

TWO LAYERS OF SELFISH-HERDS IN SPAWNING AGGREGATIONS OF CHUB
(*NOCOMIS SP.*) AND ITS NEST ASSOCIATES

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ABSTRACT

Many species have evolved to live in groups. Gregarious behavior was believed to be adaptive for whole-population survival and predator evasion until selfish-herd theory was introduced. It proposed that individuals congregate not as a method of protection for the entire population, but instead to better the individual's chance of survival, thereby using the group to benefit itself (i.e., "selfishness"). Selfish behavior is a common part of mutualisms, which are complex, dynamic interactions that often change with biotic or abiotic circumstance. Here, I investigate potential selfish behavior within the mixed-species spawning aggregations hosted by bluehead chub (*Nocomis leptocephalus*). I hypothesized that the host benefits more directly from the aggregation it supports than previous research suggests – specifically, by using nest associates to decrease its own risk of predation (creating a selfish-herd) and simultaneously forcing associate embryos into marginal nest locations while locating its own embryo in the safer locations within the nest (creating an "embryonic selfish-herd"). In Chapter 1, I investigated the adult spawning aggregation for possible selfishness by monitoring the chub's spatial location within the aggregation and vigilance behavior under varying associate abundances. I found that the host occupied the central location within the aggregation and was less vigilant when associate abundance was high. In Chapter 2, I examined the "embryonic herd" contained within *Nocomis* nests for possible embryonic selfishness orchestrated by the chub host, leading to increased embryonic survival for chub young and elevated fitness for chub parents. I found that deeper nest sections support higher embryonic survival than shallower sections, and that chub embryo make up a disproportionate percentage of embryos found in those deeper sections. I also conducted a preliminary study investigating embryo-predation by host on associate embryo which produced evidence for embryo-predation by both host and two associates on each other's offspring. Cohesively, my results support the identification of the host as a selfish participant and confirm the presence of both an adult and embryonic selfish-herd in this mutualism. This is the first study to demonstrate selfish behavior on the part of the host in this system and the first to prove the existence of an embryonic selfish-herd.

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GENERAL AUDIENCE ABSTRACT

A fish has three goals: to survive, to grow, and to reproduce. Mutualisms – when all participants experience a net benefit from interacting – arise when two or more species rely on each other to meet these objectives. In many mutualisms, however, not all participants benefit equally. Selfish-herd theory identifies those that maximize their own benefit while minimizing or negating any cost as “selfish” individuals. I examined the mutualistic mixed-species spawning aggregations hosted by a freshwater minnow, bluehead chub (*Nocomis leptcephalus*), for possible selfish behavior. I found that bluehead chubs disproportionately benefit from the aggregations they host and use the aggregation to improve their own survival, improve their offspring’s survival, and obtain food (by eating others’ offspring). Thus, I identify bluehead chub as a selfish participant and provide evidence for the existence of two selfish herds in this mutualism – one among the adult fishes and one among their young – both orchestrated by and benefiting the bluehead chub. This study is the first to identify the bluehead chub as such and rewrites our previous understanding of this mutualism, which largely denied bluehead chub as a direct beneficiary. This work contributes to the global discussion of mutualisms by attesting the complexity of these relationships and offers support for re-examination of the classification of many known interspecific interactions, such as those cursorily termed nest parasitisms.

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ATTRIBUTION

My committee members Eric Hallerman, Eugene Maurakis, and Nicole Abaid, led by my committee chair and advisor Emmanuel Frimpong, were instrumental in completing this thesis. EF was the primary editor, project design supervisor, and statistics mentor for both chapters included here. EM contributed to project design and was secondary editor for both chapters. For Chapter 1, NA assisted with project design and video analysis and contributed as an editor. For Chapter 2, EH served as editor and oversaw all genetics methods. David Haak assisted substantially with the metabarcoding work of Chapter 2. Emma Hultin edited Chapter 2.

INTRODUCTION

Interspecific interactions are complex, often context-dependent relationships that are prevalent throughout the animal kingdom and influence the dynamics of populations and communities (Bronstein 1994, 2009). Among interspecific interactions, competition lies at one extreme, as the interaction causes a net disadvantage to all participants. At the other extreme is mutualism, which Bronstein (1994) describes as a relationship where both participants experience a net benefit from the relationship. Although widespread both geographically and taxonomically, and despite recent attention (Wallin 1992, Bronstein 1994, Hoeksema and Bruna 2015, Peoples and Frimpong 2016, Silknetter et al. 2020), freshwater mutualisms remain understudied compared to other types of interspecific interactions.

Mutualisms are rarely as simple and net positive as they are described. Many mutualisms are context-dependent and involve costs as well as benefits, and often include “selfish” participants who attempt to “cheat” the mutualism to avoid or drastically reduce any cost (Bronstein 2009, Peoples and Frimpong 2016, Silknetter et al. 2020). Whereas it may be straightforward to consider mutualisms as purely positive relationships, investigating the biotic and abiotic context that surrounds a putative mutualism often results in a more precise understanding of the interaction. Understanding such context can shift perception of a mutualism to commensalism or even parasitism under extreme circumstances (Hoeksema and Bruna 2015). More recent studies have focused their questions on this complexity and found support for such shifts (Cushman and Whitham 1989, Bronstein 2009, Hoeksema and Bruna 2015, Peoples and Frimpong 2015, Peoples and Frimpong 2016, Silknetter et al. 2019).

One understudied behavior that complicates the classification of an interaction is “selfishness.” Hamilton (1971) introduced “selfish-herd theory” as an alternative explanation of the perseverance of gregarious behavior within the animal kingdom. Hamilton posited that individuals congregate not as a method of protection for the entire population, but instead to better the individual’s chance of survival, thereby using the group to benefit itself (i.e., selfishness). Partially born from the theory of marginal predation, which states that predators will attack the prey that is spatially closest to them, selfish-herd theory explains that the driving force behind aggregation is an individual’s impulse to avoid becoming that marginal prey (Hamilton 1971). Now widely cited, Hamilton’s selfish-herd phenomenon has been observed across numerous aggregating taxa (Wcislo 1984, Robinson 1985, King et al. 2012). Selfishness, however, has remained understudied among fixed mixed-species aggregators, such as communal nesters.

Although mixed-species communal nesting has been observed with some bird species (Gibson et al. 2002, Kleindorfer et al. 2009), some of the most prominent examples of this phenomenon actually belong to the North American freshwater fishes of family Leuciscidae (minnows: previously, Cyprinidae; Tan & Armbruster 2018). A common reproductive behavior utilized by members of this family is termed nest association, where one “host” species supports a spawning aggregation of two or more species of “nest associates” (Wallin 1992, Johnston and Page 1992, Johnston 1994). Leuciscid hosts can support hundreds of individual nest associates at any one time (Wallin 1989).

The most eminent hosts of nest association among leuciscids are the pebble nest-building minnows of genus *Nocomis*. Each summer, male *Nocomis* collect gravel using their mouths and build conspicuous mounded nests in the rivers and streams of eastern North America (Raney

1947, Lachner 1952, Johnston and Page 1992, Pendleton et al. 2012). Given their unique ability to manipulate their surroundings and their interconnectedness with other aquatic fauna (Johnston 1999, Moore 2006, Peoples et al. 2011, Pendleton et al. 2012, Hitt and Roberts 2012, Peoples et al. 2015, Swartwout et al. 2015, McGarvey and Veech 2018, Silknetter et al. 2020), *Nocomis* have been described as ecosystem engineers (Moore 2006, Frimpong 2018) and keystone species (Vives 1990) within the stream environments they inhabit.

Nocomis-associate interactions are considered generally mutualistic – nest associates benefit from the abundance of adequate spawning habitat offered by *Nocomis* nests and the alloparental care behaviors of the host (Wallin 1992, Johnston 1994), while the host gains reproductive success from the abundance of associate offspring (the “predation-dilution” effect; Peoples and Frimpong 2013). However, two studies have identified the mutualistic relationship between *Nocomis* and nest associates as context-dependent and identified the interaction as commensalistic or even parasitic, benefitting the associates, under certain circumstances (Peoples and Frimpong 2016, Silknetter et al. 2019). Silknetter et al. (2019) found that the interaction between bluehead chub and yellowfin shiner (*Notropis lutipinnis*) shifted from a mutualism at high associate abundance to parasitism (benefitting yellowfin shiner) at low associate abundance. Peoples and Frimpong (2016) identified low substrate availability as a factor that shifted the interaction between bluehead chub and mountain redbelly dace (*Chrosomus oreas*) from mutualistic to commensalistic in favor of mountain redbelly dace.

Despite the evidence that these interactions are more complex than currently understood, there has been virtually no research directed at understanding how *Nocomis* may selfishly use the aggregation that it hosts to holistically enhance its overall fitness. Fitness is difficult to measure directly, but can be estimated using three performance indicators – survival, growth, and

successful reproduction (Arnold 1983). Behavioral traits that promote a host's own survival – growth (used in the broadest sense to include energy acquisition for metabolic maintenance and gonad development during spawning), and successful reproduction – ultimately increase host fitness (Arnold 1983, Wootton 1998, Frimpong and Angermeier 2010). The characterization of the benefits of nest association to hosts have thus far focused only on reproductive success.

In this thesis, I investigate potential selfish behavior of bluehead chub (*Nocomis leptocephalus*, henceforth “chub”) in Toms Creek, outside Blacksburg, VA. I specifically sought to test for the presence of two separate, host-orchestrated selfish-herds: one among the adult fishes congregating on the nest, which I hypothesized increases the host's survival, and one among the embryonic offspring residing within the nest, which I hypothesized disproportionately benefits host young and thus increases host fitness. In Chapter 1, I used the host's spatial position within the aggregation and vigilance behavior relative to associate abundance to examine the possibility of an adult selfish-herd. In Chapter 2, I used DNA barcoding to identify what species' embryos are present in nests and where, and then compared these data to the relative safety of areas of the nest from predation on embryos as identified by a separate experiment. I also conducted some preliminary work investigating feeding behaviors by host and associates on the nest that implicate the use of communal spawning to promote fitness using the third performance metric – growth.

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CHAPTER 1

Bluehead chub *Nocomis leptocephalus* hosts exploit selfish-herd benefits from their
heterospecific nest associates

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ABSTRACT

Prey species often congregate in groups to detect and evade predators, yet not all group members benefit equally. We observed the nesting aggregations of bluehead chub (*Nocomis leptocephalus*), where the chub “hosts” as many as several hundred “nest associates.” We predicted that the host exploits the aggregation in a manner consistent with selfish-herd theory – specifically, by locating itself in the safest location within the aggregation (the center) and using the aggregation to reduce its own vigilance requirements. Vigilance was measured as the time it took the host, first chub female, and first individual of each associate species to return to the nest under differing associate abundances (hypothesized to relate to risk) following a simulated predator scare. Spatial position was investigated by digitizing host movement and overlaying it with the aggregation and the nest’s spawning pit area. We used information theoretic statistics to analyze the effect of aggregation characteristics on return time of the chub and its nest associates. The best-supported model included both species composition and associate abundance (risk) as predictors of return time. Hosts returned last under nearly all scenarios and took longest to return in high-risk environments. The 95% and 99% confidence ellipses of host spatial position

contained the centroids of the aggregation and spawning pits, respectively, for all nests analyzed ($n = 11$). We conclude that the host locates itself at the center of both the aggregation and the spawning pit area of the nest, and that the host uses the aggregation to reduce its own vigilance requirements. These results support our hypothesis that hosts use their nest associates to disproportionately decrease their risk of predation during spawning. This is the first study to show any mixed-species fish spawning aggregation as a selfish shoal, and to identify bluehead chub as a selfish participant in the mutualistic reproductive interactions that they host.

INTRODUCTION

Predation is a near-constant threat for most organisms in the wild (Lima and Dill 1990). Predators serve an important role in ecological systems, regulating the populations of many other species through direct consumption (Murdoch and Oaten 1975) and foster fear-based non-consumptive effects (“NCEs,” Wirsing et al. 2021), which can elicit strong responses from prey (Creel 2018, Shea et al. 2020, Wirsing et al. 2021). That is, predation pressure can change the behavior, morphology, and life history of prey species as they attempt to avoid capture (Lass and Spaak 2003, Jermacz et al. 2015). These adaptations are referred to broadly as antipredator defenses and take many forms across the animal kingdom.

One common antipredator defense behavior that assists with both predator detection and evasion is gregariousness. Many species engage in some form of group-living (e.g., insect swarms, fish schools, bird flocks, mammal herds). Such gregarious behaviors are widely adaptive for several reasons: large groups can disorient a predator and completely deter an attack, make an attack less effective, or simply reduce the energetic burden of vigilance for individuals (Magurran 1990). Vigilance is a necessary but energetically costly behavior—any time spent vigilant is time subtracted from feeding, resting, or spawning, which can negatively

impact survival and reproductive success (Beauchamp 2015, 2017). Gregariousness reduces the burden of vigilance on the individual by spreading the responsibility over the entire group. Thus, the frequency of vigilance behaviors per individual decreases as group size or abundance increases (Godin et al. 1988; Burger et al. 2000; Ward et al. 2011). Group-living therefore not only reduces direct predation risk to individuals, but also reduces the energy investment required of the individual to adequately detect potential predators. There is also evidence that mixed-species aggregations further reduce the level of vigilance required per individual to less than that required with conspecific aggregation (Stears et al. 2020).

Not all individuals that are part of a group benefit equally from group-living, however. Hamilton (1971) argues that the risk of capture is greatest along the periphery of the group, while the center of an aggregation is the safest location for an individual. The pervasive inequality of group-living due to this imbalance of risk allows some individuals to disproportionately benefit from the protection that being in a group offers and creates competition between members as they each attempt to maximize the benefits of group-living while reducing costs (Krause 1994). The individuals that succeed are identified as “selfish” (Hamilton 1971, Wade and Breden 1980). Hamilton (1971) posited that the evolution of gregarious behavior is driven by this selfishness, rather than whole-group survival (the “selfish herd”). Selfish-herd theory has been widely supported by many studies across a range of taxa (Wcislo 1984, Krause 1993, Krause 1994, Perry et al. 2008, King et al. 2012). Most studies, however, have focused on mobile groups, such as pelagic fish schools (Williams 1964, 1966). Non-mobile aggregations (i.e., communal nesters) offer a unique opportunity to study selfish behavior, because the locations and behaviors of individuals within the group are more easily observed.

Communal nesting is a well-known breeding strategy for birds (Gibson et al. 2002, Kleindorfer et al. 2009), but many fishes also reproduce in large groups (Chapman 1943, Colin 1992, Domeier and Colin 1997). Freshwater fish spawning aggregations are especially unique in that they are often heterospecific. Among fishes, when multiple species (“nest associates”) spawn on the nest of another “host” species, it is referred to as “nest association” (Johnston and Page 1992, Wallin 1992). Nest association has been observed sporadically elsewhere globally but is particularly pervasive among the North American family Leuciscidae (minnows). Four genera in this family are known to host nest associates: *Campostoma*, *Exoglossum*, *Semotilus*, and *Nocomis*.

Perhaps most famous for their hosting of nest associates are the river chubs of the genus *Nocomis*. Male *Nocomis* build mound nests out of streambed pebbles by picking up stones with their mouth and depositing them in a large, conical pile (Raney 1947, Johnston and Page 1992, Pendleton et al. 2012). Along the upstream slope of each mound, hosts excavate shallow pits upon which most spawning takes place (henceforth “spawning pit area,” Johnston and Page 1992). Their ubiquitous nests, which can grow to over a meter in diameter (Reighard 1943), attract as many as a few hundred individual nest associates (McAuliffe and Bennett 1981, Meffe et al. 1988). At least 35 minnow species, or about a third of North America’s minnow species, are considered nest associates of *Nocomis* across the range of this genus (Johnston and Page 1992).

The interaction between *Nocomis* hosts and nest associates was historically considered parasitic or commensalistic as the benefits to the host are less obvious, but there is increasing evidence of a context-dependent mutualism (Peoples and Frimpong 2013, Peoples et al. 2015, Peoples and Frimpong 2016, Silknetter et al. 2019). Research suggests that the presence of nest

associate embryos increases the survival rate of chub embryos (the “predation-dilution effect,” Wallin 1992, Johnston 1994, Peoples and Frimpong 2013). Nest associates, many of whom are lithophiles (*sensu* Balon 1975) that need clean gravel substrate for successful reproduction, benefit from the spawning habitat created by *Nocomis* (Vives 1990, Fletcher 1993, McManamay et al. 2010, Peoples et al. 2011) and the parental care behaviors of the host (Wallin 1992, Johnston 1994, Shao 1997). All tested hypotheses of fitness benefits to the host in this interaction have focused on offspring survival or broadly, reproductive success, but not survival of the spawning adults. Wallin (1989) speculated that the host may benefit from its associates through an increased vigilance for predators. However, no previous research has investigated the behavioral mechanism by which the *Nocomis* host benefits (e.g., via predation-dilution which equally benefits the host and each associate participant as a selfish herd effect disproportionately benefiting the host who is the dominant participant). In our study system, we have observed bluehead chub (henceforth “chub,” *N. leptocephalus*) routinely occupying the upstream-central position of the spawning aggregation. Hosts also appear to be less wary when hosting a large spawning aggregation as evidenced in the relative ease of approaching and photographing nests without scaring off the host for a long time.

The goal of this study was to determine whether the host is a selfish participant in this mutualism. Here, we examine whether the host disproportionately benefits from the aggregation that it supports by determining the average location it occupies on the nest and how its vigilance fluctuates in response to risk or associate abundance. We hypothesize that a host will predominantly occupy the central location within a spawning aggregation, located over the spawning pits along the upstream-central portion of its nest. We also hypothesize that a host will

remain more vigilant when associate abundance is low (expressed as a slower return to the nest after a simulated predator scare), and less vigilant when associate abundance is high.

METHODS

Study System

We conducted this study during summer 2022 in a 1-km reach of Toms Creek (N 37.261955, E -80.436715), a third-order tributary of the New River located in Montgomery County in southwest Virginia. Three nest-building leuciscids are common in this system – bluehead chub, creek chub (*Semotilus atromaculus*), and central stoneroller (*Campostoma anomalum*, henceforth “stoneroller”). Within this reach, 60-120 bluehead chub mounds are built throughout the summer spawning season (May to July) each year. Of the 11 reported associate species of bluehead chub (Pendleton et al. 2012), six are commonly seen on mounds at this site on Toms Creek, including: mountain redbelly dace (*Chrosomus oreas*), rosyside dace (*Clinostomus funduloides*), crescent shiner (*Luxilus cerasinus*), white shiner (*Luxilus albeolus*), rosefin shiner (*Lythrurus ardens*), and stoneroller (Fig. 1). In this system and across the range of *Nocomis*, there are many aerial, terrestrial, and aquatic predators that have been observed targeting chub nests. These include herons (Ardeidae), kingfishers (Alcedinidae), crayfish (Cambaridae), Eastern hellbender (*Cryptobranchus alleganiensis*, Dunn 2016), sunfishes (Centrarchidae), brown trout (*Salmo trutta*), common snapping turtle (*Chelydra serpentina*), and northern watersnake (*Nerodia sipedon*).

Predation Risk – Spatial Location

We used time-lapse video footage to assess the spatial location of the host in relation to the nesting aggregation. For a full view of the aggregation, an above-nest angle was preferred over an in-stream angle. Spawning fishes, however, are extremely wary of potential attacks from

above (Litvak 1993). Therefore, we designed a mechanism to capture video data while avoiding hanging anything large over the spawning aggregation (Fig. 2). We set up a tripod in-stream, two to three meters downstream from a nest, with a 2.5 cm x 2.5 m aluminum angle iron attached to it using tripod screws. The front end of the angle iron was positioned directly above the nest and was counterweighted on the opposite side with a bucket filled with rocks. An Afidus ATL-200S long-term time-lapse camera (1080p resolution) with polarizing filter was then secured to the front end of the angle iron. This setup was left undisturbed at each sampled nest for 2-4 hours or until the memory card filled or the charge in the batteries of the camera became depleted. Eleven nests were successfully recorded this way over the course of the spawning season.

We quantified the average occupied position of the host within a spawning aggregation by digitizing its movement across 500 (± 10) seconds of randomly selected 20-60 second clips (30 frames/second) of time-lapse footage for each nest using the DLTdv8 app (Hedrick 2008) in MATLAB (R2022b, MATLAB 2010). The back-center of the chub's head was used to represent its position when digitizing. The perimeter of the nest, spawning pit area (along the upstream slope of the nest, identified by the presence of actively spawning fishes), and spawning aggregation (perimeter including > 95% of the fishes present) were also digitized as polygons for each nest. To account for distortion, the centroids of the spawning pits and spawning aggregation polygons were calculated for each nest. We also calculated the chub's average (centroid) position across all footage from each nest and the 90%, 95%, and 99% confidence ellipses around this point. If the chub moved off-screen during a clip, those points were recorded as "Not a Number." If more than one male chub was present during a clip, the larger male (presumed to be the resident male) was tracked. We used the chub's average position, corresponding confidence

ellipses, and centroids of the spawning aggregation and spawning pits to determine whether the chub located itself at the center of the spawning aggregation.

Predator Scare — Vigilance Response Experiment

We measured vigilance as the time it took the host, the female chub, and the first individual of each associate species to return to the nest following a predator scare under different associate abundances. We manually simulated attempted aerial predator attack on 28 spawning aggregations whose associate abundances are hypothesized to determine three levels of defined risk (henceforth: low risk [> 50 associates, $n = 7$], moderate risk [$15-50$ associates, $n = 16$], and high risk [< 15 associates, $n = 5$]).

We scared each spawning aggregation by deploying a decoy bird “predator” above the nest until all fishes had vacated the nest. The decoy bird was a foamboard cutout of a bird silhouette (0.75-m wingspan) attached to a 2.5-m wooden dowel rod using monofilament line. Previous research has shown that a decoy bird silhouette effectively induces a startle response in leuciscids (Litvak 1993). At each nest, one person with a clear vantage point tallied the species present on the nest. If the fishes were disturbed while the observer was tallying species, the observer paused the operation until the fishes settled back onto the nest. On a cue, another team member emerged from a hidden location and swung the decoy over the nest until all fishes vacated the nest. Then the decoy was retracted and we recorded the time (in seconds) it took for the first individual of each species to return to the mound. Any species that had not returned after 1200 seconds (20 minutes) was considered to have vacated the nest and the experiment was terminated. Return time was $\log(x+1)$ transformed before it was used in analysis.

We used an information theoretic approach and applied model-based inference (Burnham and Anderson, 2002) to compare support for four models describing spawning aggregation attributes and their effect on return time. We fit linear mixed-effects models comparing the effect of associate species composition, associate abundance (risk), the combination of both species composition and abundance, and a null model (Table 1). Nest identity was included as a random effect nested within risk for all models. We used Akaike's Information Criterion (AIC) corrected for small sample size (AICc, Hurvich and Tsai, 1989) to calculate model weights and assess relative support for each model (weights range from 0 to 1, and sum to 1 across all competing models). Model weights were compared across all four models to find the best-supported model, that is, the model with the highest model weight. Models were fitted using Minitab 21 (Minitab LLC 2021).

RESULTS

Predation Risk – Spatial Location

On average, hosts spent approximately half their time ($44.6\% \pm 2.6\%$) off-nest. Approximately half ($47.8\% \pm 2.4\%$) of the time hosts spent on-nest ($55.4\% \pm 2.6\%$ of total time) was spent in the spawning pit area. The centroid of the spawning aggregation fell within the 95% confidence ellipse and the centroid for the spawning pit area fell within the 99% confidence ellipse of the host's location for all 11 nests included in analysis (Fig. 3). We conclude that the chub does locate itself at the center of both the aggregation and the spawning pits when on its nest, and that these areas largely overlap.

Vigilance Response to Predator Scare

Attributes of a spawning aggregation (species composition and abundance [risk level]) affected vigilance as measured by return time (Table 2). The best-supported model (model V3, w

= 0.93) included both species composition and abundance. Model V1, which included only species composition, received minimal support ($w = 0.07$, Table 2). All other models received no support (models V0, V2, $w = 0$, Table 2).

Species composition differed with abundance. At high risk, only host, mountain redbelly dace, and rosefin shiners appeared on the mound. White shiners were only present under moderate risk. Host, female chubs, crescent shiners, stonerollers, rosefin shiners, rosyside dace, and mountain redbelly dace were all present in moderate and low risk environments.

Return time differed across species (Fig. 4, Table 3). The host was last to return to the nest under all scenarios with one exception: chub females returned last under moderate risk (Fig. 4). Mountain redbelly dace returned first regardless of risk. On average across all risk levels, the host returned to the nest after $369.6 (\pm 76.0)$ seconds, which was significantly later than stonerollers (85.7 ± 102.6 seconds), rosefin shiners (53.0 ± 21.7 seconds), rosyside dace (15.8 ± 4.2 seconds), and mountain redbelly dace (4.9 ± 21.1 seconds). Bluehead chub females returned after $346.7 (\pm 156.7)$ seconds. Crescent shiners returned after $119.1 (\pm 134.8)$ seconds. White shiners returned after $84.3 (\pm 62.6)$ seconds (Fig. 4).

The host took longest to return in high-risk settings. All three species present under high risk (host, mountain redbelly dace, rosefin shiner) returned faster under moderate risk. Hosts actually returned faster under moderate risk than low risk. All other species returned faster under low risk than moderate risk (with the exception of white shiner, which was not present in low-risk environments, and stoneroller, which returned in virtually the same amount of time under both risk scenarios; Fig. 5).

DISCUSSION

This study provides new understanding of a complex freshwater mutualism between *Nocomis* hosts and nest associates. Previous research has reported that all participants of this mutualism do not benefit equally (Peoples and Frimpong 2016, Silknetter et al. 2019); our results support these findings but unlike previous studies, identify the host itself as a selfish participant within this interaction with multiple, previously unrecognized benefits. We found that the host does in fact position itself in the safest location, at the center of the aggregation over the spawning pit area. Additionally, we found that the host takes longest to return to the nest when associate abundance is low and the relative risk of predation is high. These results support our original hypotheses regarding the host's occupied position and vigilance, which stated that: 1) the host would locate itself at the center of the aggregation, and 2) the host would show increased vigilance as aggregation size decreased. When considered together, these results confirm the host uses its nest associates in a selfish manner to disproportionately increase its own chance of survival while nesting and spawning.

Species composition differed greatly across risk levels, which complicated our comparison of return times across risk levels. Only two associate species – mountain redbelly dace and rosefin shiner – were present under all risk scenarios. These species were also among the fastest to return under all risk scenarios. The affinity of these species to chub nests regardless of risk may be due to their morphology (they are shallow-bodied and small, relative to other associates), their tendency to school, or their reproductive traits. Pendleton et al. (2012) noted both of these species as strong nest associates and their reproductive traits as purely associative where their range overlaps with *Nocomis*. The only other associate species which Pendleton et al. (2012) coded in this manner was rosieside dace, which we found were also among the first to return to a nest, although they dispersed under high-risk scenarios. Together, these three species

represent the three strongest associates of *Nocomis* identified by Pendleton et al. (2012). The absence of other associate species at high risk suggests that these species may switch to spawning with other nest-building leuciscids (like creek chub or stoneroller, as shown by Peoples and Frimpong 2016) when the benefits of spawning with *Nocomis* (namely: reduced predation risk for adults and embryos) drop to equal or lower than the nests of these other leuciscids.

Perhaps the most surprising result we recorded was the host's faster return under moderate risk environments relative to low risk. While all other species returned progressively faster as risk was reduced from high to moderate to low, the host returned fastest under moderate risk, which partially counters our hypothesis. When considering only a nest's associate abundance and identity as factors contributing to perceived risk, as we did, these results also counter multiple well-cited studies that note that prey should "overestimate" risk as environmental uncertainty increases (we assume moderate-risk environments offer the most uncertainty), and vigilance should increase (Brown et al. 2014, Creel 2018, Shea et al. 2020, Wirsing et al. 2021). Other studies go even farther and report that antipredator responses decrease when predation is predictable, even if frequent, and cite the need of prey to prioritize other essential behaviors like feeding and mating, even if this slightly increases the chance of capture, as the cause (Lima and Bednekoff 1999, Foam et al. 2005). Under these assumptions, our "moderate" risk should have elicited an equal or greater antipredator response (i.e., longer return time) than either high or low risk.

However, our methods for both experiments assume that chubs build their nests in a spatially and temporally consistent manner across the spawning season, and results of new research suggests that they do not (Bustamante and Frimpong 2023, unpub. data). An increasing number of studies indicate the importance of incorporating spatial and temporal variability when

quantifying predation risk (Lima and Bednekoff 1999, Brown et al. 2013, Shea et al. 2020, Wirsing et al. 2021). Including factors in our categorization of risk during future work, like the number of active nests at our study site on any given day, how many days the nest had been active prior to observation, the aggregation's prior exposure to predators or potential predators, activity of other nest building species (i.e., potential alternative hosts for some associates), the time of day during observation, or the number of days after the spawning season began, may elucidate the host's odd behavior under what we considered "moderate" risk.

To our knowledge, this study is also the first to quantify the amount of time that the host spends on versus off its nest. We were surprised by how much time the host actually spends off-nest – almost half of its time. *Nocomis* nests are conspicuous structures that contain a high abundance of food resources (both adult fish and embryos) relative to the surrounding stream channel, and consequently attract considerable attention from predators. Floyd (2016) found that artificial chub nests received three times the predatory attention for eggs than the ambient substrate did. Therefore, perhaps it is in the chub's best interest to seek cover off-nest when it is not actively spawning or maintaining its nest, especially when the aggregation is small. In this way, the chub may reduce its risk of predation even lower than it can by occupying the central location of the aggregation, while still reaping benefits from its nest associates in other ways, like the predation-dilution effect (Wallin 1992, Johnston 1994, Peoples and Frimpong 2013) and alloparental care.

Nest association is a complicated, often mutualistic reproductive interaction. While the full complexity of the interactions between *Nocomis* hosts and their nest associates remains to be described, our study transforms the discussion of this mutualism by recognizing the *Nocomis* host as a selfish participant. We show here that the *Nocomis* host benefits directly and

disproportionately through an increased chance of survival through the presence of nest associates.

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Figure 1. Spawning aggregation on *Nocomis leptocephalus* mound in Toms Creek, VA. Species are as follows: 1) *N. leptocephalus* male, 2) *Lythrurus ardens*, 3) *Chrosomus oreas*, 4) *Luxilus cerasinus*, 5) *Campostoma anomalum*, 6) *Luxilus albeolus* (below *Clinostomus funduloides*), 7) *Clinostomus funduloides*.



Figure 2. Camera setup used to collect time-lapse footage for analyzing the host's position within the spawning aggregation in Toms Creek, Blacksburg, VA, in summer 2022.

Table 1. Competing models used to assess the effect of spawning aggregation attributes on return time (vigilance) following an aerial predator scare. Species are coded as follows: BHC-M = bluehead chub male, BHC-F = bluehead chub female, CRS = crescent shiner, SR = central stoneroller, RSD = rosyside dace, MRBD = mountain redbelly dace, RFS = rosefin shiner, WS = white shiner.

Model	Variables in model	Number of variables
<i>y = host vigilance</i>		
V0	nest	3
V1	nest, abundance (low, moderate, high risk)	5
V2	nest, species composition (BHC-M, BHC-F, CRS, SR, RSD, MRBD, RFS, WS)	10
V3	nest, species composition (BHC-M, BHC-F, CRS, SR, RSD, MRBD, RFS, WS), abundance (low, moderate, high risk)	12

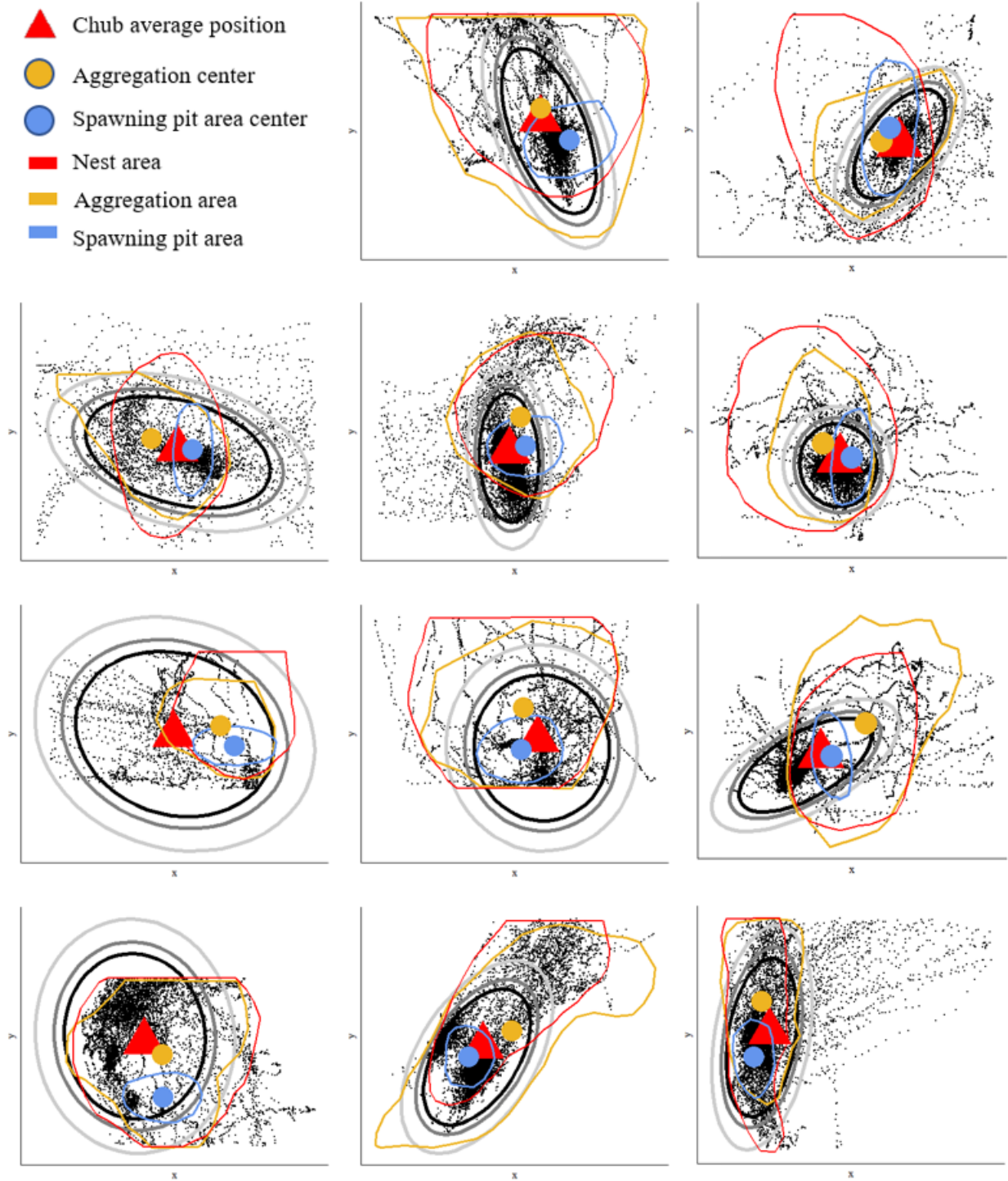


Figure 3. Scatter plots of chub movement over 500 seconds of footage on 11 nests (black points) in Toms Creek, Blacksburg, VA, in summer 2022. Confidence ellipses of average chub position (red triangle) are represented as light grey (90%), medium grey (95%), and black (99%).

Table 2. Comparison of models used to identify the effect of spawning aggregation attributes on return time to nest (vigilance) following a simulated aerial predator scare in Toms Creek, Blacksburg, VA, in summer 2022.

Model	Hypothesis	Number of parameters (<i>K</i>)	-2 Log Likelihood	<i>AIC</i> c	Δ <i>AIC</i> c	Model weight (<i>w</i>)
<i>y</i> = return time (vigilance)						
V3	Both associate species composition and abundance (~risk level) affect vigilance	12	416.1	420.15	0	0.93
V1	Species composition affects vigilance	10	421.1	425.21	5.06	0.07
V2	Abundance (risk level) affects vigilance	5	547.7	551.78	131.63	0.0
V0	Null model	3	547.4	551.46	131.31	0.0

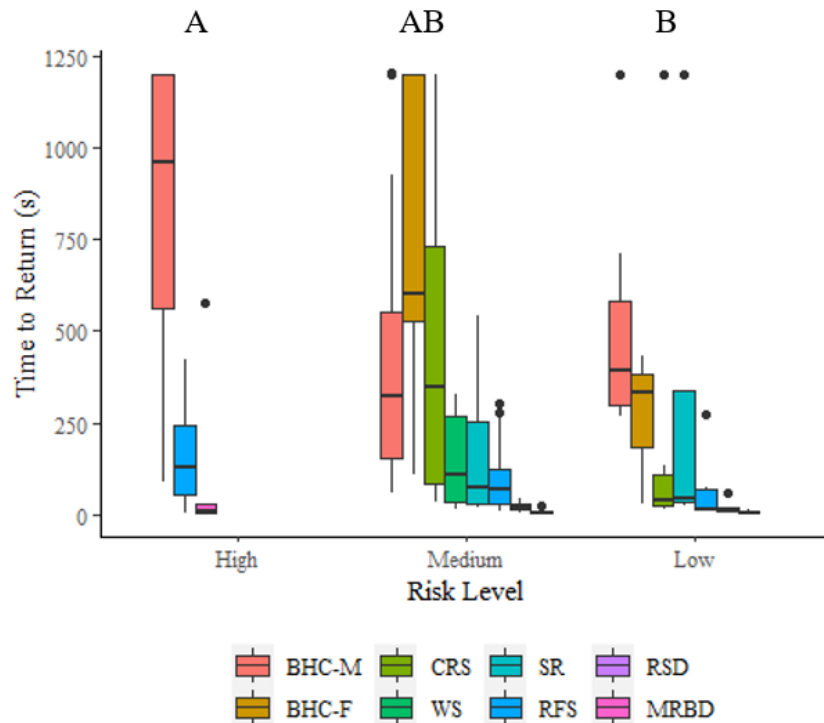


Figure 4. Species return times under different abundances (risk levels) recorded during predator scare (vigilance) experiments in Toms Creek, Blacksburg, VA in summer 2022. Letters at the top of figure indicate Tukey HSD significant difference of aggregation response to simulated predator scare between risk levels. Species codes are as follows: BHC-M = bluehead chub male, BHC-F = bluehead chub female, CRS = crescent shiner, SR = central stoneroller, RSD = rosieside dace, MRBD = mountain redbelly dace, RFS = rosefin shiner, WS = white shiner.

Table 3. Average time to return (in seconds) $\pm$ SE for each species across all risk scenarios of predator scare experiment conducted in Toms Creek, Blacksburg, VA, in summer 2022. Letters in Sig. column reflect significant differences identified by Tukey HSD test. Species are encoded as follows: BHC-M = bluehead chub male, BHC-F = bluehead chub female, CRS = crescent shiner, SR = central stoneroller, RSD = rosieside dace, MRBD = mountain redbelly dace, RFS = rosefin shiner, WS = white shiner.

Risk	Cumulative	Sig.
BHC-M	369.6 $\pm$ 76.0	A
BHC-F	346.7 $\pm$ 156.7	AB
CRS	119.1 $\pm$ 134.8	ABC
SR	85.7 $\pm$ 102.6	BC
WS	84.3 $\pm$ 62.6	ABCD
RFS	53.0 $\pm$ 21.7	CD
RSD	15.8 $\pm$ 4.2	DE
MRBD	4.9 $\pm$ 21.1	E

CHAPTER 2

Host-orchestrated embryonic selfish herds in gravel-mound nests of an iconic stream fish
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Keywords: Selfishness, *Nocomis*, Leuciscidae, DNA barcoding, mutualism, nest association

ABSTRACT

Mutualisms are complex, inherently selfish interspecific relationships, often creating “selfish-herds” as individuals compete to maximize survival. Nest association, where individuals of different species spawn on a nest created by a host species, is a mutualistic reproductive interaction characteristic of some members of the minnow Family Leuciscidae. We studied the spawning behaviors of bluehead chub (*Nocomis leptocephalus*) and its nest associates. We hypothesized that bluehead chub exploits the selfish-herd dynamic in a novel manner by arranging embryos within its nest to maximize the survival of its own offspring at the expense of the nest associates’ offspring. Model-based evidence showed that embryos were not uniformly distributed within a nest, as most ($\bar{x} = 40.0\% \pm 6.3\% \text{ SE}$) were collected from one section representing one-sixth of the nest’s total volume. Embryo-burying increased embryo survival on average by 44.0% ($\pm 9.7\%$), which disproportionately benefitted host embryo; three-quarters of all host embryo were found deeply buried in the nest, whereas only a third of all associate embryos were found in the same area. These results support our hypothesis that *Nocomis* create embryonic selfish-herds within their nests. To our knowledge, this is the first study to find support for the existence of an embryonic selfish-herd.

INTRODUCTION

Interspecific interactions are often complex relationships with beneficiaries that vary with the circumstance (Bronstein 1994a, Chamberlain et al. 2014). Frequently, these relationships are imprecisely portrayed as purely positive (mutualism) or negative (predation, parasitism, competition) without acknowledging the context-dependency of many of these interactions in the biological world (Bronstein 1994b, Chamberlain et al. 2014). Factors that may change the context of an interaction include abiotic or biotic conditions, seasonality and phenology, complexity of resource transfer, and interaction strength (Chamberlain et al. 2014, Silknetter et al. 2019). In extreme cases, these factors can change the outcome of an interaction entirely (i.e., from mutualism to parasitism; Hoeksema and Bruna 2015).

Despite the altruistic conceptualization of mutualism, where both interacting parties benefit from the interaction, mutualisms are inherently selfish interactions (Becker 1976, Eldakar and Wilson 2008). All members involved in a mutualism are seeking to maximize self-benefits while minimizing the cost they must incur to maintain the mutualism (Bronstein 1994a). Thus, animal groups – such as insect swarms, grazer herds, and fish schools – form because all members are attempting to occupy the biological low-cost, high-reward sweet spot within the system. This inherent selfishness of animal behavior is the central premise behind selfish-herd theory proposed by Hamilton (1971).

Our understanding of how the costs and benefits of mutualism change with context remains limited, due in part to the relative lack of research upon mutualisms compared to negative interactions (Bronstein 1994a, 1994b, Chamberlain et al. 2014, Peoples and Frimpong 2016). Of the studies focused on context-dependent mutualisms, few have focused on freshwater ecosystems (e.g. Brown et al. 2012, Chamberlain et al. 2014, Peoples and Frimpong 2016,

Silknetter et al. 2019, Creed et al. 2021), although nesting fishes commonly participate in mutualistic interactions (Johnston 1994a, Wisenden 1999, Peoples and Frimpong 2016, Silknetter et al. 2019). One nesting behavior seen as generally mutualistic is nest association, where one or more different species (“nest associates”) spawn in the nest of another “host” species (Johnston and Page 1992). Among North American freshwater fishes, this reproductive strategy is commonly used by some leuciscids (minnows) and centrarchids (sunfishes) (Johnston 1994a).

Among leuciscids, perhaps best known for utilizing this strategy are the pebble nest-building chubs of the genus *Nocomis* (Pendleton et al. 2012). *Nocomis* have been recognized as ecosystem engineers (Moore 2006, Frimpong 2018) and keystone species (Vives 1990) for their ubiquity, connectedness to other species (Johnston 1999, Pendleton et al. 2012, Swartwout et al. 2015, McGarvey and Veech 2018) and ability to manipulate the microenvironments of streams that they inhabit (Lachner 1952, Moore 2006, Peoples et al. 2011, Hitt and Roberts 2012, Peoples et al. 2015, Silknetter et al. 2020). Using their mouths, adult male *Nocomis* individuals build large, mounded nests out of streambed pebbles, which attract other minnows as nest associates (Raney 1947, Johnston and Page 1992, Pendleton et al. 2012). Across the geographic range of *Nocomis* in eastern and central North America, at least 35 minnow species are known nest associates of *Nocomis* (Johnston and Page 1992). Some nest associates are facultative, and may spawn in the absence of chub mounds, whereas others are obligate associates and require chub mounds for reproduction (Johnston and Page 1992, Pendleton et al. 2012).

The choice of facultative associates to forgo their ancestral mode of reproduction, broadcast spawning, in favor of spawning upon *Nocomis* nests suggests that associates receive some inherent benefit from this interaction that they cannot get from the ambient substrate

(Johnston and Page 1992, Floyd 2016). Previous studies have concluded that the structure of the nest is less important to nest associates than the parental care offered by the host *Nocomis* (Wallin 1992, Johnston 1994b). *Nocomis* prioritize embryo-burying (by continuing to add stones to their mound after spawning) over active guarding as a mode of parental care (Wallin 1989, Floyd 2016). Wallin (1989) observed bluehead chubs (*N. leptocephalus*, henceforth “chub”) adding gravel to nests up to 2-3 days after spawning. Floyd (2016) noted that chubs spent double the time on nest maintenance and burying embryos as they did on nest guarding. He also found that although chub mounds received far more attention from predators searching for embryos than the ambient streambed, the burying behaviors of the host almost entirely eliminated predation risk for buried embryos (Floyd 2016). We hypothesize that this burying behavior places embryos deeper into a mound, and thus improves survival rate. Therefore, at least one inherent benefit that nest associates gain by spawning on *Nocomis* mounds is an increase in their reproductive success.

The chub also benefits from the presence of nest associates, as the associate embryos laid on a chub mound dilute the risk of predation on chub embryo, increasing the fitness of the host (the “predation-dilution effect”; Peoples and Frimpong 2013), as well as providing additional vigilance against predators. The main costs to the host identified with nest association are the energy expended in nest construction and burying and defending associate embryos. But what are the costs to the nest associates? Two studies have found that mutualistic *Nocomis* nest association shifts to commensalism or even parasitism in favor of the associates when abiotic and biotic contexts change. In one study, the interaction between bluehead chub and yellowfin shiner (*Notropis lutipinnis*) shifted from a mutualism at high associate abundance to parasitism (benefitting yellowfin shiner through increased reproductive success) at low associate abundance

(Silknetter et al. 2019). The other study found that low substrate availability shifted the interaction between bluehead chub and mountain redbelly dace (*Chrosomus oreas*) from mutualistic to commensalistic in favor of mountain redbelly dace, as the presence of mountain redbelly dace no longer improved reproductive success of *Nocomis* in these environments (Peoples and Frimpong 2016).

If the interaction between chubs and associates is to be considered a mutualism, as previous reports suggest (Wallin 1992, Johnston 1994b, Peoples and Frimpong 2013), nest associates must also incur some cost. This cost could be imposed by the host through exploitation of the selfish-herd effect, either in the survival of adults in the spawning aggregation, the developing embryos within the nest, or both. In a related study, we are investigating the selfish-herd effect in the spawning adult aggregation. In this study, we hypothesize that the selfish-herd effect is not confined to the adult spawning aggregation, but rather extends to the “embryonic herd” contained within a nest through the spatial distribution of embryos and the potential effect of predation on embryo survival and parental fitness. Only one previous study has suggested the potential for a selfish herd in early life, albeit the free-swimming life stages of fishes, and found supporting evidence for such – McKaye and Oliver (1980) observed three pairs of a predatory catfish (*Bagrus meridionalis*) protecting young cichlids. The catfish manipulated the school of young fishes so that the catfish young were located at the center and the cichlid young were located on the periphery of the group (McKaye and Oliver 1980).

The biological context of the chub-associate mutualism foments selfishness within this system. Floyd (2016) observed that embryos buried deepest within chub mounds were safest from predators. This observation evokes two questions: how effective is the host’s burying

behavior at protecting embryos, and which species' embryos are most effectively buried? Those species would be gaining the largest fitness benefit from nest association. It would be considered selfish behavior, and create an embryonic selfish-herd, if the host exploits its own dominant role in the nesting aggregation to achieve disproportionate survival of its own offspring at the expense of nest associates.

We studied the distribution and survival from predation of embryos in bluehead chub nests to better understand the complex interactions between host *Nocomis* and nest associates. Throughout this study, we use the term “embryo” to describe situations involving one or more fertilized eggs and larvae of one species and “embryos” for similar situations involving more than one species. We addressed two objectives in this study: 1) compare predation rates between buried and unburied embryos on *Nocomis* mounds and 2) determine whether certain species predominantly benefit from the embryo-burying behaviors of the host by assessing the spatial distributions of species' embryos within mounds. We hypothesized that buried embryos experience less predation pressure than unburied embryos and that embryo of *N. leptocephalus* are located deeper in the nest than associate embryos and thus disproportionately benefit from the embryo-burying behavior of the host.

METHODS

Study System

We studied the leuciscid community of Toms Creek – a third-order tributary to the New River near Blacksburg, VA (Fig. 1). In this system, the dominant mound-building leuciscid is *N. leptocephalus*. Six *Nocomis* nest associates make frequent appearances on chub mounds in Toms Creek: mountain redbelly dace, rosieside dace (*Clinostomus funduloides*), crescent shiner (*Luxilus cerasinus*), white shiner (*Luxilus albeolus*), rosefin shiner (*Lythrurus ardens*), and

central stoneroller (*Campostoma anomalum*, henceforth “stoneroller”). Rosyface shiner (*Notropis rubellus*) is another confirmed nest associate in Toms Creek but is more common further downstream from our study reaches. Creek chubs (*Semotilus atromaculatus*) and blacknose dace (*Rhinichthys atratulus*) also make appearances, but are not widely considered nest associates (Pendleton et al. 2012). Of these species, we consider three (bluehead chub, stoneroller, white shiner) as “nest manipulators” because of their ability to pick up and/or move substrate. In our system, we have observed stoneroller and white shiner shoving and digging in substrate, in addition to the stone-moving behaviors of bluehead chub. We hypothesize the overt moving of stones is an effective mechanism for moving embryos deeper into the nest.

Our selected reach of Toms Creek is in Deerfield Park on the outskirts of Blacksburg, VA (N 37.261955, E -80.436715). This site encompasses approximately one kilometer of Toms Creek, within which 60-120 *Nocomis leptcephalus* mounds are built across the spawning season from early May to July each year. For this study, data were collected from bluehead chub mounds in Toms Creek during the 2021 and 2022 summer spawning seasons.

Embryo Predation Experiment

We conducted a field experiment to investigate the predation risk of embryos laid on chub nests and the importance of male parental care in the form of embryo-burying for embryo survival. This experiment modified a previous experiment designed by Floyd (2016).

For this study, embryos were collected from active chub nests throughout the spawning season by gently disturbing pebbles along the spawning trough and collecting drifting embryos using a wide-mouth aquarium dip net. Small pouches of skein were created by securing groups of twenty large unhatched embryos (>2mm diameter) using a fine (~1mm), soft 5x5 cm mesh

square of Atlas-Mike's Spawn Net (Atlas-Mike's Bait, Inc., Ellensburg, WA), which is typically used by steelhead fishermen to secure egg bait. This specific mesh was used because it is tight enough to contain whole unhatched embryos, but soft enough to let fishes bite (damage) embryos through it (i.e., if a fish were trying to consume the embryos). The pouches were secured to flat rocks with identifiable colored elastic bands indicating placement position. Six pouches were buried five centimeters from the nest surface and six placed on the surface directly above those buried and left for one hour (Fig. 2). A camera was installed near the nest to gather data about fish feeding behavior and to assess the effectiveness of our method to keep embryos on the nest unless disturbed or eaten by predators. After one hour elapsed, the baited stones were collected, the skein pouches opened, and the remaining embryos counted. If stones were retrieved with pouches missing, or the entire stone could not be located, these data were removed from analysis (4% of total data). This experiment was repeated 12 times during the 2022 spawning season.

Egg survival counts were converted into percentages and subjected to a two-way analysis of variance (ANOVA) using pouch horizontal position (Fig. 2A) and vertical position (Fig. 2B) as predictors, with the sampled nest treated as a random block to account for nest-level variation. A Tukey HSD test was used to identify significant differences in main effects and interactions between vertical and horizontal position combinations. JMP Pro 16.0.0 (JMP 2021) was used for statistical analysis.

Determining the Distribution of Embryos in Nest – Embryo Collection and Identification

We collected embryos from six sections of chub nests to determine embryo distribution and species composition (Fig. 3). We systematically dismantled 20 nests between May – July 2021 and 2022, selecting well-developed nests that had remained active for several days to near-incubation stage (when fishes vacate the nest and the enlargement of the nest stops, as embryos

hatch and develop into larvae). First, a rigid plexiglass sheet (~0.5m x 0.5m) was inserted vertically into the nest to separate the upstream and downstream halves. A customized plankton drift net (~30 cm x ~45 cm) was installed downstream before dismantling to catch embryos as each nest section was unpacked. A meter stick was inserted into the center of the nest to measure the height of the nest and calculate the targeted depth of each of three layers, with the targeted depth decreasing from top to bottom due to the approximate conical shape of the mounds. An additional plexiglass sheet was then inserted horizontally to the target depth to separate the top-downstream section (Section TD in Fig. 3) from the mound. This sheet was used to move the gravel of that section off the nest without shifting embryos to lower section; the gravel was sifted by hand while doing this to remove any embryos. Disturbed embryos floated downstream into the drift net, which was emptied into a separate labeled container after each section was completed. The volume of sorted gravel for each section was estimated using a 1-Liter container. These volumes were used later to adjust for variation among division of nests into the six sampling sections. This process was repeated sequentially for sections mid-downstream (Section MD), bottom-downstream (BD), top-upstream (TU), mid-upstream (MU), and bottom-upstream (BU, Fig. 3).

Embryo abundance from each section were calculated by counting eggs and larvae in a petri dish and using volumetric techniques when necessary (e.g., 1000 – 10,000+ embryos) following techniques described in Floyd (2016) and Peoples et al. (2017). A random subsample of up to 10 embryos from each nest section (a maximum of 60 embryos per nest) were retained for genetic testing with care taken to capture any observed variation in embryo size and color during subsampling. Embryos were identified to species using DNA barcoding techniques (see Appendix 2).

Determining Distributions of Embryos in Nest – Data Analysis

We used an information theoretic approach and model-based inference (Burnham and Anderson 2022) to compare support for different models describing embryo distribution within chub mounds. We compared three sets of models, one set to determine where most embryos were located within a mound and the others to determine the distribution of host embryo relative to associate embryos, and the effect of nest manipulation by the host and some associate species (stoneroller, white shiner) on embryo distribution.

We developed binomial generalized linear mixed models comparing distribution of embryos in the top-to-bottom (vertical) gradient, upstream-to-downstream (horizontal) gradient, the combination of both top-bottom and upstream-downstream gradients, and a null model (Table 1). Nest identity was the random effect in all models. Before models were fitted, imperfect nest division was corrected by adjusting embryo counts for each section of nest using gravel volumes and actual embryo counts (see Appendix 3). Akaike's Information Criterion (AIC) corrected for small sample size (AICc, Hurvich and Tsai 1989) was used to calculate model weights representing relative support for each model within a set. Model weights for all four models in each set were compared to find the best-supported model – the model with the highest model weight (weights range from 0 to 1 and sum to 1). Models were fitted using the AICcmodavg package (Mazerolle 2023) in RStudio 1.4.1717 (R Core Team 2021).

RESULTS

Embryo Predation Experiment

Results of the two-way ANOVA revealed that pouch vertical position had a significant effect on embryo survival ($F_{(1, 130)} = 22.9, p < 0.001$). After one hour, buried pouches averaged 77.3% ($\pm 4.1\%$) embryo survival overall, 26.8% ($\pm 5.6\%$) higher than the survival rate of

unburied embryos. Horizontal position did not have a significant main effect on embryo survival, but the interaction between vertical and horizontal position was significant ($F_{(2, 130.1)} = 3.7$, $p = 0.026$, Fig. 4). A Tukey HSD post-hoc test found that both the riskiest and safest locations were along the downstream side of the nest, and that on average, burying increased survival in the downstream area by 44.0% ($\pm 9.8\%$). The downstream-unburied location, the least-safe location overall ($\bar{x} = 37.1\% \pm 6.7\%$ survival), had significantly lower embryo survival than all buried locations and the upstream-unburied location, whereas the safest location was downstream-buried ($\bar{x} = 81.1\% \pm 7.1\%$ survival). Along the middle of the nest, buried embryos had a 29.5% ($\pm 9.8\%$) higher chance of survival than the unburied embryos right above them. The middle-unburied location had significantly lower embryo survival than the middle-buried and downstream-buried locations. Upstream-unburied was the safest unburied location ($\bar{x} = 65.2\% \pm 6.7\%$ survival). There was no significant difference in survival between upstream-unburied and upstream-buried. The upstream-unburied location retained, on average, 28.2% ($\pm 9.5\%$) more embryos than the riskiest downstream-unburied pouches (Table 2).

Determining the Distribution of Embryos in Nest

Model-based evidence indicates that embryos are not randomly distributed within a nest, and in fact, the bottom-upstream section contained the highest proportion of embryo collected from nests ($\bar{x} = 40.0\% \pm 6.3\%$ of total embryos; Fig. 5). The upstream sections contained a cumulative 80.4% ($\pm 4.5\%$) of total embryos (Fig. 5). Combined, the middle and bottom upstream and downstream sections combined contained 78.8% ($\pm 3.6\%$) of total embryos (Fig. 5). The model that received all support (model A3, $w = 1$) considered both horizontal and vertical gradients in embryo distribution. All other models in the set received no support (Table 3).

Genetic Analysis

Amplification of the *COI*-3 region of the mitochondrial cytochrome I gene formed a product that was approximately 700 bp long. We successfully sequenced and identified 990 embryos among 1034 total embryos subsampled from 20 nests (96%). This included 288 stoneroller (29.1%), 262 rosieside dace (26.5%), 217 bluehead chub (21.9%), 164 mountain redbelly dace (16.6%), 35 rosefin shiner (3.5%), 10 crescent shiner (1.0%), eight white shiner (0.8%), four creek chub (0.4%), and two blacknose dace (0.2%). One fantail darter (*Etheostoma flabellare*) egg was also sequenced; however, we do not believe this egg was the result of a fantail darter spawning on a mound, but rather a result of downstream drift, and this data point was thus excluded from analysis. The identification of all species except white shiner and bluehead chub was straightforward. Identity scores for white shiner samples returned both white shiner and common shiner (*Luxilus cornutus*) within one percent of each other. Similarly, our bluehead chub samples consistently returned identity scores of 100% with the closely-related bigmouth chub (*Nocomis platyrhincus*), an endemic species to the mainstem and large tributaries within the New River basin (Jenkins and Burkhead 1994), and the *Nocomis* sp. PC-2017 sample collected from Catawba Creek in the Roanoke River basin by Peoples et al. (2017) which they phenotypically identified as bluehead chub. This issue of correctly identifying closely related *Nocomis* species was first reported by Floyd (2016) and addressed by Peoples et al. (2017); however, only bluehead chub occurs in our stream reach.

Determining Embryo Distribution in Nest by Host and Manipulator versus Associate and Non-Manipulator

Model-based evidence indicates that embryos are not randomly distributed by species within a mound. The best-supported model for comparing host versus associate embryo distribution considers both horizontal and vertical position (model B3, $w = 1$). All other models

received no support (Table 3). The bottom-downstream section contained the highest percentage of chub embryo ($\bar{x} = 27.8\% \pm 6.3\%$) relative to associate embryos, while the top-downstream section contained only 4.9% ($\pm 1.8\%$) chub embryo (Fig. 5). Over three-quarters of all chub embryo were found in the middle or bottom sections. Overall, host embryo were diluted $\sim 1:4$ by associate embryos. The best model for comparing manipulator versus non-manipulator embryo distribution was C3 ($w = 0.99$), while all other competing models received little to no support (Table 3). The section that contained the most manipulator embryos was the middle-upstream section, with 58.6% ($\pm 5.8\%$) manipulator embryos. However, manipulator embryos made up the majority ($>50\%$) of embryos in all sections except top-downstream, which contained only 27.9% ($\pm 3.3\%$) manipulator embryos (Fig. 5). Stoneroller, a manipulator, made up more than a quarter of the total embryos in every section. Rosyside dace, the most prevalent non-manipulator, represented the largest percentage of embryos contained in the top-downstream section, where host and manipulator comprised less than a quarter of the total embryos in each downstream section (Table 4).

DISCUSSION

Our combined results support the existence of a host-orchestrated embryonic selfish-herd, making this the first study to find evidence for such. Previous studies have indicated that all participants in the *Nocomis*-associate reproductive mutualism are not benefitting from the interaction in the same way or to the same degree (Peoples and Frimpong 2016, Silknetter et al. 2019). The results of the current study corroborate those findings but, importantly, define several novel elements within this mutualism: 1) we verified that the burying behaviors of the host are an effective mode of parental care, and thus, we 2) identify the host as a selfish participant and the orchestrator of an embryonic selfish-herd within its nest, and 3) recognize the selfish effect of

nest manipulation by stonerollers and white shiner (in addition to the host) on embryo distribution. We also found evidence for possible opportunistic spawning on bluehead chub mounds by two leuciscids not generally regarded as associates, blacknose dace and creek chub. Overall, this study offers a refined understanding of a complex mutualism by showing both predation-dilution and selfish-herd effects at the embryonic stage in a freshwater fish nest.

Embryo-burying as Parental Care

Results from the embryo predation experiment showed that embryo-burying is an effective form of parental care for embryos in the middle, bottom, and downstream portions of a nest. These findings support our hypothesis that embryos buried within chub mounds experience less predation pressure than unburied embryos.

Although data from the embryo predation experiment for the middle and downstream nest sections strongly supported our hypothesis, our understanding of the role of parental care along the upstream portion of the nest could not be completely resolved. Whereas this area retained the overall highest survival rate for unburied embryos, there was little difference in survival rate between unburied and buried embryos in this area, indicating that the host's embryo-burying behaviors are not as effective at protecting embryos along the upstream portion of the nest. This part of the nest contains spawning pits – small indentations dug by the host where most spawning events take place (Maurakis et al. 1991). Most potential disruptions by nest manipulators occur here as well. Floyd (2016) noted that the vast majority of stoneroller feeding events occurred over the spawning trough. Therefore, the apparent short-term ineffectiveness of embryo-burying in this region of the nest may be due to the sheer number of fishes occupying the area, and the variety of behaviors involving substrate disturbance in which

they take part (including spawning, digging and eating embryos, pit fanning, etc.; Maurakis et al. 1991).

Somewhat unexpectedly, we found the highest percentage of chub embryo relative to associate embryos in the bottom-downstream section, despite the bottom-upstream section containing most of the embryos laid on the nest, as shown by this study and by Floyd (2016). In fact, bottom-downstream was the only section that contained a higher percentage of chub embryo than any other species. The disproportionate use of the bottom-downstream section and abundance of chub embryo in all sections except the top-downstream follows the pattern of nest growth (Fig. 3; Floyd 2016) and indicates that the chub may recognize both the nest's vertical and horizontal safety gradients identified by the embryo predation experiment and is actively trying to maximize survival of its embryo.

Selfish Distribution of Host Embryo

We found that an overwhelming proportion of total chub embryo were in the deeper sections of the nest. This supports our hypothesis that the host's embryo are located deeper in a nest than associate embryos and are thus less likely to be preyed upon, thereby providing evidence for the presence of a host-orchestrated embryonic selfish-herd.

Orchestrating an embryonic selfish-herd allows a host to take advantage of what is otherwise an intrusion on its nest. When considering the high fecundity of fishes and frequency of parental care, invoking an embryonic selfish-herd effect allows hosts to benefit from the alloparental care that they provide to associate young, which could otherwise easily become maladaptive (Wisenden 1999). After all, fishes can produce hundreds to thousands of eggs during one spawning event, making it virtually impossible for hosts to actively remove all

interspecific embryos from their nests as birds sometimes do, even though there is evidence that they can differentiate their own offspring from others (Yom-Tov 1980, Neff 2003). It is more energy efficient for the chub to instead use the associates' embryos to dilute or shield its embryos from predation. It would be *most* efficient if the host also uses the embryonic selfish-herd to protect its own embryo while actively consuming associate embryos while tending its nest. This possible avenue for selfishness was originally noted by Wallin (1989) and has been corroborated by our own observations in this system. We have since found supporting evidence for such (Appendix 4).

Nest Manipulation Effect

We found that species capable of manipulating nest gravel (nest manipulators) were also able to disproportionately shift their embryos deeper in nests (nest manipulation effect). From the non-random placement of manipulator embryos versus non-manipulator embryos across the nest, with manipulator embryos located deeper and in safer sections, we can infer that the host is not the only individual affecting embryo distribution. Manipulators strongly avoided placing their embryos in the top-downstream section, as hosts did, which correlates with the least safe location identified by the embryo predation experiment. No previous study has included the effect of manipulation in their analysis, yet many have suspected the manipulator we observed most frequently on nests, stoneroller, as a cheater in this mutualism (Floyd 2016, Floyd et al. 2018). A future study dedicated to formally identifying the role of stoneroller within this mutualism could prove enlightening.

DNA Barcoding

Our DNA barcoding methods elucidated far more embryonic diversity than previous attempts (Floyd 2016, Peoples et al. 2017), better represented known nest associate diversity, and offered a few unanticipated insights into the ecology of this system. We found significant differences in host-associate embryo proportions throughout the nest, which Floyd (2016) did not. Further, Floyd (2016) recorded a predominance of stoneroller embryo, while Peoples et al. (2017) recorded a predominance of chub embryo. We had no such dominating species represented in our data. This is likely due to our immediate extraction of embryos, rather than attempting to rear embryos to larvae as Floyd (2016) did, and our use of Platinum[®] *Pfx* DNA polymerase (a *Taq* known to increase amplified DNA yield, Kaboev et al. 2000) rather than *Taq* DNA polymerase (New England Biolabs) as Peoples et al. (2017) did. Additionally, our nest-dismantling methods likely contributed to the diversity of our DNA barcoding results. Previous studies sampled only what we define as the “top” sections and quoted dilution rates of host eggs as high as 84-97% (Wallin 1992, Cashner and Bart 2010). Our dilution rate was lower, around 75-80%, but was so only because we included the lower sections (which contained most host eggs) in our sampling protocol.

DNA barcoding data also revealed previously undocumented spawning activity. We identified a small percentage of blacknose dace embryo within our sample, which Pendleton et al. (2012) identified as a weak associate of bluehead chub but is not currently thought to spawn on bluehead chub mounds. These embryo were found in the top-downstream and bottom-upstream sections. Embryo of creek chub, another species not currently considered a nest associate but often seen on bluehead chub mounds and posited by some to spawn with *Nocomis* on occasion (Cooper 1980), were also identified by DNA barcoding. We identified these embryo in the top-downstream, top-upstream, and bottom-upstream sections. In Toms Creek, we have

observed adult male bluehead chubs usurping and sabotaging creek chubs by building small mounds on top of creek chub spawning pits or filling them with gravel and abandoning them. This behavior could explain the embryo identified within the bottom-upstream section, but not those found in top sections, as shown by the pattern of nest growth that we observed through embryo-larval ratios within nest sections (Appendix 1). Thus, these findings provide new evidence for possible opportunistic spawning by blacknose dace and creek chubs on bluehead chub mounds in our system.

Conclusions

Understanding and conserving mutualisms is important to the broader field of ecology and preservation of global species diversity (Bronstein et al. 2004). Although understudied, mutualisms are abundant in freshwater ecosystems, and are expressed especially well in the form of nest associations coordinated by *Nocomis* hosts (Johnston and Page 1992, Johnston 1994a, Pendleton et al. 2012). We have shown that not only do adult *Nocomis* benefit from the associates that they host, but their embryo do as well. Manipulating associates benefit from nest association to a greater degree than non-manipulators, an effect worthy of separate study. Results of our study highlight the complexity of leuciscid reproduction, introduce the existence of embryonic selfish-herds into discussion of mutualisms, and may offer support for re-examining known interspecific interactions currently classified as nest parasitisms.

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Figure 2. Typical spawning aggregation on a mound nest of *Nocomis leptocephalus* in Toms Creek, VA. Species are as follows: 1) *N. leptocephalus* male, 2) *Clinostomus funduloides*, 3) *Chrosomus oreas*, 4) *Campostoma anomalum*, 5) *Luxilus albeolus* (below *Clinostomus funduloides*), 6) *Luxilus cerasinus*, 7) *Lythrurus ardens*.

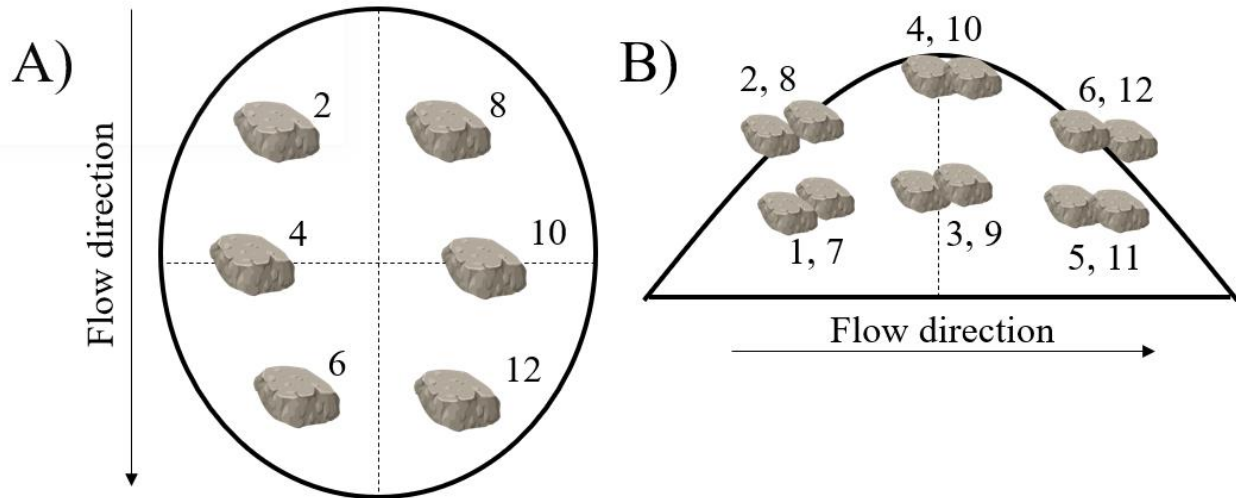


Figure 2. A) Top view of nest showing unbaited baited stone positions for embryo predation experiment. B) Side-view of nest showing buried baited stone positions (odd numbers) in relation to unbaited baited stone positions (even numbers). Numbers in both A) and B) are used consistently across nests to indicate treatment: upstream-buried = 1, 7; upstream-unbaited = 2, 8; middle-buried = 3, 9; middle-unbaited = 4, 10; downstream-buried = 5, 11; downstream-unbaited = 6, 12.

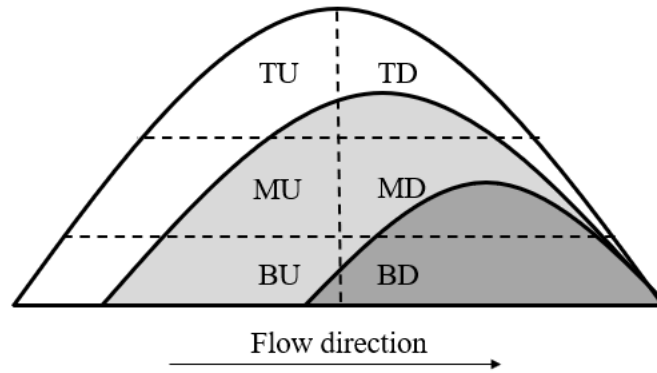


Figure 3. Side view of nest sections used in the divided-nest experiment and order of unpacking. TD = top-downstream, MD = middle-downstream, BD = bottom-downstream, TU = top-upstream, MU = middle-upstream, BU = bottom-upstream. Shading indicates nest growth upstream over several days (darkest shading indicates beginning of nest), which we derive from the embryo-larval ratios observed within nest sections (see Appendix 1). As the host continues to add gravel to the spawning trough, the nest grows both vertically and upstream. Dark dashed lines and numbers show nest division methods on nest at final size.

Table 1. Competing models used to test total embryo distributions and identity of species on spawning mounds of *Nocomis leptocephalus* in Toms Creek, VA, during 2021-2022.

Model	Variables in model	Number of parameters estimated
<i>y</i> = total embryo fractions across nest sections		
A0	nest	2
A1	nest, vertical (top, middle, bottom)	4
A2	nest, horizontal (upstream, downstream)	3
A3	nest, vertical, horizontal, vertical × horizontal interaction	7
<i>y</i> = host vs. associate embryo proportions by nest section		
B0	nest	2
B1	nest, vertical (top, middle, bottom)	4
B2	nest, horizontal (upstream, downstream)	3
B3	nest, vertical, horizontal, vertical × horizontal interaction	7
<i>y</i> = manipulator vs. non-manipulator embryo proportions by nest section		
C0	nest	2
C1	nest, vertical (top, middle, bottom)	4
C2	nest, horizontal (upstream, downstream)	3
C3	nest, vertical, horizontal, vertical × horizontal interaction	7

Table 2. Tukey HSD results for embryo predation experiment conducted in Toms Creek, VA, during summer 2021-2022 at $\alpha = 0.05$

					95% Confidence Interval	
Section (A)	Section (B)	Mean Difference (A-B)	Std. Err Dif	Sig.	Lower Bound	Upper Bound
Downstream-buried	Downstream-unburied	43.98	9.77	0.00	15.73	72.24
Middle-buried	Downstream-unburied	41.64	9.78	0.00	13.34	69.93
Upstream-buried	Downstream-unburied	34.97	9.54	0.00	7.38	62.56
Downstream-buried	Middle-unburied	31.90	9.77	0.02	3.65	60.15
Middle-buried	Middle-unburied	29.56	9.78	0.04	1.26	57.85
Upstream-unburied	Downstream-unburied	28.12	9.43	0.04	0.83	55.42
Upstream-buried	Middle-unburied	22.89	9.54	0.16	-4.70	50.48
Upstream-unburied	Middle-unburied	16.04	9.43	0.53	-11.25	43.33
Downstream-buried	Upstream-unburied	15.86	9.77	0.58	-12.39	44.11
Middle-buried	Upstream-unburied	13.51	9.78	0.74	-14.78	41.80
Middle-unburied	Downstream-unburied	12.08	9.43	0.80	-15.21	39.37
Downstream-buried	Upstream-buried	9.02	9.86	0.94	-19.51	37.55
Upstream-buried	Upstream-unburied	6.84	9.54	0.98	-20.75	34.43
Middle-buried	Upstream-buried	6.67	9.89	0.98	-21.94	35.28
Downstream-buried	Middle-buried	2.35	10.11	1.00	-26.90	31.59

Table 3. Comparison of models predicting embryo distribution on bluehead chub mounds in Toms Creek, VA, from summer 2021-2022.

Model	Hypothesis	Number of parameters (K)	-2 Log Likelihood	ΔAIC_c	AIC c	Model weight (w)
<i>y = total embryo fractions across nest sections</i>						
A3	Both vertical and horizontal gradients determine embryo distribution	7	-57098.2	0.0	114211.3	1
A1	A vertical gradient determines embryo distribution	4	-75524.0	36845.1	151056.4	0
A2	A horizontal gradient determines embryo distribution	3	-77049.4	39893.7	154105.0	0
A0	No gradient exists in embryo distribution (null model)	2	-91984.5	69761.9	183973.1	0
<i>y = host vs. associate embryo proportions by nest section</i>						
B3	Both vertical and horizontal gradients determine host and associate embryo distributions	7	-428.4	0.0	870.9	1
B1	A vertical gradient determines host and associate embryo distributions	4	-440.1	17.4	888.3	0
B2	A horizontal gradient determines host and associate embryo distributions	3	-446.0	27.1	898.0	0
B0	No gradient exists in host and associate embryo distribution (null model)	2	-449.0	31.1	902.0	0
<i>y = manipulator vs. non-manipulator embryo proportions by nest section</i>						
C3	Both vertical and horizontal gradients determine manipulator and non-manipulator embryo distributions	7	-622.0	0	1258.1	0.99
C1	A vertical gradient determines manipulator and non-manipulator embryo distributions	4	-629.9	9.7	1267.8	0.01
C2	A horizontal gradient determines manipulator and non-manipulator embryo distributions	3	-633.1	14.2	1272.3	0
C0	No gradient exists in manipulator and non-manipulator embryo distributions (null model)	2	-634.5	14.9	1272.9	0

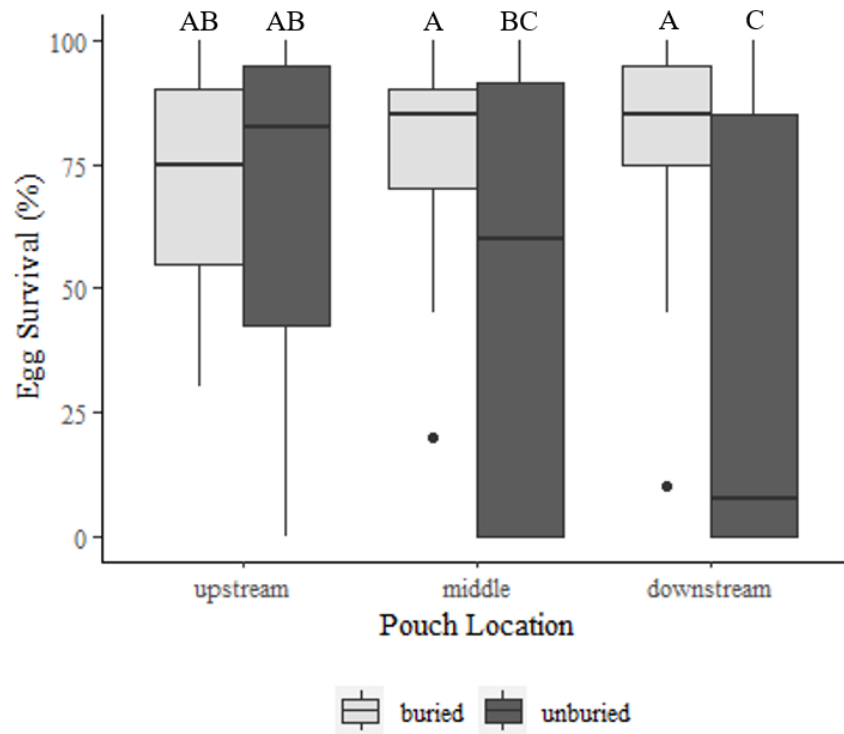


Figure 4. Embryo survival of buried and unburied treatments within three nest locations as part of the egg predation experiment conducted in Toms Creek, VA, in summer 2022. Boxplots with the same letter are not significantly different using Tukey HSD test. Points indicate outliers.

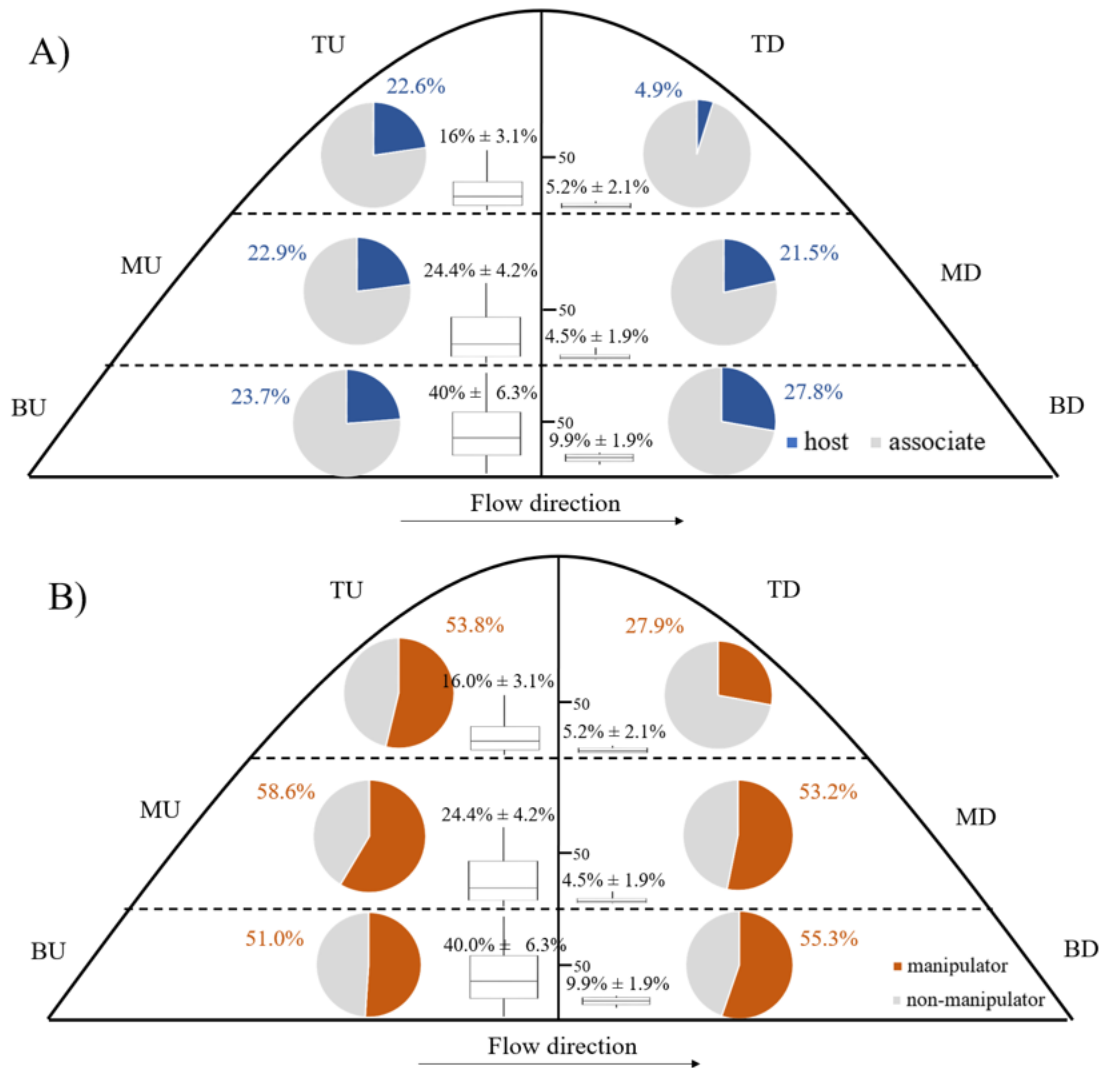


Figure 5. Embryo density within nest sections (boxplots), and relative percent host vs. associate (A) and manipulator vs. non-manipulator (B) of embryonic assemblage for each section (pie charts) within nests of *Nocomis leptocephalus* sampled from Toms Creek, VA, in 2021 and 2022. Colored text indicates host (A) or manipulator (B) percentage. Black text above boxplots indicates average $\pm$ standard error of boxplots. Sections are labeled as follows: TD = top-downstream, MD = middle-downstream, BD = bottom-downstream, TU = top-upstream, MU = middle-upstream, BU = bottom-upstream.

Table 4. Average percent occurrence of each species' embryos within in each nest section $\pm$ standard error from *Nocomis leptocephalus* nests sampled from Toms Creek, VA, in 2021-2022. Section letters align with those described in Fig. 3. Species codes are as follows: BHC = bluehead chub, CRS = crescent shiner, RSD = rosyside dace, SR = stoneroller, WS = white shiner, RFS = rosefin shiner, MRBD = mountain redbelly dace, BND = blacknose dace, CC = creek chub. Asterisk by name indicates host, plus sign indicates nest manipulators.

Section	TD	MD	BD	TU	MU	BU
BHC ^{*+}	4.9 $\pm$ 1.8	21.5 $\pm$ 6.8	27.8 $\pm$ 6.3	22.6 $\pm$ 6.0	22.9 $\pm$ 6.2	23.7 $\pm$ 5.7
CRS	0.5 $\pm$ 0.5	0.8 $\pm$ 0.8	0.5 $\pm$ 0.5	0.5 $\pm$ 0.5	3.4 $\pm$ 2.4	0.5 $\pm$ 0.5
RSD	34.2 $\pm$ 7.7	32.5 $\pm$ 8.3	27.7 $\pm$ 5.8	24.8 $\pm$ 7.3	23.7 $\pm$ 6.4	20.7 $\pm$ 5.7
SR ⁺	22.0 $\pm$ 7.5	30.3 $\pm$ 7.9	27.1 $\pm$ 6.9	31.1 $\pm$ 6.4	30.4 $\pm$ 6.3	27.4 $\pm$ 5.5
WS ⁺	1.1 $\pm$ 0.7	1.3 $\pm$ 0.9	0.5 $\pm$ 0.5	0.0 $\pm$ 0.0	5.3 $\pm$ 4.8	0.0 $\pm$ 0.0
RFS	15.0 $\pm$ 7.5	4.2 $\pm$ 2.5	3.6 $\pm$ 2.2	3.7 $\pm$ 2.7	1.9 $\pm$ 1.9	2.1 $\pm$ 1.6
MRBD	20.7 $\pm$ 7.3	9.3 $\pm$ 4.3	12.9 $\pm$ 3.1	16.7 $\pm$ 3.9	12.4 $\pm$ 2.9	23.6 $\pm$ 5.2
BND	0.5 $\pm$ 0.5	0.0 $\pm$ 0.0	0.0 $\pm$ 0.0	0.0 $\pm$ 0.0	0.0 $\pm$ 0.0	0.5 $\pm$ 0.5
CC	1.1 $\pm$ 1.1	0.0 $\pm$ 0.0	0.0 $\pm$ 0.0	0.5 $\pm$ 0.5	0.0 $\pm$ 0.0	1.6 $\pm$ 1.6

CONCLUSION

This thesis is a comprehensive look at three ecological performance metrics – growth, survival, and reproduction – that *Nocomis* derive disproportionate to those of nest associates from the spawning aggregations that they host. Historically, it was believed that the only benefit that *Nocomis* received from nest associates was indirect – an increase in fitness from heightened offspring survival (Peoples and Frimpong 2013). My research shows that *Nocomis* can no longer be considered an accidental beneficiary of this interaction, but rather the orchestrator of coevolved behaviors that produce explicit advantages for *Nocomis*.

In Chapter 1, I investigated the possibility of a selfish-herd present among the adult fishes and found support for such. I found that the chub routinely occupies the central position of the aggregation, which selfish-herd theory defines as the safest location (Hamilton 1971). I also found that the host returns to the nest last after temporary vacation when risk is perceived by the aggregation, which indicates that the host uses its nest associates to reduce its own vigilance requirements and risk of predation. This study also quantified the amount of time the host spends on versus off its nest, which was previously unknown. The small proportion of time that the chub spends on its nest suggests that the host's management of predation risk may be more complicated than the breadth of this study; perhaps the host recognizes that it can further reduce its own risk of predation by actively avoiding its own conspicuous mound when not spawning or building.

I investigated and found support for an embryonic selfish-herd in Chapter 2. With this study, I confirmed that the embryo burying behaviors of the host are an effective form of parental care through an expansion of the methods presented by Floyd (2016). Because DNA barcoding elucidated that the host's offspring reap disproportionate benefit from the burying

behaviors of the host by being located in deeper sections of the nest, I can deduce the existence of an embryonic selfish-herd. Further, this was the first study to describe the effect of nest manipulation on embryo placement within chub nests, and to define stoneroller and white shiner as nest manipulators in this system. When combined with host, nest manipulator embryos were found to follow the same pattern of distribution as host embryo, actively avoiding the superficial sections and dominating the deeper, safer compartments. I also found previously undescribed embryonic diversity with the discovery of blacknose dace and creek chub embryo on bluehead chub nests, species which are not considered nest associates of bluehead chub (Pendleton et al. 2012). Cumulatively, the results of this chapter support the existence of an embryonic selfish-herd within chub nests that is orchestrated by the host and, to a lesser extent, nest manipulators.

Additionally, I found evidence for embryo predation by both host and associate from a preliminary gut contents study presented in Chapter 2. Previous research and observation have suggested that the host and associates may be preying upon each other's embryo while spawning on a nest (Steele 1978, Wallin 1989, Floyd 2016), but this is the first study to confirm such behavior through genetic examination of gut contents. I found that the host, stoneroller, and mountain redbelly dace all consume each other's embryo, as well as embryo of the *Luxilus* and *Clinostomus* species participating in spawning aggregations. These preliminary results leave lots of room for further development. An in-depth study with revised genetic methods should be conducted to precisely quantify the frequency of embryo predation in the diets of these fishes. Such study would benefit by developing blocking PCR primers to block amplification of self-DNA and thereby investigate whether chubs eat their own embryo or purposefully avoid their own embryo as they prey on associate embryo, and likewise for associates. This would greatly contribute to the characterization of selfish participants (host or associate) in this mutualism.

The combined results of this thesis illuminate previously undescribed complexity within this mutualism and redefine our understanding of the interspecific interactions between *Nocomis* and their nest associates. Cohesively, these findings strongly support the identification of the chub as a selfish participant in a mutualism and confirm the presence of two selfish-herds within *Nocomis* spawning aggregations, both centering the host as the primary beneficiary. I present four novel contributions in this thesis, specifically providing: 1) a direct identification of a *Nocomis* host as a selfish participant, 2) recognition of a mixed-species fish spawning aggregation as a selfish shoal, 3) definitive evidence for embryo-predation in *Nocomis* spawning aggregations by both host and associates, and 4) the investigation and evincing of the existence of an embryonic selfish-herd. The complexities uncovered in the *Nocomis* host's pursuit of maximum fitness through an apparent mutualism epitomizes the apt description of mutualisms as "reciprocally exploitive interactions" (Bronstein 2001).

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APPENDIX 1

Stage of Embryos Present in Nest Sections Suggest How Nests Grow

We investigated the embryo-larval ratios contained within each nest section as a means of determining how chub nests grow over time and whether the creek chub embryo that we identified on a nest were the result of a pit sabotage or usurpation by a bluehead chub or a result of intentional spawning of creek chub. After nest division, when larvae were present within a sampled nest ($n = 10$ of 20 nests sampled), we counted larvae and unhatched embryos separately and calculated embryo-larval ratios for each nest section.

We used a generalized linear mixed model assuming binomial distribution of the proportions, with nest identity as a random effect, to compare embryo-larval ratios across nest sections. A Tukey HSD test was then used to identify significant differences in embryo-larval ratios. JMP Pro 16.0.0 (2021) was used for statistical analysis. Results of the model revealed that embryo-larval ratios differed significantly across nest sections ($F_{(5, 45)} = 5.5, p = 0.0005$). A Tukey HSD test revealed that the bottom-downstream nest section contained significantly more larvae than all other nest sections, with an average of 39.1% ($\pm 5.8\%$) larvae. The top-upstream nest section contained the lowest percent of larvae on average (6.2% $\pm 5.8\%$, Table A1).

Given that the bottom-downstream section contained the highest percentage of larvae and the top-upstream section contained the lowest percentage of larvae, we conclude that nests generally grow upwards and in the upstream direction as the host continues to add pebbles over the course of days. These results also suggest that at least some of the creek chub embryo we identified (those in the top-upstream or downstream compartments) were the result of active spawning rather than pit usurpation.

Table A1. Tukey HSD post-hoc test results comparing embryo-larval ratios across nest sections in Toms Creek, VA, across Summer 2021 and 2022 at $\alpha = 0.05$. Sections are as follows: BD = bottom-downstream, MD = middle-downstream, TD = top-downstream, BU = bottom-upstream, MU = middle-upstream, TU = top-upstream.

Section (A)	Section (B)	Mean Difference (A-B)	Std. Err Dif	<i>p</i> - value	95% Confidence Interval	
					Lower Bound	Upper Bound
BD	TU	32.85	7.32	0.001	11.06	54.65
BD	TD	30.75	7.32	0.002	8.10	52.54
BD	MD	29.45	7.32	0.003	7.65	51.24
BD	MU	26.94	7.32	0.008	5.15	48.73
BD	BU	23.72	7.32	0.026	1.93	45.51
BU	TU	9.14	7.32	0.811	-12.66	30.93
BU	TD	7.03	7.32	0.928	-14.76	28.82
MU	TU	5.92	7.32	0.965	-15.88	27.71
BU	MD	5.73	7.32	0.969	-16.07	27.52
MU	TD	3.81	7.32	0.995	-17.98	25.60
MD	TU	3.41	7.32	0.997	-18.38	25.20
BU	MU	3.22	7.32	0.998	-18.57	25.01
MU	MD	2.51	7.32	0.999	-19.29	24.30
TD	TU	2.11	7.32	0.999	-19.69	23.90
MD	TD	1.30	7.32	1.000	-20.49	23.10

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JMP®, Version 16.0.0. SAS Institute Inc., Cary, NC, 1989–2021.

APPENDIX 2

DNA Barcoding Justification and Methods

The identification of embryonic and larval fishes is difficult, especially among leuciscids due to their diversity and morphological similarity (Fuiman et al. 1983), and the lack of taxonomic keys for early life stages available in the literature (Kelso et al. 2012). Molecular methods, such as DNA barcoding, allow identification of embryonic and larval fishes to species with greater success than visual identification alone. For the leuciscids discussed in this paper, DNA barcoding methods were first developed and used successfully by Peoples et al. (2017).

For this study, DNA was extracted from each of the subsamples of embryos with the DNeasy Blood and Tissue Kit (Qiagen, Germantown, MD), using the protocols for tissue and one to two hours of incubation with Proteinase K at 56°C for lysis. Extractions occurred within three days of embryo collection. Purities and concentrations of extracted samples were confirmed with a µLite PC spectrophotometer (Biodrop, Cambridge, UK). We did not attempt to amplify samples with A_{260}/A_{280} ratios below 1.81 or concentrations below 0.5 ng/µL.

A set of four universal DNA barcoding primers were used for amplification of region *COI*-3 of the mitochondrial cytochrome I gene in our samples – VF2_t1, FR1d_t1, FishF2_t1, and FishR2_t1 (Ivanova et al. 2007). *COI* samples were amplified using the manufacturer's protocol for Invitrogen Platinum® *Pfx* DNA polymerase. Amplification was performed in a total volume of 22.4 µL, with 0.45 µL of each universal primer (10 mM), dNTP mix (2.5 mM, Lucigen), 0.4 µL bovine serum albumin (1 mg/mL, Thermo Scientific), 2.24 µL MgCl₂ (50 mM, Invitrogen) and 10x PCR reaction buffer (Invitrogen), 0.22 µL Platinum® *Pfx* DNA polymerase (Invitrogen), and 1 µL DNA template (~12.0 ng/µL). We conducted all PCR reactions on a

BioRad MyCycler using the thermocycle profile described by Peoples et al. (2017): denaturation at 94°C for 2 min; 35 cycles of: denaturation at 94°C for 30 sec, annealing at 52°C for 40 sec, and extension at 72°C for 60 sec; final extension at 72°C for 10 minutes and indefinite hold at 4°C. Amplification products were observed by agarose gel electrophoresis and ethidium bromide fluorescence under UV light.

PCR products were sequenced on an ABI 3730XL DNA Analyzer by the Genomics Sequencing Center at Virginia Tech's Fralin Life Sciences Institute. Samples were prepared for sequencing using VF2_t1 and R2_t1 primers. Raw DNA sequences were aligned using Geneious Prime (Version 2022.1.1, Biomatters, Auckland, NZ) and identified to species using the Basic Local Alignment Search Tool (BLAST; Altschul et al. 1990) to search entries in GenBank. Identity scores greater than 95% for at least 100 bp of sequence were considered indicative of successful identification.

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APPENDIX 3

Correcting for Imperfect Nest Division

Inverse weighted proportional embryo volume in section i

$$q_i = \frac{\sum_{i=1}^6 v_i}{v_i} * \frac{e_i}{\sum_{i=1}^6 e_i} * 100 \quad (1)$$

where, v_i = volume of gravel sampled from section i of nest, and

e_i = proportion of total embryo sampled from nest in section i , $i = 1, 2, \dots, 6$.

Adjusted proportional embryo volume in section i

$$E_i = \frac{q_i}{\sum_{i=1}^6 q_i} \quad (2)$$

Adjusted embryo count in section i

$$C_i = \frac{E_i}{100} * \sum_{i=1}^6 e_i \quad (3)$$

APPENDIX 4

Embryo Predation by Host and Associates

BACKGROUND

Energy acquisition can be an important consideration for fishes when spawning, as spawning is a physiologically demanding task. Unlike many other species (Greene 1919, Nadeau et al. 2010), leuciscids in mixed-species shoals are often observed to feed actively on algae and drifting material such as insects prior to the onset of spawning, while over nests, and between spawning bouts. Individuals on mounds are frequently seen rushing to consume eggs that don't settle and float off the nest. We believe, based on direct observations and photographic evidence, that active foraging on unburied and superficially buried embryo on a nest is a regular occurrence perpetrated by "all" fish on the nest and that chub as host also participates in this behavior. That is, while they may forage in the surrounding streambed for algae and macroinvertebrates, the most obvious and available source of food for the host while tending a nest are the embryos being laid on it.

Wallin (1989) was first to note this potential avenue for selfishness within chub spawning aggregations. Chubs were observed consuming associate embryo in aquaria, and "probing in the nests" in the wild (Wallin 1989). These observations have been corroborated by work reported in two theses (Steele 1978, Floyd 2016) but the potential consumption of embryo of other species has not been systematically studied or confirmed with hard evidence of gut contents analysis. If hosts consume associate embryos, or vice versa, this adds an additional layer of selfishness to this putatively mutualistic interaction. We hypothesized bluehead chub and nest associates consume each other's embryos while spawning on chub nests.

METHODS

To investigate possible embryo predation on chub nests, we collected bluehead chub and the two most common nest associates, mountain redbelly dace (*Chrosomus oreas*) and central stoneroller (*Campostoma anomalum*, henceforth “stoneroller”), from nine active bluehead chub (*Nocomis leptcephalus*) nests in Toms Creek, near Blacksburg, Virginia, across summer 2022 and then identified the species within the digested material in their guts using DNA metabarcoding. To collect fishes, we set up block nets 20-40 meters above and below each nest to separate the nest and its associates from the rest of the stream. The reach contained by the block nets was then shocked 1-3 times using a LR-24 backpack electrofisher (Smith-Root, Vancouver, WA) until depletion sampling was satisfactory. We randomly subsampled 1-6 individuals of each species/sex from the cumulative haul, humanely euthanized those fishes using an overdose of MS-222 (Topic Popovic et al. 2012) and immediately put them on ice for transportation to the laboratory, where they were dissected. Guts were removed whole (stomach, intestines) and stored in 80% ethanol at -80°C until DNA extraction (Stein et al. 2013, Jakubaviciute et al. 2017). Guts were sorted by sex, which was determined by observation of mature gonads. If sex could not be determined from morphology or the presence of mature gonads (i.e., juveniles), we combined those gut contents with those of females of the species.

To prepare for extraction, whole guts were triple rinsed with deionized and autoclaved water to remove excess ethanol and then further dissected to isolate digested material from sloughed cells from gut linings, which would produce an excess of self-DNA if included. Digested material DNA was extracted with the DNeasy Blood and Tissue Kit (Qiagen, Germantown, MD), using the protocols for tissue and 4-6 hours of incubation with Proteinase K

at 56°C for lysis. Purities and concentrations of extracted samples were confirmed with a μ Lite PC spectrophotometer (Biodrop, Cambridge, UK). We amplified and sequenced amplicons from six female chub, four male chub, three female stoneroller, four male stoneroller, four female mountain redbelly dace, and three male mountain redbelly dace samples.

Mitochondrial cytochrome oxidase I (*COI-3*) sequences were amplified using VF2_t1 and FishR2_t1 primers from Ivanova et al. (2007) to which Illumina adapter overhang nucleotide sequences were added (Forward: 5' TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG-[*COI* forward primer and sequence], and Reverse: 5' GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG-[*COI* reverse primer]). PCR was conducted according to Illumina's MiSeq manufacturers protocol. Amplification was performed in a total volume of 25.0 μ L, with 5.0 μ L of each primer (1.0 μ M), 12.5 μ L of 2x KAPA HiFi HotStart ReadyMix (Roche, Basel, Switzerland), and 2.5 μ L DNA template (5.0 ng/ μ L). The thermocycle profile used was: denaturation at 95°C for 3 min; 25 cycles of: denaturation at 95°C for 30 sec, annealing at 55°C for 30 sec, and extension at 72°C for 30 sec; final extension at 72°C for five minutes, and indefinite hold at 4°C. Amplicon size was verified by running 1.0 μ L of PCR product on a Bioanalyzer DNA 1000 chip (Agilent Technologies, Santa Clara, CA). Dual indices and Illumina sequencing adapters were added to individual amplification products using the Nextera XT Index Kit (Illumina, San Diego, CA), which were pooled into libraries (Bohmann et al. 2022). In preparation for cluster generation and sequencing, pooled libraries were denatured with NaOH, diluted with hybridization buffer, and then heat-denatured. Libraries were sequenced at the Genomics Sequencing Center at Virginia Tech's Fralin Life Sciences Institute using the MiSeq Next-Gen Sequencing platform (Illumina).

Demultiplexed raw reads were processed in the R computing environment (R Core Team 2021). The package manager Bioconductor (Callahan et al. 2016) was used to implement packages, including DADA2 1.12.1 (Callahan et al. 2016), ggplot2 3.2.1 (Wickham 2015), phyloseq 1.28.0 (Mcmurdie and Holmes n.d.), and vegan 2.5-6 (Oksanen et al. 2019). Raw reads were quality processed using methods laid out in the DADA2 tutorial (Callahan et al. 2016). The reads were first visualized using the “plotQualityProfile” function in DADA2, which allowed us to monitor for low-quality reads. No trimming was necessary, so we maintained the full ~150bp of the reads for analyses. The “learnErrors” function was then used to evaluate the error rate of the data set. The “derepFastq” function was used to combine identical sequences into unique sequences, and from this a table of amplicon sequence variants (ASVs) was generated using the “makeSequenceTable” function. Chimeric reads were removed using the “removeBimeraDenovo” function.

Classification of surviving ASVs was completed by exporting a .fasta file containing sequences for use with the python program BOLDigger (Buchner and Leese 2020) command line tools. Taxonomic identification was performed using the “ie_coi” setting which employs the Barcode of Life Data System (BOLD) COI identification engine. Associated metadata was obtained via the “add_metadata” option. Single best hits were identified by the “digger_hit” algorithm. These taxonomic annotations were combined with the ASV table, and final analyses were completed in R. Raw abundance ASV counts were obtained by subtracting self reads from the individual sample sets. All scripts, figures, and data can be found at:

<https://github.com/davidhaak/dividednestsMS>

RESULTS

ASV outputs revealed that all groups of samples submitted for amplicon sequencing had some amount of other-leuciscid DNA present in their guts (Fig. S2), but most amplicon DNA was self-DNA (Fig. S3). The greatest gut content diversity and highest ASV raw counts were found among female chub samples, including the only evidence of predation on *Luxilus* embryos. Embryos of *Nocomis*, *Campostoma*, and *Chrosomus* made up the majority of consumed items.

DISCUSSION

Our results are the first to show evidence for predation by host and associates on each other's embryo while spawning on the nests of bluehead chub. These results support the characterization of the host as a selfish participant in this mutualism and supplement our understanding of the selfish avenues utilized by the host.

The relative lack of other-DNA in host guts compared to female chubs and associate guts suggests that the consideration of who is acting selfishly in this mutualism may be more complicated than previously recognized. A future, more detailed study with a larger sample size (specifically of hosts) could render a better representation of the relative abundance of other-DNA in the guts of these leuciscids. Further, the use of blocking primers (Vestheim and Jarman 2008) could reduce the prevalence of self-DNA in sequencing and support more fine-resolution diet interpretation. A key study would be one that is able to differentiate self-DNA of the host from other *Nocomis* DNA (e.g., metagenomic sequencing from gut contents) – indicating whether the host is able to selectively choose embryos of other species or cooperating males of its own species for consumption over its own, for which other studies have found evidence with other species (Neff 2003).

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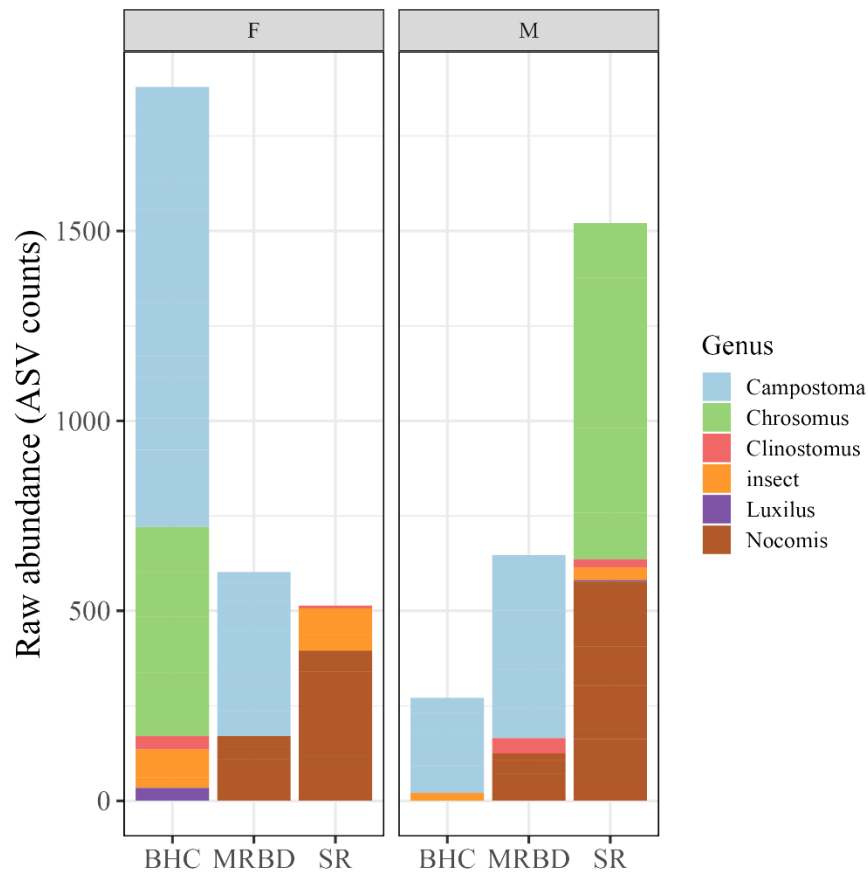


Figure S2. Raw abundance (ASV counts) of other-DNA found within the guts of female (F) and male (M) bluehead chub (BHC), mountain redbelly dace (MRBD), and stoneroller (SR) from gut samples collected from Toms Creek near Blacksburg, VA, in summer 2022. Self-DNA was removed from the bars of this plot. Based on the species diversity of Toms Creek, we assume *Campostoma* to be central stoneroller (*Campostoma anomalum*) embryo, *Chrosomus* to be mountain redbelly dace (*Chrosomus oreas*) embryo, and *Clinostomus* to be rosieside dace (*Clinostomus funduloides*) embryo. *Luxilus* is the only genus that cannot be fully resolved, as both crescent shiner (*Luxilus cerasinus*) and white shiner (*Luxilus albeolus*) are present in this system and frequently spawn with chubs.

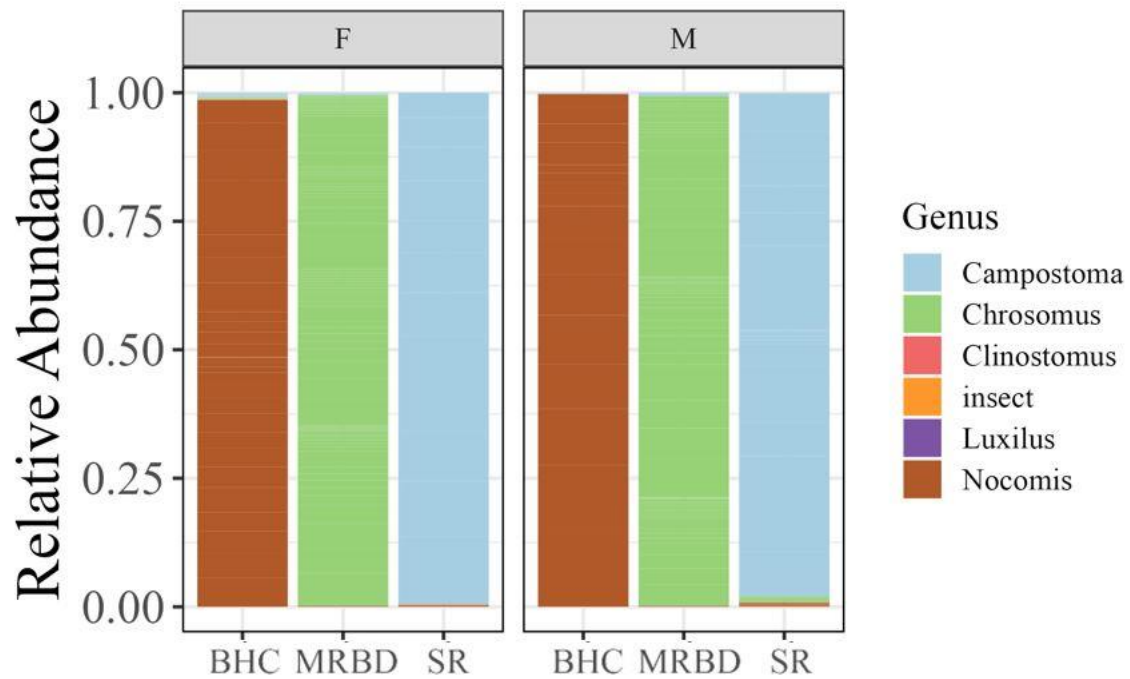


Figure S3. Relative abundance of other-DNA to self-DNA found within the guts of female (F) and male (M) bluehead chub (BHC), mountain redbelly dace (MRBD), and stoneroller (SR) from gut samples collected from Toms Creek near Blacksburg, VA, in summer 2022. Genera should be interpreted as: *Campostoma* – central stoneroller (*Campostoma anomalum*) embryo, *Chrosomus* – mountain redbelly dace (*Chrosomus oreas*) embryo, and *Clinostomus* – rosieside dace (*Clinostomus funduloides*) embryo. *Luxilus* may include crescent shiner (*Luxilus cerasinus*) and/or white shiner (*Luxilus albeolus*) as both are present in this system and frequently spawn with chubs.