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Reproductive Characteristics of the Stripetail Darter (*Etheostoma kennicotti*) Relative to Monogenean Gill Parasite Infection in Estill Fork in North Alabama

Abstract

What relationship exists between *Aethycteron* sp. gill parasite infection and the reproductive characteristics of stripetail darters, *Etheostoma kennicotti*? 450 *E. kennicotti* were collected over 11 months from Estill Fork in Jackson County, Alabama. Gonads were removed and photographed. All oocytes were counted and then classified into one of four developmental stages based on size and appearance. Gill parasites belonging to the monogenean genus *Aethycteron* were excised, photographed, and counted. Sexual dimorphism in length and mass was observed in *E. kennicotti*. The number of males found at the 25> mm SL range far outnumbered the females while almost all individuals 25< mm SL were females. A high gonadosomatic index (GSI) was found in the females, with large (2 mm) size clutch oocytes. Female *E. kennicotti* were observed to be reproductively mature starting at 19 mm SL. Prevalence and intensity of infection by *Aethycteron* sp. parasites were high in both males and females, with prevalence reaching 90% in breeding condition individuals. Two hypotheses were tested to explain these phenomena. The first is that the lack of females in the larger size categories may be the result of a high cost of reproduction resulting in near-term reproduction being paid for at the expense of long-term survival. The second hypothesis tested is that fecundity compensation in females potentially causes the early reproductive maturation. The gender imbalance is likely the result of the sheer costs involved with reproduction, indicating high investment in current reproductive success with limited longer-term survival consistent with the fecundity compensation hypothesis.

Keywords

Dactylogyrid, *Aethycteron*, Gill Parasites, Percidae

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INTRODUCTION

Symbiotic relationships are common in the natural world. Often, these relationships develop because most forms of symbiosis provide a benefit for organisms involved while also causing little or no harm. One form of symbiosis, parasitism, enables the parasite to benefit from a host organism to the host's detriment (Thomas et al., 2000). Those resources needed for an immune response necessary to combat a parasite are often drawn from other critical functions, including reproduction (Heins, 2012). This fight can prove costly for the host as using these resources has no guarantee of success. Many organisms have evolved means of bypassing an immune response by directing resources they would have spent fighting the parasite into reproduction. Examples of bypassing an immune response include Three-spined Sticklebacks, *Gasterosteus aculeatus* (see following for details), and the water flea, *Daphnia magna*, when it is exposed to the microsporidian parasite *Glucoides intestinalis* (Chadwick and Little, 2005). Water fleas hosting *G. intestinalis* have been able to alter their life histories by reproducing earlier than fleas who have not been infected.

Three-spined Sticklebacks frequently serve as an intermediate host to the tapeworm *Schistocephalus solidus* (Heins, 2012). *Gasterosteus aculeatus* become hosts by first ingesting copepods hosting the larval form of the tapeworm (Benesh, 2012). *Schistocephalus solidus* grows to adulthood while inside the host's digestive tract but requires a final host, a seabird, to reproduce (Benesh, 2012). To ensure that it reaches the digestive tract of a seabird, *S. solidus* induces its host to swim to the surface of the water where it can eventually be consumed. Parasitism by *S. solidus* poses a grave threat to the survivability and long-term reproductive success of their host. Diminished chances at reproduction later in life requires some form of compensation. Hosts may increase their fecundity by shifting energy that would have been allocated toward an immune response into producing oocytes of a greater mass and in larger number than they would have had they not been parasitized (Heins, 2012).

Million et al. (2017) summarized three hypotheses to explain the impact parasites may have on host reproduction: 1) the parasite effect on reproduction is a non-adaptive side effect of infection (Heins, 2012); 2) the effect is a direct manipulation by the parasite (Ebert et al., 2004); 3) the effect is a defensive response by the host (Heins, 2012). The strategy of fecundity compensation is in the third category of these hypotheses (Forbes, 1993), by which the host counters negative effects of the parasite by diverting energy to an alternative reproductive strategy. Fecundity compensation is termed an “adaptive non-immunological host defense”, a response that functions outside of the host's immune system (Heins, 2012). The host may delay current reproduction to put energy into future reproductive efforts (Chadwick and Little, 2005) or bring forward reproductive timing to avoid future loss of fecundity due to chronic parasite infection (Vézillier et al., 2015). Another strategy is to alter clutch size or attributes of the offspring that contribute to fitness in response to parasite infection. This may maximize offspring fitness (Schwanz, 2008; Norris et al., 2010; Hendry et al., 2016; Kaiser and Hempel, 2016) or maternal fitness (Einum and Fleming, 2000).

All fecundity compensation mechanisms may change the balance of present reproduction versus expectation of future reproduction, a distinction made and explored by Williams (1957, 1966). A female's reproductive value can be split into two components, births in the current age

class and her value in the next age class. Given this distinction, the cost of reproduction can be considered in two forms: any increase in present reproduction negatively affects survival, the value of possible future reproduction, or both. The first cost is that of survival, the second is reproductive cost (Stearns, 1992).

In this study we examined the relationship between an undescribed Monogenean gill ectoparasite belonging to the genus *Aethycteron* and its percid host, *Etheostoma (Catonotus) kennicotti*, the Stripetail Darter. We explored how this parasitism may affect reproductive fitness of individuals, especially females. Earlier work by Million et al. (2017) explored how infection by another Monogenean gill parasite, *A. moorei*, appears to influence reproductive strategy in another *Catonotus* species, *E. flabellare*, the Fantail Darter. They found that more heavily infected females produce larger clutch sizes. Our analysis of *E. kennicotti* reproduction focused on how the pattern of Monogenean infection may impact measures of reproductive strategy including host gonadosomatic index (GSI), host size at reproduction, clutch sizes, and clutch counts.

METHODS

Specimen collection and preservation

All *Etheostoma kennicotti* specimens studied in this work were collected in Estill Fork of the Paint Rock River watershed in Jackson County, Alabama (34°54'38"N, -86°10'04"W). This watershed is in the Plateau Escarpment of the Southwestern Appalachian. This region is heavily dissected plateau, and consists of narrow ridges, knobs, cliffs, and steep gorges. Streams tend to be high-gradient, fast-flowing, and rocky-bottomed. The underlying geology is diverse, as this region cuts through many layers of rock, most notably calcareous shale, limestone, and sandstone ecoregion (Griffith et al., 2001). At the collection site Estill Fork was a third order stream flowing over chert and sandstone gravel and cobble in a rural area with trees bordering most of the stream. At the collection site the stream is ~10 m wide with depth typically less than 1 m. Collections took place monthly over the 11-month period from August 2016 through June 2017. 449 fish were collected and euthanized on site using a solution consisting of 10 mL of (1:10) clove oil and 95% ethanol per every 200-300 mL of river water. Each specimen was fixed and stored in 10% phosphate buffered formalin. Fish were collected under Alabama license 2015020106874740. The Institutional Animal Care and Use Committee of the University of Alabama in Huntsville issued protocol number 2015.R001 for this project.

Specimen physical and reproductive traits

Because a fish's length and mass are indicative of its age, both measurements were taken for every specimen collected. The standard length (SL) of each specimen was recorded to 0.01 mm using Marathon digital calipers. Somatic mass was recorded in grams using an Ohaus Explorer balance. The mass of each set of excised gonads was recorded in grams. Somatic mass and gonadal mass were used to calculate gonadosomatic index (GSI), the percentage of an organism's somatic mass that can be attributed to gonadal mass. GSI was calculated using the following equation: $GSI = (Gonadal\ Mass / (Total\ Fish\ Mass - Gonadal\ Mass)) \times 100$ (Million et al., 2017). Both testes and oocytes were photographed using an Olympus SZX7 stereo microscope equipped with a 12-megapixel digital camera controlled with the cellSens software package version 1.5. All oocytes were removed from excised ovaries for counting, measurement of diameter, and assessment of

maturation. Images were made of oocytes arranged into a single layer approximately 20 mm wide on a glass slide. Multiple images were necessary for many ovaries.

Oocytes were categorized into four different stages of maturity depending on their size and appearance (Figure 1). The least mature oocytes are the smallest in diameter (< 1.00 mm) and near translucent in color belong to Stage 1 (Figure 1A). They consist of a pair of membranes, the chorion and the pellucid membrane, surrounding a clear ooplasm (Laale, 1980). Stage 2 oocytes are larger in size and have a pale-yellow coloration (Figure 1A). This is due to the accumulation of vitellogenin phospholipoglyco-proteins within the oocyte that fuel the growth of the yolk (Lubzens et al. 2009). By Stage 3 the oocytes have doubled in size from Stage 1 and appear as golden yellow spheres (Figure 1B). Stage 4 oocytes in *E. kennicotti* have previously been found to average approximately 2.0–2.1 mm in diameter (Simon, 1987). Stage 4 oocyte shape is roughly spherical with a slight invagination along one side (Figure 1C). Unlike Stage 3 oocytes they do not appear yellow in color, but their ooplasm is clear and the phospholipoglyco-proteins have coalesced into a large golden yolk (Simon, 1987). The fecundity of each female was determined by the total number of the stage 3 and stage 4 oocytes we define as clutch oocytes. These are the oocytes mature enough to be spawned in the near future. Five oocytes from each stage of five individuals each month were measured to the nearest 0.001 mm and counted using the Egg Helper program, an accessory to cellSens. These data were used to calculate mean oocyte diameters (Tarver and Tarver, 2014).

Parasite counts and calculations

Following the removal of gonads, gill parasites were excised from both male and female specimens. This was accomplished by first removing gill arches from individuals using a scalpel to remove the connective tissue surrounding the arches. A dissecting probe was used to separate the individual gill flaps and forceps were used to remove the parasites. Each parasite was photographed using cellSens version 1.5 and then stored in 10% phosphate-buffered formalin. Only fish measuring >25 mm in length had gill arches removed. Parasite load was used to determine the mean intensity and prevalence of infection. Mean intensity (the average number of gill parasites per infected specimen) and prevalence (the percentage of specimens infected) (Bush et al., 1997) were calculated per collection month. We assumed all observed parasites to be members of the same undescribed *Aethycteron* species. In similar projects with two other subgenus *Catonotus* host species, *E. flabellare* and *E. striatulum*, all observed *Aethycteron* parasites were members of a species endemic to the host species (Million et al., 2017; Million and Stallsmith, 2019).

Statistical analysis

A Chi-square test was used to test for skew in the male to female ratio. GSI values and their relationship to parasite load were tested for possible relationships using linear regression. Tests involving parasite counts were performed only on females that met the following criteria: 1) they hosted at least one gill parasite, and 2) they carried clutch oocytes. Pearson coefficient correlation tests analyzed possible significant correlations between parasite load and the sizes of clutch oocytes, and the relationship between parasite load and the number of clutch oocytes. The alpha level used for all statistical analyses was 0.05. All statistics for this study were completed using statskingdom.com.

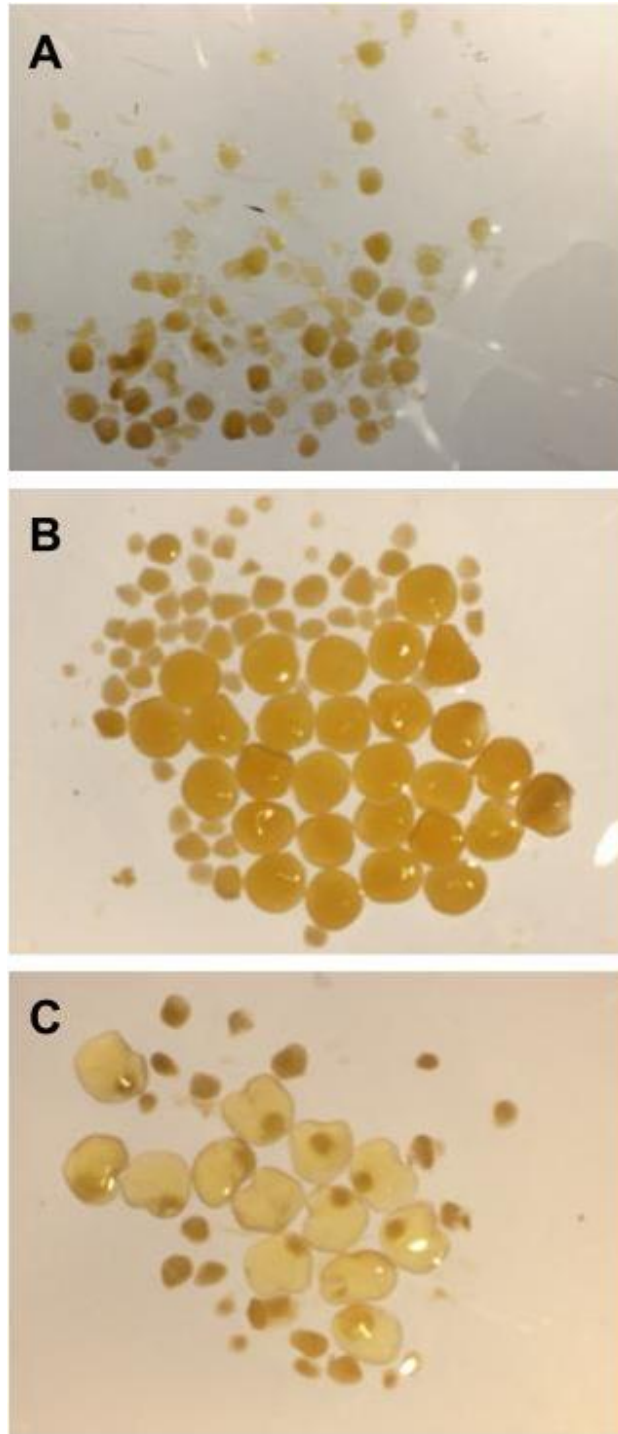


Figure 1. *Etheostoma kennicotti* oocytes in stages 1 through 4. All images are from fish collected in Estill Fork, Alabama. A) Stage 1 and 2 oocytes: Stage 1 oocytes are translucent and small in size, typically between 0.3 and 0.8 mm in diameter; Stage 2 vitellogenesis begins and the oocytes darken as the vitellogenin proteins accumulate. March 25, 2017. B) Stage 3 oocytes: increase in size to between 0.8 and 2.0 mm in diameter. Vitellogenesis is complete, or nearly complete, at this point. April 29, 2017. C) Stage 4: the vitellogenin has condensed into a yolk leaving the ooplasm clear. An obvious invagination has formed along one side of the oocyte. Images are 16 mm wide and 14 mm high. April 29, 2017.

Table 1. Mean diameter per stage of oocyte development. Oocytes were removed from 42 females from March 2017 through May 2017. Estill Fork, Alabama. n = number oocytes measured per stage.

Stage	Mean Diameter (mm)	n
1	0.73	1708
2	0.99	1716
3	1.47	778
4	1.97	64

RESULTS

Oocyte development

Oocytes removed from dissected specimens were examined and classified into one of four developmental stages based on color, size, and shape (Figure 1). Oocytes were removed and examined from 42 females from March through May. Total number of oocytes belonging to each stage was tallied and a mean diameter for each stage was calculated (Table 1). Of 4266 oocytes examined, Stage 1 and Stage 2 oocytes were the most numerous with 3424 combined. The 842 Stage 3 and Stage 4 oocytes were the clutch oocytes.

Sex differences: GSI values and sexual dimorphisms

In Figure 2 SL of all 250 reproductive individuals examined is shown by sex. Males were larger with a mean SL of 33.2 mm, and female mean SL was 28.9 mm. Mean monthly GSI values per collection month for females are shown in Figure 3. The highest mean monthly GSI values were found during March through May 2017, 8.3–20.2, with the peak in March. Monthly mean male GSI values varied between 0.3 and 0.5 (not shown). This three-month period was determined to be the breeding period of *E. kennicotti*.

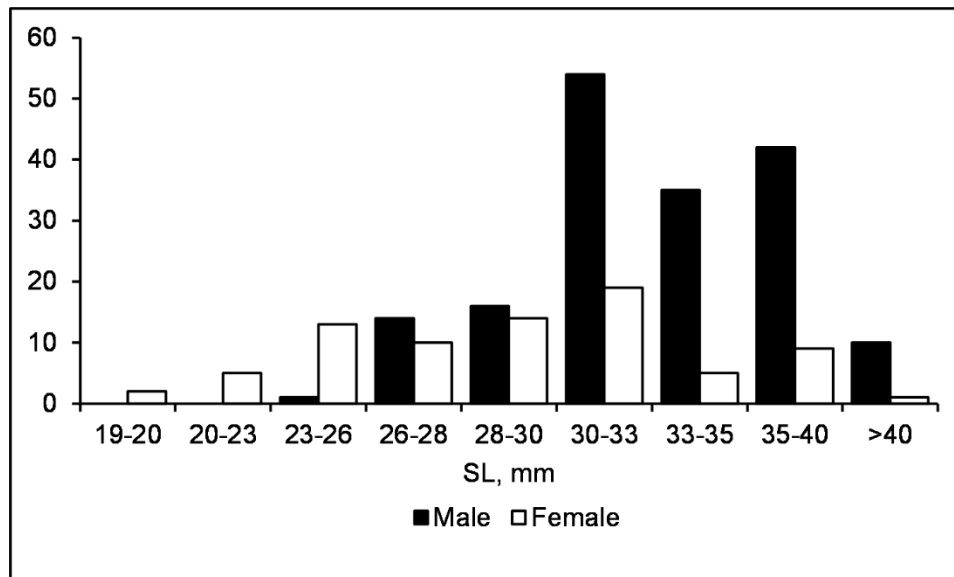


Figure 2. SL of *Etheostoma kennicotti* of reproductive size by sex, n=250. Male average SL is 33.2 mm, with a range of 25.5–47.7 mm. Female average SL is 28.9 mm, with a range of 19.31–44.8 mm. February – May 2017. Estill Fork, Alabama.

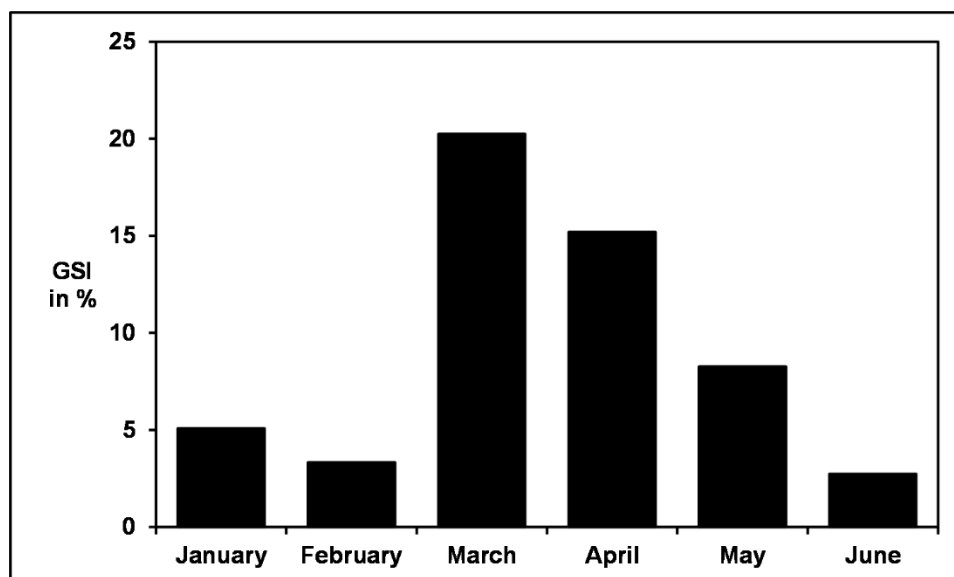


Figure 3. Mean monthly GSI of female *Etheostoma kennicotti*. 2017. Estill Fork, Alabama.

Gonadal dissections were initially performed only on specimens measuring >35 mm SL based on our experience with other *Catnotus* species. Among the 60 *E. kennicotti* exceeding this length, the male:female ratio was 5:1. Lowering the minimum dissection length to 25 mm SL resulted in more females being identified, though males were still more numerous. The male:female ratio of specimens between 25 mm and 35 mm SL was 1.95:1. The minimum length at which fish were dissected was dropped to 19 mm SL. Because no gonads were visible in fish smaller than 19 mm SL, 19 mm SL appears to be the minimal size of reproductive maturity. Sex of only 13 individuals could be determined at 19 mm SL, but all 13 were female. The smallest female carrying clutch oocytes was 19.3 mm SL in February. Of the 42 females with oocytes examined in March through May, eight were smaller than 25 mm SL and carrying clutch oocytes.

With the identification of the size of reproductive maturity, 142 males and 78 females were identified and had their gonads excised and examined. The Chi-square test for possible significant sex skew in the M:F ratio of 142:78 returned $\chi^2 (1, 220) = 18.6, p < 0.001$, showing a significant male skew in our sample. The mean SL $\pm$ SD for males was 33.3 ± 2.0 mm, and 28.9 ± 3.8 mm for females.

Parasite load and its relationships to reproduction

Parasite extraction was limited to the months of January through June 2017 and to those fish exceeding 25 mm SL in length as the extremely small size of *E. kennicotti* gill arches found in smaller fish significantly impaired our abilities to excise them. Figure 4 is an image of an *Aethycteron* that was excised from the gills of an *E. kennicotti*. Parasites examined were approximately 150 μ m long. The prevalence of infection for both sexes is shown in Figure 5. The prevalence of infection among females increased in the three spawning months of March through May, while it decreased in males. The intensity of infection is shown in Figure 6. Both sexes had high intensity of infection in January that generally decreased through the spawning months, dropping to almost nothing by June. During the peak spawning month of April females were parasitized to a much greater extent than males (Fig. 6). Twenty-eight females met our stated test

criteria of carrying at least one parasite and one clutch oocyte. No significant relationship was found between female GSI and parasite load. There is a non-significant, very small negative relationship between parasite load and mean clutch oocyte diameter, ($r(26) = .328$, $p = .215$), and also a non-significant small positive relationship between parasite load and the number of clutch oocytes carried by a female, ($r(26) = .175$, $p = .373$).

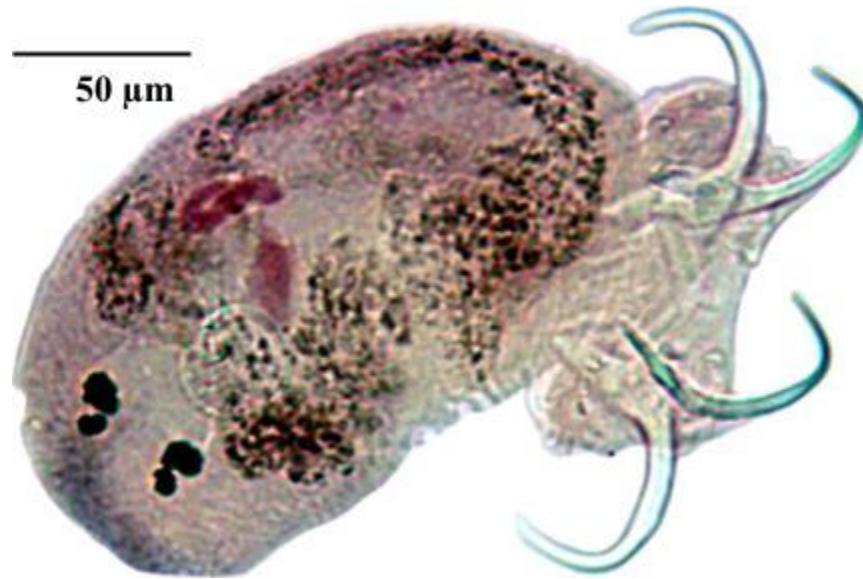


Figure 4. Undescribed species of Monogenean gill ectoparasite belonging to genus *Aethycteron*. This specimen was found attached to gills of a stripetail darter collected from Estill Fork, Alabama. April 10, 2014.

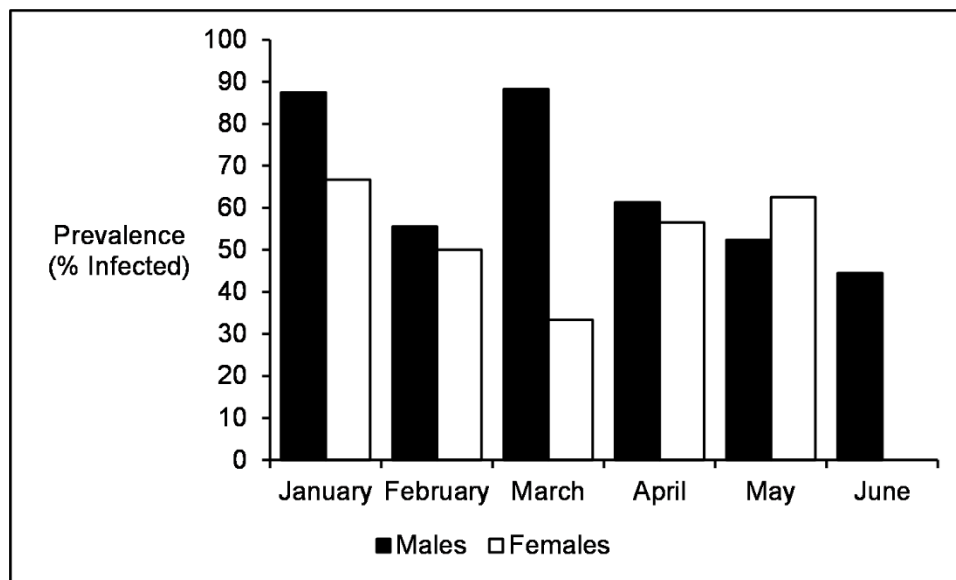


Figure 5. The prevalence of infection for both sexes of Stripetail Darter. Males are more likely to be infected by at least one *Aethycteron* parasite. Estill Fork, Alabama.

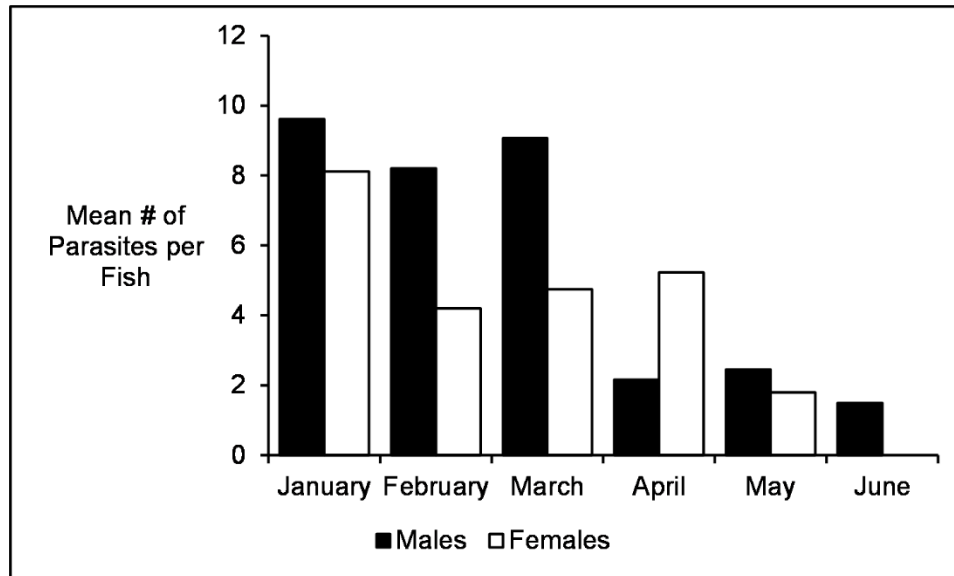


Figure 6. The intensity of infection for both sexes of Stripetail Darter. Males tend to have a higher rate of infection by *Aethycter* parasites.

DISCUSSION

The cost of reproduction hypothesis and the fecundity compensation hypothesis were both explored during this study. This was first a means of better understanding how parasitism and other conditions within their environments may affect reproduction in various species, but also to explain how the presence of parasites might alter the life cycles of their hosts. Fecundity compensation best describes what we found with *E. kennicotti*. Females spawn at a small size at age one with far fewer females than males surviving to a second year. We lack a natural reference population with which to compare the minimum size and survivorship of fecund females, but our findings are similar to what was found with females of this species in Illinois although no effort was made to examine fish for parasites (Page, 1975). No influence of parasite infection was found on female GSI, clutch oocyte size, or clutch count. This finding of small reproductive size along with high mortality of females was a surprise, not found by Million et al. (2017) in a similar study of the related *E. flabellare*. Our findings with *E. kennicotti* also differed from *E. flabellare* with lower intensity and prevalence of parasite infection. The early reproductive maturation of females, coupled with few older females, suggests a change of life history in *E. kennicotti* females such that the species shows similarities to annual fish species.

The traditional assumptions made about male mate choice are challenged if females experience a shortening of expected life span due to parasite infection. These assumptions include preference for larger females, females with more resources, healthy and parasite-free females, and more experienced females (Schlupp, 2021). All of those assumptions must be strongly altered in the population of *E. kennicotti* we examined due to the small size and young age of the females. Because *E. kennicotti* is an understudied species, with little information available concerning reproduction, we first determined how the two sexes differed before we determined what effect parasitism may have on reproduction. *Etheostoma kennicotti* are sexually dimorphic in size as would be expected of *Etheostoma* species. Females largely comprise smaller size categories, but

at sizes great than 25 mm SL male *E. kennicotti* outnumbered females (Figure 1). Because size translates to age in darter species, this lack of larger females translates into a lack of older females. Page (1975) encountered a similar phenomenon. He noted that before their first year of age, females outnumbered males 1.4:1, and continued to outnumber them 1.7:1 as adults of 1+ year. Both sexes declined in number during between the first and second years of age, but females declined at a higher rate. No individuals in that study population survived to three years. This lifespan is not uncommon among darters, but the discrepancy between the numbers of older males compared to older females, particularly when females predominate during their early lives, shows females have lower life expectancy.

High GSI values and, consequently, high reproductive investment may provide some insight into elevated female mortality. At the onset of the breeding period in March, the GSI in females reached a mean value of 20.2%. The presence of mature oocytes carried by females as small as 19 mm SL provides another clue. While studying the *E. kennicotti* population at Big Creek, IL, Page (1975) observed that females measuring greater than 30 mm SL developed clutch oocytes earlier than smaller females. In most *Etheostoma* species a fish measuring less than 27 mm SL would be considered a juvenile (Hanson and Stallsmith, 2013; Million et al. 2017; Bell et al., 2022). However, females as small as 19 mm collected from Estill Fork were observed not only to have produced clutch oocytes going into the spawning season, but, with one exception, they had produced clutch oocytes at the same rate as larger females. The fact that we did not find any sexually mature males at this small size indicates that this trade-off between fecundity and longevity exists only in the females.

The sex imbalance is likely the result of the sheer costs involved with reproduction, indicating high investment in current reproductive success with limited longer-term survival as framed by Williams (1957, 1966). The diminutive size at which this population of females makes this considerable investment in reproduction strongly suggests that this investment is made at the expense of longevity. This is a demonstration of the cost of reproduction hypothesis: a tradeoff made between longevity and reproduction. The result of this tradeoff appears to be that female *E. kennicotti* effectively have an annual life history.

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