

Cheating and Selfishness in Reproductive Interactions among Nest Associative Cyprinids

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ABSTRACT

Mutualism is an understudied interaction in ecosystems throughout the world. Within the eastern United States, one fish-fish mutualism is the nest association between *Nocomis* and other cyprinids. I assessed the role of host parental care while testing for the selfish-herd effect. Additionally, I examined multiple nest associates in order to elucidate potential cheaters. I utilized gonadosomatic index (GSI) to compare reproductive condition among the bluehead chub *Nocomis leptocephalus* and its putative nest associates in Catawba Creek, Virginia. GSI of potentially obligate associates tracked host GSI more closely than weak associates, while weak associates spawned prior to *Nocomis* spawning. Given their GSI patterns and behavior, central stonerollers *Campostoma anomalum* may be cheaters in the interaction. I used multiple experiments to test for the selfish-herd effect, the role of parental care, and how relative risk influences reproductive decisions of associates. Most eggs were located in the bottom upstream quarter of nests, and a molecular analysis revealed that stonerollers and chubs constituted the majority of identified individuals. A comparison of host-associate ratios from four nest sections failed to identify the selfish herd effect. Another experiment found that host egg covering significantly reduced egg predation. Lastly, I assessed relative egg predation risk at four potential spawning locations; predation levels did not differ significantly at any location. While GSI patterns suggest that stonerollers may be cheaters, genetic evidence indicates that stonerollers spawn on *Nocomis* nests. Because GSI does not completely assess reproduction, secondary stoneroller reproduction on *Nocomis* nests may have been overlooked.

ATTRIBUTION

Drs. Emmanuel Frimpong and Brandon Peoples were instrumental in the experimental design and analysis of this research.

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TABLE OF CONTENTS

ATTRIBUTION	iii
ACKNOWLEDGEMENTS.....	iv
LIST OF TABLES.....	vi
LIST OF FIGURES	vii
CHAPTER 1: GENERAL INTRODUCTION TO MUTUALISMS AND <i>NOCOMIS</i> NEST ASSOCIATION	1
Introduction	2
References	12
CHAPTER 2.....	19
Summary	20
Introduction	21
Methods	24
Results	26
Discussion	28
Conclusions	33
References	34
CHAPTER 3.....	42
Summary	43
Introduction	44
Methods	49
<i>Assessing relative risk</i>	50
<i>Parental care via egg burying</i>	51
<i>Divided nest experiment</i>	52
<i>Genetic analysis</i>	53
Results	54
<i>Assessing relative risk</i>	54
<i>Parental care via egg burying</i>	55
<i>Divided nest experiment</i>	55
<i>Genetic analysis</i>	56
Discussion	57
Conclusions	61
References	62
CHAPTER 4: SYNTHESIS	70
References	73
APPENDIX	74

LIST OF TABLES

Table 1.1. Expected pairwise outcomes and associated classifications of interspecific interactions. From Wootton (1998).....16

Table 1.2. The 11 Nocomis nest associates in the New River basin. Associations of rare species are in italics. Adapted from Pendleton et al. (2012).....16

Table 2.1. Pearson correlations between nonmetric multidimensional scaling (NMDS) axes and species scores. Correlation values demonstrate the relationship between species scores and the axes. Strong associates, shown in bold font, were all positively correlated with MDS2; weak associates were all negatively correlated with MDS2.....38

Table 2.2. Pairwise correlations of gonadosomatic index (GSI) values between all species. A nonparametric Spearman’s ρ test was used to determine significant correlation values. Species codes are: bluehead chub (BHC), blacknose dace (BND), crescent shiner (CRS), longnose dace (LND), mountain redbelly dace (MRB), rosyside dace (RSD), and central stoneroller (CSR). *Indicates ($p < 0.05$), †Indicates ($p < 0.1$, but > 0.05).....38

Table 3.1. Observed actions of host bluehead chub. Species codes are bluehead chub (BHCH), central stoneroller (CESR), crescent shiner (CRSH), mountain redbelly dace (MRBD), and white shiner (WHS). Percentages were calculated using observed counts of activity.....68

Table 3.2. Total number of actions and feeding events for all species documented at three potential spawning locations in Toms Creek ($n = 10$). Species codes are bluehead chub (BHCH), central stoneroller (CESR), crescent shiner (CRSH), fantail darter (FTDA), mountain redbelly dace (MRBD), rosefin shiner (RFSH), rosyside dace (RSDA), white shiner (WHS).....68

Table 3.3. Totals for all individuals identified using molecular analysis. Numbers within parentheses are percent composition for the above nest section. Nest sections are bottom upstream (BU), bottom downstream (BD), top upstream (TU) and top downstream (TD).....69

LIST OF FIGURES

Figure 1.1. The coaction compass, first described by Haskell (1949) and recreated by Bronstein (2015). Demonstrates how interspecific interactions exist along a continuum. The magnitude of the outcome increases with distance from the origin (0, 0). Any movement along the circumference of the compass (due to changing context) denotes a shift in the net outcome for one or both partners.....17

Figure 1.2. Three-stage construction of *Nocomis spp.* gravel mound: concavity (1), platform (2), and mound (3).....18

Figure 1.3 Benefits (solid arrows) and costs (dashed arrows) incurred through a two-species nest association. The thickness of the arrows denotes the degree of influence for each cost and benefit; thicker arrows denote greater influence, while thin arrows denote lesser or unknown influence. Thin edged boxes within the thick edged boxes are resources exchanged between host and associate (internal); boxes outside the thick edged box represent factors external to the nest association. Adapted from Peoples (unpublished).....18

Figure 2.1. Relationship between total length (mm) and GSI for collected individuals (n = 521) in associative groups over 18-week sampling period (9 April -8 August 2013). Associative groups are (a) chub, (b) stoneroller, (c) strong associates, and (d) weak associates.....39

Figure 2.2. Mean weekly gonadosomatic index ($\pm 2SE$) of reproductive groups (a-c) of stream fishes in Catawba Creek, Virginia from 9 April to 8 August 2013. Bluehead chub spawning began on Week 7 (22 May 2013). In above figure, bluehead chub is represented by the solid black line and the compared group is represented by the gray dashed line. Strong associates (a) include mountain redbelly dace, rosieside dace, and crescent shiner. Weak associates (c) include blacknose dace and longnose dace.....40

Figure 2.3. Species (GSI) scores (a) and time (week) scores (b) arranged within nonmetric multidimensional scaling (NMDS) ordination space based on a Bray-Curtis dissimilarity matrix (stress = 0.09). Dissimilarity between scores increases with distance within NMDS space. Strong associates (solid triangles) and weak associates (open triangles) are clearly separated along the MDS2 axis. Weeks preceding chub spawning (open circles) are grouped closely with weak associates, while weeks following chub spawning (solid circles) group closely with strong associates. Species codes: bluehead chub (BHC), mountain redbelly dace (MRB), rosieside dace (RSD), crescent shiner (CRS), blacknose dace (BND), longnose dace (LND) and central stoneroller (CSR).....41

Figure 3.1. Three-stage construction of *Nocomis spp.* gravel mound: concavity (1), platform (2), and mound (3). Male *Nocomis* excavate spawning pits on the upstream end of the platform (2).....65

Figure 3.2. Cross-section of chub nest divided into four sections: top (T) and bottom (B), upstream (U) and downstream (D). Concavity and platform added for reference.....65

Figure 3.3. Boxplots of eggs consumed at four potential spawning locations of nest associates. Location labels are: Active downstream (ADS), artificial nest (ART), Active upstream (AUS) and open substrate (OPE).....66

Figure 3.4. Boxplots of log(x+1)-transformed larval fish counts from each nest section (Nests, n=8). Ratios of chub:associates for sections displayed within each plot. Labels are as following: bottom downstream (BD), bottom upstream (BU), top downstream (TD) and top upstream (TU).....67

CHAPTER 1: GENERAL INTRODUCTION TO MUTUALISMS AND *NOCOMIS* NEST ASSOCIATION

Running Title: General introduction

Keywords: Mutualism, Nest Association, Positive Interspecific Interaction, *Nocomis*

Introduction

Interspecific interactions are ubiquitous and extremely diverse, influencing many processes and patterns within an ecosystem (Bronstein 1994). Interspecific interactions exhibit a broad range of fitness outcomes for the participants. Table 1.1 describes the spectrum of possible pairwise fitness outcomes (+, -, 0) from an interspecific interaction. Of these relationships, competition and predation have been the most heavily researched (Boucher et al. 1982, Bronstein 2009). Knowledge concerning mutualisms lags far behind other interactions, although there has been a recent surge in literature on the topic (Bronstein 2009). Mutualisms have been described in many systems, including endophyte-plant (Saikkonen et al. 1998) and insect-plant interactions (Lach 2003). Bronstein (1994) defined a mutualism as an interspecific interaction in which both partners experience a net fitness benefit. Because of the dual benefits, mutualisms are commonly defined as a strictly [+ , +] interaction (Bronstein 1994). Generally, one partner in the interaction performs some service in return for a reward from the other partner. Bronstein (2009) concluded that mutualists exchange one or a combination of three principal commodities: transport, protection, or nutrition.

Although mutualisms are often defined as a [+ , +] interaction, there is much variation in the strength and outcomes of the interaction. Bronstein (2009) defined two levels of mutualistic interactions: obligate and facultative. In obligate mutualisms, one or both mutualists are unable to persist in a system in the absence of the other. Obligate mutualisms may be highly specialized, with only a single species being able to provide the mutualistic service to the other species. Facultative mutualisms, however, are not required for the persistence of the mutualists in an ecosystem; the species may experience better fitness when co-occurring with a mutualist, but it is not necessary for their persistence. Facultative mutualisms are often much less specialized;

several species within an ecosystem may be capable of providing a necessary benefit or service (Bronstein 2009).

While the theoretical outcomes of interspecific interactions are perceived as static (Table 1.1), their actual outcomes are often plastic. The outcomes of these interactions may vary both spatially and temporally, driven by biotic, abiotic and genetic context (Hoeksema and Bruna 2015). Variation in the outcomes of interspecific interactions has been documented in many systems, including endophyte-plant associations and insect-plant interactions (Mattson and Addy 1975, Saikkonen et al. 1998). Saikkonen et al. (1998) concluded that fungal-plant interactions are highly variable and range from antagonistic to mutualistic. Haskell (1949) created the first theoretical framework to incorporate the potential plasticity of these interactions. His framework, the coaction compass, displays the possible outcomes of interspecific interactions along a continuum (Figure 1.x). Expanding on the coaction compass, several authors have incorporated outcome plasticity, or context dependency, into mutualistic interactions (Bronstein 2015, Hoeksema and Bruna 2015).

Within mutualisms, context dependency may alter the magnitude of the benefit of one or both partners; in more extreme cases, context dependency may shift the overall outcome from mutualistic to parasitic (+, -) or commensalistic (0, +) (Hoeksema and Bruna 2015). The influence of context dependency within mutualisms has been shown to be more substantial than in other interspecific interactions (Chamberlain et al. 2014). If the outcomes of mutualisms are plastic, then the strength of a mutualism (e.g. obligate, facultative) may vary at the temporal and spatial scale (Hoeksema and Bruna 2015). With both biotic and abiotic factors driving outcomes, it is vital to consider the influence of context dependency within mutualisms.

In addition to benefits, there are costs associated with nearly all mutualisms (Bronstein 2009). Members of a mutualism will seek to minimize costs by providing as little reward/service as possible to retain their partner (Bronstein 1994). Because both mutualists are attempting to maximize benefits while reducing costs, mutualisms are inherently selfish interactions. Bronstein (2009) states that natural selection favors selfish individuals that minimize or eliminate costs while still receiving the benefits of the mutualism. If more selfish individuals are being selected for, cheaters inevitably arise within mutualisms. Cheating can shift the [+ , +] outcome of mutualism towards commensalism [+ , 0] or in extreme cases, parasitism [+ , -]. Despite being beset with cheaters, many mutualisms persist (Bronstein 2001, Anderson and Midgely 2002, Jones et al. 2009). Jones et al. (2009) suggested that cheating and exploitation have been associated with mutualisms for millions of years. For example, a fig-fig wasp mutualism has persisted despite the presence of non-pollinating and parasitic wasps that exploit the fig host (Machado et al. 1996). Mutualisms can only persist when benefits outweigh costs; when the costs of cheating are greater than the benefits, the interaction would exist as a parasitism or as commensalism.

As mentioned previously, organisms are naturally selfish; when one or more species aggregate together, this selfishness can be expressed through the selfish-herd effect. The selfish-herd effect, first proposed by Hamilton (1971), states that individuals aggregate together instinctually in response to internal (herd movement) or external stimuli (predation). Individuals within a 'herd' respond to these stimuli by moving closer to the center of the 'herd' (Hamilton 1971). Within the selfish-herd effect, Hamilton (1971) concluded that predation pressure is lowest at the center and highest at the perimeter. Parrish (1989) corroborated these findings, stating that marginal predation is the driving force in aggregative behavior. Hamilton's (1971)

original example involved frogs around the edge of a pond: a geometrically one-dimensional environment with a perimeter and a center. To date, the majority of studies on the selfish-herd effect have dealt with two-dimensional systems (Goff 1984, Viscido et al. 2002, James et al. 2004, but see McKaye and Oliver 1980, Krause 1993, Couzin et al. 2002). The selfish-herd effect has been applied to three-dimensional aquatic systems, however, these studies are limited to aggregations of fish in the open water (McKaye and Oliver 1980, Krause 1993). While McKaye and Oliver worked in a three-dimensional system, the catfish and cichlid larvae were free-swimming larvae that formed a sphere with associate (cichlid) larvae on the perimeter (McKaye and Oliver 1980, McKaye 1985). This study was the first to conceive the selfish-herd effect in a three-dimensional, aquatic nest system.

Within aquatic systems, positive interactions are widespread and exist between many different taxa. Hay et al. (2004) suggested that mutualisms commonly support key species within an ecosystem and are as important as competition and predation. While the pattern is changing, the majority of mutualisms studied in aquatic systems focused primarily on invertebrates, plants and cyanobacteria (Holomuzki et al. 2010). Endosymbiotic cyanobacteria within diatoms provide nitrogen for their host, permitting diatoms to persist in unfavorable habitats (Peterson and Grimm 1992). Brown et al. (2012) defined a mutualism between crayfish *Cambarus chasmodactylus* and a branchiobdellid worm *Cambarincola ingens*; the worms provided a cleaning service on crayfish gills, resulting in increased growth and decreased mortality of the host in return for a nutritional benefit to the worms. Sandsten and Klaassen (2008) defined a predator-prey mutualism in an aquatic system; swans grazing on the tubers of the submerged plant *Potamogeton pectinatus* received nutrients from the plant while ultimately increasing the growth of *P. pectinatus*.

One well-understood mutualism involving fishes is bioturbation, the process in which the activities of one species disrupts stream substrate and releases nutrients. Bioturbation often serves as a driver of nutrient dynamics in lotic systems (Flecker 1996). Similar to terrestrial vertebrates, fishes also participate in transport and dispersal mutualisms with riparian vegetation. In flood-plain forests, fishes consuming fruits and seeds from terrestrial plants assist with seed dispersal and nutrient cycling (Goulding 1980). Goulding (1983) concluded that fish consumed more fruits and seeds than terrestrial animals in Amazonian floodplains. These transport mutualisms have been described in North American systems (Chick et al. 2003) as well as throughout the Amazonian floodplains (Galetti et al. 2008).

Mutualisms directly involving at least two species of fish are the focus of this study. One of the first fish-fish mutualisms documented is that of the bluestreak cleaner wrasse *Labroides dimidiatus* (Feder 1966). *Labroides dimidiatus* removes parasites from up to 130 host species (Grutter and Poulin 1998) and 2300 individuals (Grutter 1996) each day. Bshary and Grutter (2002) concluded that *L. dimidiatus* regularly cheats by feeding directly on the host species' live tissues. This mutualism, however, continues to persist and play a major role in coral reef ecosystems. Despite being studied for over four decades, there is still not a complete understanding of this mutualism; this demonstrates the complexity associated with mutualisms and the need to better comprehend how they influence their systems.

Nest association, a reproductive behavior in which one fish species (associate) spawns in the nest of a second species (host) (Johnston and Page 1992), is potentially mutualistic (McKaye and Oliver 1980, Goff 1984, Johnston 1994b). This interaction is not unique to North American systems; associations have been described between African cichlids and bagrid catfish (McKaye 1985, McKaye et al. 1992) and between a Japanese perch *Siniperca kawamebari* and minnow

Pungtungia herzi (Baba et al. 1990). Nest association has been described on several continents, suggesting that its influence on lotic populations and communities may be globally significant. Nest association has been documented in 34% (38 species) of all North American cyprinids whose spawning behavior is known (Johnston 1999). In North American systems, associates typically spawn in nests of cyprinid or centrarchid hosts (Raney 1947, Hunter and Hasler 1965, Goff 1984, Johnston 1994a). In both cases, associates rely on chemical cues released in the milt and ovarian fluids of the host species to induce gamete maturation and spawning (Hunter and Hasler 1965, Wisenden 1999).

Centrarchids create flat saucer shaped, defended nests in the substrate and are common hosts in nest association (Hunter and Hasler 1965, Goff 1984, Johnston 1994a). Associates in centrarchid nest association are typically cyprinids (Hunter and Hasler 1965, Johnston 1994a), however, they can serve as host for associates such as the longnose gar *Lepisosteus osseus* (Goff 1984). Nest associations have been described between bowfin *Amia calva* and their prey, the golden shiner *Notemigonus crysoleucas* (Katula and Page 1978).

Another ubiquitous nest association throughout the freshwater systems of North America is the cyprinid-cyprinid association. Common hosts in this interaction are members of the genera *Semotilus*, *Campostoma*, and *Nocomis* (Miller 1964, Johnston and Page 1992). A frequent host in the eastern United States is the creek chub *Semotilus atromaculatus*; nest associations with this host have been described for blackside dace *Chrosomus cumberlandensis*, Tennessee dace *C. tennesseensis*, and a number of other cyprinid species (Hamed et al. 2008, Mattingly and Black 2013). The central stoneroller *Campostoma anomalum* hosts *C. cumberlandensis* (Mattingly and Black 2013), Tennessee dace *C. tennesseensis* (Hamed et al. 2008), the common shiner *Luxilus cornutus* (Miller 1964), and other cyprinid fishes.

Of all interspecific reproductive interactions, *Nocomis spp.* (henceforth chub) nest association is one of the most prominent among stream fishes of the eastern United States and Canada. All seven species in *Nocomis* construct mounds for spawning (Lachner 1952, Sabaj et al. 2000). Nest associations have been described for all species in this genus (Lobb and Orth 1988, Vives 1990, Maurakis et al. 1991, Maurakis and Roston 1998). While associations with centrarchids typically involve a single associate species, chub nest association often involves multiple associates spawning with the host (Reighard 1943, Miller 1964). Sabaj et al. (2000) noted up to three associates spawning on a *Nocomis* nest, however, a genetic analysis by Cashner and Bart (2010) identified species that were not observed spawning on the nests. In addition to hosting multiple species, *Nocomis* nests provide viable spawning habitat in areas where habitat degradation and siltation are substantial (Johnston 1994b, Peoples et al. 2010).

Chubs have been described as a keystone species because their nests support numerous associates. Hitt and Roberts (2011) concluded that *Nocomis leptocephalus* and *N. platyrhynchus* were keystone species in two New River tributaries; these mound-building species allowed their nest associates to better persist and colonize headwater streams. In Wisconsin, Vives (1990) considered the hornyhead chub *N. biguttatus* to be a keystone species.

Wallin (1992) and Peoples and Frimpong (2013) both concluded that chub nest association is a mutualistic interaction. Nest associates benefit from increased brood survival (McKaye and Oliver 1980) via the parental care of the host (Johnston 1994b) and from the habitat created by the host (Cooper 1980, Vives 1990, Peoples and Frimpong 2013). Hosts benefit from reduced predation risk on their eggs in the presence of associate eggs. In the New River watershed, there are 11 different *Nocomis* nest associates (Pendleton et al. 2012). Using species reproductive traits and rarity, Pendleton et al. (2012) determined that associates could be

classified as strong (obligate or nearly so) or weak (facultative) based on life history and reproductive traits. Strong associates exhibited significant geographic overlap with *Nocomis* and were generally rare in terms of range size (Table 1.2) (Pritt and Frimpong 2010). Pendleton et al. (2012) suggested further that the conservation of these rare species may be best accomplished through the conservation of their host. Given the importance and ubiquity of *Nocomis* in eastern North America, association between a *Nocomis* host and their associates is an ideal system to study mutualism in lotic freshwater systems.

Chubs begin spawning from late April to early May (Reighard 1943). The spawning season begins with an increase in water temperature and a change in photoperiod; Miller (1964) noted an abundance of river chub *Nocomis micropogon* nests when water temperature reached 16°C. Normally, chubs place nests in the heads or margins of riffles with moderate flow (Lobb and Orth 1988, Maurakis et al. 1991). Chubs construct nests both diurnally (Maurakis et al. 1991) and nocturnally (Maurakis and Woolcott 1996). In a three-stage process (Figure 1.2), male chubs construct large gravel mounds on which they spawn (Reighard 1943; Maurakis et al. 1991). The male chub first excavates a pit in the streambed, builds a platform in the concavity, and continues to add gravel to the platform throughout the spawning process (Maurakis et al. 1991). Gravel size ranges greatly from 6.0 mm to 23.0 mm (Maurakis et al. 1991).

Male chubs excavate spawning pits on the upstream end of their nest; when a female chub enters the trough, she releases her eggs to be fertilized by the male (Maurakis et al. 1991). The male then fills the pit with gravel, covering the fertilized eggs. The male chub continues to cover additional eggs and provide parental care for several days until abandoning the nest (Wallin 1992). The eggs then hatch and fry hide in the gravel mound until their yolk sacs are completely absorbed. Throughout the spawning process, nest associates gather at the nesting site

and spawn on the nest; the chub host also covers the associate eggs with stones. While many of *Nocomis* associates actively spawn on the nest, host females and associates opportunistically consume eggs. Reighard (1943) observed possible egg predation of female river chubs on a nest. Sabaj et al. (2000) noted post-nuptial male stonerollers on active chub nests that did not appear to be spawning. Even though associates have been observed on active chub nests, it is unclear which species are spawning and which species are there as egg predators (Cashner and Bart 2010).

In this putative mutualism, hosts receive reduced egg predation and increased brood survival; Johnston (1994a) proposed that the dilution effect is the mechanism driving this interaction, Johnston (1994a) defined the dilution effect as the chance that a particular individual being consumed by a predator is reduced by the presence of other individuals. The host benefits from having higher ratios of associate eggs to host eggs, decreasing the probability of predation on host eggs (Johnston 1994a). Peoples and Frimpong (2013) corroborated the presence of the dilution effect in *Nocomis* nest association. In order to encourage this dilution, hosts should be increasingly apathetic or even conniving towards associate spawning after they have completed their own spawning events. Cashner and Bart (2010) found that egg composition on *Nocomis* nests consist of up to 83% associate eggs; Wallin (1992) found that associate eggs accounted for 93% of eggs on chub nests. The potential costs and benefits for both host and associate are numerous, illustrating the complexity of this interaction (Figure 1.3). Understanding the underlying mechanisms driving this interaction will further the knowledge of nest association and mutualisms as a whole.

The three-dimensional aspect of chub nests makes this study system unique. As the host deposits gravel over the spawning trough on the upstream end of the nest, the nest expands both

vertically and upstream (Figure 1.2). With this nest expansion, there is much variation in the suitability of spawning habitat on the nest. The nest may provide protection (from predators) advantage due to both depth and distance from the periphery of the nest. The eggs placed on the nest first will be eventually the deepest in the nest and the relative risk of predation should be lowest. The eggs placed later (e.g. associate eggs) are potentially at a greater risk of predation. The chub nest association would demonstrate the selfish-herd effect during the spawning and incubation process. As a modification of Hamilton's original hypothesis, the safest location on a three-dimensional chub nest may be the spawning trough at the upstream end of the nest, where most spawning on the nest takes place and the chub spends most of its time when on the nest. As the host deposits gravel and the nest grows upstream, eggs are slowly buried and the position of the fertilized eggs is shifted towards the bottom center of the nest.

In this thesis, I investigated *Nocomis* nest association for potential cheaters as well as several other underlying mechanisms facilitating this interaction for both host and associates. In Chapter 2, I utilized gonadosomatic index (GSI) to evaluate reproductive condition among the bluehead chub *N. leptocephalus* and all putative nest associates in Catawba Creek, Virginia. I found that GSI for obligate associates was correlated with host GSI in the weeks prior to and immediately following the onset of nest construction. Conversely, reproductive condition of facultative associates suggests that these associates are reproducing in the weeks prior to *Nocomis* spawning. In Chapter 3, I examined the relative risk of egg predation at several potential associate spawning locations. According to the results, egg predation rates on actively guarded chub nests do not differ significantly from the open substrate or unguarded, artificial nests. I used the eggs collected from this experiment to determine whether or not *Nocomis* hosts benefit from the selfish herd effect. Additionally, I conducted a separate experiment to assess the

effect of host parental care on egg predation rates. I found that parental care via egg covering significantly reduced egg predation rates on chub nests. These results suggest that egg covering is essential for reducing egg predation rates.

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Table 1.3. Expected pairwise outcomes and associated classifications of interspecific interactions. From Wootton (1998).

		<i>Species 1 influence on fitness of Species 2</i>		
		<i>+</i>	<i>0</i>	<i>-</i>
<i>Species 2 influence on fitness of Species 1</i>	<i>+</i>	<i>+,+</i> Mutualism	<i>+,0</i> Commensalism	<i>-,+</i> Predation
	<i>0</i>	<i>+,0</i> Commensalism	<i>0,0</i> Neutralism	<i>-,0</i> Ammensalism
	<i>-</i>	<i>-,+</i> Parasitism	<i>-,0</i> Ammensalism	<i>-,-</i> Competition

Table 1.4. The 11 Nocomis nest associates in the New River basin. Associations of rare species are in italics. Adapted from Pendleton et al. (2012).

Species	Association Strength
Rosyside Dace	<i>Strong</i>
Rosefin Shiner	<i>Strong</i>
Mountain Redbelly Dace	<i>Strong</i>
Saffron Shiner	<i>Strong</i>
Rosyface Shiner	Strong
Crescent Shiner	<i>Strong</i>
White Shiner	<i>Strong</i>
Blacknose Dace	Weak
Swallowtail Shiner	Weak
Central Stoneroller	Weak
Longnose Dace	Weak

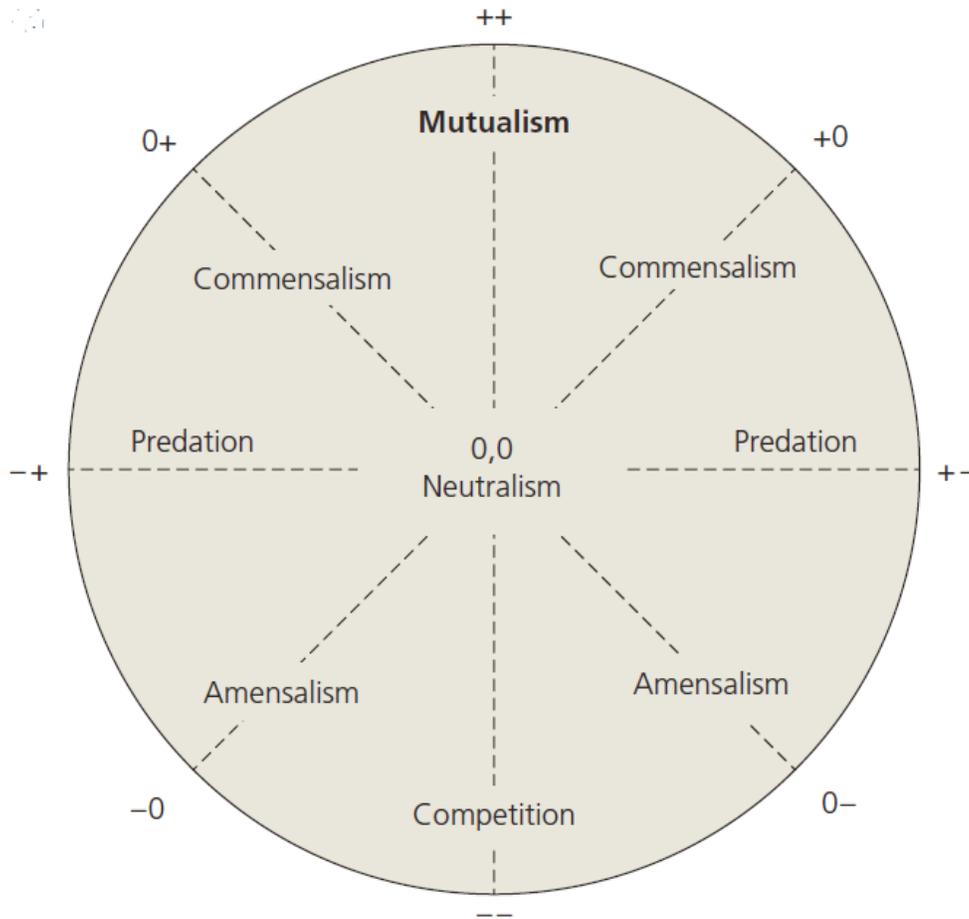


Figure 1.1. The coaction compass, first described by Haskell (1949) and recreated by Bronstein (2015). Demonstrates how interspecific interactions exist along a continuum. The magnitude of the outcome increases with distance from the origin (0, 0). Any movement along the circumference of the compass (due to changing context) denotes a shift in the net outcome for one or both partners

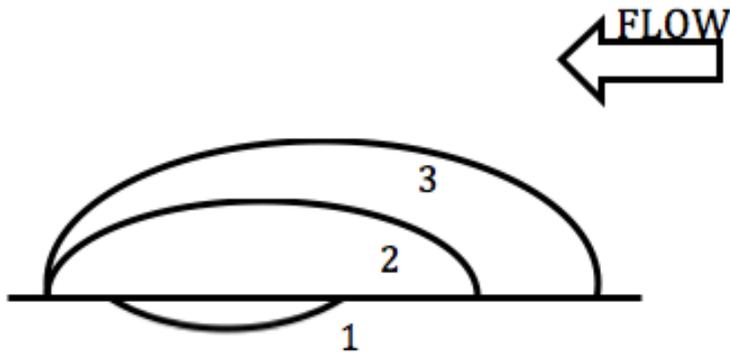


Figure 1.2. Three-stage construction of *Nocomis spp.* gravel mound: concavity (1), platform (2), and mound (3).

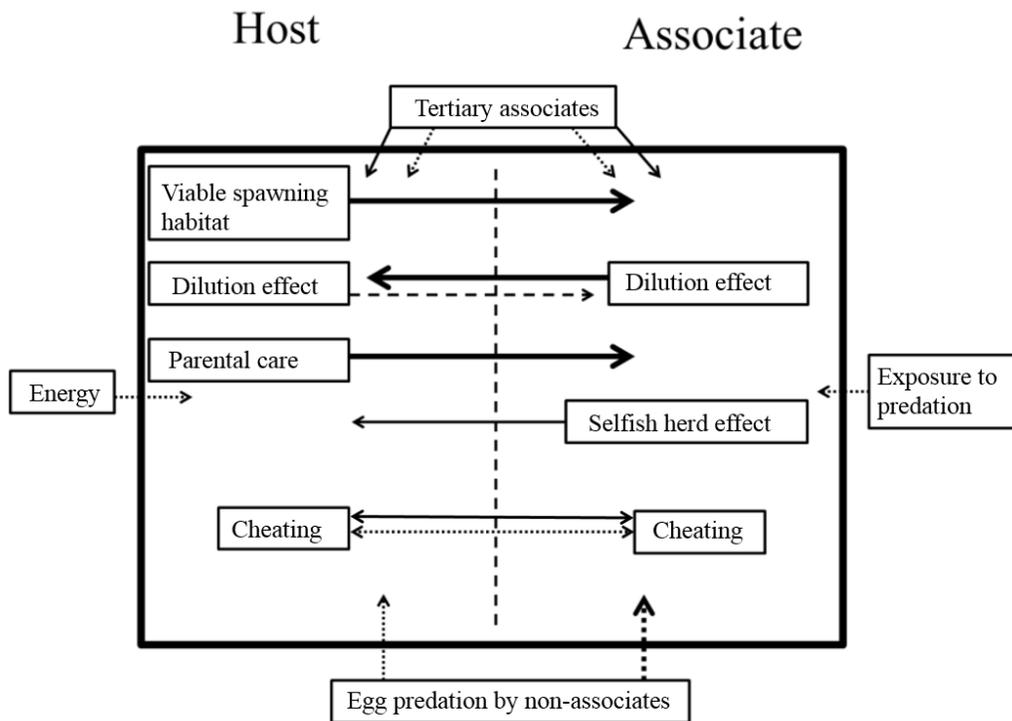


Figure 1.3 Benefits (solid arrows) and costs (dashed arrows) incurred through a two-species nest association. The thickness of the arrows denotes the degree of influence for each cost and benefit; thicker arrows denote greater influence, while thin arrows denote lesser or unknown influence. Thin edged boxes within the thick edged boxes are resources exchanged between host and associate (internal); boxes outside the thick edged box represent factors external to the nest association. Adapted from Peoples (unpublished).

CHAPTER 2

Running Title: Cheating in *Nocomis* nest association

Keywords: Mutualism, Nest Association, Positive Interspecific Interaction, Cheating, Gonadosomatic Index, *Nocomis*

Summary

1. Costs and benefits dictate the outcomes of biotic interactions; if the net outcome is beneficial for all participants, the interaction is considered mutualistic. Cheaters often evolve alongside mutualists, however, their effect on mutualisms is poorly understood. Understanding how cheating influences fitness outcomes of these interactions will help elucidate how mutualisms structure populations and communities.
2. Using multivariate analysis, I assessed a time series of gonadosomatic index (GSI) values to detect patterns in the reproductive timing of the bluehead chub *Nocomis leptocephalus* and its nest associates in a Virginia stream. Additionally, a nonparametric Spearman's ρ test was used to contrast reproductive condition among species. Complementary field observations were conducted to document the onset of chub reproduction, spawning events, and other behaviors.
3. Reproductive condition for all obligate associates was positively correlated with the chub host, whereas facultative associates were negatively associated with host GSI. Within groups (e.g. obligate, facultative), GSI of all species was significantly correlated with the GSI of other group members. All associates considered facultative spawned prior to the onset of *Nocomis* spawning.
4. One facultative associate, the central stoneroller *Campostoma anomalum*, spawned before chubs and was documented on all nests. These results indicate that stonerollers may be cheaters within *Nocomis* nest association.
5. Our research demonstrates that GSI can be used to effectively assess reproductive relationships among cyprinid communities in the eastern United States. Additionally, GSI trends may elucidate potential cheaters within *Nocomis* nest association,

Introduction

Participants in interspecific biotic interactions incur costs and benefits. Interactions are mutualistic when benefits outweigh costs to all participants. Mutualism has been documented in many systems, including endophyte-plant systems (Saikkonen et al. 1998, Hoeksema et al. 2010), insect-plant systems (Machado et al. 1996, Anderson and Midgely 2002, Lach 2003, Chamberlain and Holland 2009) and many other interactions (Boucher et al. 1982, Grutter 1995, Hay et al. 2004). Mutualisms are also abundant among aquatic invertebrates (Brown et al. 2012), microorganisms, and vertebrates throughout the world (McKaye 1985, Baba et al. 1990, Johnston 1994b, Grutter 1995, Hay et al. 2004, Peoples and Frimpong 2013).

Cheaters (a species or individuals within a species benefitting from the interaction without incurring costs) commonly occur in mutualisms (Bronstein 2009, Jones et al. 2009). While some cheaters abandoned their mutualistic tendencies over time (Bronstein 2001), other mutualists elect to exploit a mutualism when presented with the opportunity (Bronstein 2001, Bshary and Grutter 2002). The bluestreak cleaner wrasse *Labroides dimidiatus* typically removes ectoparasites from other fishes, however, *L. dimidiatus* opportunistically cheats by feeding on the living tissue of their partners (Bshary and Grutter 2002). Whereas cheaters may not completely invalidate mutualisms, they may alter the balance between costs and benefits for participants (Anderson and Midgely 2002). Hay et al. (2004) suggested that mutualisms commonly support key species within aquatic ecosystems and are as important as competition and predation. Given the ubiquity and significance of mutualisms, knowledge of how cheating determines interaction outcomes is a vital step toward understanding how mutualisms influence populations and communities.

Cheating and its influence on mutualisms have been thoroughly investigated only in a few systems such as figs-fig wasps (Machado et al. 1996, Anderson and Midgely 2002), yucca-

yucca moths (Pellmyr et al. 1996), mycorrhizae (Jones and Smith 2004) and cleaner fishes (Bshary and Grutter 2002). However, mutualisms in freshwater systems are generally poorly understood (Johnston 1994b, Brown et al. 2002, Galleti et al. 2008, but see Holomuzki et al. 2010), and even less is known about the role of cheating in those mutualisms (Skelton et al. 2013). One commonly mutualistic interaction is nest association, a reproductive strategy in which individuals of one fish species (nest associates) spawn in nests constructed by host species (Johnston and Page 1992). Normally, hosts contribute parental care (cost) in exchange for increased egg survival (benefit); associates incur increased egg predation (cost) in exchange for the host's nest and parental care that may ensure overall survival of offspring (benefit). Nest association is common on at least three continents (McKaye 1985, Baba et al. 1990, Johnston and Page 1992). In North American systems, associates are typically members of Cyprinidae and spawn in nests constructed by members of Centrarchidae or other cyprinid hosts (Hunter and Hasler 1965, Johnston and Page 1992).

Probably the most common nest association in North America occurs with *Nocomis* hosts. *Nocomis* construct gravel mounds for spawning that are used by over 30 nest associate species throughout North America. In most systems, at least three nest associates utilize *Nocomis* nests during spawning (Raney 1947). Associates of *Nocomis*, which do not care for broods, benefit from increased brood survival via the parental care of the host (Johnston 1994a) and from the unique physical characteristics of nests in exchange for a potential increase in egg predation (cost) (Vives 1990, Johnston 1994a). Because *Nocomis* can also benefit from a dilution effect (reduced predation risk on their eggs in the presence of associate eggs) in exchange for parental care (cost), nest association in this system is often mutualistic (Johnston 1994b, Peoples and Frimpong 2013).

Nocomis nest association provides an ideal study system for examining the role of cheating in freshwater mutualisms because the degree to which associates require their host for reproduction differs among species. Within the upper Catawba Creek system, the bluehead chub *Nocomis leptocephalus* is the primary nest builder. Seven putative associates of varying strength co-occur with the host. The obligate associates (species believed to require a nest-building host to spawn) include mountain redbelly dace *Chrosomus oreas*, rosefin shiner *Lythrurus ardens*, crescent shiner *Luxilus cerasinus* and rosieside dace *Clinostomus funduloides* (Pendleton et al. 2012). Given their reliance on *Nocomis* for reproductive success, it is unlikely that obligate associates frequently exploit this interaction. Central stoneroller *Campostoma anomalum*, longnose dace *Rhinichthys cataractae* and blacknose dace *R. atratulus* are all classified as facultative associates; these associates typically utilize simple open-substrate broadcast spawning, but opportunistically spawn as nest associates (Johnston and Page 1992, Pendleton et al. 2012). While these species have been observed on nests, it is unclear how often these associates spawn with *Nocomis* hosts (Cooper 1980, Jenkins and Burkhead 1994, Sabaj et al. 2000, Cashner and Bart 2010) and which (if any) are potential cheaters.

It is possible that some species spawn prior to the onset of *Nocomis* nesting, and then visit nests to feed on eggs. If these species are present on nests and are not driven away by tending male *Nocomis*, they could receive benefits (food) without incurring costs (contributing to host dilution effect), and thus be cheating. Identifying the roles played by each species in this mutualism will yield a better understanding of this system in particular, and of freshwater mutualisms in general. Specifically, this research provides insight into the effect of association strength on the likelihood of cheating and how cheating may alter the outcome of this interaction.

Using gonadosomatic index (GSI) as a measure of reproductive condition, we collected samples of *N. leptocephalus* and seven known nest associates from Catawba Creek, Virginia over 18 weeks. We used nonmetric multidimensional scaling (NMDS) to compare reproductive condition among species. The goal was to elucidate the potential for cheating among *Nocomis* nest associates exhibiting a gradient of association strength from facultative to nearly obligate. Given their strong reliance on *Nocomis* for reproduction, GSI of obligate associates should track closely to *Nocomis* throughout the spawning season. Alternatively, facultative associates may exhibit shifts in gonadal condition independent of *Nocomis*. Accordingly, we hypothesize that associates whose GSI does not track *Nocomis* closely, but are observed frequently visiting nests, exhibit the highest potential for cheating.

Methods

The study site for this experiment is Catawba Creek, a 4th Strahler-order tributary to the James River in Roanoke County, Virginia, USA (80.074686° W, 37.391944° N). Catawba Creek is an ideal site because it is a mid-size Appalachian stream representative of the range of the host, the bluehead chub *Nocomis leptocephalus*. Additionally, nearly all of the co-occurring cyprinids have been classified as nest associates of *N. leptocephalus*. Strong associates in this system include mountain redbelly dace *Chrosomus oreas*, rosyside dace *Clinostomus funduloides*, rosefin shiner *Lythrurus ardens*, and crescent shiner *Luxilus cerasinus*; weak associates include central stoneroller *Campostoma anomalum*, blacknose dace *Rhinichthys atratulus* and longnose dace *R. cataractae* (Pendleton et al. 2012). From April to August 2013, we collected weekly samples of bluehead chubs and their nest associates using backpack electrofishing; these samples represented the sexually-mature populations of *N. leptocephalus* and their associates. Due to heavy rainfall events in June and July, we were unable to sample on

weeks 5, 9, 13, and 14. We identified all individuals to species in the field and released any species not used in the experiment. We kept females for analyses because they exhibit greater and more variable range of gonadal mass throughout the spawning season (Jennings et al. 2012). Because some associates are locally rare, we sought to collect at least 5 sexually-mature females (per species, based on secondary sex characteristics) per visit. We preserved all samples in formalin for laboratory processing.

Additionally, we conducted daily, qualitative surveys to determine the onset of chub spawning. Two workers surveyed for chub nests, walking on either side of the stream and wearing polarized sunglasses to reduce surface glare. When observers located an active nest, they watched each nest for at least 30 minutes and noted all species observed on the nest and any activities (e.g. feeding, spawning, defense). Given the conspicuity of chub nests, we are confident no nests went overlooked.

Prior to processing, we soaked samples in water overnight to remove formalin. We measured all individuals to the nearest mm (total length) and recorded wet weight (g). We extracted gonads and recorded their wet weight (g). Additionally, we recorded the wet weight (g) of viscera and the empty body cavity. We stored gonads and empty body cavities in 70% ethanol. We calculated gonadosomatic index (GSI) for each individual using Nikolsky's (1963) equation:

$$\frac{\textit{gonadal weight}}{\textit{total weight}} \times 100$$

We then calculated average weekly gonadosomatic index (GSI) values for each species. Because some associates were rarer than others, we then pooled GSI values based on strength of association (*sensu* Pendleton et al. 2012); these groupings included strong associates, weak associates, stoneroller, and chub. By grouping species, the increased sample size allowed us to better visualize weekly GSI trends. In order to account for missing weeks, we filled sampling

gaps by averaging GSI values from the preceding and following collections. Using the average weekly GSI for each species, we performed a multivariate, pairwise correlation to assess relationships among species. A nonparametric Spearman's ρ test was then used to test for significant relationships among the pairwise correlations.

We used nonmetric multidimensional scaling (NMDS) to identify similarities in reproductive condition among weeks and species. We performed a two-dimensional NMDS in R v.3.0.3 using the *vegan* package (*metaMDS* function) on a matrix of Bray-Curtis dissimilarities. We interpreted axis scores based on their correlations with the weekly GSI values. We plotted both “time scores” (rows, weeks) and “species scores (columns, species) in ordination space (two-dimensional space depicting dissimilarity among species' GSI values and weeks), and used plots to visually examine for patterns among reproductive groups.

Results

We collected a total of 521 individuals of eight species over 18 weeks. Because rosefin shiner was not consistently present in collections, we omitted it from the analysis. We assessed the potential impact of individual size (TL, mm) for all individuals to ensure that GSI values were not biased by variation in fish size (see Brown and Murphy 2004) (Figure 2.1). Chubs began spawning on 22 May 2013 (Week 7). Out of approximately 20 observed nests, obligate associates and stonerollers were present on all nests. Mountain redbelly dace, rosieside dace, and crescent shiner frequently spawned on active chub nests; spawning events for obligate associates were regularly documented, with mountain redbelly dace regularly spawning on nests. We consistently observed stonerollers on chub nests, but never observed any spawning events. Instead, large male stonerollers (identified by larger body size and presence of tubercles) regularly excavated pits and burrowed into the substrate, possibly to feed on eggs. We never

observed any female stonerollers in the vicinity of a chub nest. Weak associates (blacknose dace and longnose dace) rarely congregated on chub nests; only a single blacknose dace visited a chub nest, but did not spawn or consume eggs. Gonadosomatic index of chubs and strong associates decreased in the week following the onset of chub spawning.

Gonadosomatic index (GSI) of longnose and blacknose dace began to decrease four weeks prior to chub spawning, and GSI of stonerollers decreased dramatically in the week before chub spawning (Figure 2.2). Furthermore, GSI of strong associates increased consistently until the week after chubs began spawning (Figure 2.2).

Time scores were separated in ordination space based on timing (before or after the onset of chub spawning) along the MDS2-axis. Most weeks before chub spawning demonstrated negative MDS2 values, while the weeks following the onset of chub spawning had positive MDS2 values (Figure 2.3). Week 6 demonstrated a positive MDS2 value, probably because of its proximity to the onset of chub spawning. Week scores on this axis were negatively correlated with all weak associates, but were most strongly correlated with longnose dace ($r = -0.74$); this axis was positively correlated with all strong associate species, but most strongly bluehead chub ($r = 0.49$) and crescent shiner ($r = 0.64$) (Table 2.1). NMDS species scores separated along the MDS2-axis based on association strength. Strong associates demonstrate positive MDS2 values, while weak associates demonstrate negative MDS2 values. Bluehead chub and strong associates grouped closely, indicating similarity in spawning dates. Importantly, stonerollers grouped more closely with weak associates.

The Spearman's ρ test revealed a weak relationship between GSIs of chub and crescent shiner ($p = 0.095$) (Table 2.2). While not significant, chubs also demonstrated a suggestive relationship with mountain redbelly dace ($p = 0.106$) and rosyside dace ($p = 0.135$). Pairwise

correlations among strong associates were all statistically significant (Table 2.2). Similarly, all relationships between weak associates (including stonerollers) were significant (Table 2.2). The significant, pairwise relationships within groups further justify the grouping of species by association strength.

Discussion

This study seems to be the first to assess reproductive condition across an entire cyprinid community using GSI. Through nonmetric multidimensional scaling, we found that the GSI patterns varied among strong and weak associative groups. The GSI of all groups fluctuated erratically after chubs began spawning, though this may be explained by the reproductive strategies of the associates. Many of the associates are batch spawners and may reproduce multiple times between April and late June (Jenkins and Burkhead 1994). Our sampling strategy may have collected individuals in various reproductive states, obscuring GSI patterns in later weeks. While fish size and season may influence GSI values (Brown and Murphy 2004), total length (mm) of collected individuals remained constant throughout the sampling period (Figure 2.1). The initial drop in GSI, however, is a strong indicator of a spawning event. Chubs demonstrated a decrease in GSI during week 7, followed by strong associates in week 8. Alternatively, weak associates and stonerollers seemed to spawn independently over the several weeks before the onset of *Nocomis* spawning. The absence of longnose dace and blacknose dace on chub nests, coupled with an early drop in GSI, suggests that they are not nest associates within the study system. NMDS scores for these weak associates grouped closely with weeks 1-6, indicating further that both species spawn before *Nocomis*.

Given their presence on nests, differing GSI patterns and potential egg foraging behavior, stonerollers may be exploiting *Nocomis* nest association as cheaters. We documented male

stonerollers on every nest observed, but we never witnessed stoneroller spawning events in over 20 hours of collections and observations; throughout our observations, female stonerollers were largely absent from chub nests. Larger post-nuptial males commonly excavated pits and burrowed into the substrate, suggesting foraging behavior or spawning preparation. Spawning stonerollers have been documented on chub nests (Reighard 1943) and their larvae have been identified on nests (Peoples et al., pers. comm), though most studies have not observed spawning activity. Miller (1964) suggested that stonerollers had already spawned prior to chubs. Sabaj et al. (2000) noted post-nuptial male stonerollers on chub nests possibly foraging for eggs. Jenkins and Burkhead (1994) suggested that stonerollers forage for eggs in the nests of other species. Stonerollers are also known to spawn in rainbow trout *Oncorhynchus mykiss* redds; in high abundances, stoneroller pit excavation destroyed redds and greatly reduced the reproductive success of rainbow trout in Great Smoky Mountain National Park (Lennon and Parker 1960).

In this system, stonerollers began spawning a week before *Nocomis* began nest construction. Their presence on nests suggests that they may be there to forage for eggs, although there is the possibility that they are preparing for another round of reproduction. Given the findings of previous studies (Reighard 1943, Lennon and Parker 1960), it is possible that large male stonerollers were excavating spawning pits on nests, not foraging for eggs. Alternatively, stonerollers may exhibit multiple life history tactics throughout their lifespan. Large, tuberculate males may construct pits and spawn independently of *Nocomis*, visiting nests afterwards to forage for eggs. Smaller male stonerollers may be incapable of excavating a spawning pit and elect to spawn on chub nests. The latter strategy is evidenced by Sabaj et al. (2000), who documented small male stonerollers interrupting spawning clasps between a *Nocomis* hosts and females.

The role of stonerollers as cheaters or associates may be driven by context. Bronstein (2009) argued that the outcome of mutualisms varies based on biotic and abiotic context. Stonerollers typically begin spawning when water temperature reaches 10.8°C (with peak spawning between 13-15°C), whereas chub spawning begins around 13°C (Jenkins and Burkhead 1994). If water temperature rises slowly, stonerollers may opt to spawn independently. Spring 2013 was particularly cold in Catawba, Virginia, with snowfall recorded as late as April 4th. With a delayed increase in water temperature, stonerollers in Catawba Creek likely elected to spawn before chubs began nesting. Within NMDS space, stoneroller scores are closely grouped with the week scores prior to chub spawning; this indicates further that stonerollers spawned independently from chubs. Alternatively, warmer springs may increase the probability that stonerollers spawn on chub nests. Abiotic context (e.g. water temperature) may determine whether or not a stoneroller exploits this mutualism. While it is unclear whether or not stonerollers are cheaters in Catawba Creek, the outcome of the chub-stoneroller interaction appears to be influenced by abiotic context; stonerollers are more likely to cheat this nest association when the onset of spawning for both species overlap.

Initial drop in GSI for strong nest associates closely followed that of the chub host. Strong associates were recorded on every chub nest and were never observed exhibiting behavior resembling egg foraging (e.g. excavating nest substrate). NMDS scores for strong associates were grouped with both the host scores and the week scores following the onset of chub spawning. Additionally, correlations between the chub host and strong associates were all marginally significant. The positive correlation between host GSI and strong associate GSI corroborates that strong associates require a host for reproduction; species reliant on a host for

reproduction are less likely to exploit an association. Similar to *Nocomis*, strong associates typically begin spawning when water temperatures reach 13°C (Jenkins and Burkhead 1994).

The obligate-facultative continuum of associative behavior may be an important contributor to whether or not species function as cheaters in mutualisms. Bronstein (1994) suggested that the outcomes of facultative mutualisms are more variable than outcomes of obligate mutualisms. Weak associates often form facultative mutualisms with hosts in which reproductive success is not entirely dependent on the host, but may be improved by the interaction (Bronstein 2009). Both mutualist behavior and ecological context can shift the outcome of facultative mutualisms from mutualistic to antagonistic (Thompson 1988, Bronstein 1994). Within chub nest association, the availability of viable spawning habitat and/or spring water temperature may shift the behavior of weak associates and consequently, the interaction outcome. Alternatively, strong associates often establish obligate mutualisms with chubs and depend on them for reproductive success. Furthermore, the presence of cheaters in a mutualism often augments the necessity of obligate mutualists (Ferrière et al. 2007). If a partner is able to reduce instability within an environment (e.g. creating spawning substrate), this increases the importance of a mutualistic interaction (Burns 1993). By constructing nests, chubs provide a consistent and predictable supply of spawning habitat for associates. Associates that have coevolved with a host rely on their services; this facilitates the establishment of obligate mutualisms (Addicott 1984, Burns 1993). Strong associates that require a chub host to spawn are unlikely to exploit the system because of their dependency on a host for reproductive success.

Additional benefits gained by the *Nocomis* host may reduce the potential effect of cheating in this association. One such benefit, the dilution effect, reduces the probability of host eggs being consumed when in the presence of associate eggs. This mechanism has been

documented in nest associations between smallmouth bass *Micropterus dolomeiu* and longnose gar *Lepisosteus osseus* (Goff 1984) and between green sunfish *Lepomis cyanellus* and redbfin shiner *Lythrurus umbratillis* (Johnston 1994b). Peoples and Frimpong (2013) also presented evidence that the dilution effect is an important mechanism in *Nocomis* nest association. Associate eggs constitute the vast majority of eggs on a chub nest, with values ranging from 84% (Cashner and Bart 2010) to 97% (Wallin 1992). The presence of so many associate eggs may completely negate the effect of egg predation on host's egg survival.

The results of this study demonstrate the need for a better understanding of the role each species plays in nest association. While nonmetric multidimensional scaling (NMDS) effectively grouped species by time and association strength, GSI patterns were unable to clearly track reproductive patterns in the later weeks. This may be due in part to small samples size; future studies using GSI to track reproductive condition in cyprinids should optimize model strength by increasing sample size. The methods and analysis used in this study may be readily used to assess reproductive relationships among cyprinids in other freshwater stream systems. Previously, GSI has been used to assess the reproductive traits of single cyprinid species (Johnston and Knight 1999).

This study suggests that stonerollers spawn before chubs and may be visiting nests to forage for eggs, although evidence suggests from other studies suggests that stonerollers sometimes spawn on chub nests. A broad genetic analysis of eggs found on chub nests (e.g. Cashner and Bart 2010) will grant better insight into which species are truly spawning on nests and which species are foraging for eggs. This study provides novel insight into mutualisms in freshwater nest association; the evidence from this study illustrates that association strength (e.g. strong, weak) influences the likelihood of cheating for nest associates. While the results from this

study may not directly apply to other systems, it demonstrates that both biotic and abiotic context may drive the actions of weak associates. Future investigation will further elucidate the identity and effects of cheaters in these understudied systems.

Conclusions

Overall, our findings demonstrate that the reproductive condition of obligate associates are linked with *Nocomis* hosts. Conversely, facultative associates seem to be spawning independently in the weeks before chubs. This is corroborated by both the Pearson correlation and the arrangement of species and weeks in NMDS space. Reproductive condition of obligate associates was positively correlated with *Nocomis* along the NMDS2-axis. Additionally, chubs and obligate associates were tightly grouped with the weeks following chub spawning within the NMDS space. With only one season of GSI data, the conclusions of this study are tentative; several years of reproductive data would provide a more concrete perspective of reproductive relationships. The techniques used in this study can be readily applied to any system where reproductive relationships are unclear. By utilizing the information gained from this study, cyprinid communities may be managed much more efficiently.

While it is still unclear which, if any, associates are exploiting this mutualism, our findings provide evidence that association strength influences the potential for cheating. Because obligate associates require the presence of a host for reproductive success, they are less likely to exploit the relationship; this is corroborated by their positive correlation with *Nocomis* GSI. Given these findings, certain facultative associates may be more likely to be cheaters within the system. Both members of the genus *Rhinichthys* spawned independently of *Nocomis* and were never documented on the nest; this suggests that they do not rely on a *Nocomis* significantly for reproductive success and are not visiting the nest to forage for eggs. Conversely, the GSI patterns

for the central stoneroller suggests that they may spawn in the weeks before *Nocomis*. Stonerollers were documented on all nests, though no spawning events were ever observed. Given their pervasive presence on chub nests, stonerollers are the most likely candidate for cheating within the mutualism. In addition to our findings, an extensive genetic analysis is required to determine whether or not stonerollers are depositing eggs on chub nests. If stoneroller eggs are extensively documented on *Nocomis* nests, then they may not be cheating this mutualism as our findings suggest; additional research is required to explore the potential for multiple reproductive strategies among male stoneroller size classes.

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Table 2.1. Pearson correlations between nonmetric multidimensional scaling (NMDS) axes and species scores. Correlation values demonstrate the relationship between species scores and the axes. Strong associates, shown in bold font, were all positively correlated with MDS2; weak associates were all negatively correlated with MDS2.

Species	MDS1	MDS2
Bluehead Chub	0.29	0.49
Mountain Redbelly Dace	0.87	0.31
Rosyside Dace	0.73	0.33
Crescent Shiner	0.55	0.64
Blacknose Dace	0.55	-0.51
Longnose Dace	0.42	-0.74
Central Stoneroller	0.74	-0.50

Table 2.2. Pairwise correlations of gonadosomatic index (GSI) values between all species. A nonparametric Spearman's ρ test was used to determine significant correlation values. Species codes are: bluehead chub (BHC), blacknose dace (BND), crescent shiner (CRS), longnose dace (LND), mountain redbelly dace (MRB), rosyside dace (RSD), and central stoneroller (CSR). *Indicates ($p < 0.05$), †Indicates ($p < 0.1$, but > 0.05)

	BHC	BND	CRS	LND	MRB	RSD	CSR
BHC	1						
BND	0.195	1					
CRS	0.366 [†]	0.155	1				
LND	-0.293	0.475 [*]	-0.349	1			
MRB	0.419	0.360 [†]	0.656 [*]	0.188	1		
RSD	0.188	0.060	0.537 [*]	0.005	0.771 [*]	1	
CSR	0.002	0.646 [*]	-0.004	0.658 [*]	0.511	0.338	1

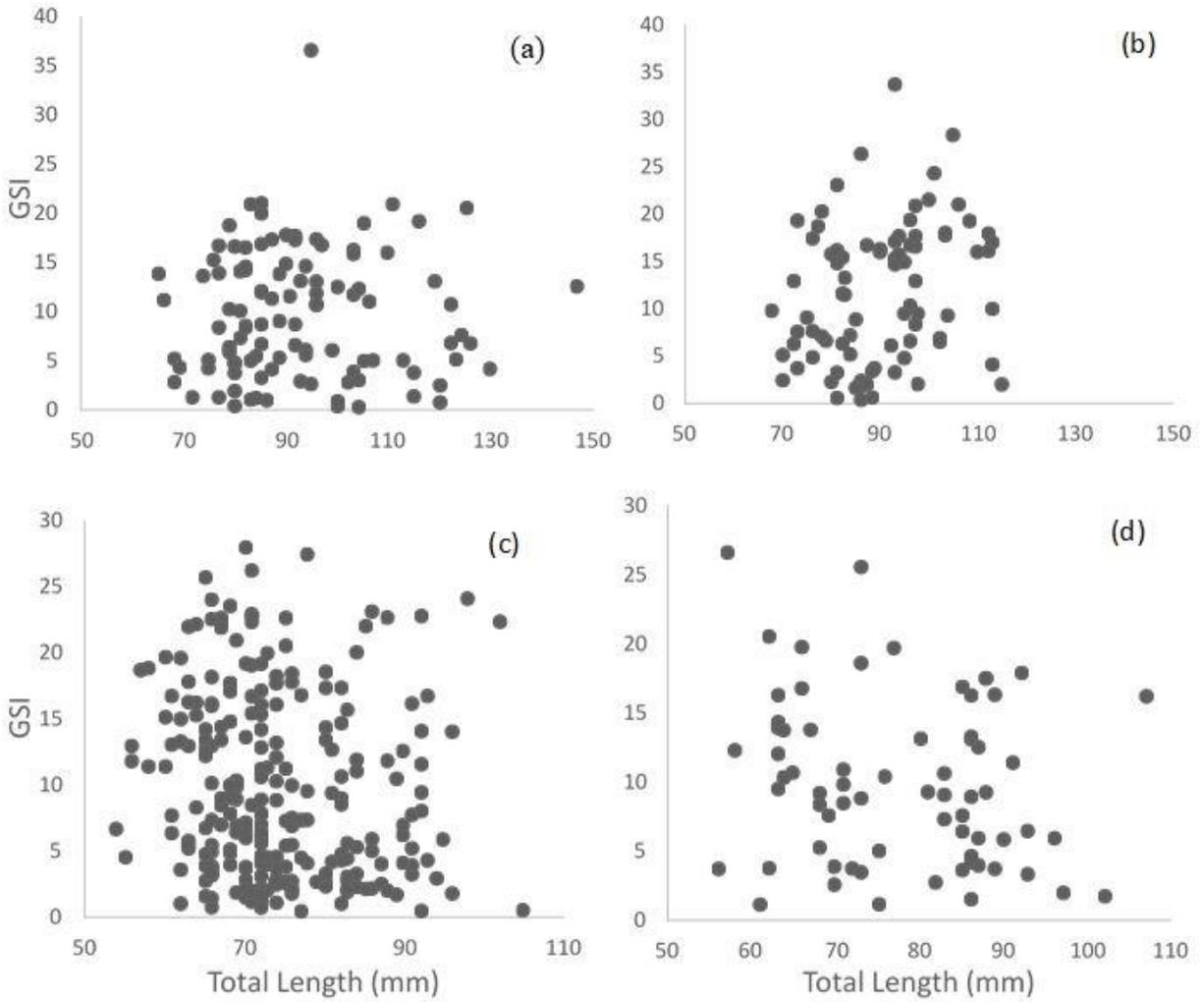


Figure 2.1. Relationship between total length (mm) and GSI for collected individuals ($n = 521$) in associative groups over 18-week sampling period (9 April -8 August 2013). Associative groups are (a) chub, (b) stoneroller, (c) strong associates, and (d) weak associates.

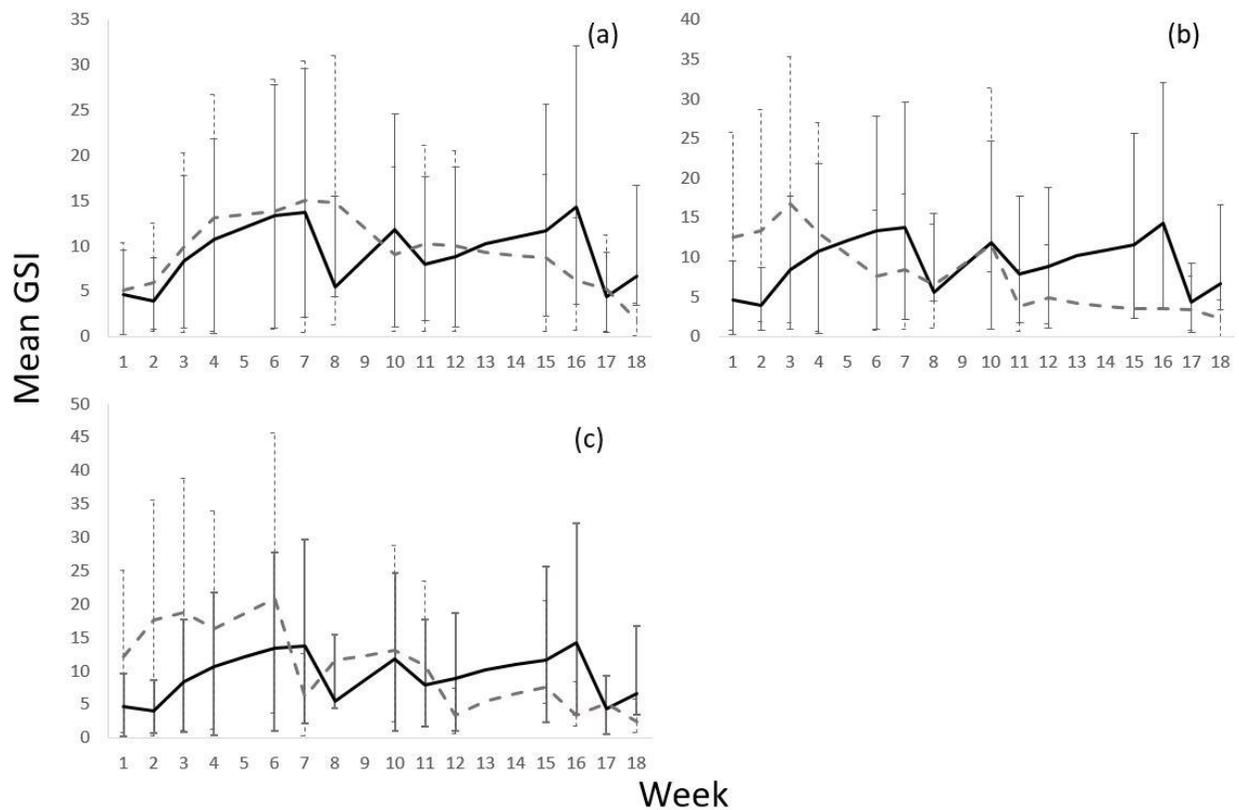


Figure 2.2. Mean weekly gonadosomatic index ($\pm 2SE$) of reproductive groups (a-c) of stream fishes in Catawba Creek, Virginia from 9 April to 8 August 2013. Bluehead chub spawning began on Week 7 (22 May 2013). In above figure, bluehead chub is represented by the solid black line and the compared group is represented by the gray dashed line. Strong associates (a) include mountain redbelly dace, rosieside dace, and crescent shiner. Weak associates (c) include blacknose dace and longnose dace.

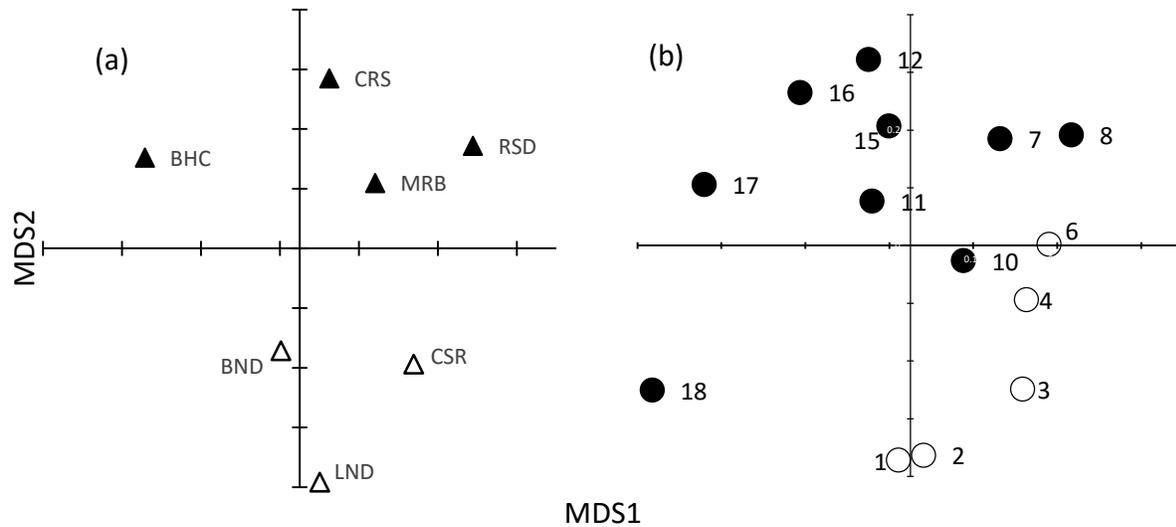


Figure 2.3. Species (GSI) scores (a) and time (week) scores (b) arranged within nonmetric multidimensional scaling (NMDS) ordination space based on a Bray-Curtis dissimilarity matrix (stress = 0.09). Dissimilarity between scores increases with distance within NMDS space. Strong associates (solid triangles) and weak associates (open triangles) are clearly separated along the MDS2 axis. Weeks preceding chub spawning (open circles) are grouped closely with weak associates, while weeks following chub spawning (solid circles) group closely with strong associates. Species codes: bluehead chub (BHC), mountain redbelly dace (MRB), rosieside dace (RSD), crescent shiner (CRS), blacknose dace (BND), longnose dace (LND) and central stoneroller (CSR).

CHAPTER 3

Authors: Stephen Floyd, Brandon K. Peoples, Emmanuel A. Frimpong and Eric Hallerman

Running Title: Selfishness, risk, and parental care

Keywords: Mutualism, *Nocomis*, Nest Association, Positive Interspecific Interaction, Selfish Herd Effect, Relative Risk, Parental Care, DNA Barcoding

Summary

1. In eastern North America, one common, often mutualistic, interaction among stream fishes occurs between a *Nocomis* host and other cyprinid species collectively called nest associates. Despite its prevalence, there has been limited exploration of the underlying mechanisms that make the *Nocomis* nest association adaptive.

2. We assessed the risk of egg predation by placing an egg-covered cobble at four potential associate spawning locations. Results from a one-factor ANOVA with a block effect suggest that relative risk did not differ significantly among locations. Behavioral observations of the host/associate found that 72.9% of all activity was documented on the natural nest, with 31% of host activity being covering the spawning trough. Checking for exposed eggs and feeding constituted 87% of stoneroller activity.

3. A second study assessing the role of parental care via egg burying demonstrated that predation was significantly lower for buried eggs compared with eggs deposited on the surface. Correspondingly, a third study revealed that the majority of eggs were located in the bottom, upstream section of *Nocomis* nests.

4. Lastly, a genetic analysis of larvae hatched in the laboratory from eggs collected from four nest sections was conducted. The majority of individuals were identified as central stoneroller (70%) or bluehead chub (23%). Unexpectedly, few putative strong associates were identified in the genetic analysis.

5. These experiments demonstrate the importance of parental care via egg burying. The host positions the majority of eggs in the bottom of the nest, reducing the relative egg predation risk for both host and associates.

Introduction

Participants in interspecific interactions accrue both costs and benefits. Mutualism, one such interaction that results in a net benefit for both participants, is common in nature. Despite its ubiquity, knowledge of mutualism lags far behind that of competition and predation (Bronstein 2009). Mutualisms in freshwater systems are particularly understudied (Johnston 1994b, Brown et al. 2002, but see Holomuzki et al. 2010). One commonly mutualistic interaction among stream fishes is nest association, a reproductive strategy in which individuals of one fish species (nest associates) spawn in nests constructed by host species (Johnston and Page 1992). A common nest association occurs between *Nocomis* hosts (Cyprinidae) and several cyprinid associates—over 35 species in North America. While *Nocomis* nest association is common, there has been limited exploration of the underlying mechanisms that make it adaptive; existing studies have determined that parental care is vital for nest associates, while hosts benefit from the dilution effect (Wallin 1992, Johnston 1994a, b, Shao 1997a, b, Peoples and Frimpong 2013). Knowledge of these mechanisms will promote both a better understanding of nest association and of freshwater mutualisms in general.

Nest associates may benefit from the unique physical characteristics of chub nests, or from the parental care provided by hosts. Adult male *Nocomis* construct conspicuous gravel mound nests for spawning, selecting a narrow range of substrate sizes and current velocities to maximize aeration while reducing flow and the potential of fungal growth (Maurakis 1998, Wisenden et al. 2009, Peoples et al. 2014). *Nocomis* can facilitate associate reproductive success because their nests are often the only source of unsilted, concentrated gravel (Vives 1990, McManamay et al. 2010, Peoples et al. 2011) available for nest associates, which are lithophilic spawners (requiring clean gravel substrate, *sensu* Balon 1975). By facilitating reproductive

success, *Nocomis* hosts alleviate limitations on associate recruitment in systems where spawning habitat is limited (Vives 1990). To construct nests, male *Nocomis* first excavate a pit in the streambed; they then build a platform and continue to add gravel to the platform throughout the spawning process (Figure 3.1, Maurakis et al. 1991). Nest diameter averages about 70 centimeters and nest height can reach beyond 13 cm (Raney 1947, Sabaj et al. 2000). Males excavate spawning pits on the upstream end of nests, and both chubs and associates deposit eggs in these pits throughout the spawning process lasting several days. Hosts cover the spawning pit with gravel after individual spawning events. As this process is repeated, nests become increasingly larger and eggs are buried deep in nests, potentially safe from egg predators. Associates may continue to spawn for several days after the male *Nocomis* has finished spawning and abandoned the nest. Tending male *Nocomis* may also chase away potential egg predators (Hankinson 1932, Vives 1990). The ancestral spawning mode of most nest associates is simple broadcast spawning (Johnston and Page 1992). By utilizing nest association, associates can achieve lower egg predation relative to open broadcast spawning (Johnston 1994a).

Despite having alternative reproductive tactics, nest associates consistently spawn on chub nests. This suggests that certain factors associated with chub nests alleviate egg predation risk for these associates. To determine whether associates are attracted to the unique physical characteristics of nests or the host-provided parental care, researchers have presented known associates with artificial, unattended nests. Associates failed to aggregate over unguarded nests, and eggs outside nests or in unguarded nests were preyed upon quickly, demonstrating that parental care is vital for brood survival (Wallin 1992, Johnston 1994a, Shao 1997). The common conclusion offered by these studies is that associates benefit from host parental care, not the physical structure of the nest. While these studies clearly demonstrate the importance of parental

care, they do not directly negate the role of nest physical structure. A host will not reproduce or provide parental care without its nest, and associates will not spawn with hosts who do not create a nest. Therefore, the benefits of parental care are inaccessible in the absence of a host nest. However, nests are highly conspicuous and attract potential egg predators. The conspicuity of a nest might make it a worse place for brood survival than on open substrate, unless a host actively defends the brood. Accordingly, we examined relative predation risk at multiple potential associate spawning locations: open substrate, artificial/unguarded nests, and natural/guarded nests. We hypothesized that a guarded nest is likely the safest place for associates to deposit eggs. However, being equally conspicuous and lacking a vigilant host, we hypothesized that an unguarded nest is likely the most perilous place to deposit eggs.

Nocomis hosts provide parental care in two ways: nest guarding and egg burying. During the spawning process, hosts display aggressive behavior to any individual perceived as a threat. This aggression is typically focused towards conspecifics, but can also be directed towards nest associates (Miller 1964, Maurakis et al. 1991, Herrington and Popp 2004). The nest guarding behavior of sunfishes is well-documented; a vigilant sunfish host readily defends the entirety of their saucer-shaped nests and significantly reduces egg predation (Hunter and Wisby 1961, Johnston 1994a, Shao 1997a). Unlike sunfish hosts, *Nocomis* are unable to efficiently guard the entire nest. Instead, *Nocomis* focus the majority of parental care over their spawning pits (Vives 1990). Thus, the conclusions of nest guarding drawn from sunfish hosts may not be comparable in this system. However, hosts typically ignore associates and focus on depositing gravel over the spawning pit (Miller 1964). Because these nests can be covered with hundreds of associates, protecting nests from individual egg predators may be futile (Wallin 1989). Whereas several studies have addressed the importance of *Nocomis* parental care (Wallin 1992, Johnston 1994a),

none have investigated the relative importance of nest guarding or active defense and egg burying.

The construction and maintenance of a nest is energy intensive, however, *Nocomis* hosts permit associates to utilize their nest; which factors of this interspecific mutualism offset the energy costs for the host? Hosts often (but not always, *see* Baba et al. 1990 and Fletcher 1993) benefit from nest association. Several studies suggest that hosts benefit from a “dilution effect”, reduced predation risk of host eggs caused by high proportions of associate eggs (Goff 1984, Wallin 1992, Johnston 1994b, Peoples and Frimpong 2013). Another potential mechanism benefitting hosts is the “selfish-herd effect”, in which individuals at the center of a group experience lower predation probability than those at the periphery (Hamilton 1971). Few studies have sought to explicitly examine the selfish herd in nest associations (Goff 1984), and mixed evidence for this mechanism has been presented (McKaye and Oliver 1980, Johnston 1994b). McKaye et al. (1992) concluded that the selfish-herd effect was present in an interspecific brood between cichlids and the catfish *Bagrus meridionalis*. Host *B. meridionalis* permitted cichlid young to join their young, however, forced them to the periphery of the brood; survival of *B. meridionalis* young was six times higher when cichlid young were present on the periphery (McKaye 1985). This application of the selfish-herd effect took place after the spawning and hatching of the eggs, whereas chub nest association would demonstrate the selfish herd effect during the spawning and incubation process. This mechanism would be evident in *Nocomis* nest association if host eggs were situated most remotely (in terms of depth) and closest to defended locations relative to distribution of associate eggs.

Nocomis nesting presents a unique system for examining the selfish-herd effect because of the three-dimensional nest structure. All previous studies of egg survival from mixed-brood

nests have focused on host species that construct shallow saucer-pit nests (*e.g. Lepomis, Micropterus*), which lack a substantial vertical component. Because *Nocomis* typically begin spawning before associates, and because associates continue spawning well after tending male *Nocomis* have abandoned nests, it is possible that host eggs would become concentrated in the bottom-upstream of the nest as the male *Nocomis* continues to bury eggs deposited in the spawning pit at the upstream end of the nest. Accordingly, associates spawning after chubs would be forced to deposit eggs on top of host eggs, allowing *Nocomis* to benefit from the selfish herd effect (and are presumably also the dilution effect).

Although the identity of *Nocomis* nest associates is well documented, few studies have sought to identify eggs or larvae from nests using molecular techniques (Cashner and Bart 2010, Peoples et al., unpublished), Cashner and Bart (2010) identified eggs collected from *Nocomis* nests using restriction fragment length polymorphisms (RFLP) on the mtDNA ND2 gene. While this was an effective method for identifying cyprinid eggs to species, DNA barcoding has recently emerged as a proficient molecular technique. Genetic identification of fishes is most effectively completed using the cytochrome oxidase (COI) gene from mitochondrial DNA (Ivanova et al. 2007). Amplification and sequencing of the COI gene can be accomplished with universal or species-specific primers in polymerase chain reactions (PCR) (Ivanova et al. 2007). Sequences can then be compared with species-specific DNA sequences from a taxonomic database (*e.g.* GenBank) (Kress and Erickson 2012, Moran et al. 2015) to identify fishes to species. DNA barcoding is both an efficient and cost-effective technique for identifying larval fishes (Peoples et al., unpublished). Using DNA barcoding, we identified individuals collected from four different sections of *Nocomis* nests. This approach allows us to identify which

associates are spawning on nests; additionally we also gain insight into the spatial arrangement of host/associate eggs while testing for the selfish-herd effect in this putative mutualism.

The goal of this study is identify the underlying mechanisms that make nest association adaptive for both *Nocomis* and associates. We accomplished this goal by addressing three main objectives: (1) to evaluate relative egg predation risk at four potential spawning locations, (2) to compare egg predation rates between buried and non-buried eggs on *Nocomis* nests, and (3) to investigate *Nocomis* nest association for the presence of the selfish-herd effect using molecular techniques. We hypothesized that associates spawn on guarded nests because risk of egg predation is lowest relative to spawning on open substrate or unguarded nests. Additionally, we hypothesized that egg burying significantly reduce segg predation rates on nests. Given their ability to manipulate egg position on nests, we hypothesized that *Nocomis* hosts may benefit from the selfish-herd effect by further reducing the threat of egg predation.

Methods

We conducted this study in May 2014 in Toms Creek, a tributary to the New River in Montgomery County, Virginia (80°27'35"W 37°14'41"N). The Bluehead Chub *Nocomis leptocephalus* is the most common host in smaller tributaries (up to 5th order) to the New River, and was the focal host of this study. Strong associates at this site included Mountain Redbelly Dace *Chrosomus oreas*, Crescent Shiner *Luxilus cerasinus*, White Shiner *L. albeolus*, Rosefin Shiner *Lythrurus ardens*, and Rosyside Dace *Clinostomus funduloides*; weak associates in Toms Creek include Central Stoneroller *Campostoma anomalum*, Blacknose Dace *Rhinichthys atratulus* and Longnose Dace *R. cataractae* (Pendleton et al. 2012).

Assessing relative risk

We collected eggs from an active chub nest, then glued ten eggs to four small, flat cobbles (10 eggs x 4 cobbles). Preliminary trials confirmed that eggs glued to cobble did not over-harden and could be consumed by foraging egg predators. When we located an active nest, we used rinsed gravel from an abandoned chub nest to create an artificial nest. We constructed the artificial nest within 2 meters of the natural nest in similar mesohabitat (Lobb and Orth 1988), current velocity and depth (10-50 cm) (Bolton et al. 2015). We then excavated a spawning trough on the upstream end of the artificial nest according to dimensions described by Sabaj et al. (2000). We also chose a proximal location on the open substrate with similar mesohabitat, depth, and substrate. We then placed one of the prepared cobbles at the open substrate location and in the spawning pit of the artificial nest. We placed the final two cobbles on the upstream (spawning pit) and downstream ends of the natural nest. We placed eggs specifically in the spawning pits because both host and associates deposit their eggs in this location. Because the host focuses on guarding the upstream end of their nest, we placed eggs on the downstream end of the nest to compare predation levels on the nest. We lightly covered each experimental cobble with gravel so that eggs were not completely exposed to egg predators. We conducted a total of ten replications of this experiment.

A team of two observers monitored activity over the course of 45 minutes and recorded all host and associate activity (e.g., feeding events, spawning, egg covering, and nest defense). Observations were recorded in three-minute intervals; any activity occurring during the three-minute period was recorded (Appendix A). We conducted these observations to improve current understanding of host and associate behavior, as well as to assist in identifying potential cheaters in this mutualism. We defined nest defense as any aggression or territoriality exhibited towards

another individual. For hosts, we classified digging the spawning pit and moving gravel as nest maintenance (Wallin 1992). We defined checking as any foraging behavior that did not result in feeding (e.g., moving rocks in order to dislodge eggs). One observer monitored the two cobbles on the natural nest and the chub host; the second observer monitored the cobbles on the artificial nest and the open substrate. Any other species observed on the nest was also recorded. All nests were video recorded to supplement observations. After the observation period, we recovered the cobbles and recorded the number of eggs that had been eaten, representing egg predation at each location. Final remaining egg counts were $\log(x+1)$ -transformed and subjected to a one-way analysis of variance (ANOVA) with a blocking factor to identify differences in predation risk among the four spawning locations.

Parental care via egg burying

To examine the role of parental care via egg burying, we exposed eggs collected from an active bluehead chub nest to two different treatments: buried and surface. For this experiment, we used 20 eggs per cobble, and two cobbles per nest on nine nests. At each nest, we buried one cobble five centimeters below the surface of the spawning trough; to ensure recovery, we marked its location using a piece of colored wire. We positioned a second cobble at the surface of the nest and slightly covered it with gravel to simulate eggs that would have deposited and fallen slightly between surface gravel on nests. We staggered the position of the cobbles so the one on the surface would not shield the buried cobble from predation, and randomized which cobble would be placed in the left or right position. After two hours, we recovered the cobbles and recorded egg survival; eggs were placed between 9-10 A.M. and collected between 11-12 P.M. We used a paired t-test to compare egg predation rate at the two burying depth treatments.

Divided nest experiment

To examine differences in egg density among different sections of the nest, we collected eggs from each of four sections on eight nests (Figure 3.2). To isolate the upstream and downstream halves, we first inserted a flat panel (~0.5m x 0.5m) vertically through the center of the nest. Before disturbing the nest substrate, we positioned a mesh bag (~0.5m x 0.5m, 1-mm mesh-size) directly downstream of the nest. Using the depth measurement for each nest to distinguish the top and bottom sections, we gently sifted through small amounts of gravel from the top downstream (TD) section of the nest; we carefully disturbed the substrate and allowed stream flow to carry eggs into the mesh bag. This method greatly reduced the chance that eggs would fall into the bottom section; we are confident that this method minimized any transfer of eggs to other sections. We recorded the gravel volume (L) of each section to be used as a covariate in statistical analysis. We repeated this process for each of the four sections. Because chubs spawn on the upstream ends of nests (Sabaj et al. 2000), dividing the nest in this manner allowed us to isolate the upstream end while accounting for the upper half of the nest where associates have been observed spawning (Vives 1990; Wallin 1992). Lastly, we counted the eggs from each section. We brought all collected eggs back to the laboratory and reared them to the larval stage; the larval fish were then sacrificed and preserved to be identified using molecular techniques.

Using $\log(x+1)$ transformed fish counts, we conducted a general linear model (GLM) using one treatment (nest division), one blocking effect (replication), and one covariate (gravel volume). We used the gravel volume for each section as a covariate to determine if imperfect division of nests affected larval fish counts. We used a *post hoc* Tukey-Kramer test to compare larval fish counts among nest divisions.

Genetic analysis

A subsample of larval fishes from each section was randomly selected for genetic analysis. We isolated DNA from preserved whole larval fish using a DNeasy Blood and Tissue Kit (Qiagen). We assessed the quality and quantity of DNA using a μ Lite PC spectrophotometer (Biodrop, Cambridge, UK). Sequences for the mitochondrial *COI* gene were amplified using a combination of four universal primers (VF2_t1, FR1d_t1, FishF2_t1, and FishR2_t1) targeting the *COI-3* region of the *COI* gene (Ivanova et al. 2007). We performed PCR amplification using a modified protocol of Ivanova et al. (2007). PCR reactions had a volume of 22 μ L, including 14.7 μ L of ultrapure water, 2 μ L of 5xPCR buffer (10 mM KCl, 10 mM (NH₄)₂SO₄, 20 mM Tris-HCl (pH 8.8), 2mM MgSO₄, and 0.1% Triton X-100), 1.8 μ L MgCl₂ (25mM), 0.1 μ L of each dNTP (10 mM), 0.4 μ L of BSA (bovine albumin), 0.1 μ L of *Taq* DNA Polymerase (New England Biolabs), 0.4 μ L of each primer cocktail (10 mM), and 1 μ L of DNA template (mean conc. 62 ng/ μ L). We conducted all reactions on a BioRad MyCycler with a thermocycle profile of: 94°C for 2 minutes, 35 cycles of 94°C for 30 seconds, 52°C for 40 seconds, and 72°C for 1 minute, with a final extension for 10 minutes at 72°C. For samples with low DNA quantity and suboptimal DNA quality, we attempted to amplify template DNA using Platinum[®] *Pfx* DNA polymerase (Life Technologies Corp.) using the manufacturer's suggested protocol.

All PCR products were sequenced at Virginia Bioinformatics Institute (VBI) using the BigDye Terminator Cycle Sequencing Kit v. 3.1 on an ABI3730 DNA sequencer; sequencing reactions were conducted using FishF2_t1 and FishR2_t1 primer cocktails (sensu Ivanova et al. 2007). Raw sequences received from VBI were edited in Sequencher (v. 4.5, Applied Biosystems). Edited sequences were compared with existing entries in GenBank (<http://www.ncbi.nlm.nih.gov/nucleotide/>) using the Basic Local Alignment Search Tool (Altschul

et al. 1990). Only sequences with homology scores greater than 90% were considered indicative of a particular species.

The proportion of larval *Nocomis* and associates were log transformed ($\log(x + 1)$). Using these values, we calculated the ratio of host to associates for each section on all nests. We conducted an ANOVA to determine if nest division had a significant effect on chub-associate ratios.

Results

Assessing relative risk

The 2-way ANOVA revealed a significant block effect ($F_{8,24} = 3.12, p = 0.015$). However, egg location had no significant effect ($F_{3,24} = 0.39, p = 0.759$) on egg survival. After the observational period, an average of 6.44 ± 3.23 eggs had been consumed on the open substrate, as well as 6.33 ± 2.38 consumed on the artificial nest. Similar values were recorded on the upstream (6.77 ± 2.19 eggs consumed) and downstream (6.11 ± 2.21 eggs consumed) ends of the active nest (Figure 3.3). Chub host activity was focused exclusively on the upstream end of their nest. Hosts were either digging spawning troughs (21% of activity), spawning (13%) or placing gravel over deposited eggs (31%) (Table 3.1). Additionally, hosts demonstrated some aggression towards conspecifics (7% of activity) and stonerollers (14% of activity) (Table 3.1). Host aggression towards conspecifics primarily involved chasing away other tuberculate males, perceived as potential spawners. Similarly, aggression towards stonerollers was focused on large males. Out of 491 actions of associates, 358 (72.9%) were recorded on the natural nest. On the artificial nest and open substrate, 97 (19.8%) and 36 (7.3%) actions were recorded, respectively (Table 3.2).

Large male stonerollers and chubs constituted the majority of feeding events at all locations; the majority of feeding chubs appeared to be post-nuptial females and immature males. Checking and apparent feeding accounted for 87% of stoneroller activity; the vast majority of these observations occurred over the spawning trough of the natural nest. Only four possible stoneroller spawning events were recorded. Strong associates were rarely observed feeding on natural nests, although one crescent shiner was observed possibly feeding on eggs on a natural nest; this represents a minute percentage (0.2%) of all observations. At least one strong associate species was always present at nests; the majority of strong associate behavior involved mating, searching, or defending territories on nests. This analysis demonstrated that egg predation levels did not differ at any of the four spawning locations. Additionally, it revealed that chub hosts focused efforts on maintaining the spawning trough and covering any deposited eggs; potential egg predators appeared to be conspecifics and large male stonerollers.

Parental care via egg burying

Buried eggs experienced much lower predation rate than eggs on the surface. The difference between surface and buried treatment was highly significant ($t_{1,8} = 4.95, p=0.001$). After 2 hours, buried eggs had 16.6 ± 0.7 remaining per cobble, while eggs placed on the nest surface had 8.2 ± 1.9 left per cobble.

Divided nest experiment

Most eggs were concentrated in the bottom-upstream portions of nests. Both nest section ($F_{3,20} = 7.88, p=0.001$) and block (nest) ($F_{7,20} = 2.73, p=0.037$) had significant effects on the number of eggs present in each section (Figure 2.4). Gravel volume had no effect on the number of eggs per section ($F_{3,20} = 0.33, p=0.571$). The Tukey-Kramer means comparison revealed that

the bottom upstream (BU) section had significantly more eggs than all other sections, which did not differ from one another.

Genetic analysis

Of the three nests evaluated for genetic analysis, we attempted to identify 144 individuals; 141 of these fish were successfully identified (98%). Half (49%) of the samples did not amplify with *Taq* DNA Polymerase (New England Biolabs), however, nearly all of these samples successfully amplified when Platinum[®] *Pfx* DNA polymerase (Life Technologies Corp.) was used.

For successfully amplified samples, read length ranged from 275 to 716 bp, with modal lengths of 701 and 703 bp. Queries in GenBank resulted in 1 to 8 COI sequences over 90% identity, though most queries yielded 4-5 sequences. Identification of most species was straightforward, specifically for *Campostoma anomalum* and both *Luxilus* species. Identification of *Nocomis*, however required more analysis. Many samples were identified as bigmouth chub *Nocomis platyrhynchus*, which is endemic to the New River drainage (Jenkins and Burkhead 1994). While *N. platyrhynchus* is present in lower Toms Creek, *N. leptocephalus* is the only *Nocomis* present in upper Toms Creek. Thus, *Nocomis* larvae could only be confidently identified to genus. Through these methods, larval fish for all nests combined were identified as 100 *C. anomalum*, 33 *Nocomis* sp., 5 *Luxilus albeolus*, 2 *L. cerasinus*, and 1 *Lythrurus ardens* (Table 2.2). Mountain redbelly dace *Chrosomus oreas*, arguably the most abundant nest associate, was not identified on any of the three nests. The analysis of variance (ANOVA) demonstrated that nest section had no significant effect on the chub-associate ratio ($p = 0.142$). *Nocomis* was located primarily in the bottom two sections of the nests, though one individual was identified in the top upstream (TU) section.

Discussion

Relative predation risk did not differ among the experimental spawning locations, suggesting that associates do not increase brood survival via reduced egg predation when spawning with *Nocomis* hosts, if substrate was amply available and eggs were not buried; eggs on artificial nests and open substrate experienced predation rates relatively equal to those on both ends of the natural nest. Whereas predation rates were equal on open substrate and artificial nests, there were nearly three times more actions (e.g. checking, feeding) recorded on the artificial nests compared to the open substrate. This supports our hypothesis that the open substrate is the least conspicuous place for associates to deposit their eggs. However, predation at these locations was more variable than on natural nests. Eggs on open substrates and artificial nests were typically undetected, however, all eggs were consumed when discovered; the variability in egg consumption at these two locations may be a result of their proximity to an actively guarded nest. Conversely, eggs on the natural nest always experienced some predation pressure. This was initially counterintuitive because all previous studies have found that associates experienced better egg survival when spawning with a host than they would have experienced if spawning without a host (Johnston 1994a, Shao 1997). Shao (1997a) found that golden shiner *Notemigonus crysoleucas* eggs experienced significantly higher predation levels when placed on the open substrate; the parental care of their host, pumpkinseed *Lepomis gibbosus* alleviated egg predation on their nest (Shao 1997). Johnston (1994b) found that egg predation increased significantly with the removal of a green sunfish *Lepomis cyanellus* host. These studies all involved sunfish hosts, which do not bury eggs deposited in their nest. The conclusions of these studies differed from our observations because our experimental design did not account for the impact of parental care via egg burying. Because of their conspicuity,

guarded nests attract a large congregation of associates; Wallin (1989) noted up to 300 associates on a *N. leptocephalus* nest. Egg predation inherently increases in the presence of more associates. Given the results, the design of the first experiment failed to incorporate egg covering as a form of parental care. If nest guarding had no significant effect on predation levels, then parental care via egg covering may be the critical form of host parental care.

Over time, the inefficiency of nest guarding may have led to the adoption of an alternative strategy: egg burying. Our behavioral observations from this study indicate that actual nest defense accounts for less than half the time adult male chub spends on maintenance of a spawning trough and burying eggs; this suggests that hosts prioritize egg covering over nest guarding throughout the spawning process. Wallin (1989) found that *N. leptocephalus* continue to add gravel to their nests for up to 2-3 days after they finish spawning. The results of experiment 2 reaffirm that egg covering is a vital factor increasing associate egg survival. Eggs just below the surface of nests experienced significantly higher predation levels than eggs buried in the substrate. Because hosts constantly bury eggs, this activity can reduce predation levels on both host and associate eggs. Additionally, the third experiment revealed that the majority of eggs are located in the bottom upstream section of the nest. Originally deposited in a surface spawning trough, burying efforts by the host left the eggs in this location. The lower predation levels, coupled with the highest density of eggs at the bottom of the nest, suggest that host and associate benefit from host eggs burying. While Johnston (1994a) and Wallin (1992) stress the importance of host parental care, we believe that egg burying is more effective at reducing egg predation than combative nest guarding.

The lack of spawning events at the artificial nest and open substrate may be associated with the absence of necessary chemical cues at these locations. Hosts emit specific chemical cues

(via ovarian fluid and milt) when spawning; these cues have been shown to be necessary for gamete maturation and reproduction for their nest associates (Hunter and Hasler 1965, Wisenden 1999). Given that spawning events were only observed on the natural nest, *Nocomis* nest associates likely require the presence of chemical cues to successfully spawn.

In North America, inconclusive evidence of the selfish-herd effect has been demonstrated in associations between Smallmouth Bass *Micropterus dolomeiu* and Longnose Gar *Lepisosteus osseus* (Goff 1984) as well as between Green Sunfish *Lepomis cyanellus* and Redfin Shiner *Lythrurus umbratilis* (Johnston 1994b). With the majority of eggs in the bottom upstream section of the nest, these eggs are least vulnerable to potential egg predators. Given the ability to manipulate the position of eggs within the nest, chub hosts may be positioning their eggs in an even safer position than associate eggs. The results of the genetic analysis, however, indicate that there is no significant difference in host-associate proportions throughout the nest. The absence of certain obligate associates (namely *Chrosomus oreas*) suggests that unfavorable laboratory conditions greatly reduced egg survival. These obligate associates rely heavily on the clean gravel substrate and conditions created by *Nocomis* hosts (Peoples et al. 2010, Pendleton et al. 2012). Upon removal, the eggs of obligate associates may have been unable to survive. If the majority of obligate associate eggs did not persist until the larval stage, then the host-associate proportions in this study may not be representative of natural systems. Conversely, stonerollers, a facultative nest associate, constituted nearly 70% by count of all identified species on the nests. Given the lack of obligate associates and the abundance of stoneroller larvae, we conclude that the laboratory conditions were unsuitable for the more sensitive species. In the future, we plan to revise our approach to ensure more adequate aeration of eggs. Alternatively, the genetic analysis could be conducted using fertilized eggs collected from *Nocomis* nests (*sensu* Cashner and Bart

2010). With a more representative population of host and associates, we could more conclusively assess this nest association for the selfish-herd effect.

Nest association is vital for the reproductive success of many North American cyprinids (Johnston and Page 1992). This study demonstrated the importance of parental care via egg covering in *Nocomis* nest association, as well as the intrinsic value of nest structure. With the exception of stonerollers, these nest associates are broadcast spawners in the absence of a host. Associates can only benefit from egg covering when spawning with *Nocomis* hosts. Thus, chub nests maximize brood survival for both hosts and their associates. Additionally, reduced survival of associate eggs further suggests the importance of the microhabitat created by *Nocomis* hosts. Peoples et al. (2010) suggested that the clean gravel substrate on these nests may be the only viable spawning habitat in heavily silted systems. At the community level, Hitt and Roberts (2011) concluded that nest association was an important factor in species persistence and colonization. In the eastern United States, *Nocomis* nest association is vital for the persistence of many cyprinid communities, particularly in systems afflicted by heavy siltation and anthropogenic disturbances.

Conclusions

The findings in the first experiment suggest that the relative risk of egg predation did not differ significantly at any of the potential associate spawning locations. If this were valid, then associates should readily spawn independently of their *Nocomis* hosts. Alternatively, I concluded that the experiment failed to incorporate the role of host parental care via egg burying into the design. With the majority of eggs located in the bottom upstream section of the nest, the host's efforts to bury eggs directly reduces predation levels on both host and associate eggs. With the vast number of individuals visiting the nest, egg burial is the most efficient way for *Nocomis* to combat potential egg predators.

Results from the genetic analysis, however, are much less conclusive. I surmise that many of the obligate associate eggs were lost during the incubation period. Out of 141 individuals, only 8 were identified as obligate associates. Within the Toms Creek system, mountain redbelly dace *Chrosomus oreas* is the most common associate documented on *Nocomis* nests; not a single *C. oreas* was identified during the analysis. It is probable that poor environmental conditions during the incubation process led to total mortality for obligate associate eggs. Furthermore, *Nocomis* and *Campostoma* eggs may have been more resilient under stressful conditions; this may explain the disparity between my findings and the findings of Cashner and Bart (2010) and Wallin (1992). I plan to conduct a follow-up genetic analysis in the future to better assess the species composition on chub nests in the study system. If feasible, I will conduct the analysis using eggs (*sensu* Cashner and Bart 2010) to minimize the loss of individuals due to poor environmental conditions. Alternatively, I will reassess my incubation techniques in order to minimize the potential for fungal infection or poor aeration.

Without viable results from the genetic analysis, I am unable to confidently determine whether or not the selfish-herd effect is present in this interaction. While the selfish herd effect was not expressly identified in this nest association, the results support the hypothesis that it may be present. Larval fish counts revealed that the majority of individuals were located in the bottom upstream section of the nest, and the genetic analysis revealed that the highest ratios of chub-associates were located in the two bottom sections. These results corroborated our hypothesis that host eggs would be concentrated in the bottom sections of the nest, due primarily to the host's ability to manipulate the nest substrate and reposition their eggs deeper in the nest. As mentioned previously, this is the first study to assess *Nocomis* nest association as a three-dimensional system. A previous study (Cashner and Bart 2010) identified species composition in this association, however, our genetic analysis and experimental design permitted us to assess both the species composition and their distribution throughout the nest.

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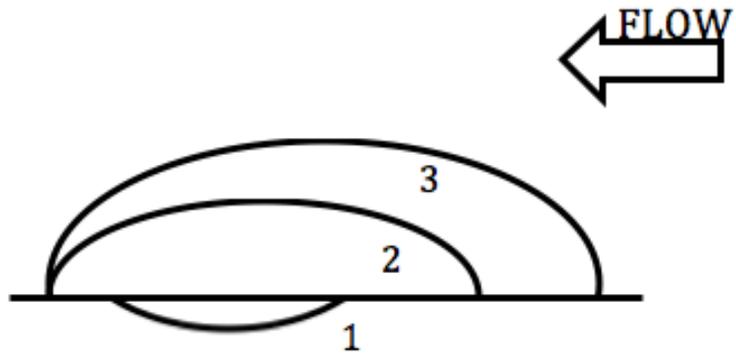


Figure 3.1. Three-stage construction of *Nocomis spp.* gravel mound: concavity (1), platform (2), and mound (3). Male *Nocomis* excavate spawning pits on the upstream end of the platform (2).

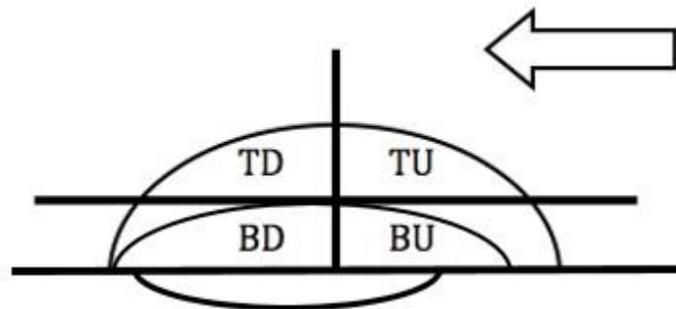


Figure 3.2. Cross-section of chub nest divided into four sections: top (T) and bottom (B), upstream (U) and downstream (D). Concavity and platform added for reference.

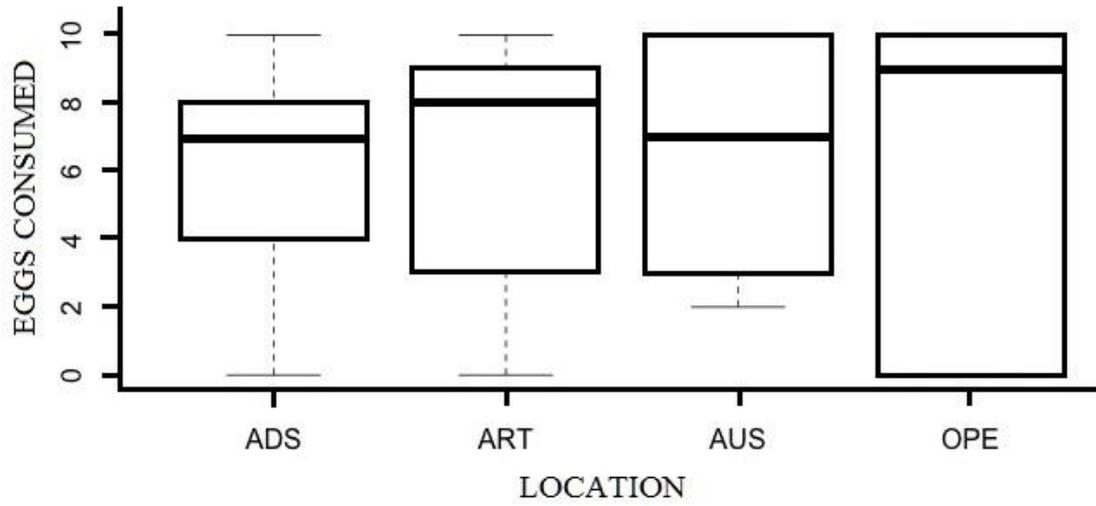


Figure 3.3. Boxplots of eggs consumed at four potential spawning locations of nest associates. Location labels are: Active downstream (ADS), artificial nest (ART), Active upstream (AUS) and open substrate (OPE).

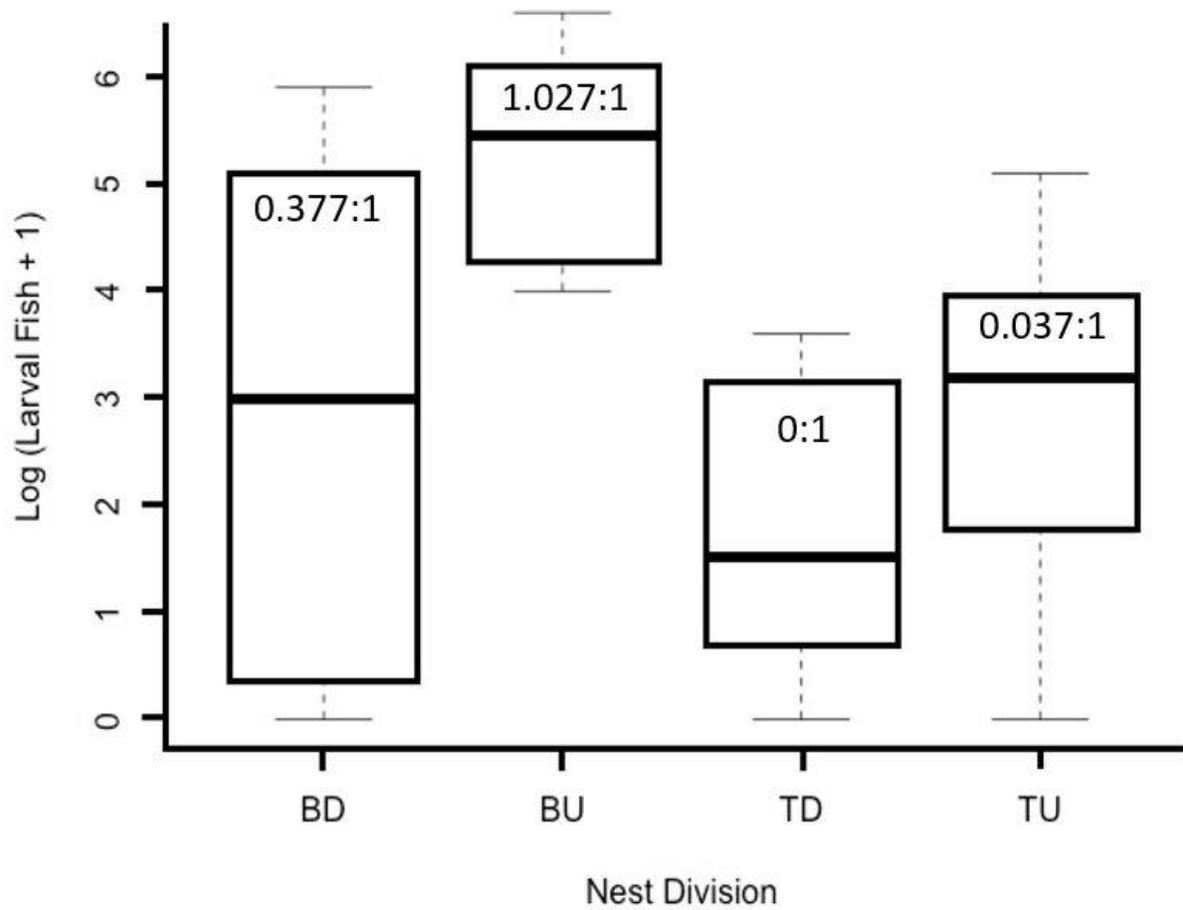


Figure 3.4. Boxplots of log(x+1)-transformed larval fish counts from each nest section (Nests, n=8). Ratios of chub:associates for sections displayed within each plot. Labels are as following: bottom downstream (BD), bottom upstream (BU), top downstream (TD) and top upstream (TU).

Table 3.1. Observed actions of host bluehead chub. Species codes are bluehead chub (BHCH), central stoneroller (CESR), crescent shiner (CRSH), mountain redbelly dace (MRBD), and white shiner (WHSH). Percentages were calculated using observed counts of activity.

Activity	Count	Percentage of Activity
Absent	18	7.11
Aggression (BHCH)	17	6.72
Aggression (CESR)	36	14.23
Aggression (CRSH)	1	0.40
Aggression (MRBD)	1	0.40
Aggression (WHSH)	9	3.56
Digging spawning trough	54	21.34
Feeding	2	0.79
Hovering	3	1.19
Maintenance	79	31.23
Mating	33	13.04
Total Actions	253	100.00

Table 3.2. Total number of actions and feeding events for all species documented at three potential spawning locations in Toms Creek (n = 10). Species codes are bluehead chub (BHCH), central stoneroller (CESR), crescent shiner (CRSH), fantail darter (FTDA), mountain redbelly dace (MRBD), rosefin shiner (RFSH), rosieside dace (RSDA), white shiner (WHSH).

Species	Artificial Nest		Natural Nest		Open Substrate	
	Total Actions	Feeding	Total Actions	Feeding	Total Actions	Feeding
BHCH	39	21	39	23	14	13
CESR	39	24	211	106	8	7
CRSH	0	0	19	1	2	1
FTDA	1	0	1	0	0	0
MRBD	3	1	51	0	0	0
RFSH	12	0	5	0	0	0
RSDA	2	0	4	0	0	0
WHSH	1	0	28	0	12	0
	97	46	358	130	36	21

Table 3.3. Totals for all individuals identified using molecular analysis. Numbers within parentheses are percent composition for the above nest section. Nest sections are bottom upstream (BU), bottom downstream (BD), top upstream (TU) and top downstream (TD).

Species	BU	BD	TU	TD	Total
<i>Campostoma anomalum</i>	36 (63.2%)	31 (70.5%)	24 (85.7%)	9 (75%)	100
<i>Nocomis sp.</i>	21 (36.8%)	11 (25%)	1 (3.6%)	-	33
<i>Luxilus albeolus</i>	-	2 (4.5%)	-	3 (25%)	5
<i>Luxilus cerasinus</i>	-	-	2 (7.1%)	-	2
<i>Lythrurus ardens</i>	-	-	1 (3.6%)	-	1

CHAPTER 4: SYNTHESIS

In this research, I investigated several key mechanisms influencing *Nocomis* nest association: parental care, relative risk, and cheating. Using a combination of observational, manipulative and molecular approaches, I was able to draw several novel conclusions. First, gonadosomatic index (GSI) revealed a direct connection between nest association strength and correlation with *Nocomis* reproductive condition. Both the Pearson correlation and nonmetric multidimensional scaling (NMDS) suggest that reproductive patterns of potentially obligate associates are closely linked to host reproductive patterns. Alternatively, facultative associates appear to be spawning separately from chubs; both *Rhinichthys* species spawned nearly a month before chubs, while stonerollers appeared to spawn the week before the onset of chub spawning. Our behavioral observations from Chapter 3 corroborate the stonerollers patterns, with the vast majority of their activity on nests indicating feeding; no stoneroller spawning events were observed.

These studies worked to expand current knowledge concerning mutualism theories, specifically the role of cheating and the influence of mutualism strength. My research revealed that, within *Nocomis* nest association, these two concepts appear to be linked. In Chapter 2, I found that strong (nearly obligate) nest associates did not attempt to cheat the chub host, while one weak (facultative) associate, the central stoneroller, may be cheating the system. Additionally, I found that two other weak associates, the blacknose and longnose dace, do not appear to interact with *Nocomis* when spawning in the Catawba Creek system; this may be due in part to context dependency. Hoeksema and Bruna (2015) concluded that the importance of mutualisms, specifically facultative mutualisms, may vary from across systems. The association between these two associates and a *Nocomis* host may be required outside of the Catawba

Valley. Because context (biotic and abiotic factors) varies greatly over the range of *Nocomis*, it is critical to assess association strength when exploring novel, potentially mutualistic interactions.

While it appears that stonerollers may be cheaters or egg predators, this conclusion is contradicted by the abundance of stoneroller eggs identified during the genetic analysis. If stonerollers are truly cheaters within this interaction, why are they depositing eggs? One explanation may be that GSI does not fully measure reproduction for a species. GSI can identify major spawning events, however, it is unable to identify smaller spawning events. Stonerollers may spawn independently of *Nocomis*, however, some individuals may elect to spawn again on chub nests. Alternatively, stonerollers may elect whether or not to spawn on chub nests depending on context. Bronstein (2009) concluded that the outcome of mutualisms, particularly facultative mutualisms, varies based on context. Temperature may influence the onset of stoneroller reproduction and, contextually, their use of chub nests for spawning. Initial spawning temperature for stonerollers in Virginia is 13°C, two degrees lower than the initial temperature for the bluehead chub (Jenkins and Burkhead 1994). After particularly harsh winters or cold springs, water temperature may not reach optimal temperature until late April or early May. Peoples et al. (2015) documented stonerollers excavating spawning pits in May 2014, two days before the onset of chub spawning. Several obligate associates (rosyside dace, rosefin shiner and mountain redbelly dace) also aggregated over these stoneroller pits (Peoples et al. 2015). After chubs began spawning, the stoneroller pits were completely abandoned for *Nocomis* nests. Throughout the spawning process, stonerollers were frequently documented on chub nests. If the abiotic conditions (e.g. temperature, photoperiod) do not reach an optimum before the onset of chub reproduction, then stonerollers may elect to spawn on chub nests. We collected eggs for

genetic analysis in Spring 2013, which was preceded by a particularly long winter; this may explain the extensive presence of stonerollers in our analysis.

Relative risk for nest associates appears to be driven by the host's ability to cover/bury eggs in the nest. In Chapter 3, I conclude that eggs buried in the nest substrate experience significantly lower predation rates than eggs near the surface. Furthermore, the majority of eggs were positioned in the bottom two sections of the nest. Thus, the inherent physical structure of the nest and the parental care provided by the *Nocomis* host greatly reduce egg predation risk relative to that of the open substrate or an artificial nest.

While the genetic analysis was unable to elucidate the selfish-herd effect in *Nocomis* nest association, we plan to reassess our procedure and attempt the analysis again in the future. With such high mortality rates in the incubation process, poor aeration may have increased the susceptibility of fungal infection in the aquariums. If we can minimize the risk of mass mortality during this period, we can better assess this interaction for the selfish-herd effect. One alternative would be to conduct the genetic analysis directly on the eggs (*sensu* Cashner and Bart 2010). This would minimize the risk of mass mortality, however, DNA isolation from eggs can be a difficult process.

One future experiment that would assist in determining potential cheaters would be a stomach content analysis of species aggregating over chub nests. By collecting individuals of each species, we could more accurately determine which (if any) are actively foraging for eggs. Use of molecular techniques would be the most efficient method to identify whether or not individuals are regularly consuming eggs. Both associates and female chubs will opportunistically consume a free-floating egg, however, the presence of many eggs in an individual's stomach suggests that they are actively feeding on eggs.

This research illuminates the complexity that is inherent in interspecific, mutualistic interactions. For *Nocomis* nest association, biotic and abiotic factors influence the behavior and reproductive decisions of the chub and its associates. The novel use of GSI as a tool to monitor the reproductive condition of an entire cyprinid community can easily be applied to any stream system in eastern North America; in particular, GSI could be used to identify reproductive relationships between any of the seven *Nocomis* species and their cooccurring cyprinids.

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APPENDIX

Appendix A: Protocol and data sheets from relative risk assessment in Chapter 3.

Time	Active Nest – flow end ()	Active Nest – lee end ()	Artificial Nest – flow end ()	Open substrate ()
1 st scan	Sp_____ Actn S C F			
Time:	Sp_____ Actn S C F			
2 nd scan	Sp_____ Actn S C F			
	Sp_____ Actn S C F			
3 rd scan	Sp_____ Actn S C F			
	Sp_____ Actn S C F			
4 th scan	Sp_____ Actn S C F			
	Sp_____ Actn S C F			
5 th scan	Sp_____ Actn S C F			
	Sp_____ Actn S C F			
6 th scan	Sp_____ Actn S C F			
	Sp_____ Actn S C F			
7 th scan	Sp_____ Actn S C F			
	Sp_____ Actn S C F			
8 th scan	Sp_____ Actn S C F			
	Sp_____ Actn S C F			
9 th scan	Sp_____ Actn S C F			
	Sp_____ Actn S C F			
10 th scan	Sp_____ Actn S C F			
	Sp_____ Actn S C F			
11 th scan	Sp_____ Actn S C F			
	Sp_____ Actn S C F			
12 th scan	Sp_____ Actn S C F			
	Sp_____ Actn S C F			
13 th scan	Sp_____ Actn S C F			
	Sp_____ Actn S C F			
14 th scan	Sp_____ Actn S C F			
	Sp_____ Actn S C F			
15 th scan	Sp_____ Actn S C F			
	Sp_____ Actn S C F			
End time:	Status: exposed partially buried completely buried Survival: # eggs left = (record significantly broken eggs as eaten)	Status: exposed partially buried completely buried Survival: # eggs left = (record significantly broken eggs as eaten)	Status: exposed partially buried completely buried Survival: # eggs left = (record significantly broken eggs as eaten)	Status: exposed partially buried completely buried Survival: # eggs left = (record significantly broken eggs as eaten)

Species seen on the nest: _____

‘Egg stealing’ Experiment Protocol:

- Select an active nest and have a bucket full of nest-size gravel ready for artificial nest.
- Select an active nest and find a good location where activities on the nest can be watched from stream bank.
- Select an open substrate patch within 5-10 ft of the active nest.
- Pour gravel in a pile within 5-10 feet of active nest and open substrate patch to simulate a new chub nest.
- Partially hide each of the egg masses (10#/mass) with gravel on each of the experimental units. The chub nest should have 2 egg masses, one at the chub-end/flowward end and the other at the leeward end. Eggs should be at the chub end on the artificial nest. *Eggs should be slightly depressed but exposed enough to be found by actively searching fish.*
- Train a camera on each of the 3 experimental units (Omit if turbidity is a problem).
- Assign each crew member to one unit to scan for activities and record data. [Use a separate datasheet for each crew member]
- Allow up to approximately 5 minutes for the turbidity to clear and fish to return and begin recording observations
- Observe what species (if possible) and their action. *Action codes are on datasheet.*
- A different person should observe the resident chub and record actions of the chub on a separate datasheet
- Scan for a total of 45 minutes or end the experiment after 5 consecutive scans of no action on any of three experimental units.
- At takedown, first observe the status of the egg mass for each unit and record.
- Also record the number of eggs remaining for each unit.
- Record all fish species seen on the nest

Host Male Observation:

Time	Location (circle 1)	Location on nest	Action, if tending nest	Other Action (if on nest)
1 st scan Time:	On nest Returning Away from nest	Flowward Leeward N/A (i.e., Away from nest)	Picking/Dropping rock Moving Inactive	Aggressive to _____ Sp Mating Other _____
2 nd scan	On nest Returning Away from nest	Flowward Leeward N/A (i.e., Away from nest)	Picking/Dropping rock Moving Inactive	Aggressive to _____ Sp Mating Other _____
3 rd scan	On nest Returning Away from nest	Flowward Leeward N/A (i.e., Away from nest)	Picking/Dropping rock Moving Inactive	Aggressive to _____ Sp Mating Other _____
4 th scan	On nest Returning Away from nest	Flowward Leeward N/A (i.e., Away from nest)	Picking/Dropping rock Moving Inactive	Aggressive to _____ Sp Mating Other _____
5 th scan	On nest Returning Away from nest	Flowward Leeward N/A (i.e., Away from nest)	Picking/Dropping rock Moving Inactive	Aggressive to _____ Sp Mating Other _____
6 th scan	On nest Returning Away from nest	Flowward Leeward N/A (i.e., Away from nest)	Picking/Dropping rock Moving Inactive	Aggressive to _____ Sp Mating Other _____
7 th scan	On nest Returning Away from nest	Flowward Leeward N/A (i.e., Away from nest)	Picking/Dropping rock Moving Inactive	Aggressive to _____ Sp Mating Other _____
8 th scan	On nest Returning Away from nest	Flowward Leeward N/A (i.e., Away from nest)	Picking/Dropping rock Moving Inactive	Aggressive to _____ Sp Mating Other _____
9 th scan	On nest Returning Away from nest	Flowward Leeward N/A (i.e., Away from nest)	Picking/Dropping rock Moving Inactive	Aggressive to _____ Sp Mating Other _____
10 th scan	On nest Returning Away from nest	Flowward Leeward N/A (i.e., Away from nest)	Picking/Dropping rock Moving Inactive	Aggressive to _____ Sp Mating Other _____
11 th scan	On nest Returning Away from nest	Flowward Leeward N/A (i.e., Away from nest)	Picking/Dropping rock Moving Inactive	Aggressive to _____ Sp Mating Other _____
12 th scan	On nest Returning Away from nest	Flowward Leeward N/A (i.e., Away from nest)	Picking/Dropping rock Moving Inactive	Aggressive to _____ Sp Mating Other _____
13 th scan	On nest Returning Away from nest	Flowward Leeward N/A (i.e., Away from nest)	Picking/Dropping rock Moving Inactive	Aggressive to _____ Sp Mating Other _____
14 th scan	On nest Returning Away from nest	Flowward Leeward N/A (i.e., Away from nest)	Picking/Dropping rock Moving Inactive	Aggressive to _____ Sp Mating Other _____
15 th scan	On nest Returning Away from nest	Flowward Leeward N/A (i.e., Away from nest)	Picking/Dropping rock Moving Inactive	Aggressive to _____ Sp Mating Other _____