Where to place your eggs: the effects of conspecific eggs and water depth on oviposition decisions in bluefin killifish

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Abstract

In species with external development, egg placement is expected to impact the fitness of females and males via offspring survival. Both environmental and social cues influence the placement of eggs. In nest building fishes with male parental care, females frequently prefer to lay eggs in areas where eggs are already present. Most studies on female oviposition strategies have focused on species where males build nests and care for the eggs. However, few studies have examined oviposition strategies in species lacking parental care. This study tested whether female bluefin killifish *Lucania goodei* prefer to lay eggs in spawning substrates that already contain eggs from other females. The unique aspect of this study is that *L. goodei* is a non-nest building species with no parental care and high levels of iteroparity. Females preferred to lay eggs in areas where eggs were already present but these effects decreased with increasing clutch size. We suggest that females prefer to lay small bouts of eggs in areas already containing eggs of other females, but that in nature, they distribute these eggs across multiple males and locations. By doing so, females may increase the probability of offspring survival via either the dilution effect (reduced individual probability of predation due to increased group size) or the selection of 'good locations' for offspring development.

Introduction

Egg placement in species with external development affects the probability of egg survival, and, thus, the fitness of the parents. As such, natural selection should favor a female's oviposition strategies that maximize the survival of their offspring. Egg placement is likely to be influenced by environmental parameters such as temperature (Teletchea *et al.*, 2009), oxygen levels (Takegaki & Nakazono, 1999; Rombough, 2007) and the productivity of the body of water the fish are in (Johnston & Leggett, 2002).

Female egg placement may also be influenced by social conditions. In many fish species, females prefer to spawn with males whose nests already contain eggs (Ridley & Rechten, 1981; Unger & Sargent, 1988; Petersen, 1995; Forsgren, Karlsson, & Kvarnemo, 1996; Alonzo, 2008; Matsumoto *et al.*, 2008). In fact, males of some species raid the nests of neighboring males for the sole purpose of stealing eggs (Rohwer, 1978; Mrowka, 1987; Jones, Ostlund-Nilsson, & Avise 1998). There have also been limited reports of egg stealing in a few bird species (Gaston, Deforest & Noble, 1993; Fernandez & Reboreda, 1995), although these occurrences are rare. Subsequent research focusing on fish has found that females frequently prefer males with clutches of eggs over males with no eggs (reviewed in Brown & Laland, 2003).

Three non-mutually exclusive hypotheses have been put forth to explain the evolution of female preference for males with eggs in the nest. First, eggs laid among other eggs may face a smaller risk of predation or cannibalism if some eggs should be eaten, because the risk is spread out among the entire nest (termed the 'dilution effect') (Rohwer, 1978). Second, females may be copying the choice of other females by using the already-laid eggs as an indication of female choice and, thus, the female reduces her search costs by not having to independently assess each prospective mate (Ridley, 1978; Goldschmidt, Bakker, & Feuth-De Bruijn, 1993). Third, eggs in a male's nest may reflect his parental ability (Pampoulie, Lindström & St Mary, 2004; Lindström, St Mary & Pampoulie, 2006). The parental investment hypothesis predicts that the male is more likely to invest greater parental care in larger (and, thus, more valuable) clutches and, therefore, the female is ensuring that the male will properly care for the eggs (Coleman, Gross & Sargent, 1985). Similarly, the presence of eggs in the nest may indicate that the male is a successful parent (Ridley, 1978; Sargent, 1988). An early review of the first two hypotheses by Jamieson (1995) found less support for the copying hypothesis than for the egg survival hypothesis, but suggested that they are not mutually exclusive.

Most studies have focused on species which build nests and care for the eggs, but not all fish build nests or provide parental care. Some fish only defend temporary territories in

which eggs are laid. Laying eggs in territories that already contain eggs could still be advantageous because egg placement can have strong impacts on fitness (through one or more of the hypotheses previously mentioned). Yet, studies on female oviposition strategies have overlooked species with simple territoriality but no male care. The current study explores whether the female preference to oviposit eggs in areas already containing other eggs is unique to systems with nest building and male parental care. To do this, we use the bluefin killifish *Lucania goodei* which does not provide parental care (Fuller & Travis, 2001) or build a nest. Males aggressively defend small patches of vegetation in which females oviposit, but only for short periods of time (Fuller, 2001). Fertilization is external, and females will typically spawn with multiple different males within a single day (Arndt, 1971; R. C. Fuller, pers. obs.). Males will defend their temporary territories from non-breeding females. However, males are not very aggressive towards females in spawning condition and, even when they are aggressive, males are not able to force females to mate. In behavioral trials, males normally follow females and do not appear to force them to a particular spawning location (K. E. McGhee, pers. obs.). Thus, it is typically the decision of the female as to where she spawns (Fuller, 2001) and who she spawns with (McGhee, Fuller & Travis, 2007).

The goal of the current study was to determine whether female bluefin killifish *L. goodei* prefer to lay eggs in spawning 'mops' (spawning substrates) that already contained eggs. To do this, we allowed fish to spawn in tanks containing a variety of spawning mops – some that contained eggs previously spawned by another fish and some that were empty of eggs – and subsequently noted the placement of the new eggs. Because this species lacks male parental care, the third hypothesis for the evolution of female oviposition preferences for nests already containing eggs (the good parent hypothesis) is not relevant to this species. If females prefer to lay eggs in spawning substrates containing eggs, then this suggests that females obtain benefits from either the dilution effect or through the potential benefits of mate copying.

Materials and methods

Study system

The bluefin killifish *L. goodei* is a small freshwater fish found in rivers and springs in the south-eastern part of the US. In the wild, spawning mainly occurs from January to September, although year-round spawning is possible (Foster, 1967) and happens readily under laboratory conditions. Eggs are about 1.3 mm in diameter (Crawford & Balon, 1994) and stick to the spawning substrate because of attached adhesive filaments. Eggs are laid throughout the water column, from floating vegetation to bottom vegetation around 1.5 m in depth (Fuller, 2001). Both sexes will engage in egg cannibalism (Breder & Rosen, 1966; Fuller & Travis, 2001; Sandkam & Fuller, in press).

Collection and animal husbandry

The fish were collected from the Wakulla River, Wakulla County, FL, in October 2008. Fish were collected using seines and dip nets and transported back to the University of Illinois (Champaign, IL).

The fish were initially housed in mixed-sex 114 L (30 gallons) rectangular glass tanks in a greenhouse. At least 5 days before experimentation, animals were moved into a temperature-controlled fishroom where the experiment was conducted. Here, the fish were housed in mixed-sex 75 L (20 gallons) glass holding tanks. Bluefin killifish are highly iteroparous in both the field and the lab. They spawn multiple times a day and may spawn daily when in good condition. Females cannot be held in isolation from males for long periods of time because they become egg bound. Thus, it seems unlikely that any reproduction that occurs in the holding tank prior to the experimental trial would significantly affect our results. All of the fish, both in the greenhouse and fishroom, were fed daily *ad libitum* with a mixture of TetraMin® tropical flake food, Tetra® Micro Crab granules, frozen bloodworms and frozen brine shrimp (*Artemia* spp.). Additionally, on the day of a trial, the fish in the holding tank were fed an extra quantity of this mixture about 15–30 min before the trial began with the goal of prompting the females to lay eggs and to reduce the chances that any of the eggs would be eaten during the trial (individuals will eat both their own and other individual's eggs).

Experimental procedure

To determine whether *L. goodei* prefer to spawn their eggs in areas already containing eggs, pairs of fish were allowed to spawn in tanks containing four spawning mops. Each mop was created by tying 10–12 pieces of green yarn together. These mops have been used in other studies of killifish (Fuller & Travis, 2004; McGhee *et al.*, 2007) and the fish readily spawn in them. We manipulated the position of the mops (top vs. bottom) and the presence of eggs (empty vs. full), such that two top mops (one full and one empty) floated near the surface of the water and two bottom mops (one full and one empty) lay at the bottom of the tank. Full mops were created by placing them into a stock tank and removing the mops once fish laid eggs on them. The fish in the stock tank were from the same location, but had been collected at a different time. The age and developmental stage of the eggs in the full mops was not controlled for and eggs in the full mops were haphazardly assigned for each trial. The empty mops were placed in a tank without fish as a control. Thus, for each trial, there was one top mop with eggs, one top without eggs, one bottom mop with eggs, and one bottom without eggs. Each trial had four new mops. None were reused.

For all trials, the full mops had at least eight eggs present (mean ± SE = 9.87 ± 0.22, range = 8–13). The location of each egg in the two full mops was marked with a small piece of green sewing thread tied to the yarn right next to the egg

and the two full mops were made to have an equal number of eggs by removing any excess eggs. The number of eggs in each mop was counted twice to ensure that handling the mops did not cause any eggs to be dislodged. The empty mops were double-checked to ensure no eggs were present and then marked with an equal number of pieces of sewing thread placed in haphazard locations on the mop. The top mops were fixed into place in one of two specific locations at opposite sides of the tank with a small piece of tape (to prevent the mops from coming in contact with each other) and the bottom mops were gently placed on the bottom of the tank in one of two other, opposing locations (to prevent the mops from coming in contact with each other). The location of each of the mops of the same type (top or bottom) was haphazardly assigned before the start of the trial.

For each trial, one male and one female were removed from a holding tank and placed in a tall 40 L (10 gallons) all-glass hexagonal tank (45 cm $H \times 37$ cm $L \times 32$ cm W). Two-thirds (four of the six sides) of the tank were covered by a black plastic garbage bag to visually isolate the fish from neighboring tanks. The tank was illuminated by a full-spectrum bulb on a 14 L:10 D light cycle. The tank was filled with de-chlorinated, buffered tap water and was aerated using a small air stone connected to an air pump. The water was not changed, but fresh water was added as necessary to maintain a (roughly) constant water level for all of the trials. Immediately after placing the pair of fish into the experimental tank, we covered it with cardboard, thus allowing the fish to both acclimate to the new tank and to minimize any activity (spawning or eating eggs) while they were acclimating. After 1 h, the cardboard was removed and the fish were allowed to spawn for one additional hour. All trials were started in the morning with fish being moved to the cardboard-covered tank between 09:50 and 11:40 h.

Once the trial was over, the fish were carefully removed from the hexagonal tank, and the number of eggs in each of the four mops was counted. Each of the eggs was recorded as either 'new' (newly laid during the trial) or 'old' (which were the original eggs on the full mops marked with the sewing thread). Because eggs can be eaten and the remnants do not always remain, only whole, intact eggs were counted. Each mop was checked twice to ensure that no eggs were overlooked. We also measured egg cannibalism of 'old' eggs. 'Old' eggs that were not present after a trial ended were considered to have been eaten. We could only measure cannibalism on 'old' eggs because there was no way to determine how many newly laid eggs were consumed by the fish before the end of the trial. Females were only used once. Of 41 males, only one was used twice, but he was used several months apart. There was no mortality during a trial or over the course of the entire 8 months. Upon completion of the trial, the fish were placed into tanks containing conspecifics used in previous trials, and these tanks were maintained according to standard IACUC protocol (#0515). In total, 41 trials were conducted, 30 of which successful.

Statistical analysis

The goal of the analysis was to determine whether egg placement was affected by position of the mops (top or bottom) and by whether the mops contained eggs (full or empty). To do this, we used a general linearized model in SAS Proc Genmod that considered the effects of position, presence of eggs and their interaction on the number of eggs placed in each of the substrates. The dependent variable was the number of new eggs laid in each of the mops in each of the trials. Because the data were non-normally distributed ($W = 0.673$, $P < 0.001$), we used a maximum likelihood model that assumed a negative binomial distribution with log link function. The negative binomial distribution is appropriate for count data and can handle zeros (Littell, Stroup & Freund 2002). A type 3 analysis was used to examine the effect of removing each of the terms from the model. To ensure that the analysis took account of the structure of the data, the 'repeated' statement was used with trial as the repeated measure. This ensures that trials are units of observation and avoids any pseudoreplication and non-independence of the data by accounting for the correlation among repeated measurements within the trial (Littell et al., 2002). Since the trials were conducted over 8 months, the model was initially analyzed using a 'month' term, but the model was then re-run without the 'month' term because it was found to be not significant (see 'Results'). The results were qualitatively identical regardless of whether month was left in the model. Only successful trials were considered in the statistical analysis. A trial was considered successful if at least one new egg was present upon completion of the trial.

Egg placement may differ depending on how many eggs were laid. To account for this, we used a Spearman correlation to test whether the proportion of eggs laid in full mops (i.e. substrates with eggs) was affected by the total number of eggs laid. Additionally, we asked whether egg cannibalism differed as a function of the total number of eggs laid and the position of the eggs. To do this, we performed two analyses. A linear regression was used to test whether the number of 'old' eggs eaten was influenced by the number of eggs in the mop. A signed rank Wilcoxon test was used to determine whether there was a difference in the number of 'old' eggs eaten between the top and bottom mops. All analyses were carried out using SAS (v. 9.1, SAS Institute Inc., Cary, NC, USA).

Results

A total of 41 trials were conducted between January and August of 2009. Of these, 30 were successful. The negative binomial model provided a good fit to the data, and there was no evidence of overdispersion (deviance/d.f. = 0.927).

Females preferred to lay eggs in mops that contained eggs and also laid more eggs lower in the water column in the bottom substrates (Fig. 1). Both position (top or bottom) and presence of eggs (full or empty) affected the placement of the egg (presence of eggs $\chi^2 = 5.84$, d.f. = 1, $P = 0.0157$;
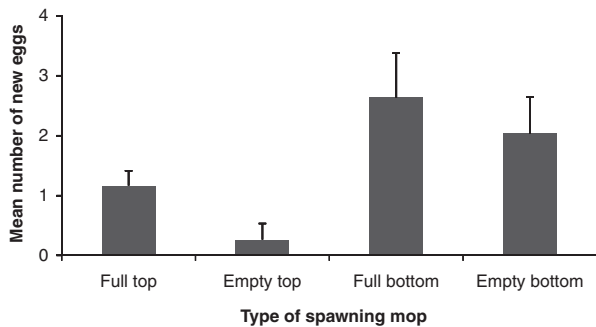
Figure 1 Mean number of new eggs laid in each type of spawning mop (*n* = 30). Error bars represent one standard error.
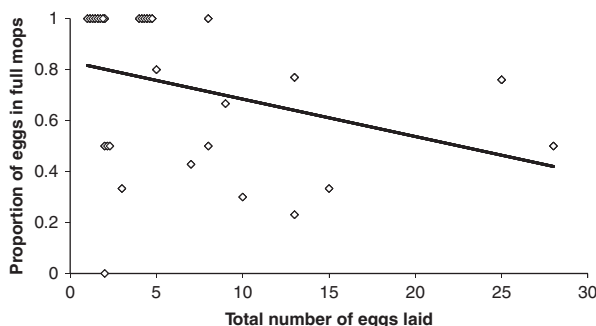
Figure 2 Relationship between the proportion of eggs in full spawning mops and the total number of eggs laid. For better visualization, overlapping data points have been 'jittered' (i.e. a nominal value was added to overlapping data points such that the data points on the graph no longer overlap).

position $\chi^2 = 6.04$, d.f. = 1, $P = 0.0140$). There was no statistically significant interaction between position and presence of eggs ($\chi^2 = 2.46$, d.f. = 1, $P = 0.1168$).

There was a negative relationship between the number of eggs in substrates containing eggs and the total number of eggs spawned. The proportion of eggs laid in the full substrates decreased with the total number of eggs laid ($R_s = -0.496$, $P = 0.0053$, $n = 30$, Fig. 2).

The number of eggs in the mops did not affect the number of 'old' eggs eaten ($F_{1,29} = 0.51$, $P = 0.4798$). However, there was differential cannibalism as a function of location. There was a larger number of total 'old' eggs eaten from the bottom mops than from the top mops ($S = 65$, $P = 0.0182$).

Discussion

There are three main findings from this study. First females preferred to lay eggs in mops that already contained the eggs of other females. While such preferences have already been documented in other teleosts, to the best of our knowledge they have not been demonstrated in a species that lacks male parental care and does not build a nest. Hence, one of the explanations for female preference to lay eggs in areas already containing eggs (the good parent hypothesis) cannot explain the evolution of this behavior. Second, while females prefer to lay eggs in areas already containing eggs, this preference decreased as the total number of eggs increased. The implication of this result is that females do not place all of their eggs in a single location. Third, more eggs were laid on the bottom substrates, but more eggs were eaten from these substrates. We discuss these findings below.

The current study suggests that females prefer to lay eggs in areas that already contain eggs, at least to a limited extent. Taken in isolation, this observation would suggest that females prefer to group their eggs in one location. However, field observations and the breeding biology of *L. goodei* indicate that they are extremely iteroparous (Fuller, 2001). Females in spawning condition will spawn every day for ~2 weeks (Breder & Rosen, 1966). It is not uncommon for females to lay seven to 14 eggs each day, but only release a couple of eggs per spawning event. Hence, females must spawn multiple times each day to distribute their eggs. Field observations suggest that females distribute their eggs across multiple males. Research in a polyandrous frog species found that females spread eggs over the nests of several males had higher offspring survival, suggesting that the frogs use this oviposition strategy as insurance against the possibility of nest failure (Byrne & Keogh, 2009). This oviposition strategy may serve as insurance against the possibility of nest failure.

How do we reconcile the fact that females prefer to lay eggs in areas that already contain eggs with the natural history which suggests that females distribute their eggs across multiple males and locations? First, we propose that egg mortality in the field is high and that this leads to selection to disperse eggs widely over time and space. Previous work has shown that both cannibalism and egg predation is high (Fuller & Travis, 2001). Second, we propose that there are benefits to be had by laying small numbers of eggs in areas already containing eggs. These two ideas account for the finding that female *L. goodei* preferred to lay their eggs in substrates already containing eggs, but that this preference decreased with increasing clutch size. The question remains as to how females benefit from placing their eggs in areas where eggs are already present.

The dilution hypothesis states that eggs laid among other eggs may face a smaller risk of predation or cannibalism if some eggs should be eaten, because the risk is 'spread out' among the entire nest (Rohwer, 1978). However, the benefits of the dilution hypothesis are greatest when a small number of eggs are added to a larger mass, and this may also explain the decreased female preference with increasing clutch size. The benefits that accrue through the dilution effect decrease as individuals lay higher numbers of eggs at any given location and, thus, the more eggs an individual adds to a group of eggs, the lower the benefit received from the dilution effect. However, this explanation assumes the entire clutch is not consumed. If a fish consumes an entire clutch upon detection, then the dilution hypothesis does not hold. Under this scenario, lumping eggs with other eggs might increase the probability of detecting the clutch. In the

bluefin killifish system, it is unknown how many eggs are typically eaten by the most common predators. In aquaria, males will often consume all of their eggs if given a long enough time (Fuller & Travis, 2001; Sandkam & Fuller, in press), but the extent of predation (from both conspecifics and heterospecifics) in the wild is not currently known.

Another possibility is that females are choosing good locations, but not good parents. The presence of older, developing eggs (that have not been preyed upon) may indicate that the present location is good at concealing eggs from conspecific and heterospecific predators (King & Withler, 2005). It may also indicate that the male, himself, is not a particularly voracious egg cannibal, although, as mentioned previously, all *L. goodei* males in aquaria will consume all their eggs if given a long enough time (Fuller & Travis, 2001; Sandkam & Fuller, in press). Complete egg consumption is known in other species as well, although several factors may influence the decision to cannibalize (Chin-Baarstad, Klug & Lindström, 2009; Svensson, Lissaker & Mobley, 2010). Regardless, the idea is that the presence of eggs indicates a high-quality location for eggs to develop.

A third hypothesis is that females are reducing their search costs by copying the choice of other females by using the already-laid eggs as an indication of a previous female's choice (Ridley, 1978, Goldschmidt et al., 1993, Goulet & Goulet, 2006). This is unlikely given the high levels of female iteroparity and the fact that females spawn with multiple males, oftentimes on the same day. Direct female inspection of males would appear less costly than poking around aquatic vegetation in an attempt to determine whether eggs are present. Males continually court females as they inspect spawning substrates, and male harassment may represent a significant cost to females in this system (McGhee et al., 2007).

Finally, we note that females spawned more eggs on the bottom substrates, but that the fish were most likely to consume eggs from these substrates. Similar results have been found by Sandkam & Fuller (in press) who found that cannibalism rates were higher on mops located lower in the water column. The implication of these data is that cannibalism will result in a higher proportion of fish eggs developing at shallower depths in the water column. However, these data need to be interpreted cautiously. *Lucania goodei* tend to be found at greater depths than other fish in our sites, such as *Gambusia holbrooki* and *Heterandria formosa*. The actual effects of total egg predation on the location of developing eggs in *L. goodei* require field studies.

In summary, the preference to lay eggs where other eggs are already present is not unique to species that build nests and provide parental care. We found that *L. goodei* prefer to oviposit their eggs in areas that already contain eggs, but that this preference decreases as the total number of eggs laid increases. While this study was not designed to test any of the specific hypotheses directly, these findings combined with the natural history of this species suggest two possible hypotheses to explain this behavior. First, females may prefer to lay eggs where there are other eggs if this reduces their risk of predation via the dilution effect. Alternatively, the presence of developing eggs may indicate a 'good location' that increases the probability of egg survival.

Acknowledgments

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