, allocation of paternity was skewed, with the predominant male siring 59–100% of broods (Figure 11). We found no evidence of sires

contributing to broods of more than one female, and we found no evidence for a correlation between mating success and reproductive success (i.e. female Bateman gradient) in *D. temminckii* (Figure 12).

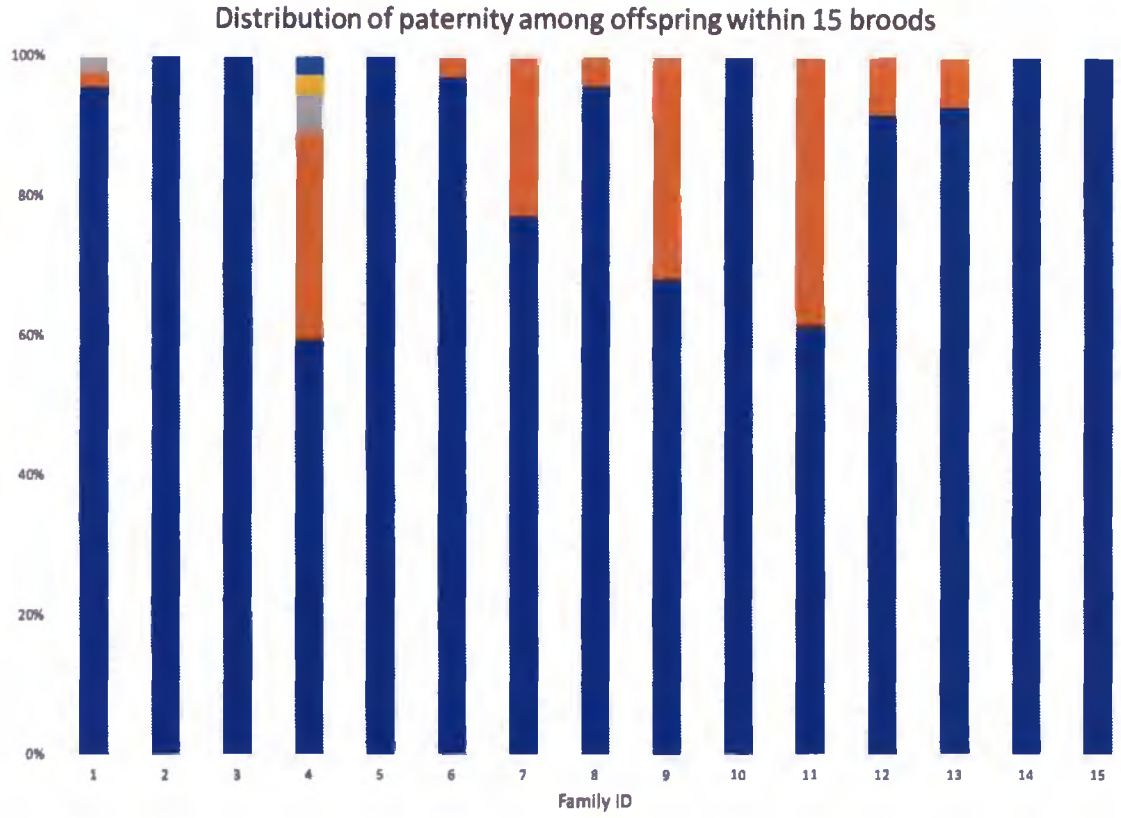

Figure 11. Relative paternal contribution to *D. temminckii* brood. Paternity was assigned to *D. temminckii* broods. Each bar represents all the offspring of a single female and the colors represent the contribution of each genetically distinct sires.

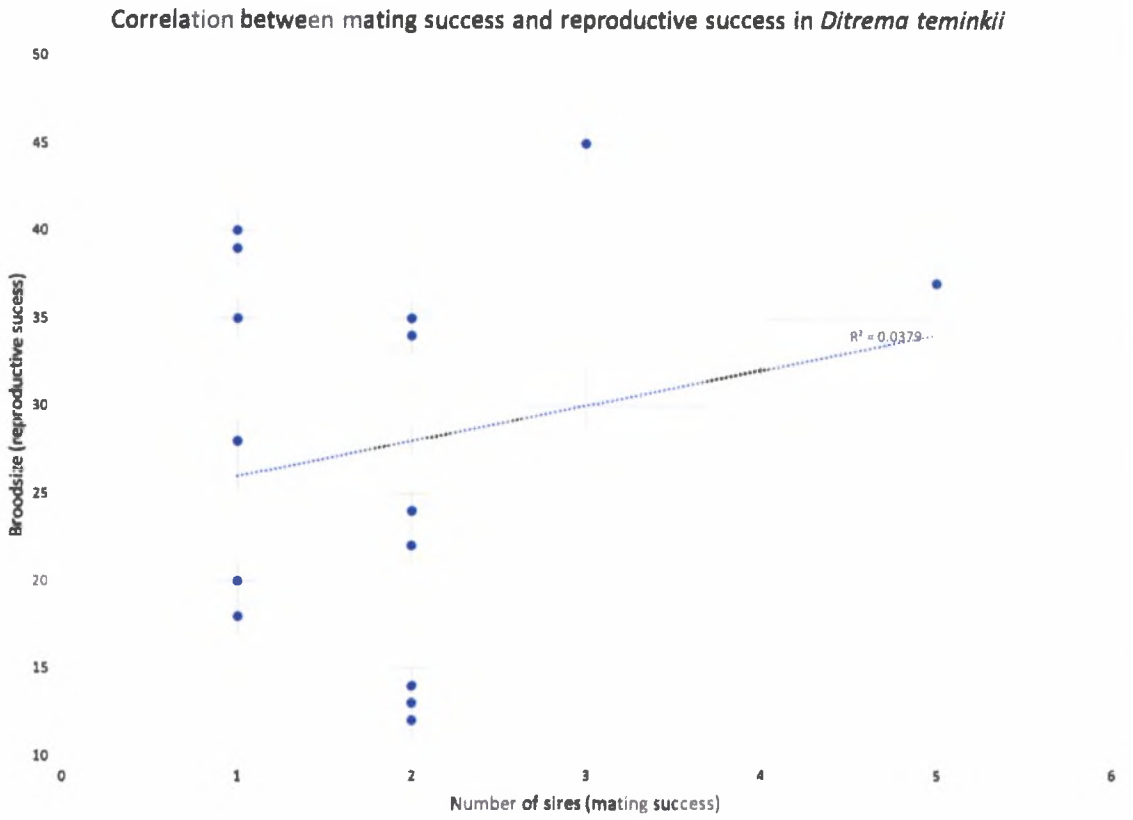

Figure 12. Correlation between mating success and reproductive success of *D. temminckii* inferred from 15 pregnant females. We found no correlation between mating success and reproductive success.

Comparing allelic variation between mates and sires

To evaluate whether the number of sires is a reasonable estimate for the number of mates, we collected 27 *D. temminckii* females that were sexually mature (i.e. >145mm SL and likely to have mated), toward the end of the mating season (from mid to late November). As a positive control for detection of sperm donors (i.e. mates) in the uterine sac, we set the criterion that, the allelic profile of the mother (all maternal alleles detected in mother's fin clip) must be successfully detected in the uterine sac sample. One concern

was that DNA concentration could be biased due to an overrepresentation of maternal tissue in the uterine sac extractions, resulting in allelic peaks that were either imprecise or distorted due to variation in peak height.

Unfortunately, four of the six loci did not amplify consistently from the uterine sac samples and we were only able to reliably score six of 27 individuals exhibiting known maternal alleles at the remaining two loci. By happenstance, each of these individuals also exhibited unique paternal alleles (i.e. non-maternal) indicating successful detection of mates as well as maternal alleles. All seven loci amplified successfully from offspring within broods, but we only used data from the same two loci for the comparative analyses. While variation from only two loci results in low power, our goal was to make an even comparison between the number of mates and sires before and after fertilization. From these two loci (Dte2 and Dte5), we found no significant difference in the combined number of alleles between mates and sires ($t=1.3882$, $p = 0.1825$, Figure 13), suggesting that the number of mates is not greater than the number of sires in this species. The number of alleles from mates ranged from four to seven ($\bar{x}=5.66$, $n=6$), while the number of alleles from sires of developing broods ranged from four to 13 ($\bar{x}=6.6$, $n=15$, Figure 13).

While there is no way to estimate the number of offspring that might occur before fertilization within uterine sacs, brood size is correlated with body size in surfperches. Therefore variation in the number of mates and sires may be associated with female body

size (15 pregnant females ranged from 145 – 196 mm SL, Figure 14). To compare females of similar size, we removed the females larger than 165 mm SL from the "sires" estimate for comparison of females from the same age and size class , resulting in six and seven females from 145-165 mm SL, corresponding to approximately one year of age (see Nakazono *et al.* 1981), for estimates of allelic diversity in mates and sires, respectively. Again, we found no significant difference in the number of alleles between mates and sires in the one-year-old females of similar sizes ($\bar{x}$= 6.29, n=7, t= 1.3451, p= 0.2212, Figure 15).

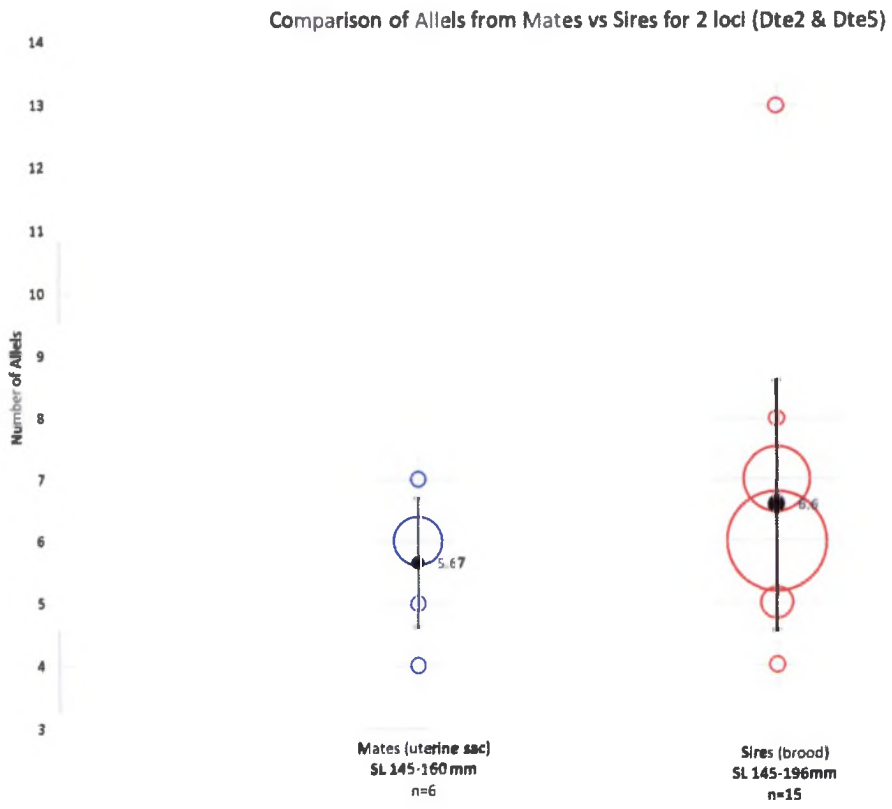

Figure 13. Comparison of the number of alleles from mates vs sires for two loci (Dte2 & Dte5) in *D.temminckii*. The diameter of the circle represents the frequency of each observation. Alleles contributed

by mates (uterine sacs blue, $\bar{x}$=5.66, n=6) and sires (brood, red, $\bar{x}$= 6.6 n=15). We did not detect a statistical difference between the two (t=1.3882 , p = 0.1825).

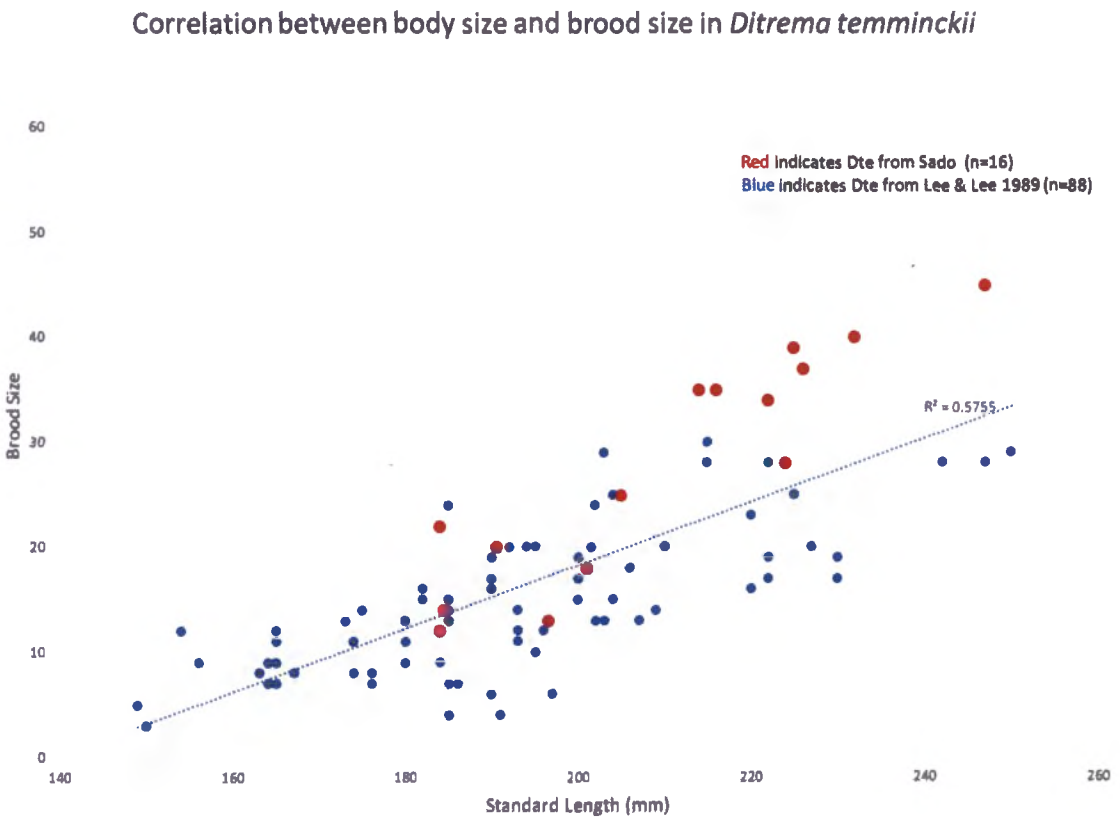

Figure 14. The correlation between brood size and female body size (standard length, mm) in *D.temminckii* from Sado island (red, n=16) and Lee and Lee (1989).

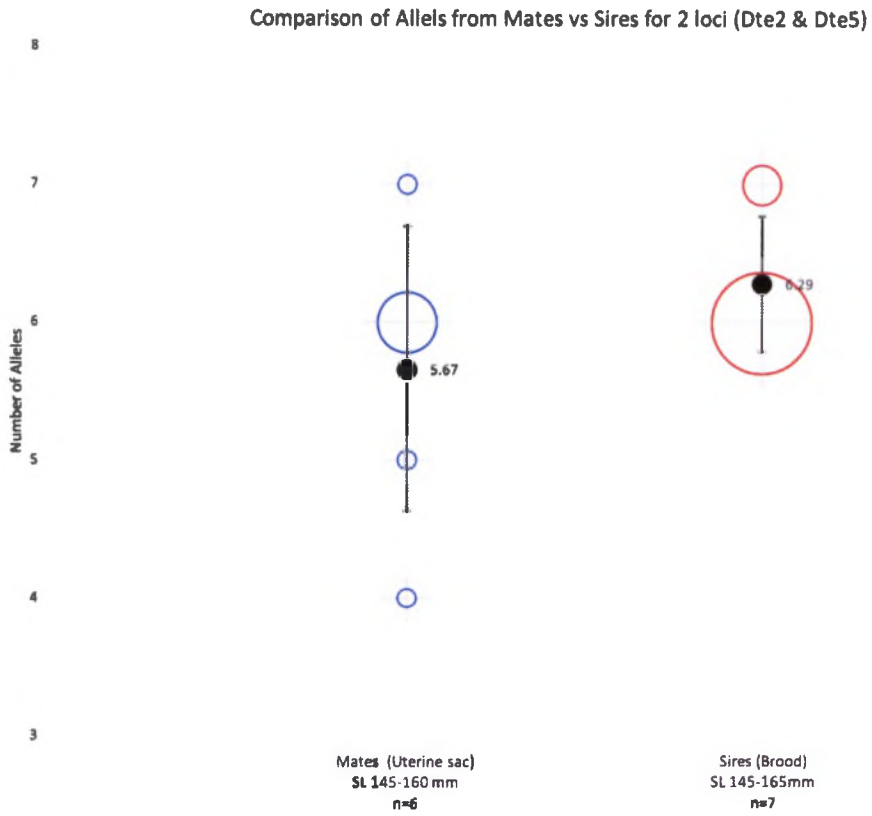

Figure 15. Comparison of the number of alleles from mates vs sires for two loci (Dte2 & Dte5) in *D.temminckii* of one-year-old females SL 145-165mm. The diameter of the circle represents the frequency of each observation. Alleles contributed by mates (uterine sacs, blue, $\bar{x}$=5.66, n=6) and sires (brood, red, $\bar{x}$=6.28, n=7). We did not detect a statistical difference between the two (t=1.3451, p= 0.2212).

Discussion

Multiple paternity and mating system in D.temminckii compared to other surfperches

Interestingly, the prevalence of multiple paternity in *D. temminckii* broods is lower than other species of surfperches examined to date, which was also noted by Takagi et al. (2008a). We also found that fewer sires fertilize Dte broods compared to other surfperches, with the majority of broods sired by a single male (40%) or two males (47%). In broods that were sired by multiple males, allocation of paternity was skewed

based on the formula adapted from Pamilo (1993) where the sum of the proportion of paternity squared is greater than 0.5 (Pamilo 1993; Boomsma and Sundström 1998), with the majority of offspring within broods sired by a single male. This suggests cryptic female choice of sires or sperm competition. However, we do not know the order of mating and fertilization, therefore we cannot evaluate differences between mate choice, postcopulatory selection and/or sperm competition. One possibility is that females that have mated with multiple males may have followed a trade-up hypothesis of initially mating with a male to ensure fertilization and being selective with additional mates encountered (Jennions and Petrie 2000). In this scenario, the mate that contributes sperm closest to ovulation of mature oocytes gets the most paternity. This could occur via sperm competition, or reduced sperm viability of previous mates, but either way, this implies that females have a mechanism to manipulate paternity allocation.

Is the number of sires a good estimate for the number of mates in D. temminckii?

There are three possible outcomes in the comparison of allelic variation between mates and sires.

1) *If the number of mates exceeds the number of sires,* then some mates did not receive paternity, and more importantly, the number of sires is not a good estimate for the number of mates when determining female Bateman gradients.
2) *If the number of mates is less than the number of sires,* then the only possible explanation is that females were not finished mating. We determined that the

reproductive season of *D. temminckii* in our population corresponds to GSI data from previous studies (Mizue 1961; Nakazono et al. 1981), and females collected for this study were near the end of mating. We also noticed a reduced number of males displaying courtship behaviors suggesting that most males had mated and moved away from the shallow mating grounds and the number of alleles detected in uterine sacs represents females that have finished mating.

3) *If there is no difference in the number of mates and sires,* then estimating the number of sires when inferring Bateman gradients is a reasonable practice (for this species). We found no significant difference between the number of mates and sires in *D. temminckii* off Sado Island. While we had limited power to detect allelic variation due to low sample size and only two loci, we excluded other loci from estimates of sires to make the data comparable. We further examined the number of alleles in females of similar body size and still found no significant difference in the number of alleles. Our comparison of one-year-old females captured the first time that female has ever mated which removes any possible caveat in the mating strategy of *D. teminckii* such as extended sperm storage from previous mating seasons. Importantly, the low number of alleles in both uterine sacs and broods is consistent with expectations given that we found so few sires that actually contribute to broods in this species.

Comparing mating strategies among surfperches

Multiple paternity has been observed in the six surfperch species examined to date representing all major clades, indicating that polyandry is a conserved trait in surfperches. The prevalence of multiple paternity varies between the eastern and western Pacific surfperches. In the eastern Pacific surfperches, multiple paternity is more prevalent with more sires per brood, occurring in 100% of the families evaluated in *Embiotoca jacksoni*, *E. lateralis* (Reisser *et al.*, 2009), and *Hyperprosopon anale* (LaBrecque *et al.*, 2014), 92% of *Hysterocarpus traski* (Liu *et al.*, 2013), and 96% of *Cymatogaster aggregata* examined (Liu & Avise, 2011) (Table 1). Alternatively, multiple paternity was observed in only 60% of the *D.temminckii* broods, which was consistent in two different populations (this study and Takagi *et al.* 2008). Further, this is the first study to estimate the total number of sires within broods. With a range of 1-5 sires per brood (n=15)), and high proportions of broods with only one or two sires (average number of sires was 1.86), *D. temminckii* appears to have a different mating strategy compared to Western Pacific surfperches.

There are many genetic and non-genetic benefits to polyandry including ensuring fertilization (i.e. against sterile males or limited sperm), reducing costs associated with male coercion, increased genetic diversity/bet-hedging, and permitting post-copulatory mate choice (trade up/cryptic female choice) (see (Jennions and Petrie 2000; Parker and Birkhead 2013). These benefits align with behaviors and allocation of paternity observed in surfperches. In the eastern Pacific clade of surfperches dense mating aggregations form in which females get mobbed by males, (personal observation and Warner & Harlan,

1982), it is likely that females mate multiply to avoid male aggression as well as increase genetic diversity and ensure fertilization. In *D. teminckii* males guard small territories on rocky outcrops, that are visited by prospective females, suggesting a strong role for direct female mate choice on males.

The skewed paternity in *D. temminkii* is consistent with post-copulatory selection on males (cryptic female choice or sperm competition), and post-copulatory selection that results in paternity bias can result in restricted gene-flow similar to assortative mating (Jennions and Petrie 2000). Surfperches are sexually dimorphic with a genital papilla and a modified anal fin in males which may facilitate internal fertilization. In the genus *Ditrema,* males have a genital papilla and a modified anal fin similar to other surfperches. Additionally, they have elongated fin rays posterior to the anal fin modification which appears more ornamental than functional that is only present in this genus. The elongated anal fin rays may be associated with female selection on males leading to "sexy sons" in a Fisherian model (Fisher 1930; Lande 1981). When comparing mating strategies among surfperches, there is stronger the female selection and few sires in broods in the Western Pacific *D. temminkii,* which is different from other species of surfperch found in the Eastern Pacific. Little is known about the two congeners of *D. temminkii*, but they have similar meristics and morphometrics (including the elongated anal fin morphology), with only slight variation in body color to distinguish species (Katafuchi and Nakabo 2007). Examining reproductive strategies in a comparative context is necessary to understand

how unique mating strategies have evolved and can be crucial in understanding how these systems arose.

References

Ala-Honkola O, Friman E, Lindstrom K (2011) Costs and benefits of polyandry in a placental poeciliid fish Heterandria formosa are in accordance with the parent-offspring conflict theory of placentation. J Evol Biol 24: 2600-2610 doi 10.1111/j.1420-9101.2011.02383.x

Ali OA, O'Rourke SM, Amish SJ, Meek MH, Luikart G, Jeffres C, Miller MR (2016) RAD Capture (Rapture): Flexible and Efficient Sequence-Based Genotyping. Genetics 202: 389-400 doi 10.1534/genetics.115.183665

Anderson EC, Garza JC (2006) The power of single-nucleotide polymorphisms for large-scale parentage inference. Genetics 172: 2567-2582 doi 10.1534/genetics.105.048074

Andersson M, Iwasa Y (1996) Sexual selection. Trends Ecol Evol 11: 53-58 doi https://doi.org/10.1016/0169-5347(96)81042-1

Andersson MB (1994) Sexual selection. Princeton University Press

Andrews KR, Adams JR, Cassirer EF, Plowright RK, Gardner C, Dwire M, Hohenlohe PA, Waits LPJMer (2018) A bioinformatic pipeline for identifying informative SNP panels for parentage assignment from RAD seq data

Arnold SJ, Duvall D (1994) Animal Mating Systems - a Synthesis Based on Selection Theory. American Naturalist 143: 317-348 doi Doi 10.1086/285606

Avise JC, Liu JX (2011) Multiple mating and its relationship to brood size in pregnant fishes versus pregnant mammals and other viviparous vertebrates. Proc Natl Acad Sci U S A 108: 7091-7095 doi 10.1073/pnas.1103329108

Baltz DM (1984) Life-History Variation among Female Surfperches (Perciformes, Embiotocidae). Environmental Biology of Fishes 10: 159-171 doi Doi 10.1007/Bf00001123

Baltz DM, Knight EE (1983) Age, Growth, Reproductive Characteristics, and Seasonal Depth Distribution of the Spotfin Surfperch, Hyperprosopon-Anale. California Fish and Game 69: 97-104

Bateman AJ (1948) Intra-sexual selection in Drosophila. Heredity (Edinb) 2: 349-368

Becher SA, Magurran AE (2004) Multiple mating and reproductive skew in Trinidadian guppies. Proc Biol Sci 271: 1009-1014 doi 10.1098/rspb.2004.2701

Bennett DE, Wydoski RS (1977) Biology of the redtail surfperch (Amphistichus rhodoterus) from the central Oregon coast

Bernardi G (2005) Phylogeography and demography of sympatric sister surfperch species, Embiotoca jacksoni and E. lateralis along the California coast: historical versus ecological factors. Evolution 59: 386-394

Birkhead TR (2010) How stupid not to have thought of that: post-copulatory sexual selection. Journal of Zoology 281: 78-93 doi 10.1111/j.1469-7998.2010.00701.x

Blacket M, Robin C, Good R, Lee S, Miller AJMER (2012) Universal primers for fluorescent labelling of PCR fragments—an efficient and cost - effective approach to genotyping by fluorescence12: 456-463

Boomsma JJ, Sundström L (1998) Patterns of paternity skew in Formica ants. Behavioral Ecology Sociobiology 42: 85-92 doi 10.1007/s002650050415

Carlisle JG, Schott JW, Abramson NJ (1960) The barred surfperch (Amphistichus argenteus Agassiz) in southern California. Department of Fish and Game, Print. Division, Documents Section

Catchen J, Hohenlohe PA, Bassham S, Amores A, Cresko WA (2013) Stacks: an analysis tool set for population genomics. Mol Ecol 22: 3124-3140 doi 10.1111/mec.12354

Cummings ME (2004) Modelling divergence in luminance and chromatic detection performance across measured divergence in surfperch (Embiotocidae) habitats. Vision research 44: 1127-1145

Cummings ME (2007) Sensory trade - offs predict signal divergence in surfperch. Evolution 61: 530-545

Darwin CR (1871) *The descent of man, and selection in relation to sex.*, London

DeMartini EE (1969) A correlative study of the ecology and comparative feeding mechanism morphology of the Embiotocidae (surf-fishes) as evidence of the family's adaptive radiation into available ecological niches. Wasmann J Biol 27: 177-247

Dierkes P, Taborsky M, Achmann R (2008) Multiple paternity in the cooperatively breeding fish Neolamprologus pulcher. Behavioral Ecology and Sociobiology 62: 1581-1589 doi 10.1007/s00265-008-0587-3

Evans JP, Magurran AE (2000) Multiple benefits of multiple mating in guppies. Proc Natl Acad Sci U S A 97: 10074-10076 doi 10.1073/pnas.180207297

Feldheim KA, Gruber SH, Ashley MV (2004) Reconstruction of parental microsatellite genotypes reveals female polyandry and philopatry in the lemon shark, Negaprion brevirostris. Evolution 58: 2332-2342

Fernandez ME, Goszczynski DE, Liron JP, Villegas-Castagnasso EE, Carino MH, Ripoli MV, Rogberg-Munoz A, Posik DM, Peral-Garcia P, Giovambattista G (2013) Comparison of the effectiveness of microsatellites and SNP panels for genetic identification, traceability and assessment of parentage in an inbred Angus herd. Genet Mol Biol 36: 185-191 doi 10.1590/S1415-47572013000200008

Fisher RA (1930) The genetical theory of natural selection. Clarendon Press, Oxford, England

Garant D, Dodson JJ, Bernatchez L (2001) A genetic evaluation of mating system and determinants of individual reproductive success in Atlantic salmon (Salmo salar L.). J Hered 92: 137-145

Gardiner DM (1978) Cyclic changes in fine structure of the epithelium lining the ovary of the viviparous teleost, Cymatogaster aggregata (Perciformes: Embiotocidae). Journal of morphology 156: 367-379

Gerlach NM, McGlothlin JW, Parker PG, Ketterson ED (2012) Reinterpreting Bateman gradients: multiple mating and selection in both sexes of a songbird species. Behavioral Ecology 23: 1078-1088 doi 10.1093/beheco/ars077

Goldberg SR, Ticknor WC, Jr. (1977) Reproductive cycle of the pink surfperch, Zalembius rosaceus (Embiotocidae). Fish Bull NMFS/NOAA, 75(4), 882-884, (1977)

Hauser L, Baird M, Hilborn R, Seeb LW, Seeb JE (2011) An empirical comparison of SNPs and microsatellites for parentage and kinship assignment in a wild sockeye salmon (Oncorhynchus nerka) population. Mol Ecol Resour 11 Suppl 1: 150-161 doi 10.1111/j.1755-0998.2010.02961.x

Jennions MD, Petrie M (2000) Why do females mate multiply? A review of the genetic benefits. Biol Rev Camb Philos Soc 75: 21-64

Johannes RE (1978) Reproductive strategies of coastal marine fishes in the tropics. Environmental Biology of Fishes 3: 65-84 doi 10.1007/bf00006309

Johnson PCD, Haydon DT (2007) Software for Quantifying and Simulating Microsatellite Genotyping Error. Bioinformatics and Biology Insights 1: 71-75

Jones AG, Arguello JR, Arnold SJ (2002) Validation of Bateman's principles: a genetic study of sexual selection and mating patterns in the rough-skinned newt. Proc Biol Sci 269: 2533-2539 doi 10.1098/rspb.2002.2177

Jones AG, Rosenqvist G, Berglund A, Arnold SJ, Avise JC (2000) The Bateman gradient and the cause of sexual selection in a sex-role-reversed pipefish. Proc Biol Sci 267: 677-680 doi 10.1098/rspb.2000.1055

Jones B, Walsh D, Werner L, Fiumera A (2009) Using blocks of linked single nucleotide polymorphisms as highly polymorphic genetic markers for parentage analysis. Mol Ecol Resour 9: 487-497 doi 10.1111/j.1755-0998.2008.02444.x

Jones OR, Wang J (2010) COLONY: a program for parentage and sibship inference from multilocus genotype data. Mol Ecol Resour 10: 551-555 doi 10.1111/j.1755-0998.2009.02787.x

Katafuchi H, Nakabo T (2007) Revision of the East Asian genus Ditrema (Embiotocidae), with description of a new subspecies. Ichthyological Research 54: 350-366 doi 10.1007/s10228-007-0410-6

Kells VA, Rocha LA, Allen LG (2016) A Field Guide to Coastal Fishes: From Alaska to California. JHU Press

LaBrecque JR, Alva-Campbell YR, Archambeault S, Crow KD (2014) Multiple paternity is a shared reproductive strategy in the live-bearing surfperches (Embiotocidae) that may be associated with female fitness. Ecol Evol 4: 2316-2329 doi 10.1002/ece3.1071

Lande R (1981) Models of speciation by sexual selection on polygenic traits. Proc Natl Acad Sci U S A 78: 3721-3725 doi 10.1073/pnas.78.6.3721

Lee JS, An CM, Chin P (1996) Copulation and Embryonic Development of the Viviparous Teleost Surfperch, Ditrema temmincki. Korean Journal of Fisheries and Aquatic Sciences 29: 26-34

Lee JS, Lee TY (1989) Reproductive cycle and embryonic development within the maternal body of viviparous teleost Ditrema temmincki (Bleeker). Bulletin of National Fisheries University of Pusan Natural Sciences Pusan 29: 37-51

Lee JS, Lee YD (1996) Early gonadogenesis and sex differentiation in the viviparous teleost, Ditrema temmincki. Korean Journal of Fisheries and Aquatic Sciences 29: 35-43

Levine BA, Smith CF, Schuett GW, Douglas MR, Davis MA, Douglas ME (2015) Bateman-Trivers in the 21st Century: sexual selection in a North American pitviper. Biological Journal of the Linnean Society 114: 436-445 doi 10.1111/bij.12434

Liu JX, Avise JC (2011) High degree of multiple paternity in the viviparous Shiner Perch, Cymatogaster aggregata, a fish with long-term female sperm storage. Mar Biol 158: 893-901 doi 10.1007/s00227-010-1616-0

Liu JX, Tatarenkov A, O'Rear TA, Moyle PB, Avise JC (2013) Molecular evidence for multiple paternity in a population of the Viviparous Tule Perch Hysterocarpus traski. J Hered 104: 217-222 doi 10.1093/jhered/ess105

Longo G, Bernardi G (2015) The evolutionary history of the embiotocid surfperch radiation based on genome-wide RAD sequence data. Mol Phylogenet Evol 88: 55-63 doi 10.1016/j.ympev.2015.03.027

Longo GC, Bernardi G, Lea RN (2018) Taxonomic revisions within Embiotocidae (Teleostei, Perciformes) based on molecular phylogenetics. Zootaxa 4482: 591-596

Mastretta-Yanes A, Arrigo N, Alvarez N, Jorgensen TH, Pinero D, Emerson BC (2015) Restriction site-associated DNA sequencing, genotyping error estimation and de novo assembly optimization for population genetic inference. Mol Ecol Resour 15: 28-41 doi 10.1111/1755-0998.12291

Miller DJ, Lea RN (1972) Guide to the coastal marine fishes of California. UCANR Publications

Mizue K (1961) Studies on Ditrema temmincki, 1. Records of oceanographic works in Japan

Munroe KE, Koprowski JL (2011) Sociality, Bateman's gradients, and the polygynandrous genetic mating system of round-tailed ground squirrels (Xerospermophilus

tereticaudus). Behavioral Ecology and Sociobiology 65: 1811-1824 doi 10.1007/s00265-011-1189-z

Murua H, Saborido-Rey F (2003) Female reproductive strategies of marine fish species of the North Atlantic. Journal of Northwest Atlantic fishery science 33: 23-31

Nakazono A, Tateda Y, Tsukahara H (1981) Mating Habits of the Surfperch, *Ditrema temmincki*. Japanese Journal of Ichthyology 28: 122-128 doi 10.11369/jji1950.28.122

Neff BD, Pitcher TE, Ramnarine I (2008) Inter - population variation in multiple paternity and reproductive skew in the guppy. Molecular Ecology 17: 2975-2984

Nielsen LA, Johnson DL (1983) Fisheries techniques. Bethesda, Md. : American Fisheries Society, Bethesda, Md.

Pamilo P (1993) Polyandry and allele frequency differences between the sexes in the ant Formica aquilonia. Heredity 70: 472

Parker GA, Birkhead TR (2013) Polyandry: the history of a revolution. Philos Trans R Soc Lond B Biol Sci 368: 20120335 doi 10.1098/rstb.2012.0335

Peakall R, Smouse PE (2006) GENALEX 6: genetic analysis in Excel. Population genetic software for teaching and research. Molecular Ecology Notes 6: 288-295 doi 10.1111/j.1471-8286.2005.01155.x

Peakall R, Smouse PE (2012) GenAlEx 6.5: genetic analysis in Excel. Population genetic software for teaching and research--an update. Bioinformatics 28: 2537-2539 doi 10.1093/bioinformatics/bts460

Pruden D (2000) Final Report 1991-2000 Southern Oregon Surfperch Studies. Oregon Department of Fish and Wildlife

Reisser CM, Beldade R, Bernardi G (2009) Multiple paternity and competition in sympatric congeneric reef fishes, Embiotoca jacksoni and E. lateralis. Mol Ecol 18: 1504-1510 doi 10.1111/j.1365-294X.2009.04123.x

Rousset F (2008) genepop'007: a complete re - implementation of the genepop software for Windows and Linux. Molecular ecology resources 8: 103-106

Serbezov D, Bernatchez L, Olsen EM, Vollestad LA (2010) Mating patterns and determinants of individual reproductive success in brown trout (Salmo trutta) revealed by parentage analysis of an entire stream living population. Mol Ecol 19: 3193-3205 doi 10.1111/j.1365-294X.2010.04744.x

Soucy S, Travis J (2003) Multiple paternity and population genetic structure in natural populations of the poeciliid fish, Heterandria formosa. J Evol Biol 16: 1328-1336

Takagi M, Sakai K, Taniguchi N (2008a) Direct evidence of multiple paternities in natural population of viviparous Japanese surfperch by allelic markers of microsatellite DNA loci. Fisheries Science 74: 976-982 doi 10.1111/j.1444-2906.2008.01615.x

Takagi M, Sakai K, Taniguchi N (2008b) Isolation and characterization of 13 microsatellite markers for the viviparous surfperch Ditrema temmincki (Embiotocidae) and

cross-species amplification. Mol Ecol Resour 8: 1030-1033 doi 10.1111/j.1755-0998.2008.02145.x

Tamura E, Honma Y, Kitamura Y (1981) Seasonal-Changes in the Thymus of the Viviparous Surfperch, Ditrema-Temmincki, with Special Reference to Its Maturity and Gestation. Japanese Journal of Ichthyology 28: 295-303

Thrasher DJ, Butcher BG, Campagna L, Webster MS, Lovette IJ (2018) Double-digest RAD sequencing outperforms microsatellite loci at assigning paternity and estimating relatedness: A proof of concept in a highly promiscuous bird. Mol Ecol Resour doi 10.1111/1755-0998.12771

Warner RR, Harlan RK (1982) Sperm Competition and Sperm Storage as Determinants of Sexual Dimorphism in the Dwarf Surfperch, Micrometrus-Minimus. Evolution 36: 44-55 doi Doi 10.2307/2407965

Westphal MF, Morey SR, Uyeda JC, Morgan TJ (2011) Molecular phylogeny of the subfamily Amphistichinae (Teleostei: Embiotocidae) reveals parallel divergent evolution of red pigmentation in two rapidly evolving lineages of sand-dwelling surfperch. J Fish Biol 79: 313-330 doi 10.1111/j.1095-8649.2011.03011.x

Wourms JP (1981) Viviparity - the Maternal-Fetal Relationship in Fishes. American Zoologist 21: 473-515

Zeh JA, Zeh DW (2001) Reproductive mode and the genetic benefits of polyandry. Animal Behaviour 61: 1051-1063 doi 10.1006/anbe.2000.1705