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TESTING FOR THE EXISTENCE OF SMALL NESTING MALES IN A NATURAL POPULATION OF BLUEBANDED GOBIES, LYTHRYPNUS DALLI.

Joseph Bush

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Under the Direction of Matthew Grober, PhD

ABSTRACT

In protogynous fishes, smaller individuals tend to be female and only the largest individuals are male. In *Lythrypnus dalli* the small population of mini-males that are found in the wild are typically alternative males that mimic females to gain access to the nest. However, recent work has shown that small juveniles can develop into mini-nesting males in a laboratory setting. We tested whether or not some of the small males in the wild are actually mini-nesting males by comparing the content of the accessory gonadal structure (AGS), a prostate-like gland that is known to have different functions in nesting and mini-males. Over half of the mini-males had AGS contents typical of nesting males as well as larger dorsal fins and longer genital papillae than the alternative males. These findings add to our understanding of the role of the environment in regulating sexual phenotype in *L. dalli*.

INDEX WORDS: Bluebanded Goby, Mini males, Nesting males

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JOSEPH BUSH

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JOSEPH BUSH

Committee Chair: Matthew Grober

Committee: Walter Walthall

Aaron Roseberry

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Office of Graduate Studies

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Georgia State University

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1 INTRODUCTION

1.1 Introduction

Sexual selection plays a critical role in understanding the divergence in morphology between males and females of a given species, and has produced large differences between males and females, such as bright colors or the presence of antlers. These changes are not well explained by natural selection, as they can at times seem to decrease an individual's ability to survive. Sexual selection, where individuals struggle for access to the opposite sex rather than to simply better survive, can often explain these sexually dimorphic characteristics (Darwin, 1874; Huxley, 1938a, 1938b). Intersexual selection, one component of sexual selection, explains traits that make the individual more appealing to the other sex, such as bright colors. Other differences are shaped by intrasexual selection, where characteristics that can enhance competition over mates are preferential (Huxley, 1938a, 1938b). For example, larger and stronger males are more likely to win a territorial challenge over a smaller, weaker male. If male mating success is determined, in part, by territory quality, then intrasexual interactions can have a significant impact on reproductive success and thus fitness.

Since large males can have much higher reproductive success by excluding small males from access to females (Huxley, 1938a, 1938b), some smaller males have evolved alternative reproductive tactics to increase their reproductive success in the face of the overwhelmingly poor odds of winning a physical competition with large males. Several different alternative mating tactics in fish have been described. In the North American Sunfish, smaller males will mimic females and thereby gain temporary access to the nest, other females, and most importantly, their eggs (Gross, 1979, 1982). Alternatively, smaller male Coho Salmon will attempt to gain access to female nests by "sneaking." By hiding behind boulders they can attempt to sneak into the nest and release their milt right after the female spawns (Gross, 1985). Although these males are small adults, that doesn't necessarily mean that they are inferior males. It may be instead that they grew quickly as juveniles and use these tactics early in adulthood until they are able to grow larger and compete successfully with other large males (Gross, 1985).

Since sex is fixed at birth in most species and both sexes grow as they mature, there is a large overlap in size between the sexes, even if one sex is larger in adulthood. Some hermaphroditic species have an advantage in that they are more plastic and can choose the sex that maximizes reproductive success based on their current characteristics, such as size (Warner, Robertson, & Leigh, 1975). The size-advantage model predicts that in a protogynous hermaphroditic system, larger individuals have higher reproductive success as males and smaller individuals have higher reproductive success as females (Ghiselin, 1969; Warner, 1975). So while in non-hermaphroditic species size may not always be a reliable indicator of sex, even if on average one sex is larger, in hermaphrodites size is often robustly associated with sex. One of the hallmarks of protogynous hermaphroditic species is the absence of males at smaller sizes and a strong skew towards males in the largest size classes. Generally speaking, the Bluebanded Goby follows this model in that smaller individuals of 17-25mm standard length (SL) are female and larger individuals of larger size tend to be male (Mary, 1993, 1994). In addition to larger SL, nesting males have longer first dorsal fin rays. The genital papilla (GP) is also dimorphic, with females having a short blunt GP and nesting males having a longer more pointed GP (Wiley, 1976).

Previous work in *L. dalli* has established the existence of alternative males (Drilling & Grober, 2005). These "mini-males" tend to be approximately 17-25mm SL, the general size range of females, but have a GP typical of nesting males. Nesting male gobies have an accessory gonadal structure (AGS) that contains mostly mucins, which are used for laying down trails of mucus filled with sperm (Scaggiante, Mazzoldi, Petersen, & Rasotto, 1999). The AGS of mini-males, however, is filled with sperm in addition to the sperm already in the testes (Drilling & Grober, 2005). Because of sperm competition with the nesting male, it is advantageous for the mini-male to produce and store large amounts of sperm to increase the odds of successful fertilization.

Not all small fish with male typical papilla are alternative males. Solomon-Lane and colleagues have shown that mini-nesting males developed in laboratory reared groups of 4-5 juveniles that lack adults (Solomon-Lane et al, in press). In order to determine if mini-nesting males are present in wild populations, we examined AGS contents of a small sample of field caught mini-males. We also looked at several sex typical traits to determine if they were associated with differences in AGS content / phenotype.

2 METHOD

2.1 General methods

L. dalli were collected off the shores of Catalina Island, California during the reproductive season of 2014 (California Fish and Game permit SC-11879). Fish were caught using hand nets while SCUBA diving and were later euthanized using tricaine methanesulfonate (MS-222). The fish were then stored in a solution of 4% paraformaldehyde at 4°C. Mini-males were separated into groups after analyzing AGS on the basis of sperm content (N = 11 total). Although multiple traits are usually used to differentiate between groups, AGS contents clearly indicate whether the fish is a nesting male or alternative male (Drilling & Grober, 2005).

2.2 Gonad sectioning

To determine the contents of the AGS, fish were sectioned using a cryostat at a thickness of 60-70 μm. DAPI florescent staining was used to reveal the presence of sperm in the AGS. Because DAPI reveals the presence of nuclei there should be a large amount of signal with the presence of sperm and no signal with the presence of mucins. Sections were mounted using Fluoroshield Mounting Medium with DAPI (abcam). Sections were brought up to room temperature and then several drops of the mounting medium were applied directly to the tissue. The slides were left to stand for 5 minutes and then coversliped, viewed under a microscope

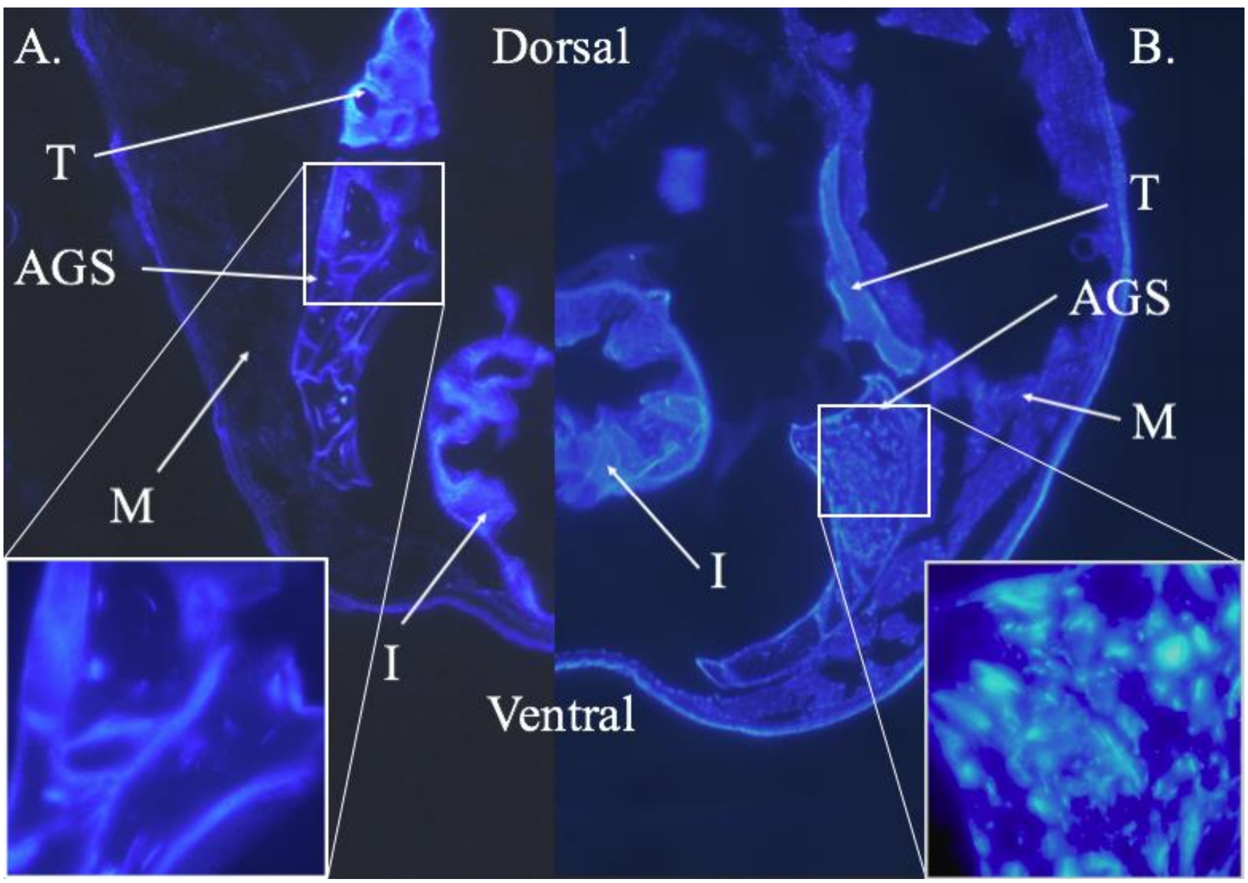

Figure 1. DAPI staining of nuclei and thus the presence of sperm in mini-nesting male (A) and alternative male (B). T = testes, AGS = accessory gonadal structure, I = intestinal tract, M = muscle. Insets reveal AGS contents at higher magnification.

and digital images were acquired. The content of the AGS was assigned numerical ranks on the basis of how much sperm was present in the largest chamber (Fig. 1). Rank 1 was 0-20% sperm; rank 2 was 20-50% sperm; rank 3 was 50-80% sperm; rank 4 was 80-100% sperm.

2.3 Morphological measurements

Genital papilla morphology is measured using a ratio of length to width at the halfway point of length (Fig. 2). Because the female typical papilla is blunter, they have a L:W ratio of around 1. Male papillae are long and pointy and typically have a L:W ratio greater than 2 (but always in excess of 1.4).

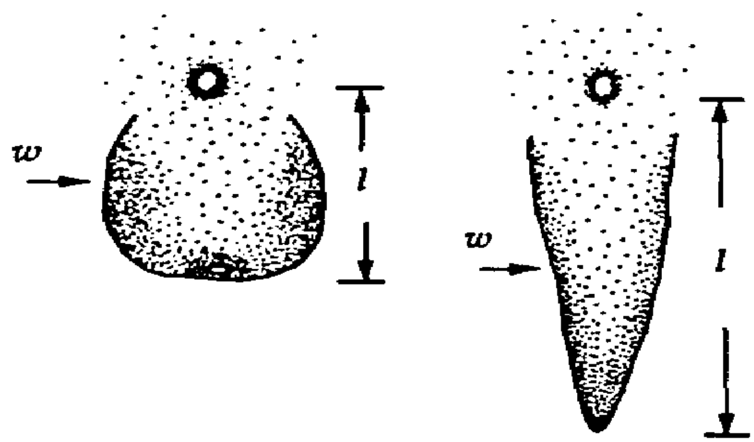

Figure 2. Genital Papilla Morphologies. Left: typical female papilla, blunt-tipped; Right: typical male papilla, pointed. Taken from St. Mary 1993.

Body length was measured as Standard Length; the distance from the tip of the snout to the caudle peduncle. Dorsal fin height was measured from the base of the dorsal fin to the tip (Fig. 3). The dorsal fin rays have a ball at the tip, which were used to determine if the fin had been damaged, impacting the measurement. If the first dorsal fin ray was damaged, the next intact ray was used. Dorsal fin area was measured as the total area around the margin of the extended dorsal fin. All measurements were taken using images processed with ImageJ (NIH).

Statistical comparisons of traits between the groups were made using a t-test in Microsoft Excel 2016. Since there was only one rank 2 fish and it was more similar to rank 1 than rank 4, the AGS rank 2 fish was included in the mini-nesting males group. Compairisons of fin size were run using a 2 tailed t-test because there was no *a priori* expectation of outcome. Comparisions of GP L:W ratio were run using a 1 tailed t-test because we expected the mini-nesting males to have a larger L:W ratio to assist in laying sperm trails.

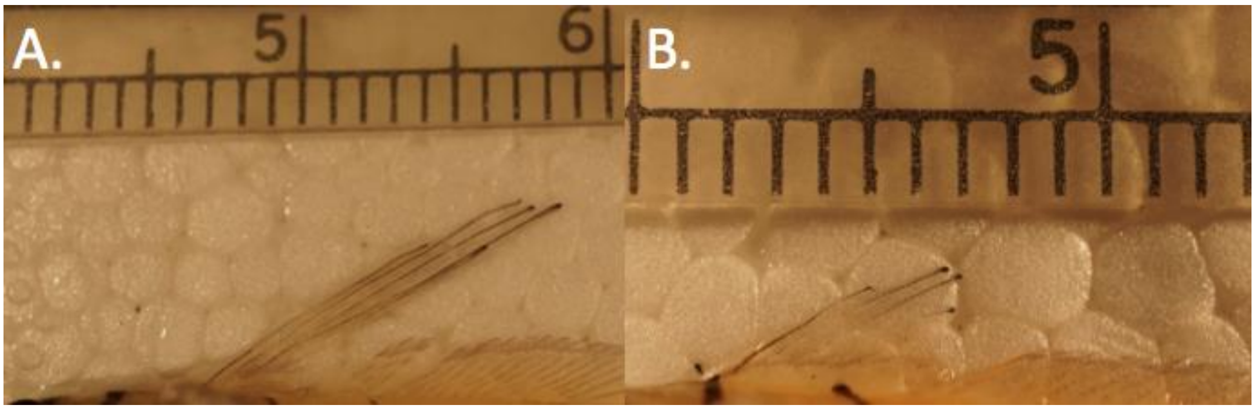

Figure 3. Comparison of dorsal fin of a mini-nesting male on the left (A) and a mini-male on the right (B). Mini-nesting males have much larger dorsal fins on average than mini-males. Ruler above the dorsal fins is in centimeters. Fish A and B had similar SL (24.23mm and 25.57mm respectively)

3 RESULTS

3.1 Histology

Staining of sectioned gonads with DAPI revealed that the AGS was either mostly filled with sperm or mostly filled with mucins. Three of the fish had sperm filled AGS's typical of alternative males, while 7 had a mucin filled AGS typical of nesting males (Fig. 1 & 4). There was one fish that had an AGS score of 2 with approximately half the AGS being filled with sperm.

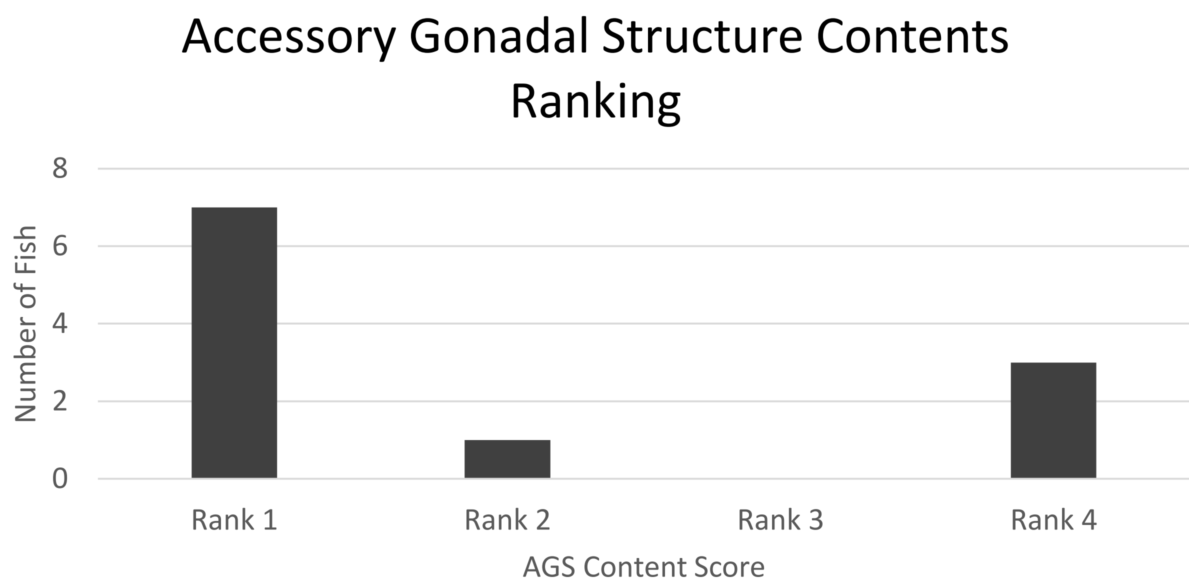

Figure 4. Number of fish with each AGS content score. Rank 1 is 0-20% sperm; rank 2 is 20-50% sperm; rank 3 is 50-80% sperm; rank 4 is 80-100% sperm.

3.2 Morphological measurements

Dorsal fin height ($p = 0.01$) and dorsal fin area ($p = 0.01$) differed significantly between alternative males and mini-nesting males, with the latter having larger and longer dorsal fins (Fig. 3 & 5). There was a substantial difference in papilla L:W ratio with mini-nesting males having more male-biased papilla ratio relative to alternative males ($p = 0.03$; Fig. 5).

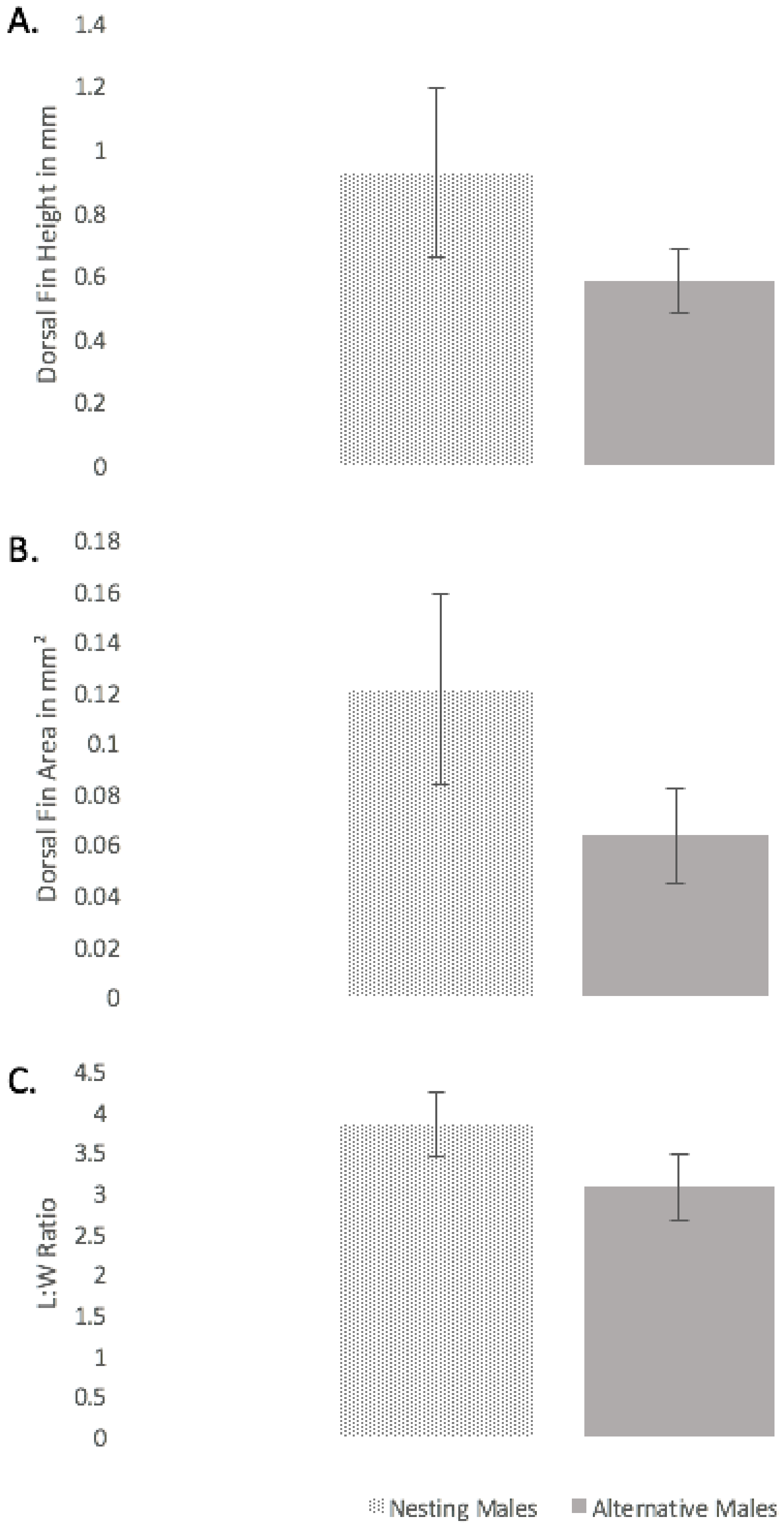

Figure 4. Comparison of average (±SEM) of (A) dorsal fin height, (B) dorsal fin area, and (C) genital papilla L:W ratio between mini-nesting males and alternative males.

4 DISCUSSION

Consistent with previous research that demonstrated laboratory-reared juveniles can develop into mini-nesting males in the absence of adults (Solomon-Lane et al, in press), our data show quite clearly that this phenomenon can also occur in the wild. Because of the small percentage of mini-males in the larger *L. dalli* population, our sample size was limited, but of the mini-males collected, over half of them were mini-nesting males. The majority of the small males had AGS's filled with less than 20% sperm, indicative of nesting males. This suggests that there are opportunities in nature for very small males to establish / maintain a nest. Interestingly, one fish had an AGS with between 20-50% sperm indicating that the fish was likely transitioning between the male morphs. This possibility has been demonstrated by previous research (Lorenzi & Grober, unpublished data).

There is a robust correlation between AGS content and genital L:W ratio. Nesting males have more pointed papilla than alternative males, which can aid in laying down mucus trails, something alternative males do not require (Grober & Grober, unpublished data). These mini-nesting males are young adults who are just acquiring these sexual traits, which may explain why the statistical analysis does not quite reach $p<0.05$. Nesting males also have larger dorsal fins than alternative males, which is likely due either to intrasexual or intersexual selection. Large dorsal fins can be used in aggressive displays against other males (Hastings, 1991), however they might also be used to attract females (Fisher & Rosenthal, 2007).

One mechanism that may be driving these differences in phenotypes is different hormone levels. Mini-males have higher testosterone (T) levels than 11-ketotestosterone (KT) or estradiol (E_2) and mini-males have a lower T:KT ratio than nesting males or females in the brain, muscles, and reproductive tissues (Bass & Grober, 2001; Pradhan, Solomon-Lane, & Grober, 2014).

Because mini-males make up less than 5% of the *L. dalli* population (Drilling & Grober, 2005), performing studies that determine the exact mechanisms that regulate different polymorphs of males is difficult.

In the wild, most juveniles live in close proximity to adults, with only about 15% living separately (Solomon-Lane et al., in press). Because the mini-nesting male strategy only works in the absence of mature adults, we would expect a higher percentage of alternative males in our sample. However, 63% of our sample is comprised of mini-nesting males and only 27% is alternative males. One possible explanation for this is that these mini-nesting males were found in a different quality habitat. Typically, *L. dalli* are found in highest densities near Crowned sea urchins *Centrostephanus coronatus* (Drilling & Grober, 2005; Hartney & Grorud, 2002). By staying near the spins of the sea urchins, they gain protection from predation. Therefore, in a habitat with few sea urchins there may be only a few adults around. This would allow for a higher percentage of small mini-nesting males to establish small peripheral territories that are not productive enough to attract the attention of larger nesting males or large females.

Our current understanding of sexual plasticity in *L. dalli* is that the environment plays a key role in regulating sex. The most dominant, and often largest, fish become male, while the smaller, submissive fish become or remain female (Rodgers, Earley, & Grober, 2007). Our data suggest that some environmental conditions can provide the opportunity for very small males to establish small territories and dominant status social status, and thus under some environmental conditions some young *L. dalli* can develop into mini-nesting males. Our data from wild caught fish support the conclusion that, from an early age, *L. dalli* can pick the sex that maximizes their reproductive success (Solomon-Lane et al, in press).

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