The evolution of kin recognition

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Graduate Program in Biology
A thesis submitted in partial fulfillment of the requirements for the degree in Doctor of Philosophy
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THE EVOLUTION OF KIN RECOGNITION

(Thesis format: Integrated Article)

by

Timothy John Alexander Hain

Graduate Program in Biology

A thesis submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy

The School of Graduate and Postdoctoral Studies
The University of Western Ontario
London, Ontario, Canada

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Abstract

The discovery that many animals are promiscuous has challenged the importance of Hamilton’s Rule because it reduces the net benefits of helping nestmates. To resolve this challenge, biologists have investigated animals’ abilities to determine degrees of relatedness among individuals using kin recognition mechanisms. I conducted a literature review and found that most animals use one of two mechanisms: “familiarity” whereby kin are remembered from interactions early in life, such as in a nest, or “phenotype matching” whereby putative kin are compared to a template of what kin should look, smell, or sound like based on relatives encountered during early life or on one’s own phenotype (called “self-referent phenotype matching”). Theory suggests that familiarity should evolve when being born together is a reliable cue of relatedness, and phenotype matching should evolve when familiarity is unreliable. However, the conditions favouring the evolution of one of these mechanisms over the other has been largely unstudied. In my thesis, I begin to fill this gap using two promiscuous fish species, bluegill (Lepomis macrochirus) and guppies (Poecilia reticulata), and suggest other life history (brood size) and environmental (predation) factors that might influence recognition mechanism. In bluegill, I show that kin recognition can lead to enhanced anti-predator shoaling behaviour, and that high promiscuity, which causes low levels of relatedness within broods, leads to the expression of self-referent phenotype matching. In guppies, I use six guppy populations to show that average brood size and predation regime cannot explain kin recognition mechanism, and in contrast to my findings in bluegill, brood relatedness does not explain recognition mechanism, but it is correlated with the intensity of recognition. Furthermore, I use phylogenetic analysis to show that recognition mechanism is not evolutionarily constrained in guppies. Together, my thesis provides new data on the factors that influence kin recognition mechanism and moves the field beyond the simple observation of what species recognize relatives to the ultimate questions of how these mechanisms evolve.

Keywords

Behavioural ecology, bluegill, evolution, fish, guppy, kin recognition, kin selection, multiple mating, phylogeny, promiscuity, social behaviour
Co-Authorship Statement

A version of Chapter 2 was published in the *Journal of Fish Biology* with Bryan Neff as co-author. Dr. Neff funded the project, contributed to study design, provided advice on statistical analysis, and offered editorial comments on the manuscript.

A version of Chapter 3 was published in *Current Biology* with Bryan Neff as co-author. Dr. Neff provided funding for the project, helped design the study, contributed paternity and relatedness data, provided advice on statistical analysis and gave editorial comments on the manuscript.

A version of Chapter 4 was published in *Molecular Ecology* with Bryan Neff as co-author. Dr. Neff funded the project, contributed to study design, provided gravid female guppies for analysis of paternity, gave advice on statistical analysis, and offered editorial comments on the manuscript.

A version of Chapter 5 was published in *Behavioral Ecology* with Shawn Garner, Indar Ramnarine, and Bryan Neff as co-authors. Dr. Garner assisted with paternity analysis, developed simulations in the statistical analysis, and gave editorial comments on the manuscript. Dr. Ramnarine provided facilities and local expertise on Trinidadian guppy populations, assisted in sample collection, and provided editorial comments on the manuscript. Dr. Neff provided funding for the project, helped design the study, gave advice on statistical analysis and gave editorial comments on the manuscript.

Chapter 6 has been submitted to the *Canadian Journal of Zoology* and was co-authored with Shawn Garner, Indar Ramnarine, and Bryan Neff. Dr. Garner assisted with genetic analysis, contributed to experimental design, developed the simulation in the statistical analysis and provided additional statistical help, and contributed to the writing of the manuscript. Dr. Ramnarine provided facilities and equipment for sample collection and provided local expertise on Trinidadian guppy populations. Dr. Neff provided funding for the project, helped design the study, gave comments on the statistical analysis and contributed editorial comments on the manuscript.
Acknowledgments

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The wider Western graduate student community has been instrumental in the development of my research abilities. I would like to thank Paul Mensink and Julie Faure-Lacroix in particular for helpful discussion on my work.

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<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>A</td>
<td>Ambiguous recognition mechanism</td>
</tr>
<tr>
<td>C</td>
<td>Context-based recognition mechanism</td>
</tr>
<tr>
<td>F</td>
<td>Familiarity recognition mechanism</td>
</tr>
<tr>
<td>PM</td>
<td>Phenotype matching recognition mechanism</td>
</tr>
<tr>
<td>RA</td>
<td>Recognition alleles recognition mechanism</td>
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### Bluegill breeding protocol

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>C1</td>
<td>Cuckolder male 1</td>
</tr>
<tr>
<td>F1, F2</td>
<td>Female 1 and female 2</td>
</tr>
<tr>
<td>P1</td>
<td>Parental male 1</td>
</tr>
</tbody>
</table>

### Guppy populations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>LG</td>
<td>Lower Guanapo River</td>
</tr>
<tr>
<td>LO</td>
<td>Lower Oropouche River</td>
</tr>
<tr>
<td>PA</td>
<td>Paria River</td>
</tr>
<tr>
<td>TN</td>
<td>Tunapuna River</td>
</tr>
<tr>
<td>UA</td>
<td>Upper Aripo River</td>
</tr>
<tr>
<td>UY</td>
<td>Upper Yarra River</td>
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### Other abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>AMP</td>
<td>Adenosine monophosphate</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>bp</td>
<td>Base pair length</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>eqn</td>
<td>Equation</td>
</tr>
<tr>
<td>EST</td>
<td>Eastern Standard Time</td>
</tr>
<tr>
<td>$H_e$</td>
<td>Expected heterozygosity</td>
</tr>
<tr>
<td>$H_o$</td>
<td>Observed heterozygosity</td>
</tr>
<tr>
<td>MHC</td>
<td>Major histocompatibility complex</td>
</tr>
<tr>
<td>MS-222</td>
<td>Tricaine methanesulfonate</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>$R$</td>
<td>Relatedness</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>SD</td>
<td>Standard deviation</td>
</tr>
<tr>
<td>SE</td>
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Chapter 1

1 General introduction

1.1 Historical background

Helping behaviours that benefit a recipient at a cost to the donor are widespread in nature, but their very existence is puzzling. Why would animals be willing to sacrifice their time, energy, or opportunities for reproduction or indeed, even their lives, to help another individual? Modern biologists have noted that such altruism was problematic to Charles Darwin’s theory of natural selection because such costly behaviours should be purged from a population (reviewed by Nowak et al. 2010). Indeed, Darwin discussed these altruistic behaviours in some of his most important works, attempting to explain sterility in ants (Darwin 1859) and volunteer soldier behaviour in humans (Darwin 1871). In doing so, Darwin put forward group selection arguments, for example, that tribes would be more successful in battle if they had more selfless warriors. However, Darwin seemed unsatisfied by his explanation for this behaviour, because the “sympathetic and benevolent” individuals who performed the self-sacrificial behaviour of fighting in wars were unlikely to leave more offspring than individuals who did not engage in such behaviour (Darwin 1871). Further research was required to explain altruism.

Biologists have now proposed several possible explanations for the persistence of apparently altruistic behaviours. These explanations include reciprocal altruism (Trivers 1971), in which one individual helps another individual with the expectation of being helped at a future time; policing (Clutton-Brock and Parker 1995), in which non-cooperators are punished, often by more dominant individuals; and kin selection (Fisher 1930; Haldane 1932), in which an individual helps relatives survive and reproduce, thereby indirectly passing on the genes they share by descent. In particular, the formalization of kin selection theory by Hamilton (1964) greatly advanced our understanding of social behaviour. Hamilton’s great insight was that a helping behaviour should evolve if the product of the relatedness coefficient of the individuals involved (that is, the probability that two individuals share an allele because of a common ancestor) and the benefit to the
recipient of performing the behaviour exceeds the cost to the donor of performing the behaviour \((i.e. \ R \times b > c)\). This inequality has come to be known as Hamilton’s rule.

Hamilton’s paper was largely devoted to explaining the evolution of eusociality in social insects, wherein sterile castes of workers forgo reproduction to assist in the rearing of relatives. Biologists have subsequently shown that relatedness is important in the evolution of a large number of social behaviours (reviewed by Foster 2009), and kin selection theory has been used to explain cooperative breeding (Hatchwell et al. 2014), alarm calls (Sherman 1977; Wheeler 2008), alloparental care (Andersson and Waldeck 2007), colony formation (Mehdiabadi et al. 2006), and cooperative gregarious behaviours for purposes of courting potential mates (Petrie et al. 1999; Shorey et al. 2000), or feeding (Brown and Brown 1996; Gerlach et al. 2007). In spite of criticism of kin selection theory (Nowak et al. 2010), it has become one of the most pervasive and useful theories for understanding the evolution of behaviour (Breed 2014).

To gain kin selective benefits, an individual must direct helping behaviours towards relatives. Hamilton (1964) first suggested the idea of direct kin recognition in which relatives are recognized based on their phenotypic traits such as appearance or odour, but immediately noted that such “sophisticated” discrimination need not evolve. Instead, he proposed that individuals could direct their helping behaviours towards individuals near their home. However, the discovery that many females mate multiply (reviewed by Parker 1970; Birkhead and Møller 1998) revealed that being born together or occupying the same nest did not reliably indicate full-sibling kinship. This discovery meant that an animal that followed Hamilton’s simple decision rule of helping individuals close to the nest could inadvertently help less-related individuals. These recognition errors would erode the benefits of helping and might prevent the evolution of helping behaviours unless populations also evolve accurate kin recognition mechanisms.

1.2 Recognition mechanisms

Biologists have described a variety of recognition mechanisms that would allow individuals to direct helping behaviours to related recipients. Holmes and Sherman (1982)
outlined four potential mechanisms: location, association (often referred to as “familiarity”), phenotype matching, and recognition alleles. Subsequent authors (e.g., Mateo 2004) have updated the terminology of Holmes and Sherman (1982) by broadening the “location” recognition mechanism to include all context-based cues, but the definitions of these mechanisms have otherwise remained largely the same. Below I describe the four primary kin recognition mechanisms and the conditions under which they would reliably identify kin.

1.2.1  Context-based cues

Kin recognition by context-based cues is similar to the simple decision rule for helping kin that was first described by Hamilton (1964), and they are expected to evolve when some observable variable reliably correlates with kinship. For example, individuals may treat anyone in the vicinity of their natal nest as related, or a male may remember having mated with a particular female. He could use his memory of his reproductive history with that female to treat any of her offspring as kin (Mateo 2004). Clearly, although context-based cues are reliable in some situations, the context in which they are reliable is often narrow.

1.2.2  Familiarity

Familiarity is a kin recognition mechanism based on prior association (Mateo 2004). When familiarity is used, individuals remember the phenotypes of other individuals encountered in situations normally correlated with kinship (for example, at the natal nest), and later treat these individuals as kin (Holmes and Sherman 1982). In general, familiarity is reliable when a population has low dispersal or non-overlapping generations (so that all kin are familiar), and low levels of multiple mating (leading to low variance in relatedness both within and among broods), such that being born together is a reliable indicator of kinship.
1.2.3 Phenotype matching

Phenotype matching is a kin recognition mechanism whereby individuals form a ‘kin template’ based on the appearance, odour, or sound produced by family members encountered during early development. Later, individuals compare the phenotype of putative kin to the kin template, and treat these individuals as related if there is a close match (Holmes and Sherman 1982; Mateo 2004). Self-referent phenotype matching is a special case in which the kin template is formed using one’s own phenotype, and allows the discrimination of kin and non-kin even when individuals are born into broods of mixed relatedness in promiscuous species (Mateo 2004). However, testing for self-referent phenotype matching is difficult in practice, because it requires that individuals have no exposure to other reliable cues of kinship during their early development. This is prohibitively difficult in species that have internal gestation and live births.

1.2.4 Recognition alleles

The fourth recognition mechanism described by Holmes and Sherman (1982) is recognition alleles. When recognition alleles are used as a recognition mechanism, an allele at a single locus has three functions: 1) to express itself phenotypically; 2) to enable bearers to recognize the allele or its effect; and 3) cause bearers to favour individuals carrying that allele. An advantage of recognition alleles over other recognition mechanisms is that there is no learning component, so unfamiliar relatives can be recognized (Mateo 2004), and even species with limited cognitive abilities can recognize their relatives.

Although theoretically possible, there are several factors that limit the evolution of recognition alleles. First, Holmes and Sherman (1982) pointed out that this type of recognition mechanism can lead to conflict with the rest of the genome. Specifically, when individuals of low genetic relatedness share the same copy of the recognition allele, the rest of the genome that does not benefit from the recognition allele’s effect could evolve to suppress the activity of the recognition allele (Alexander and Borgia 1978). Second, individuals that use recognition alleles are expected to have a large number of recognition
errors where kin who do not have the preferred allele are treated as non-kin, and unrelated individuals who do have the preferred allele are treated as kin. Whereas kin recognition by phenotype matching can allow a range of phenotypes to be treated as kin, recognition alleles allow recognition of kin only with a narrow range of phenotypes. Third, loci used as recognition alleles need to be highly polymorphic (Grosberg and Quinn 1986) to reduce the likelihood of incorrectly accepting non-kin individuals as related, but the question of how this diversity in genotypes is maintained remains unanswered. In addition to these theoretical issues with recognition alleles, there are also practical difficulties in testing for this recognition mechanism. To conclusively demonstrate recognition alleles, all other mechanisms must be ruled out (Holmes and Sherman 1982). Tests of self-referent phenotype matching and recognition alleles both require removing other cues of kinship, and thus have very similar experimental methods. Thus, unless the effect of a candidate recognition allele is tested directly, authors of most kin recognition studies that control for the effect of familiarity are likely to conclude that phenotype matching was used for discrimination rather than recognition alleles. Because of this practical limitation to tests of recognition alleles, it may be productive to consider recognition alleles as a special case of phenotype matching where only a single locus is used to form the kin template. Indeed, Jansen and van Baalen (2006) have shown that some of the theoretical problems with the stability of recognition alleles are resolved if more than one locus is involved in signaling genotype – a state that more closely resembles phenotype matching than recognition alleles.

1.3 Recognition mechanisms across species

Biologists have performed many studies on the expression of kin recognition mechanisms across a variety of species. In general, a taxon is expected to evolve the recognition mechanism that is least costly to develop, provided it can reliably discriminate kin from non-kin. However, there has been little examination of the prevalence of different recognition mechanisms or the conditions under which they evolved. I thus conducted a literature review with the objectives of: 1) determining the evidence for each kin
recognition mechanism in nature; 2) understanding if there are patterns in recognition mechanism across taxa; and 3) establishing if there are consistent ecological or life history factors that explain the evolution of one mechanism over another. Published literature is notoriously biased towards positive results (Jennions and Møller 2002) and greater research effort (McKenzie and Robertson 2015; Wurtsbaugh et al. 2015), thus the purpose of this search was to illustrate the state of our collective knowledge on kin recognition and not to make direct comparisons between groups or make statements on the importance of one mechanism over another. By performing this search, I intended to provide observational data that could inform scientific hypotheses.

I performed a search for “kin recognition” on the Web of Science that included all references that appeared as of October 1, 2015, and found 596 papers that featured a test of the ability to recognize kin in one or more species. There were 513 cases where kin were recognized, and 131 cases where they were not. Many species have been studied multiple times. For example, house mice (Mus musculus) have had their ability to recognize kin studied in 22 different published papers. 21 of those studies found discrimination between kin and non-kin and 1 study did not. I categorized each species as recognizing kin if at least one study found discrimination between kin and non-kin, and categorized the species as not recognizing kin if no studies found such discrimination. Using this methodology, 287 species have been shown to recognize kin and 88 species have not.

In cases with kin recognition, I also recorded the recognition mechanism used, which was either stated explicitly by the study’s authors, or was inferred by me based on the experimental methods. Some studies were not designed to test a specific recognition mechanism – for example, an individual may be given the choice of associating with a familiar/related group or an unfamiliar/unrelated group. In that example, recognition could not be attributed to either familiarity or phenotype matching. In these cases, I recorded that the species recognizes kin, but the recognition mechanism was “ambiguous.” A total of 44 species, most commonly those studied in more than one context, use more than one recognition mechanism, so I recorded them as using multiple recognition mechanisms. The house mouse, for example, has been shown in separate studies to use familiarity, phenotype matching, and recognition alleles, and was thus recorded as using each mechanism.
1.3.1 Results of literature review

Table 1.1 shows the breakdown of kin recognition abilities among taxa. Because most species studied (162 of 287 species that recognize kin) are vertebrates, I have categorized vertebrates to their class. Among non-vertebrates, insects have also been broadly studied, thus I present their results separate from non-insect arthropods. However, non-arthropod invertebrate animals have not been thoroughly investigated, so I have grouped their results together as “other.” This polyphyletic group includes bacteria, protists, tunicates, gastropods, and other invertebrates. Interestingly, the discovery that plants can alter their investment in tissues depending on the relatedness of their neighbours (Dudley and File 2007) has led many researchers to investigate the ‘social behaviour’ of plants as it relates to relatedness, and now the kin recognition abilities of 23 plant species have been studied.
Table 1.1 Kin recognition studies summarized by taxon. Non-insect arthropods include 16 species of arachnids and 3 crustaceans. ‘Other’ includes bacteria, protists, tunicates, gastropods, and a variety of other invertebrates. For species that recognize kin, recognition mechanisms are categorized as: C = context-based recognition, F = familiarity, PM = phenotype matching, RA = recognition alleles, A = ambiguous. Note that the total number of species that recognize kin does not equal the sum of the categories because some species use multiple mechanisms.

<table>
<thead>
<tr>
<th>Taxon</th>
<th># of species that recognize kin</th>
<th># of species that do not recognize kin</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total C F PM RA A</td>
<td></td>
</tr>
<tr>
<td>Amphibians</td>
<td>26 0 2 19 0 6 7</td>
<td></td>
</tr>
<tr>
<td>Birds</td>
<td>33 2 12 14 1 9 18</td>
<td></td>
</tr>
<tr>
<td>Fish</td>
<td>21 0 8 17 3 1 1</td>
<td></td>
</tr>
<tr>
<td>Mammals</td>
<td>70 1 32 33 1 20 6</td>
<td></td>
</tr>
<tr>
<td>Reptiles</td>
<td>12 1 3 4 0 5 1</td>
<td></td>
</tr>
<tr>
<td>Insects</td>
<td>80 1 12 48 1 26 33</td>
<td></td>
</tr>
<tr>
<td>Non-insect arthropods</td>
<td>12 0 3 4 0 7 7</td>
<td></td>
</tr>
<tr>
<td>Plants</td>
<td>15 0 0 15 0 0 8</td>
<td></td>
</tr>
<tr>
<td>Other</td>
<td>18 0 0 12 7 3 7</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>287 5 72 166 13 77 88</td>
<td></td>
</tr>
</tbody>
</table>

1.3.2 Prevalence of recognition mechanisms

Although there is empirical support for all four recognition mechanisms, there are considerable differences in how frequently each mechanism is represented in the literature. Familiarity, which is used by 72 species (25.1% of species shown to recognize kin), and phenotype matching, used by 166 species (57.8%), are highly represented, whereas context-based cues (5 species, or 1.7%) and recognition alleles (13 species, or 4.5%) are much less common. Although a large number of species (77 species) were studied in a way that did not allow me to categorize the recognition mechanism, based on the experimental design, the most likely were either familiarity or phenotype matching. The results of this literature review show that familiarity and phenotype matching are commonly used to recognize kin.
It is probable that context-based recognition mechanisms are understudied and that more species would be found to use this mechanism if researchers designed their studies to test for the use of these cues in kin recognition. Nest-building birds, for example, typically provide food to young hatchlings found in their nest and do not provide food to nestlings outside their nest. Parents of these species thus recognize their offspring by looking at who is in their nest – a context-based cue of recognition. However, this recognition mechanism is useful in only a narrow range of situations, and for example, one bird species, the black-legged kittiwake (*Rissa tridactyla*) stops relying on locational cues to recognize its offspring as the nestlings age and become more mobile (Cullen 1957). A species that uses location as a context-based cue for recognition thus might evolve other recognition mechanisms to discriminate kin from non-kin in additional contexts.

The low frequency of recognition alleles could either be a true representation of how uncommon the mechanism is, or could instead be explained by the methodological difficulty in identifying candidate recognition alleles and then testing for them. I found support for recognition alleles comes mainly from “other” species, which include one sea sponge, one yeast, one protist, and four tunicates. These species are very small, allowing cell-to-cell contact, where a gene product expressed on the cell membrane of one individual could conceivably come in direct contact with a complementary gene product on the cell membrane of a second individual. Consistent with this possibility, the earliest study to discover recognition alleles found that a highly polymorphic histocompatibility locus was responsible, and this locus was important in colony fusion in a tunicate (Grosberg and Quinn 1986). Although cell-to-cell contact is less feasible in larger species, there are parallels in this recognition mechanism in vertebrates. The major histocompatibility complex (MHC) has been shown to be important in discriminating related from unrelated individuals in salmonids (Olsén et al. 1998; Rajakaruna et al. 2006) and mice (Penn and Potts 1998). Interestingly, the MHC does not meet the classic criteria of a recognition allele (Holmes and Sherman 1982) because it is unlikely that the MHC locus codes for the preferential treatment of individuals sharing the same allele, although it is possible that this function is performed by a linked gene. These studies suggest that the histocompatibility gene products, whose major role is discriminating between self- and non-self, can be used
in discriminating between kin and non-kin as well, though this function is most likely in small organisms.

1.3.3 Patterns across taxa

Caution should be used when making general statements about the ability of one taxon over another’s ability to recognize kin based on the data presented in Table 1.1. However, it is tempting to make a few observations about these results.

First, we see that familiarity is not expressed in plants or the “other” taxa. This absence could be because of limited study in these species, or it could be because plants and “other” species are missing the cognitive abilities to remember familiar individuals. This question remains unanswered in kin recognition research.

Second, as a general pattern, we see that although phenotype matching is used in all taxa, some taxa tend to use phenotype matching much more than familiarity, while other taxa have a close-to-even split in the number of species that use one mechanism over another. Amphibians (19 species vs. 2 species) and insects (48 species vs. 12 species) in particular tend to use phenotype matching instead of familiarity, whereas birds (14 species vs. 12 species) and mammals (33 species vs. 32 species) do not favour the expression of phenotype matching over familiarity. Although there is a bias towards phenotype matching as a recognition mechanism in amphibians and insects, it is interesting that both familiarity and phenotype matching are expressed in all vertebrate classes as well as in arthropods. Together, these data suggest that recognition mechanism is not evolutionarily fixed within taxa. However, it is not clear if the bias towards phenotype matching in amphibians and insects is an artefact of the research methodology or if the bias has a biological explanation. That is, the bias could be caused by shared ecological or life history factors that favour phenotype matching over familiarity, or alternatively, the evolution of recognition mechanism could be slowed by phylogenetic constraints. By comparing within a closely-related taxon, a phylogenetic test could eliminate many confounding life history variables.
and allow biologists to assess the evolvability of recognition mechanisms to determine if ecology or phylogeny best explains the observed mechanism.

### 1.3.4 Factors influencing evolution of recognition mechanism

The reason why insects and amphibians tend to use phenotype matching much more than birds or mammals is unclear, and the fact that most data on predictive variables are incomplete makes the task of identifying the most important factors difficult. Two factors that may contribute to this relationship are differences in cognitive ability or in average brood size. Many insects and amphibians have smaller brains than mammals and birds (Crile and Quiring 1940; Gillooly and McCoy 2014). If brain size is correlated with cognitive function, it is likely that insects and amphibians simply cannot remember familiar broodmates and must instead rely on comparing putative kin to a template. A second possibility is that the large family size of many amphibians and insects (e.g. Inger and Bacon 1968; Bourke 1999; Ferguson-Gow et al. 2014) relative to mammals and birds (Gilbert 1986; Charnov and Morgan Ernest 2006; Jetz et al. 2008) prevents amphibians and insects from remembering all of their family members. Instead, phenotype matching could be a less cognitively-expensive means of recognizing relatives because it does not require remembering a large number of individuals. These two explanations are not mutually exclusive, and in fact they complement each other. However, there are not data available that would allow a thorough test of other factors such as degree of multiple mating, dispersal distance, or lifespan, that are also potentially important in the evolution of recognition mechanism. Thus, a study that examines variation in recognition mechanism while controlling for phylogenetic history is needed to further our understanding of the evolution of kin recognition mechanisms.

### 1.4 Study species

I investigated the ability of individuals to recognize kin and the factors affecting recognition mechanisms in two fish species, bluegill (*Lepomis macrochirus*) and guppies
(Poecilia reticulata). Both species have promiscuous mating systems, wherein both males and females mate with multiple partners (Houde 1997; Neff 2001). A result of this promiscuity is that individuals may encounter relatives from outside of their natal family group, and that there is variation in the level of relatedness of individuals within a natal group. Thus, in both species, a direct kin recognition mechanism is necessary for individuals to discriminate between full-siblings and less related individuals. However, differences in ecology and life history of these species lead to bluegill being more amenable than guppies in the investigation of some research questions, and guppies being more amenable than bluegill in the investigation of others. Thus, my thesis examines the recognition mechanisms of both species.

1.4.1 Bluegill

Bluegill are a member of the Centrarchidae family, and are a temperate freshwater fish widespread throughout North America (Scott and Crossman 1998). I conducted my fieldwork on the bluegill population at Lake Opinicon in eastern Ontario (44°34′N, 76°19′W), which has been studied continuously since 1977 (Colgan et al. 1979). During the reproductive season, male bluegill sweep the substrate with their caudal fins to construct tightly-packed nests within colonies of up to 150 nests (Gross and MacMillan 1981). These males attract females to their nest to spawn, and parental care is provided exclusively by males. Over the course of a care-giving period that lasts 7-10 days, nest-tending “parental” males defend their brood against predators, aerate the eggs by fanning their tails over the clutch, and remove fungus and dead eggs to prevent disease (Rodgers et al. 2012). While providing care, males do not actively forage and may lose up to 10% of their body weight (Coleman and Fischer 1991). This costly period of care has made it profitable for discrete alternative reproductive strategies to evolve, in which some males fertilize eggs but do not provide parental care (Dominey 1980; Gross 1982). In contrast to parental males, “cuckolder” males mature precociously, do not build nests or provide care, and opportunistically intrude on parental males in the act of spawning with females using one of two tactics: sneaker or satellite. Sneaker males are the youngest and smallest
cuckolders, stealing fertilizations from the parental male by stationing themselves on the periphery of nests, and quickly entering the nest to release sperm while the parental male spawns with a female (Gross 1982). Satellite males are older cuckolders and are closer in size to adult female bluegill (Gross 1982), and for this reason are often called mimics (Dominey 1980; Neff and Svensson 2013). These males adopt the colouration and behaviour of female bluegill to deceive parental males into perceiving that they have two females in their nest. Satellite males typically position themselves between a parental male and a female, and release their sperm when females ‘dip’ horizontally to release eggs, thereby stealing fertilizations from the nest-tending parental male. In this population, parental males mature at an age of 7 years. Cuckolders reach maturity at 2 years of age, and are believed to transition into satellite males at an age of 4 years (Gross 1982). Cuckolders intrude on approximately 10% of female dips (Fu et al. 2001), but because cuckolders release more sperm than the parental male and satellites have an advantaged spawning position over parentals (Stoltz and Neff 2006), cuckolders win in sperm competition and fertilize an average of approximately 20-25% of all the eggs in the population (Neff and Clare 2008; Garner and Neff 2013).

The high level of cuckoldry in bluegill leads to variation in the level of relatedness among nestmates. A bluegill larva that hatches in a cuckolded nest will have nestmates that share a father and a mother (i.e. are full-siblings), or only a mother (i.e. are half-siblings). Indeed, because up to nine females visit each nest (Hain and Neff 2006), larvae may also have nestmates that share only a father (i.e. are half-siblings), or share neither a father nor a mother (are unrelated). This natural variation in relatedness among nestmates offers an opportunity to test the recognition of kin and discrimination against non-kin in an ecologically relevant setting. Furthermore, because parental males tend to fertilize the majority of eggs in their nest, their offspring are expected to be more related to their nestmates, on average, than the offspring of cuckolders. This asymmetry in relatedness could lead to differences in the ability of parental-sired and cuckolder-sired larvae to recognize kin, and offers a unique opportunity to test the evolution of recognition mechanisms among individuals sired by males of alternative reproductive strategies.
As an externally-fertilizing fish, bluegill offer an advantage over other species in the investigation of some questions related to kin recognition. Specifically, it is possible to manipulate cues of relatedness from the moment of fertilization. As described in section 1.2.3 above, to definitively demonstrate self-referent phenotype matching, an individual’s rearing environment must be manipulated so that there are no other reliable cues of kinship encountered during development (Hauber and Sherman 2001). Such manipulations are especially difficult in internally-fertilizing animals, in which the mother is always and siblings are occasionally encountered during gestation as well as the moments after birth. For example, Mateo and Johnston (2000) performed one of the best experimental tests of self-referent phenotype matching. In that test, the authors scrambled cues of kinship in newborn golden hamsters (*Mesocricetus auratus*) by cross-fostering individuals between nests within twelve hours of birth. Although these cross-fostered hamsters later discriminated between unfamiliar siblings and unfamiliar non-siblings, the conclusion that these hamsters had used of self-referent phenotype matching was criticized because the authors could not rule out the possibility that newborn hamsters had learned relatedness cues in the first hours after birth (Hare et al. 2003). Because bluegill fertilize eggs externally, I can scramble cues of relatedness beginning at the moment of fertilization and effectively test for self-referent phenotype matching, an opportunity that is not available in internally-fertilizing animals.

1.4.2 Guppies

Guppies are a small live-bearing fish native to rivers and streams of northern South America and the island of Trinidad (Houde 1997). Guppies have long been described as promiscuous based on their behaviour (Houde 1987) because males court females continuously and visit many females within short time periods (Baerends et al. 1955; Farr 1975). Although females are receptive for only two or three days in each reproductive cycle of 25-30 days, the high number of males who court receptive females or attempt sneak copulations during this period suggests that females also mate multiply (Houde 1997). Indeed, parentage tests made possible by the design of genetic markers has confirmed that
both females (Kelly et al. 1999) and males (López-Sepulcre et al. 2013) mate with multiple individuals. Females can store sperm from previous matings, and mixed broods are generated with sperm from old and recent matings (Hildemann and Wagner 1954). Multiple mating by guppy females means that guppies are born with full-siblings (*i.e.* have mother and father in common) and maternal half-siblings (*i.e.* have mother in common), and females’ ability to store sperm means that guppies may encounter unfamiliar full-siblings or half-siblings born at a different time. Furthermore, because males mate with multiple females, guppies may encounter paternal half-siblings (*i.e.* have father in common). This creates situations in which guppies may need to discriminate between kin and non-kin, and makes guppies a good species for the study of kin recognition. Indeed, authors have already found that guppies recognize kin in contexts such as juvenile shoaling behaviour (Griffiths and Magurran 1999) and inbreeding avoidance as adults (Daniel and Rodd 2015).

Although internal fertilization in guppies makes this species unfit for tests of self-referent phenotype matching, they do have an advantage over bluegill for the study of kin recognition in at least one respect. Specifically, guppies have emerged as a model system for the study of evolution because they have repeatedly evolved life history and behavioural traits in response to differences in predation pressure across populations, particularly in Trinidad (Reznick and Endler 1982; Reznick et al. 1990; Magurran 2005; Reznick et al. 2008). Biologists are fascinated by this variation, and have explored many traits that differ among populations, including male colouration (Houde and Endler 1995), brood size (Reznick and Endler 1982), shoal size (Magurran and Seghers 1991), mate choice (Endler and Houde 1995), lifespan (Reznick et al. 1996), the frequency of sneak copulations (Magurran and Seghers 1994), and the frequency of multiply-sired broods (Kelly et al. 1999; Neff et al. 2008). For many populations, these characters are well-described, creating opportunities for researchers to test relationships between traits. This knowledge of different populations allows me to test the effects of several candidate ecological factors on the expression of kin recognition, improving our understanding of what influences the evolution of kin recognition mechanism.
Because of guppies’ emergence as a model system in evolutionary biology, genetic tools have been developed to help understand phylogenetic relationships among populations. Indeed, phylogenies of guppy populations have been constructed based on allozymes (Carvalho et al. 1991), mitochondrial DNA (Alexander et al. 2006), and microsatellites (Suk and Neff 2009). These phylogenies allow us to understand how often the expression of traits transition between populations. By mapping recognition mechanism on such a tree, I can understand how often recognition mechanism transitions over time, and using a molecular clock, I can estimate the speed at which these transitions occurred. Controlling for phylogeny allows researchers to ask questions about the effect of one variable of interest on the expression of another trait (Harvey and Purvis 1991). Thus, phylogenetic trees are valuable tools that allow us to ask new questions about the evolution of kin recognition mechanism.

1.5 Research objective

The major objective of this thesis is to develop a more thorough understanding of the evolution of kin recognition. The development of kin selection theory has greatly advanced our understanding of social behaviour (Hamilton 1964), but the advent of molecular markers has revealed that many family groups are comprised of individuals of mixed-relatedness (e.g. Birdsall and Nash 1973). This mixed relatedness challenges the importance of Hamilton’s rule in nature, because group members are not necessarily related. In my thesis I test the idea that kin selection might continue to operate in the face of multiple mating through the evolution of kin recognition mechanisms. My literature review in section 1.3 showed that many species across a variety of taxa have been investigated for their kin recognition abilities. However, I found that although recognition has been observed many times, there is a paucity of empirical studies that test what ecological and life history variables favour the evolution of one recognition mechanism over another. In my thesis, I investigate the relationship between various ecological and life history variables (particularly the degree of multiple mating) and recognition mechanism, and I use guppies – a well-studied species in evolutionary questions – to test
the evolvability of recognition mechanisms over a short time-scale. In doing so, I aim to move the field of kin recognition beyond the observation of recognition mechanisms used to identify relatives to the ultimate questions of how these mechanisms evolve.

1.6 Thesis structure

My thesis is comprised of five data chapters, which were designed as distinct studies to be submitted for independent publication. Chapters 2 - 5 have been published, and Chapter 6 has been submitted for review. I use two fish species, bluegill and guppies, to study the mechanisms and evolution of kin recognition to begin to assess the generality of the patterns I found.

1.6.1 Kin recognition and bluegill

In the first two data chapters of my thesis, I use bluegill to look at a potential benefit of associating with kin, and at the kin recognition mechanisms used by bluegill. In both chapters, I take advantage of the alternative reproductive strategies of bluegill to compare behaviours of parental-sired and cuckolder-sired larvae.

In chapter 2 of my thesis (“Kinship affects innate responses to a predator in bluegill sunfish Lepomis macrochirus larvae”; Hain and Neff 2009), I look for a potential benefit of associating with kin versus associating with groups of mixed relatedness. I use in vitro fertilization techniques to create broods of known parentage, and I form groups comprised of either ten full-siblings or of mixed broods comprised of two full-sibling families of five individuals each. I then introduce a predator odour cue to these groups and observe the change in cohesiveness of the shoal in response to this cue. I further test for differences in the shoaling response between parental-sired and cuckolder-sired larvae. Shoaling closely together is an anti-predator response in bluegill, and closely-spaced shoals are expected to have enhanced survival (Chipps et al 2004).
In chapter 3 ("Promiscuity drives self-referent kin recognition"; Hain and Neff 2006), I use bluegill to look at the kin recognition mechanisms of larvae sired by males from alternative reproductive strategies. I first test the hypothesis that the offspring of parental males are more related to their nestmates than the offspring of cuckolder males. I then test the hypothesis that because of this asymmetry in relatedness among the offspring of parental and cuckolders, cuckolder-sired larvae are more likely to use phenotype matching than parental-sired larvae. Also in this chapter, I take advantage of external fertilization in bluegill to scramble cues of relatedness among nestmates to test for self-referent phenotype matching.

1.6.2 Kin recognition and guppies

In chapters 4 - 6 of this thesis, I use guppies to look at the relationship between kin recognition mechanism and various ecological or life history variables, including the degree of multiple mating by females. I then map the observed recognition mechanisms onto a phylogenetic tree.

In chapter 4 ("Multiple paternity and kin recognition mechanisms in a guppy population"; Hain and Neff 2007), I test the ability of a guppy population to recognize kin. Previously, guppies from a population exposed to high levels of predation had been shown to use familiarity to recognize kin (Griffiths and Magurran 1999). Here, I tested the recognition mechanism of individuals from a population exposed to low levels of predation to see if recognition mechanism, like many traits in guppies (Magurran 2005), differs among predation regimes. Briefly, I used guppies to determine the degree of multiple mating, used dichotomous choice trials to test the population’s kin recognition mechanisms, and I looked at the relatedness of natural shoals of adult guppies from this population to examine what opportunities these guppies would have for kin-directed social behaviours in the wild.

In chapter 5 ("Multiple mating predicts intensity but not mechanism of kin recognition"; Hain et al. 2016), I extend the analysis of the previous chapter by testing the
kin recognition mechanisms of six guppy populations. I then test the relationship of these recognition mechanisms to three ecological or life history variables (degree of multiple mating, predation regime, and brood size) to determine if any of those variables can explain the observed variation in recognition mechanism. In particular, I test the kin recognition hypothesis by examining the relationship between brood relatedness (a measure of a female’s degree of multiple-mating) and preference for kin across populations.

In chapter 6 (“Evolution of kin recognition mechanisms in a fish”), I map the recognition mechanisms of the six guppy populations observed in chapter 5 onto a phylogeny developed with mitochondrial sequence data, thereby illustrating how recognition mechanism has evolved over time and across populations. Using a novel simulation, I test if phylogenetic inertia determines the recognition mechanism observed in each population. I also apply a molecular clock to estimate the speed of evolution of these recognition mechanisms.

Together, chapters 5 and 6 address a major problem in the study of the evolution of kin recognition, which I describe in section 1.3 of this thesis. By comparing recognition mechanisms within, rather than across species, I minimize variation in many covarying factors such as cognitive ability or phylogenetic history that obscure the relationship between recognition mechanism and ecological variables of interest.

1.6.3 Summary

Finally, in chapter 7 I summarize my findings and discuss how my data advance our understanding of how kin recognition has evolved, and offer directions for future study, particularly in human health. By taking a comparative approach within and among species, my thesis broadens our understanding of the evolution of kin recognition, and provides a test of the kin recognition hypothesis.
1.7 References


Chapter 2

2 Kinship affects innate responses to a predator in bluegill larvae

Naïve kin groups and mixed-family groups of bluegill (Lepomis macrochirus) were exposed to a novel predator cue. Bluegill larvae responded by increasing shoal cohesiveness in kin groups but not in mixed-family groups; moreover, larvae sired by males of the ‘cuckolder’ life history tended to have an enhanced ability to respond to direct cues of kinship versus larvae sired by males of the ‘parental’ life history, who instead appeared to respond to cues of life history rather than relatedness per se. The increased shoal cohesion among related individuals likely confers a survival benefit and indicates that the anti-predatory shoaling response is innate in bluegill.

2.1 Introduction

The formation of animal groups has long been of interest to biologists (Welty 1934; Tinbergen 1953). Groups may form for the purpose of increasing the efficiency of foraging or mate choice, but perhaps most importantly, groups improve predator defence (Bertram 1978). Improved predator defence may come from risk dilution, improved overall vigilance, or increased predator confusion (Hamilton 1971; also reviewed by Pitcher and Parrish 1993; Godin 1997). For example, in silvery minnows (Hybognathus nuchalis Agassiz), solitary individuals are readily captured by largemouth bass (Micropterus salmoides Lacépède), whereas capture time was considerably longer when the minnows were in shoals (Landeau and Terborgh 1986). Furthermore, when one or two minnows were experimentally manipulated to look different from the other individuals in the shoal,

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1 A version of this chapter has been published and is presented here with permission from the Journal of Fish Biology.

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the bass took less time to capture the minnow (Landeau and Terborgh 1986). This result highlights the importance of looking similar to shoalmates and the resultant confusion effect that the similarity can have on predators.

Grouping with relatives may be especially effective in minimizing individuals’ susceptibility to predation. Anti-predator behaviours such as predator-searching and predator-inspection involve cooperation among group members (Pitcher and Parrish 1993; Godin 1997), and kin selection theory predicts that groups of related individuals will be more cooperative than groups of unrelated individuals (Hamilton 1964). Kin groups should also benefit from an increased predator confusion effect because related individuals tend to have more similar phenotypes than unrelated individuals (e.g., Rajakurana et al. 2006).

The mechanisms individuals use to form groups in response to a predator have also received considerable attention. Broadly, these mechanisms can be innate or learned. For example, Chinook salmon (*Oncorhynchus tshawytscha* Walbaum) have an innate component to their behaviour as naïve individuals show characteristic anti-predator behaviours when exposed to predator cues for the first time (Berejikian et al. 2003; also see Hawkins et al. 2007; Scheurer et al. 2007). On the other hand, in steelhead trout (*Oncorhynchus mykiss* Walbaum), individuals reared in isolation from predators have poor anti-predator responses when first exposed to a predator, but show an improved response after being exposed to a combination of a predator chemical extract and conspecific alarm cues (Berejikian et al. 1999). These data suggest that anti-predator behaviour can also have a significant learned component.

In this study, the anti-predator behaviour of bluegill (*Lepomis macrochirus* Rafinesque) in response to a novel predator cue was investigated. Bluegill are endemic to North America and have paternal care of the eggs and larvae (Lee et al. 1980). Once the larvae leave the nest, however, they are subjected to predation by other fishes as well as cnidarians (*Hydra canadensis* Rowan) (Gross and MacMillan 1981; Elliot et al. 1997). In response to a threat of predation, juvenile bluegill can form shoals and it has been shown that tighter shoal cohesion reduces susceptibility to predation (Chipps et al. 2004). Here, I generated full-sibling and mixed-sibling groups using in vitro fertilization techniques and
examined the role of kinship on shoal cohesion in naïve larval bluegill in response to a predator odour cue. I predicted that in comparison to mixed-sibling groups, full-sibling groups would respond more strongly to the predator cue by shoaling more closely together. These data allow us to determine the importance of kinship on predator defense and whether or not bluegill larvae have an innate anti-predator behaviour response.

2.2 Methods

2.2.1 Study species

The study was conducted using the bluegill population found in Lake Opinicon, Ontario, Canada (44° 16’ N, 76° 30’ W). The Lake Opinicon population has a long history of behavioural studies (eg. Gross and Charnov 1980; Hain and Neff 2006). In Lake Opinicon, adult bluegill males are characterized by discrete life histories termed “parental” and “cuckolder” (Gross and Charnov 1980). Parentals mature at 7 years of age, construct nests, court and spawn with females, and provide sole parental care to the developing larvae. In contrast, cuckolders mature precociously at the age of 2 years and opportunistically steal fertilizations from the nest-tending parental. The offspring of cuckolders have previously been shown to actively discriminate between odours of kin and non-kin and prefer to associate with kin, but offspring of parentals do not appear to use a direct kin recognition mechanism to discriminate between kin and non-kin (Hain and Neff 2006).

2.2.2 Sample collection and husbandry

In June 2006 and 2008, swimmers equipped with snorkeling gear conducted daily surveys of bluegill breeding activity in Lake Opinicon. When spawning was discovered, mature parentals, cuckolders and females were collected opportunistically using snorkeling gear and dip nets and transported by boat to the aquarium facilities at the Queen’s University Biological Station, which sits on the lake’s shore. These fish were used to create families using *in vitro* fertilization techniques as described in Neff and Lister (2007). Briefly, sperm was collected in 2 ml syringes from cuckolder and parental males by applying pressure to the gonad region of the abdomen. Eggs were collected from gravid
females by applying gentle pressure to her abdomen. Eggs were then fertilized with sperm from either a cuckolder or a parental male and then reared in 500 mL glass jars filled with lake water and equipped with a small airstone. Each female and each male was used only once to form a family. Fifty percent water changes were conducted daily until larval swim-up (5-8 days post-hatch), which signals the onset of exogenous feeding.

2.2.3 Behavioural trials

Anti-predator response trials were conducted in brown translucent tanks measuring 40.9 cm × 28.2 cm × 15.0 cm filled to a depth of 8.0 cm with water from Lake Opinicon. Each tank was visually divided using a horizontal grid of 28 equally-sized rectangles (arranged as 4 × 7) positioned beneath the tank. On the first day of exogenous feeding, ‘Pure’ and ‘Mixed’ groups were formed by transferring larvae of known pedigree to trial tanks using a small plastic pipette. Pure groups consisted of 10 full-siblings sired by either a parental or cuckolder male. Three types of mixed groups of 10 larvae were created: a group of the ‘Mixed Parental’ type consisted of 5 full-siblings sired by a parental male and 5 full-siblings sired by a second parental male; a group of the ‘Mixed Cuckolder’ type consisted of 5 full-siblings sired by a cuckolder male and 5 full-siblings sired by a second cuckolder male; and a group of the ‘Mixed life history’ type consisted of 5 full-siblings sired by a parental male and 5 full-siblings sired by a cuckolder male. Within all mixed groups, the two sets of full-siblings were themselves unrelated (i.e. the sets had different fathers and mothers). Families used in the trials were used once to form pure groups and a maximum of twice to form mixed groups. The larvae were then allowed to acclimate for approximately 24 h. One hour before a trial began, a male pumpkinseed (Lepomis gibbosus L., a known predator of bluegill larvae: Gross and MacMillan 1981; Neff 2003) was placed in a tank filled with 10 L of lake water. This water conditioned by the pumpkinseed served as a predator cue in the trials. A trial began by recording the grid coordinates of each larva. Then, for ‘control’ treatments, 100 mL of unconditioned lake water was added to the centre of the tank, and for ‘predator’ treatments, 100 mL of predator-conditioned water was added to the centre of the tank. Pilot trials were conducted to determine that a volume of 100 mL was small enough that the introduction of the cue would not disturb the fish in the tank, but was a large enough to induce a response (as in Hain and Neff 2006). The grid coordinates
of each larva were read by a naïve observer to a stenographer at 20 s and 80 s after the addition of the cue. The times when I recorded the location of the fish were chosen to give one initial measure after sufficient time for the larvae to respond to the odour cue, and another measure one minute later to give an indication of how stable these groups were over time. The locations of the most active larvae were recorded first, followed by the location of less active or stationary larvae, and this process typically took no more than a few seconds per time step. All groups were subjected to both treatments on consecutive days with approximately half of the trials (n = 31) starting with the control treatment and the other half (n = 30) starting with the predator treatment. Fifty percent water changes were performed between trials using water from Lake Opinicon.

The distance between pairs of larvae was calculated as the square-root of the sum of the square of the horizontal grid reference position of larva ‘A’ minus the horizontal grid reference position of larva ‘B’ and the square of the vertical grid reference position of larva ‘A’ minus the vertical grid reference position of larva ‘B’: \( ((H_A-H_B)^2 + (V_A-V_B)^2)^{1/2} \), where the H and V refer to the horizontal and vertical positions, respectively. For each individual, the distance to the third-nearest neighbour was determined and then averaged across each individual in a trial to determine the trial’s ‘shoal dispersion index’. A group with a high shoal dispersion index can be interpreted as being more spread out than a group with a low shoal dispersion index.

2.2.4 Statistical analysis

For the analysis of shoaling behaviour, two paired t-tests were used to compare the shoal dispersion index at the beginning of trials (ie. day 1 and day 2) for trials that began with either the control or predator treatments. This analysis was performed to test if conducting the predator treatment first had any residual effect on shoal dispersion the following day when the control treatment was conducted. For the analysis of shoaling behaviour, separate repeated-measures ANOVAs were conducted on the control and predator treatments. To test the hypothesis that kinship affects shoal cohesiveness in response to a predator, the shoal dispersion index at each of the three time steps (before, 20 s after, 80 s after the introduction of the cue) was entered as the repeated measure, and
degree of kinship (pure or mixed) and day (first or second day of trials) were entered as fixed factors. Day was included in the ANOVA as a fixed factor to statistically control for any effect of trial day (and treatment order) on shoaling dispersion. Within each time step, a post-hoc comparison between pure and mixed groups was done using a one-tailed independent samples t-test. For the predator treatments, two additional repeated-measures ANOVAs were performed to compare mixed groups to pure groups for both the parental and cuckolder life histories, using kinship and day as fixed factors. Statistics were performed using SPSS version 16.0 (SPSS Inc., Chicago, IL, USA) or JMP version 4.0.4 (SAS Institute, Cary, NC, USA).

2.3 Results

There was no residual effect of the predator cue on shoaling behaviour at the beginning of trials on day 2 – when the control treatment was performed first, there was no significant difference between Day 1 and Day 2 in the shoal dispersion index before the addition of the cue (paired t-test: \( t_{30} = 0.51, P = 0.62 \)). Similarly, when the predator treatment was performed first, there was no significant difference between Day 1 and Day 2 in the shoal dispersion index before the addition of the cue (paired t-test: \( t_{29} = 0.62, P = 0.54 \)). Thus, being exposed to the predator cue on Day 1 did not result in groups that were more clumped on Day 2 at the beginning of the trial.

The results of the repeated-measures ANOVA are summarized in Table 2.1. In the control treatment, the addition of the cue had no significant effect on the shoal dispersion index (Table 2.1, Fig. 2.1a). Pure groups and mixed groups all responded similarly to the addition of the cue. However, in the predator treatment there was a significant effect of timing on the shoal dispersion index, indicating that the addition of the predator cue resulted in the larvae associating more closely (Fig. 2.1b). There was a significant main effect of the level of kinship on the shoal dispersion index, with pure groups less dispersed than mixed groups (Table 2.1, Fig. 2.1b). There was a trend towards an interaction between timing and kinship on the shoal dispersion index, which can be explained by pure groups becoming less dispersed than mixed groups over time following the addition of the odour
cue (one tailed t-test comparing pure groups and mixed groups; Before addition of cue: \( t_{59} = 0.95, p = 0.17 \); 20s after: \( t_{59} = 1.36, P = 0.090 \); 80s after: \( t_{59} = 1.74, P = 0.044 \)). Additionally, parental-sired and cuckolder-sired larvae responded to the predator cues differently when in mixed groups versus pure groups. Specifically, there was no difference in the shoal dispersion index for parental-sired larvae when in mixed groups versus pure groups (Table 2.2, Fig. 2.1b), but there was an interaction effect of time and kinship on the shoal dispersion index for cuckolder-sired larvae (Table 2.2). Both mixed and pure cuckolder-sired groups tended to become less dispersed immediately following the addition of the predator cue, but the mixed cuckolder-sired groups tended to become more dispersed than the pure cuckolder-sired groups 80s after the addition of the predator cue (Fig. 2.1b). There was also a significant interaction of time and day on the shoal dispersion index for cuckolder-sired larvae (Table 2.2). This interaction seemed to be driven by the small number of pure trials conducted on day 1, which become less dispersed after the addition of the cue (mean dispersion index before cue = 2.09 vs. mean dispersion index 80s after cue = 1.11, \( n = 3 \)), while in contrast, on day 2 the pure group tended to become more dispersed after the addition of the cue (mean dispersion index before cue = 1.66 vs. mean dispersion index 80s after cue = 1.94, \( n = 8 \)).
Table 2.1 Summary of repeated measures ANOVA for shoal dispersion index in bluegill (Lepomis macrochirus) larvae exposed to a predator cue. The cohesiveness of bluegill shoals was measured at three time steps in response to the control treatment (no predator cue) and the predator treatment (predator odour cue). The fixed factors in the analysis are kinship (full-siblings or mixed relatedness) and day that the treatment was performed.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Variable</th>
<th>$F$</th>
<th>df</th>
<th>$P$</th>
</tr>
</thead>
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<td>2, 56</td>
<td>0.57</td>
</tr>
<tr>
<td></td>
<td>Kinship</td>
<td>0.065</td>
<td>1, 57</td>
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</tr>
<tr>
<td></td>
<td>Day</td>
<td>1.09</td>
<td>1, 57</td>
<td>0.30</td>
</tr>
<tr>
<td></td>
<td>Kinship × day</td>
<td>1.00</td>
<td>1, 57</td>
<td>0.37</td>
</tr>
<tr>
<td></td>
<td>Time × day</td>
<td>1.01</td>
<td>2, 56</td>
<td>0.37</td>
</tr>
<tr>
<td></td>
<td>Time × kinship</td>
<td>0.46</td>
<td>2, 56</td>
<td>0.50</td>
</tr>
<tr>
<td></td>
<td>Time × kinship × day</td>
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<td>2, 56</td>
<td>0.91</td>
</tr>
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<td>Predator</td>
<td>Time (repeated measure)</td>
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<td>2, 56</td>
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</tr>
<tr>
<td></td>
<td>Kinship</td>
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<td>1, 57</td>
<td>0.047</td>
</tr>
<tr>
<td></td>
<td>Day</td>
<td>1.72</td>
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<td></td>
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<td>1, 57</td>
<td>0.13</td>
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<tr>
<td></td>
<td>Time × day</td>
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<td></td>
<td>Time × kinship</td>
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<td>Time × kinship × day</td>
<td>2.63</td>
<td>2, 56</td>
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Table 2.2 Summary of repeated measures ANOVA for shoal dispersion index in parental- and cuckold-sired bluegill (*Lepomis macrochirus*) larvae exposed to a predator cue. The cohesiveness of bluegill shoals was measured at three time steps in response to the control treatment (no predator cue) and the predator treatment (predator odour cue). The fixed factors in the analysis are kinship (full-siblings or mixed relatedness) and day that the treatment was performed.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Variable</th>
<th>$F$</th>
<th>df</th>
<th>$P$</th>
</tr>
</thead>
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<td>1, 20</td>
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</tr>
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<td>Kinship × day</td>
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<td>1, 20</td>
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<td>2, 19</td>
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<td></td>
<td>Time × kinship</td>
<td>0.32</td>
<td>2, 19</td>
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</tr>
<tr>
<td></td>
<td>Time × kinship × day</td>
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<td>2, 19</td>
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<tr>
<td></td>
<td>Day</td>
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<tr>
<td></td>
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<tr>
<td></td>
<td>Time × day</td>
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<td>2, 18</td>
<td>0.023</td>
</tr>
<tr>
<td></td>
<td>Time × kinship</td>
<td>4.55</td>
<td>2, 18</td>
<td>0.025</td>
</tr>
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<td></td>
<td>Time × kinship × day</td>
<td>3.38</td>
<td>2, 18</td>
<td>0.057</td>
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Figure 2.1 Shoal dispersion index for bluegill (*Lepomis macrochirus*) larvae after the addition of (a) lake water or (b) a predator cue. In the lake water (control) treatment, there were no significant differences among groups. In the predator cue treatment, pure full-sibling groups associated more closely than mixed groups. Error bars represent ± 1 standard error.
2.4 Discussion

I found that bluegill larvae shoal more cohesively when subjected to an odour cue of a potential predator (pumpkinseed, *L. gibbosus*). Given that these larvae had never before been exposed to pumpkinseed, or any other predator, these results are strong evidence of an innate ability of bluegill larvae to recognize the odour and respond as if it is a potential predator. Furthermore, full-sibling groups of bluegill larvae, but not groups of mixed relatedness, reacted to the predator cue by increasing their shoal cohesiveness. Interestingly, the effect of kinship was dependent on the sire’s life history (cuckolder vs. parental).

Numerous fishes have been shown to increase shoal cohesion in response to a predator (eg. Botham et al. 2006; Pink et al. 2007). Presumably, this increased cohesion reduces predation efficiency and leads to increased survivorship of shoal members (Chipps et al. 2004; but see Ruxton et al. 2007). In bluegill, for example, individuals in open water habitats tend to shoal more closely together than those in littoral habitats, and the increased cohesion reduces predation intensity by largemouth bass (*M. salmoides*; Chipps et al. 2004). Because bluegill larvae move to open waters once they leave the nest (Garvey et al. 2002), the increased shoal cohesion detected after the introduction of a predator odour no doubt is an effective anti-predator response. Interestingly, unlike pure kin groups, mixed-family groups did not appear to reduce their shoal dispersion. Although the hypothesized benefits from an increase in shoal cohesion in the presence of a predator exist for both kin groups and mixed groups, additional kin selective benefits may exist for kin groups from predator inspection because kin benefit from performing the behaviour even in the absence of reciprocity (Griesser et al. 2006, also see Croft et al. 2006). Furthermore, the confusion effect is enhanced when members of a shoal are composed of similar phenotypes (Ranta et al. 1994; Godin 1997). Given that closely-related individuals tend to look and smell more similar than unrelated individuals (e.g., Rajakurana et al. 2006), shoaling with kin would serve to increase the confusion effect for predators.

The difference between parental-sired and cuckolder-sired larvae in their reaction to the predator cue is interesting and consistent with previous studies. Here, the group
dispersion of parental-sired larvae was the same regardless of whether the group was composed entirely of full-siblings or was of mixed parentage, but the dispersion of cuckold-sired larvae differed based on the relatedness of the group. Previous work has shown that bluegill larvae sired by cuckolders but not parentals discriminate between the odour of full siblings and unrelated conspecifics using a mechanism referred to as self-referencing (Hain and Neff 2006). In contrast, larvae sired by parentals tend to be highly related to their nestmates; and could form kin groups simply by continuing to associate with nestmates after swim-up, which is a form of indirect kin recognition (see discussion in Hain and Neff 2006; also Mateo 2004). The data in the present study suggest that parental-sired larvae may identify kin based on odour cues related to their sire’s life history as opposed to kinship per se. This mechanism would be reliable in nature because multiple parentals rarely spawn eggs in the same nest (Neff 2001). In contrast, cuckold-sired larvae can differentiate between groups of full-siblings and groups of mixed relatedness, and associate more closely with full-sibling groups. This direct recognition mechanism would be required in nature to associate with kin because multiple cuckolders routinely spawn in a single nest (Stoltz and Neff 2006).

There has been much debate, particularly among aquaculture biologists, over the relative importance of learning versus an innate ability in recognizing predators. Early evidence suggested that fish were unable to recognize a predator innately (Thompson 1966; Goodyear 1973; Berejikian 1995). However, more recent studies have shown that several fishes can recognize a predator innately and respond defensively (e.g., Alemadi and Wisenden 2002; Berejikian et al. 2003; Hawkins et al. 2007; Scheurer et al. 2007), and that there may be an additional learned component to predator recognition (eg. Olla and Davis 1989; Berejikian 1995). In this study, there was evidence of an innate response to predator odours by bluegill larvae. In Lake Opinicon, bluegill larvae hatch in the littoral region and on swim-up head to deeper, open water (Garvey et al. 2002). Switching habitats exposes the larvae to novel predators (Keast and Harker 1977) and thus it is probable that survival of the larvae depends on their ability to recognize a predator and respond appropriately to the type of threat presented even when they have never before been exposed to that threat. Bluegill larvae may additionally modify their innate anti-predator response after repeated
exposure to a predator in a way that is consistent with learning, but this has yet to be examined. Conceivably, as has been shown in other fishes such as steelhead trout (*O. mykiss*; Berejikian 1995) and fathead minnows (*Pimephales promelas* Rafinesque; Ferrari et al. 2005), bluegill larvae may have both innate and learned components to their anti-predator shoaling response.

In summary, kin selection has been a major advancement in biologists’ understanding of social behaviour (Hamilton 1964). Although many animals have been shown to recognize kin (Mateo 2004), the particular benefits gained by fish are only beginning to be understood (reviewed by Ward and Hart 2003; also see Greenberg et al. 2002; Gerlach et al. 2007). The results of this study suggest that not only is an anti-predator response innate in bluegill, larvae use kin recognition to increase shoal cohesion when exposed to a predator cue. Specifically, cuckolder-sired larvae use a direct recognition mechanism whereas parental-sired larvae appear to use a life-history based recognition mechanism. The increased cohesion should lead to reduced predation intensity (Chipps et al. 2004). Thus, kin selection appears to influence anti-predator behaviour in bluegill larvae.

### 2.5 References


Chapter 3

3 Promiscuity drives self-referent kin recognition

3.1 Summary

Kin selection theory has been one of the most significant advances in our understanding of social behaviour (Hamilton 1964; Michod 1982; Trivers 1985). However, the discovery of widespread promiscuity in mating systems has challenged the evolutionary importance of Hamilton’s rule because it reduces the benefit to helping nestmates (Burke et al. 1989; Jennions and Petrie 2000; Wilson and Hölldobler 2005). This challenge would be resolved if promiscuous species evolved a self-referent kin recognition mechanism that enabled individuals to discriminate between kin and non-kin (Holmes and Sherman 1982; Hauber and Sherman 2001; Mateo 2004). Here I take advantage of a rare asymmetry in the level of sperm competition among males of alternative life histories in the bluegill sunfish (Lepomis macrochirus). I show that, as a consequence of this asymmetry, offspring of “parental” males have a high level of relatedness to nestmates whereas offspring of “cuckolder” males have a low level of relatedness to nestmates. In support of the resolution to the apparent conflict between promiscuity and kin selection, I find that offspring of parentals do not use a direct recognition mechanism to discriminate among nestmates, whereas offspring of cuckolders use kin recognition by self-referent phenotype matching to differentiate between kin and non-kin. Using this dichotomy in recognition mechanism, I estimate that the fitness cost of utilizing kin recognition by self-referent phenotype matching is equivalent to a relatedness ($R$) of at least 0.06. These results provide

\[ \text{Citation: Hain TJA, Neff BD. (2006) Promiscuity drives self-referent kin recognition. Curr. Biol. 16: 1807-1811.} \]
compelling evidence for adaptive use of kin recognition by self-referent phenotype matching and confirm the importance of kinship in social behaviour.

3.2 Results and Discussion

Here I use the bluegill sunfish (*Lepomis macrochirus*) to test the association of promiscuity with kin recognition by self-referent phenotype matching (hereafter referred to as “self-referent kin recognition”). With self-referent kin recognition (euphemistically referred to as the “armpit effect” (Dawkins 1982)), individuals compare phenotypic cues of putative kin to their own phenotype to determine the degree of relatedness. This mechanism contrasts to familiarity, in which individuals instead learn phenotypic cues of conspecifics encountered during early development and remember these individuals as kin (Holmes and Sherman 1982; Hauber and Sherman 2001; Mateo 2004). Bluegill are native to lakes and rivers of North America. The population studied here is found in Lake Opinicon, Ontario, Canada (44° 38' N, 76° 19' W), where males are characterized by a discrete life history polymorphism termed “parental” and “cuckolder” (Dominey 1980; Gross 1982). Parentals construct nests, court and spawn with up to 9 females (range = 1-9, mean = 5; B.D. Neff unpublished data), and provide sole care for developing larvae. Cuckolders instead mature precociously and specialize in stealing fertilizations from parentals. Genetic paternity analysis has shown that a parental fertilizes an average of about 80% of the eggs in his nest, largely by excluding cuckolders during spawning (Neff 2001; Neff 2004a). Cuckolders fertilize the remaining 20% of the eggs and do this in part by opportunistically intruding into the nests of multiple parentals during spawning. An average of approximately 10% of mating events between parental males and females includes sperm competition with a cuckolder male (Fu et al. 2001).

The difference in the levels of paternity between the two male life histories should lead to an asymmetry in the relatedness of nestmates of parentals’ offspring versus cuckolders’ offspring. Using microsatellite loci and pair-wise relatedness calculations, I indeed found that within the nests examined (n = 38 nests with parentals, 35 nests intruded on by cuckolders) the average relatedness of parentals’ offspring was over three times that of
cuckolders’ offspring (median parental \( R = 0.30 \), median cuckold \( R = 0.09 \); Mann-Whitney \( U = 152.5 \), \( n = 73 \), \( P < 0.001 \); Fig. 3.1). Thus, because of this higher relatedness among nestmates, a parental’s offspring could gain more kin selective net benefits than a cuckolder’s offspring simply by associating with and helping a random nestmate (Hamilton 1964). Such benefits may include reduced aggression and increased cooperation within shoals of fish, which in bluegill and other fishes have been shown to lead to increased foraging efficiency and growth rate (Brown and Brown 1996; Dugatkin and Wilson 1992). Kin discrimination may be particularly important in such foraging contexts when there is an optimal group size that limits membership, or when food resources are limited and must be shared among group members. Conversely, to gain a similar kin selective benefit, a cuckolder’s offspring would have to actively discriminate among nestmates. Because cuckolders’ offspring are always in broods of mixed parentage with potential kin dispersed throughout the nest (DeWoody et al. 1998), only self-referent kin recognition would provide a reliable mechanism to distinguish kin from non-kin (Holmes and Sherman 1982; Hauber and Sherman 2001; Mateo 2004). Location and familiarity (learning), two other reported kin recognition mechanisms (Cullen 1957; Sharp et al. 2005), would not reliably allow discrimination of kin and non-kin.
Figure 3.1 Average pairwise relatedness of nestmates sired by cuckolders (grey bars) or parentals (black bars). Each vertical bar represents the number of nests containing the given brood relatedness value. Arrows represent the median relatedness value for each life history. Cuckolder offspring were more than three times less related to nestmates than were parental offspring.
I used *in vitro* fertilization and two-choice behavioural trials to determine the kin recognition mechanisms employed by offspring of parentals and offspring of cuckolders. I predicted that because of the reduced broodmate relatedness of cuckolder-sired larvae versus parental-sired larvae, cuckolder-sired larvae would use self-referencing to recognize their relatives, while parental-sired larvae would not. The two-choice trials presented pairs of larvae with the choice of associating with odour cues of broods differing in their degree of relatedness or familiarity, but not both, relative to the focal larvae. Specifically, four types of trials were conducted: (1) familiar full sibling versus unfamiliar full sibling; (2) unfamiliar full sibling versus unfamiliar non-kin; (3) unfamiliar full sibling versus unfamiliar half sibling; and (4) unfamiliar half sibling versus unfamiliar non-kin. These trials enabled us to test for the independent roles of familiarity and relatedness (and the degree of relatedness) in kin recognition.

Consistent with the hypothesis, offspring of parentals did not discriminate between the odours of unfamiliar full-siblings and unfamiliar non-kin (mean of differences between kin and non-kin = -0.06 ± 0.16 SE; Wilcoxon Z = 0.55, n = 15, P = 0.58), unfamiliar full-siblings and unfamiliar half-siblings (mean of differences = 0.07 ± 0.14; Z = 0.53, n = 11, P = 0.59), unfamiliar half-siblings and unfamiliar non-kin (mean of differences = 0.24 ± 0.18; Z = 1.31, n = 11, P = 0.19), or unfamiliar full-siblings and either unfamiliar half-siblings or non-kin (mean of differences = -0.01 ± 0.11; Z = 0.04, n = 26, P = 0.97; Fig. 3.2a). Thus, I could rule out that offspring of parentals use self-referent kin recognition. Furthermore, I found that offspring of parentals do not use familiarity as they did not discriminate between the odours of familiar full-siblings versus unfamiliar full-siblings (mean of differences = 0.09 ± 0.28; Z = 0.20, n = 10, P = 0.84).

In contrast, although offspring of cuckolders did not discriminate between odours of familiar full-siblings versus unfamiliar full-siblings (mean of differences = 0.24 ± 0.19; Wilcoxon Z = 1.19, n = 10, P = 0.24) or unfamiliar half-siblings and unfamiliar non-kin (mean of differences = -0.07 ± 0.16; Z = 0.56, n = 20, P = 0.57), they did prefer to associate with odours from unfamiliar full-siblings versus unfamiliar half siblings or non-kin (mean of differences = 0.63 ± 0.18; Z = 2.64, n = 16, P = 0.008; Fig. 3.2b). Thus, offspring of cuckolders do not use familiarity as a recognition mechanism and do not discriminate
between half-siblings and unrelated individuals. They do discriminate between full-siblings and all other less related individuals. It is unlikely that cuckolder offspring simply prefer the odour of any cuckolders’ offspring because in all full-sibling versus paternal half-sibling trials where cuckolders sired both stimulus broods \((n = 5)\), focal larvae still preferred to associate with full-siblings (Binomial test: \(P = 0.03\); see Fig. 3.2b).
Figure 3.2 Association preference for full sibling odours by (a) parentals’ offspring or (b) cuckolders’ offspring. Preference was calculated as the average count of larvae in the full sibling association zone minus average count of larvae in the non-kin (black bars) or half-sibling (grey bars) association zone. Trials are arranged in order of decreasing preference for full sibling odour.
However, this experiment could not definitively rule out the possibility that these larvae had instead formed their kin template using cues from their nestmates and not themselves (Hare et al. 2003). Thus, I conducted another experiment in which I scrambled the cues of kinship for offspring of cuckolders to confirm that these larvae use self-referent kin recognition. I accomplished this by generating focal fish from broods mixed at fertilization, consisting of two full-sibling families, one sired by a cuckolder and the other sired by a parental, and rearing these larvae together throughout their lives. The focal larvae were then presented in pairs with odours from ‘pure’ unfamiliar full siblings and ‘pure’ unfamiliar non-kin (Appendix A).

In these mixed brood trials, when there was at least one larva sired by a cuckolder in the focal pair (in which case an asymmetry in association preference is expected), there was a significant preference for associating with the odour from the pure cuckolder-sired brood versus the pure parental-sired brood (mean of differences = 0.50 ± 0.12; Wilcoxon $Z = 2.54, n = 11, P = 0.011$; Fig. 3.3). This latter result remained significant when corrected for multiple comparisons (corrected $\alpha = 0.0125$). A kin template formed from nestmates would not allow a cuckolder’s offspring to differentiate between the two referent odours because both odours were present in its nestmates since fertilization. Thus, my results cannot be explained by environmental or learned cues for kin recognition (Carlin and Hölldobler 1983; Sharp et al. 2005), but instead conclusively demonstrate that offspring of cuckolders use self-referent kin recognition.
Figure 3.3 Association preference for odours from the cuckolder-sired broods. Preference was calculated as the average count of larvae in the cuckolder-sired association zone minus average count of larvae in the parental-sired association zone. Trials involved focal pairs of larvae that were either full sibling offspring of a cuckolder (black bars) or one offspring of a cuckolder and one offspring of a parental (grey bars). Trials are arranged in order of decreasing preference for cuckolder-sired odour.
My analysis also enabled us to determine the potential fitness cost of utilizing self-referent kin recognition. First, by examining trials involving offspring of cuckolders, I found that in 4 out of 14 trials that involved full sibling versus unrelated referents, the focal pair incorrectly associated with the unrelated referent (see Figs. 3.2, 3.3). This represents a maximum error rate of 28%; the actual error rate may be lower because I do not know if the focal pair chose to associate with the unrelated referent. Using this error rate, a cuckolders’ offspring would be expected to associate with on average an individual of relatedness 0.36 (= 0.28 × 0 relatedness + 0.72 × 0.5 relatedness). Thus, a cuckolders’ offspring could increase its kin selective benefits by as much as 4-fold (= 0.36 / 0.09) by actively discriminating kin from non-kin. If a parentals’ offspring discriminated kin from non-kin with the same accuracy as a cuckolders’ offspring, it too could expect to associate with on average an individual of relatedness 0.36. By randomly associating with a nestmate, the level of relatedness would instead be 0.30 (see Fig. 3.1). Thus, a parentals’ offspring could increase its kin selective benefits by only 1.2 times (= 0.36 / 0.30) by using self-referent kin recognition. Because offspring of parentals do not use self-referent kin recognition (see Fig. 3.2a), my data suggest that there is a fitness cost in excess of a relatedness value of 0.06 (= 0.36 - 0.30).

It is likely that both types of offspring possess the genetic architecture for self-referent kin recognition and the differential expression represents phenotypic plasticity. A portion of a parental’s offspring become parentals themselves and as adults use self-referent kin recognition in the context of parental care (Neff 2003; Neff and Sherman 2005). The differential gene expression in the larva could be mediated by RNA or transcription factors released by the spermatozoa into the ovum as has been recently discovered in several mammals (Ostermeier et al. 2004; Krawetz 2005). In bluegill, spermatozoa of cuckolders have more ATP than do those of parentals, and have more ATP than is required to travel to and fertilize eggs (Burness et al. 2004). Given that ATP is a precursor to cyclic-AMP, an important signal transmitter implicated in many cellular activities (Schramm and Selinger 1984), it is possible that ATP is one of the transcription factors involved in the differential expression.
The evolution of social behaviour, which is the association and interaction with conspecifics, has interested biologists for decades, and in many species such behaviour can be explained by kin selection (Hamilton 1964; Michod 1982; Trivers 1985). Although kin selection has been one of the most significant advances in our understanding of social behaviour, its importance in explaining altruism has recently been challenged with the discovery of widespread promiscuity in mating systems because promiscuity reduces the benefit to helping nestmates (Burke et al. 1989; Jennions and Petrie 2000; Wilson and Hölldobler 2005). Several other studies have attempted to quell this challenge by demonstrating an association between promiscuity and self-referent kin recognition (Petrie et al. 1999; Hauber et al. 2000; Mateo and Johnston 2000; Jacob et al. 2002; Buchan et al. 2003). However, my study is among the first to provide evidence of self-referent kin recognition that cannot be explained by learning in utero or indirect recognition (Hare et al. 2003; Sherman and Neff 2003). Furthermore, although self-referent kin recognition should evolve in promiscuous species because in these species other mechanisms cannot reliably identify kin (Holmes and Sherman 1982; Hauber and Sherman 2001; Mateo 2004, but see Jansen and van Baalen 2006), to date there has been little empirical evidence directly linking promiscuity with the mechanism. Here, I used an intra-specific approach that eliminates phylogeny as a potential confounding variable (Harvey and Pagel 1991) and showed that an asymmetry in promiscuity between alternative male reproductive life histories is associated with differential expression of self-referent kin recognition in their offspring. These data suggest that promiscuity, and specifically its consequence on the relatedness of nestmates, is a driving force behind the expression of self-referent kin recognition. This recognition mechanism allows individuals to discriminate between kin and non-kin even when nestmates are not reliably kin. Future studies will investigate context-dependent kin recognition, the ontogeny of self-referent kin recognition in larvae sired by parentals, and the benefits of kin discrimination by larvae.
3.3 Experimental procedures

3.3.1 Relatedness calculation

I calculated average relatedness within a nest using microsatellite analysis of larvae and putative parents collected in June 1996 (Neff 2001; Neff 2004a). Genotypes were determined for an average of 46 larvae (range = 43-48) from 38 nests at 11 microsatellite loci. For each nest, each larva was first assigned to either the nest-tending parental or a cuckolder by exclusion methods (Neff 2001). Next, the larva’s mean relatedness to all other larvae within the nest was calculated using the formula (eqn. 6) developed by Queller and Goodnight (1989). These data were then used to determine the mean level of relatedness within nests for parental-sired larvae and for cuckolder-sired larvae.

3.3.2 Experimental fish

During the summer of 2005, swimmers equipped with snorkelling gear conducted daily surveys of breeding activity along the littoral zone of the northern edge of Lake Opinicon. When spawning was discovered, mature parentals, cuckolders and females were netted opportunistically and transported by boat to aquarium facilities at the Queen’s University Biological Station, which resides on the lake’s shore. These fish were used to generate offspring via in vitro fertilization (Neff 2004b). Full and half siblings were generated by fertilizing 100 eggs from either one or each of two females in 500 mL glass jars with milt from either a parental or cuckolder. Full-sibling fertilizations were performed in duplicate, and one replicate was used for focal fish and the other replicate was used to provide an ‘unfamiliar’ odour source. This design ensured that I could control for the effects of familiarity as a recognition mechanism because I could select referent odours that the focal fish had never come into contact. Mixed broods were generated by dividing a jar in half with a removable barrier. Eggs from one of two females were placed on either side of the barrier and one batch was fertilized with milt from a parental and the other batch was fertilized with milt from a cuckolder. Five minutes after fertilization, when sperm have ceased activity (Burness et al. 2004), the barrier was removed and the eggs were gently mixed. As above, replicate families of ‘pure’ full-siblings were also generated for both
families in the mixed broods to serve as unfamiliar full sibling referent odours (Appendix A).

3.3.3 Recognition trials

Behavioural trials were conducted between 10:00 and 17:30 EST within four days of post larval swim-up (i.e. when the larvae switch to exogenous feeding and are free swimming). The two-choice trial aquarium measured 34.4 cm × 18.9 cm × 20.4 cm (l × w × h) and was filled with fresh lake water to a depth of 8.1 cm. Two-10 cm association zones were defined at either end of the tank (Appendix A); the remaining 14.4 cm defined the middle, neutral zone. A trial began by placing two larvae from the same brood into the centre of the tank. Simultaneously, water conditioned by one of two broods differing from the focal fish in either relatedness or familiarity (but not both) was introduced at a distance of 5 cm from either end of the aquarium at a rate of 6.6 ± 1.5 ml/min (SD). The conditioned water was taken directly from the jars that had contained the referent brood for 16-23 h, and the side that the referents were placed on was determined by flipping a coin. Fish, including bluegill, have a well-developed olfactory system that has been shown to be involved in mate choice and kin recognition (Olsén et al. 1998; Milinski et al. 2005; Neff and Sherman 2005). Two focal larvae were used because preliminary trials with only a single focal larva revealed erratic and agitated swim behaviour by the larva consistent with a flight response. This behaviour was not displayed by pairs of larvae. Each trial lasted 5.0 min during which an observer who was naïve to the sources of conditioned water recorded the number of focal fish that were in the neutral or either association zones at 10 s intervals. All analyses examined the average of the counts of the scan samples for the second half of trials to ensure that there was sufficient time for odour cues to accumulate in the test aquarium and for focal larvae to assess these cues (Neff and Sherman 2005). For the trials involving mixed broods, I used microsatellite loci and exclusion paternity techniques to determine the paternal origin of the two focal larvae (methods in Neff 2001). Focal fish were never used in more than one trial and between trials the lake water was changed and the aquarium was cleaned with ethanol.
3.4 References


Evolution. 43: 258-275.


Chapter 4

4 Multiple paternity and kin recognition mechanisms in a guppy population

Help directed toward kin (nepotism) is an important example of social behaviour. Such helping behaviour requires a mechanism to distinguish kin from non-kin. The prevailing kin recognition hypothesis is that when familiarity is a reliable cue of relatedness, other mechanisms of recognition will not evolve. However, when familiarity is an unreliable cue of relatedness, kin recognition by phenotype matching is instead predicted to evolve. Here I use genetic markers to show that guppies (*Poecilia reticulata*) from a population in a tributary of the Paria River in Trinidad are characterized by a high degree of multiple mating with 95% of broods having more than one sire and some dams having offspring sired by six males. These levels of multiple mating are the highest reported among live-bearing fishes. The mean relatedness of brood-mates was 0.36 (as compared to 0.5 for full-siblings). Therefore, familiarity does not seem to be a reliable mechanism to assess relatedness. Using two-choice behavioural trials, I find that juveniles from this population use both phenotype matching and familiarity to distinguish kin from non-kin. However, I did not find strong evidence that the guppies use these mechanisms to form shoals of related individuals as adults, which is similar to results from other guppy populations in Trinidad. The use of both familiarity and phenotype matching is discussed in the context of the Paria River guppy population’s mating system and level of predation. Overall, these data provide support for the kin recognition hypothesis and increase our understanding of the evolution of kin recognition systems.

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4.1 Introduction

The evolution of altruistic behaviour has intrigued biologists for decades, and in many species such behaviour has evolved through kin selection (Hamilton 1964; Wilson 1975; Trivers 1985). However, widespread promiscuity in animal mating systems has challenged the importance of kin selection in explaining altruism (Burke et al. 1984; Jennions and Petrie 2000). This challenge would be resolved if promiscuous species evolved a mechanism to discriminate between kin and non-kin. Two mechanisms of direct kin recognition are familiarity and phenotype matching (Holmes and Sherman 1982; Sherman et al. 1997; Hauber and Sherman 2001). When kin recognition by familiarity is used, individuals learn phenotypic cues of conspecifics encountered during early development and “remember” these specific individuals as kin. When kin recognition by phenotype matching is used, individuals instead learn the phenotypic cues of their rearing associates (or their own cues) and use these cues to form a “kin template.” Individuals later compare phenotypic cues of putative kin to the template and, based on the similarity of the cue to the template, determine the degree of relatedness of the individual (Holmes and Sherman 1982). So, in phenotype matching, specific individuals are not remembered as kin. Self-referencing is the special case of phenotype matching where individuals use their own cues to form their kin template. The prevailing kin recognition hypothesis is that phenotype matching should evolve only when familiarity is an unreliable cue of genetic relatedness, as can be the case in promiscuous mating systems (Holmes and Sherman 1982; Sherman et al. 1997; Hauber and Sherman 2001). Here I genetically determine mating patterns in a population of the guppy (*Poecilia reticulata*) and then detail the kin recognition mechanisms used by individuals within the population. I compare my data to results from a previous study of a population characterized by high-predation to test for the predicted association between promiscuity and kin recognition by phenotype matching.

The guppy is a livebearing fish with internal fertilization and a non-resource based promiscuous mating system (Houde 1997; Magurran 2005). Northern Trinidad, where the guppy is most commonly studied, is a mountainous region with waterfalls that restrict movement of aquatic organisms from downstream to upstream populations. Waterfalls not only exclude larger guppy predators from upstream locales, but also restrict gene flow from
downstream to upstream populations, which has resulted in genetic differentiation and variation in guppy behaviour within short geographic distances (Reznick and Endler 1982; Crispo et al. 2006). The level of predation risk, evaluated on the basis of what predators are present, is one ecological variable used to explain variation in guppy characters, including life history (Reznick and Endler 1982) and mate choice (Breden and Stoner 1987). Guppies from both high predation and low predation populations tend to have strong site fidelity (Reznick et al. 1996; Croft et al. 2003), but in high predation populations, mortality is as much as 2.5 times higher than in low predation populations (Reznick et al. 1996), which greatly increases the likelihood of overlapping generations in low predation populations versus high predation populations. Consequently, in populations with low predation, the probability of meeting an older or younger sibling from a different brood is high and phenotype matching should be a more reliable mechanism of kin recognition than familiarity. Conversely, in populations with high predation, the probability of meeting a sibling from a different brood is low, and, barring multiple mating, familiarity should be a reliable mechanism of kin recognition (Holmes 1986). Indeed, a study on the Lower Tacarigua River, which is characterized by high predation and low multiple mating (Reznick et al. 1996; Evans and Magurran 2001) showed that guppies use familiarity and not phenotype matching to recognize kin (Griffiths and Magurran 1999).

In contrast, tributaries of the Paria River are characterized by low predation risk to guppies (Reznick et al. 1996) and a laboratory study of descendents from the Paria River showed that broods are sired by multiple males, with the most successful male siring only about two-thirds of the brood (Pitcher et al. 2003; also see Kelly et al. 1999). In this case, familiarity reliably indicates a level of kinship of only half-siblings ($R = 0.25$), but it does not provide a reliable indicator of the actual level of relatedness (i.e. a brood-mate could be either 0.25 or 0.5 related). In addition because individuals in tributaries of the Paria River typically are found in small pools, it is likely that individuals will come into contact with unfamiliar paternal half-siblings and possibly unfamiliar half- or full-siblings from their mothers’ other broods. Thus, phenotype matching would be required to distinguish between full- and half-sibling brood-mates and to distinguish between unfamiliar kin and unrelated individuals.
Here I test for kin recognition by phenotype matching and familiarity mechanisms in a population from a tributary of the Paria River. First, I determine the level of multiple mating within a natural population from the Paria River tributary using microsatellite paternity analysis to assess the mean relatedness of brood-mates, and hence the importance of kin recognition by phenotype matching rather than recognition by familiarity. Second, I perform two-choice behavioural experiments to observe the preference of juvenile guppies for potential shoaling partners of different levels of either relatedness or familiarity. My expectation was that these guppies would be able to use phenotype matching to recognize kin. Finally, I test one application of kin recognition in the wild, that is, if adult shoals are structured on the basis of kinship. I did this test in part to compare to a previous study that found the relatedness within shoals was not significantly different from relatedness within the entire population for two Trinidadian rivers where guppies are subject to high predation (Russell et al. 2004).

4.2 Methods

4.2.1 Multiple mating and paternity assignment

In May 2005, gravid female guppies were collected from a tributary of the Paria River in northern Trinidad and isolated in individual tanks until they gave birth. Newborn guppies and females were then euthanized. Fin clips from females and entire body tissue of juveniles were preserved in 95% ethanol within 24 h of parturition.

The parentage of each juvenile was determined using microsatellite DNA analysis at three loci (Pre1, Pre13, Pre15; locus heterozygosities are shown in Table 4.1 and allele frequencies are shown in Fig. 4.1; primer sequences are published in Paterson et al. 2005). First, DNA was isolated from the females as well as the juveniles using a proteinase K digestion (Neff et al. 2000). I then used a Whatman-Biometra T1 Thermocycler to amplify the microsatellites with the following program: 60 s at 92°C; 15 cycles of 30 s at 92°C, 30 s at 60°C, and 30 s at 72°C; and 34 cycles of 30 s at 92°C, 30 s at 55°C and 30 s at 72°C. Each 10 µL PCR reaction contained ~75 ng of total DNA, 3 mM MgCl2, 1× PCR buffer
(Fisher), 0.25 mM of each deoxynucleotide (Fisher), 0.25 units Taq DNA polymerase (Fisher) and 0.25 µM of each forward and reverse primer (Invitrogen life technologies), and the forward primer was fluorescently labelled (Beckman Coulter). PCR products were run following standard protocol for the CEQ 8000 Genetic Analysis System (Beckman Coulter). Offspring were assigned to parents using COLONY version 1.2, a parentage assignment program that reconstructs putative sires based on a maximum likelihood method (Wang 2004). For this analysis, I set the typing error rate at the suggested 0.025 (Wang 2004). Average relatedness was then determined for each brood by determining the mean of all pairwise relatedness comparisons within a brood.
Table 4.1 Summary statistics for ten microsatellite loci in parentage and adult shoal structure analyses, including number of individuals scored (n), number of alleles, observed heterozygosity (Hₐ), and expected heterozygosity (Hₑ).

<table>
<thead>
<tr>
<th>Locus</th>
<th>Analysis</th>
<th>n</th>
<th>No. Alleles</th>
<th>Hₐ</th>
<th>Hₑ</th>
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<td>0.83</td>
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</table>

* denotes a significant deviation from Hardy-Weinberg equilibrium (P < 0.001; Raymond and Rousset 1995).
Figure 4.1 Allele frequency distributions for (a) three microsatellite loci used in the multiple mating and paternity assignment analysis, and (b) eight microsatellite loci used in the adult relatedness in shoals analysis.
4.2.2 Juvenile behavioural trials and kin recognition

Guppies used in the behavioural trials were descendants of individuals caught in a tributary of the Paria River. For the duration of the study, guppies were kept in tanks containing a bottom layer of neutral-colour gravel with water temperature maintained at 24-26°C and on a 12 h : 12 h light-dark cycle to simulate natural tropical conditions (Houde 1997). A single male was mated to either one or two (a ‘mating triad’) virgin females over the course of 7 days. Guppies within a brood born from the same mother were thus full-siblings, and guppies born from the other mother within a mating triad were paternal half-siblings.

Newborn guppies were isolated within 24 h of birth and were reared in visual and chemical isolation until they were large enough to be marked by tail clipping (mean = 33.6 days, range = 22-48 days). After the isolation period, six or eight guppies – three or four from each of two kin groups – were anaesthetized (using 15 mg MS-222 in 50 mL water) and marked according to kin group by cutting and removing either the top third or bottom third of the guppy’s caudal fin. Following Griffiths and Magurran (1997), the fish were then combined into a single rearing tank for 12-15 days before the trials began. Because guppies have been shown to shoal preferentially with tank-mates after a period of 12 days (Griffiths and Magurran 1997), guppies in these rearing tanks were assumed to be familiar with one another. Thus, each rearing tank contained familiar full-siblings and familiar half-siblings or familiar non-kin.

Four types of dichotomous choice trials were performed: Full-sibling versus unrelated, full-sibling versus half-sibling, half-sibling versus unrelated (where both choices were either familiar or unfamiliar), and familiar versus unfamiliar (where both choices were either full-siblings or unrelated). In a trial, a focal fish was presented with pairs of ‘stimulus’ guppies on either side of a test tank differing either in the level of relatedness or familiarity (but not both), and given the choice of associating with either group. Each focal fish was also used as ‘stimulus’ fish in either one or two trials.
The test arena was a tank (19 cm × 34 cm × 20 cm, with water depth of 15 cm) divided into three compartments by two transparent, porous plastic sheets that allowed visual and chemical communication between compartments (Griffiths and Magurran 1999). The centre compartment, which contained the focal fish, was 18 cm in length, and was further divided into two peripheral ‘association zones’ 5 cm in length, and a centre ‘neutral zone’ of 8 cm. The outer compartments were 8 cm in length, and housed a pair of guppies corresponding to one treatment of relatedness or familiarity.

A focal fish was allowed 15-30 min before the trial began to settle and explore the arena. Each trial lasted 15 min. Both the number of times that the focal fish switched from associating with one group to the other group, and the time spent within each association zone were recorded. During a trial, if the focal fish was entirely within the association zone, or had its gill slits within this region with its head oriented towards the barrier, it was said to be associating with the fish on that side. If the focal fish did not associate with both groups, or if any of the fish in the test arena displayed courtship behaviour (e.g. a characteristic sigmoid display) during the course of the trial, the trial was discarded from further analysis. Guppies are considered juveniles until about age 70 days when, for example, males start producing sperm (Evans et al. 2002). Thus, because all these guppies were younger than 70 days of age, I considered them a priori to be juveniles. Fish were used only once as a focal fish. Water changes were performed in the test tank between trials to remove any olfactory cues from previous trials.

I compared the percentage of time spent associating with the familiar (or more related) stimulus to the percentage of time spent with the unfamiliar (or less related) stimulus using Wilcoxon signed-rank tests. This non-parametric test was used because the data were not normally distributed. The significance level was set at $\alpha = 0.05$ and tests were performed using SPSS (version 14.0).
4.2.3 Adult relatedness in shoals

In December 2006, I captured entire shoals using seine nets from two pools within the tributary of the Paria River. The two pools were connected by a 3 m stream. There were no guppies caught by seine net or otherwise observed within 25 m upstream or downstream of the pools. Fish were euthanized by an overdose of clove oil and preserved in 95% ethanol for subsequent genetic analysis.

The genotypes of each guppy caught were determined at eight microsatellite loci (Pre1, Pre8, Pre9, Pre15, Pre26, Pre39, Pre92, Pre171; allele frequencies are shown in Fig. 4.1; primers are published in Becher et al. 2002 and Paterson et al. 2005). I followed the DNA extraction and PCR protocol described above but with minor modification: an initial step of 94°C for 3 min, and then 35 cycles of 30 s at 94°C, 30 s at 53°C (for Pre39 and Pre171; 56°C for Pre92, 60°C for Pre1, 62°C for Pre15 and Pre28; and 65°C for Pre8 and Pre9), 30 s at 72°C and a final elongation at 72°C for 7 min. This PCR protocol provided equivalent or better amplification than the previous protocol. PCR product was run following the standard protocol for the CEQ 8000 Genetic Analysis System. Relatedness values among individuals were calculated using ML-RELATE (Kalinowski et al. 2006). To test the hypothesis that guppy shoals are composed of relatives, for each shoal I determined the mean relatedness of pairs within the shoal (within-shoal relatedness) and compared this to the mean relatedness of pairs where one individual is in the shoal and one individual is outside the shoal (outside-shoal relatedness) using a paired t-test.

4.3 Results

4.3.1 Multiple mating and paternity assignment

I genotyped 23 broods from females that were inseminated in natural populations, of which parentage was successfully assigned to 22 broods. For one brood the COLONY program failed to resolve paternity because there were multiple, equally probable solutions. The average brood size for the 23 broods was 12.9 individuals (range = 3-40). I detected an average of 3.0 sires per brood (range = 1-6, n = 22). The average relatedness within a brood
was 0.36 (range = 0.29-0.5; Table 4.2). Brood size and number of sires were positively correlated ($r = 0.79, n = 22, P < 0.001$). Similar results were found when the locus that deviated from expected Hardy-Weinberg equilibrium proportions was omitted (data not shown; see Table 4.1 for Hardy-Weinberg results).

Table 4.2 Multiple mating for 22 broods of guppies from a tributary of the Paria River in Trinidad. Brood size (n), mean relatedness of brood-mates ($R$), and the percentage of the brood sired by up to six putative males as calculated by COLONY vers. 1.2 (Wang 2004) are provided.

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<tr>
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<th>Brood size (n)</th>
<th>Relatedness ($R$)</th>
<th>Sire 1 (%)</th>
<th>Sire 2 (%)</th>
<th>Sire 3 (%)</th>
<th>Sire 4 (%)</th>
<th>Sire 5 (%)</th>
<th>Sire 6 (%)</th>
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NB. The percentage of the brood fertilized by each sire may not sum to 100% due to rounding error.
4.3.2 Juvenile behavioural trials and kin recognition

A total of 144 recognition trials were conducted to distinguish between the preference for familiar or related fish as shoalmates. Though all fish used in the trials appeared to be immature based on colouration (Evans et al. 2002), courtship behaviour occurred in 13 trials, and these were discarded from further analysis. An additional 7 trials were discarded because the focal fish had not sampled both pairs (i.e. did not cross the centre line), and 5 trials were discarded because a stimulus fish escaped from its side of the tank. Fifty-nine trials tested the preference for full-siblings versus unrelated individuals, 17 tested the preference for full-siblings versus half-siblings, 13 tested the preference for half-siblings versus unrelated individuals, and 30 tested the preference for familiar versus unfamiliar individuals.

Focal fish had no preference for full-siblings over unrelated individuals when the focal fish was familiar with both stimulus groups (mean ± SE full siblings = 53.4 ± 5.6%; unrelated = 46.6 ± 5.6%; Z = 0.57, n = 22, P = 0.57). However, when both stimulus groups were unfamiliar with the focal fish, the focal fish had a significant preference for full-siblings versus unrelated individuals as shoaling partners (mean ± SE full siblings = 58.8 ± 4.2%; unrelated = 41.2 ± 4.2%; Z = 2.02, n = 37, P = 0.043). This preference for full-siblings remained significant when the full-sibling versus unrelated tests were pooled (Z = 2.02, n = 59, P = 0.043; Fig. 4.2). There was a trend for focal fish to prefer familiar fish to unfamiliar fish as shoaling partners when both stimulus groups were full-siblings of the focal fish (mean ± SE familiar = 60.0 ± 7.5%; unrelated = 40.0 ± 7.5%; Z = 1.48, n = 14, P = 0.14), and when both stimulus groups were both unrelated to the focal fish (mean ± SE familiar = 59.7 ± 6.1%; unrelated = 40.3 ± 6.1%; Z = 1.40, n = 16, P = 0.16). This preference for familiar fish as shoaling partners was significant when the data were pooled and both stimulus groups were either full-siblings or unrelated to the focal fish (Z = 1.96, n = 30, P = 0.049; Fig. 4.2). Focal fish also preferred full-siblings over half-siblings (12 out of 17 trials; Z = 0.781, n = 17, P = 0.44) and half-siblings over unrelated individuals (9 out of 13 trials; Z = 1.503, n = 13, P = 0.13; Fig. 4.3), but neither of these results were statistically significant. However, these two results were statistically significant when the data were combined with a binomial test (21 out of 30 trials: P = 0.021), which suggests
that guppies from this population can distinguish between individuals differing in relatedness of 0.25.

There was no correlation between the age difference of the two stimulus groups and the percentage of time spent associating with the more related group (mean of age difference = 2.9 d; range = 0-8 d; \( r = 0.03, n = 89, P = 0.79 \)). Thus, the preferences by focal fish for kin or familiar individuals could not be explained by matching for age (or body size insomuch as size and age are correlated; Grether et al. 2001). I also tested for an effect of sex bias in the stimulus groups on association time in both full-sibling versus unrelated and familiar versus unfamiliar trials. Neither males nor females showed discrimination between groups on the basis of how many more males there were in one stimulus group than in the other group (\( P > 0.2 \) for all). There was no correlation between the age of the focal fish and the time spent associating with groups that had more males in either sex (\( P > 0.2 \) for all).
Figure 4.2 Relative association times ± SE of juvenile guppies from a tributary in the Paria River in Trinidad for full-siblings over unrelated individuals (n = 59), or familiar individuals over unfamiliar individuals (n = 30). The dashed line represents the expectation of 50% association time with either stimulus group. Association time was calculated as time spent associating with one stimulus shoal as a percentage of the total time spent associating with either stimulus shoal in a 15-minute trial.
Figure 4.3 Relative association times ± SE of juvenile guppies from a tributary in the Paria River in Trinidad for (a) full-siblings over half-siblings (n = 17), or (b) half-siblings over unrelated individuals (n = 13). The dashed line represents the expectation of 50% association time with either stimulus group. Association time was calculated as time spent associating with one stimulus shoal as a percentage of the total time spent associating with either stimulus shoal in a 15 min trial.
4.3.3 Adult relatedness in shoals

Across the two pools, I caught 59 adults from 16 natural shoals (mean shoal size = 3.7; range = 1-9). There was no difference in the mean relatedness of fish from the two pools ($t_{764} = .919, P = 0.36$). The overall relatedness for the population, calculated as the mean of all pairwise relatedness values, was 0.095 (Appendix B). Sixteen percent of all pairs in the population were more closely related than half-siblings ($R = 0.25$). There was no significant difference between the mean within-shoal relatedness ($R = 0.090$) and the mean outside-shoal relatedness ($R = 0.087$) ($t_{10} = 0.04, P = 0.97$; Appendix C). The mean multi-locus heterozygosity for the population was 0.32 (range = 0.12-0.62).

4.4 Discussion

I used microsatellite markers to find that 95% of broods collected from a tributary in the Paria River (Trinidad) were sired by more than one male and 50% of broods were sired by more than two males. Within the family Poeciliidae this level of multiple paternity is high (Luo et al. 2005; Soucy and Travis 2003). Previous studies of poeciliids have found that the percentage of multiply mated females ranges from 23% in Poeciliopsis monacha (Leslie and Vrijenhoek 1977) to 90% in Gambusia holbrooki (Zane et al. 1999). Furthermore, an average of 3.0 sires per brood is also the highest yet reported among poeciliids (reviewed in Soucy and Travis 2003).

The paternity data indicate that multiple mating among guppies within tributaries of the Paria River is much higher than has been previously reported. Kelly and colleagues (1999) reported that only approximately 20% of Paria broods had more than one sire. This discrepancy may be explained in three ways. First, Kelly and colleagues used a conservative approach of counting unique paternal alleles to detect multiple mating. For example, two sires were detected only if there were either three or four paternal alleles in a brood. I used a more sophisticated and powerful program called COLONY that reconstructs putative sires based on a maximum likelihood method (Wang 2004). This program could infer a multiply sired brood when only two unique paternal alleles were observed in a brood when, for example, the two alleles deviated significantly from the
expected Mendelian inheritance ratio of 1:1. The program was not available to Kelly and colleagues. Second, the broods examined by Kelly and colleagues were smaller than the broods examined here (their mean brood size was 7.0 whereas the mean brood size here was 12.9). I found a significant positive correlation between brood size and the number of sires detected. Thus, assays of small broods may have missed additional sires that would be detected in large broods. Third, Kelly and colleagues used a single microsatellite locus with relatively low variation (4 alleles) to detect multiple paternity. Here I used three loci with greater variation (10-13 alleles per locus), which increases the likelihood of detecting multiple sires. Indeed, using the paternal allele counting method of Kelly and colleagues, the probability of detecting a multiply mated brood in their analysis was estimated to be 0.363 and in my analysis it was 0.987 (see Neff and Pitcher 2002).

My study was able to detail the recognition mechanisms used by guppies from a tributary of the Paria River. Based on my paternity data of natural broods, I determined that the average relatedness within a brood was $R = 0.36$. If broods continue to associate post-parturition, this level of relatedness may be sufficiently high for familiarity to be a reliable method of distinguishing kin from non-kin. Indeed, I have shown that juvenile Paria guppies choose shoaling partners based in part on familiarity. However, because guppies in tributaries of the Paria River typically are found in small pools, predation rates are low, adult sex ratio is female-biased (1.7:1; authors’ unpublished data; also see Rodd and Reznick 1997) and each female mates with an average of 3.0 males (this study), it is likely that individuals will come into contact with unfamiliar paternal half-siblings as well as unfamiliar half- or full-siblings from their mothers’ previous or subsequent broods. Thus, phenotype matching is expected to evolve as a kin recognition mechanism (Holmes and Sherman 1982; Sherman et al. 1997; Hauber and Sherman 2001). Consistent with this hypothesis, I also found that juvenile Paria guppies preferred to associate with related over unrelated individuals, independent of their level of familiarity. Thus, my data show that these guppies are able to use both familiarity and phenotype matching recognition mechanisms.

The use of both familiarity and phenotype matching as kin recognition mechanisms in Paria guppies is perhaps surprising. Individuals clearly require phenotype matching to
distinguish between unfamiliar siblings and unrelated individuals or between familiar full- and half-siblings. It is less clear why they would also use familiarity. Familiarity may be used because it is more reliable (i.e. less error prone) or because it is cognitively ‘cheaper’ to utilize than phenotype matching. To my knowledge, there are no data available on the sophistication of the neurology needed to perform either mechanism. Furthermore, shoaling with familiar individuals may have added benefits outside of kin selection such as reciprocal altruism (Wilkinson 1984; Trivers 1985). Guppies have been shown to have stable social networks (Croft et al. 2004), which facilitates the development of reciprocal altruism (Milinski 1987). Reciprocal altruism may be important in the context of foraging and predator inspection (Croft et al. 2006).

It is unlikely that Paria guppies were using familiarity developed in utero and not phenotype matching in some of my trials. First, the preference of guppies for paternal half-siblings over unrelated individuals could not be explained by familiarity because all individuals were unfamiliar (i.e. gestated separately). Second, Griffiths and Magurran (1997) have previously shown in another population of guppies that familiarity with shoalmates develops only after 12 days of association post-parturition. The guppies used in this experiment were separated within 24 hours of birth. Furthermore, a kin template formed based on the phenotypes of brood-mates is likely to provide a heterogeneous signal (e.g. mean relatedness ranged from 0.29 to 0.5 across broods; see Table 4.2) and is unlikely to allow discrimination between individuals of differing relatedness. Thus, phenotype matching, and specifically self-referencing, is the most likely mechanism to explain some of the patterns of discrimination observed in my study.

This study was not designed to test for a particular cue used in kin recognition by phenotype matching. However, the relationship between odour phenotype and genotype at loci associated with the major histocompatibility complex (MHC) makes MHC a strong candidate for providing cues of kinship (Penn 2002; Milinski et al. 2005). For example, juvenile Arctic charr, Salvelinus alpinus, discriminate between shoalmates based on differences in MHC (Olsén et al. 1998). Furthermore, MHC has been implicated as a cue of kin recognition in several other taxa, including mice (Manning et al. 1992) and rats
(Brown et al. 1987). Thus, MHC may be a recognition cue involved in phenotype matching in guppies, but this remains to be explored.

Little is known about the specific benefits gained by juvenile guppies from shoaling preferentially with kin. However, it is known that in two species of salmon, *Salmo salar* and *Oncorhynchus mykiss*, juveniles in shoals of related individuals grow faster and have fewer antagonistic behaviours than in shoals of unrelated individuals (Brown and Brown 1996). Guppies may similarly benefit by shoaling with kin. However, the behavioural interactions of relatives in shoals, and the specific benefit to shoaling with kin, such as increased growth rate, has yet to be investigated in the guppy.

Although the Paria guppies I studied can recognize kin, I found no evidence of kin structure in adult shoals. This is perhaps surprising given that I found that a large number of close relatives are present within the population; for example, 16% of pairs were more related than half-siblings (see Appendix B). The absence of kin structure in adult shoals may in part be explained by reduced shoaling behaviour by adults. Shoaling is a common defence against fish predation (Pitcher and Parrish 1993) and adults in the Paria River population are subjected to low predation. These results are consistent with other studies from the Quare River and Lower Tacarigua River populations (Russell et al. 2004). It is possible that adult guppies do not shoal with kin because they are actively seeking mates.

Finally, the contrasting results from this study and that of Griffiths and Magurran (1999) provide support for the kin recognition hypothesis put forward by Holmes and Sherman (1982). The Paria River tributary and Lower Tacarigua River differ in critical aspects of their ecology and mating system, which should lead to the evolution of different recognition mechanisms. Guppies from the Lower Tacarigua River are characterized by broods that are sired predominantly by a single male, with one male typically siring about 96% of the brood (Evans and Magurran 2001). This population also experiences high predation (Reznick et al. 1996). As such, unfamiliar full-siblings are unlikely to be encountered and familiarity with brood- and shoal-mates provides a reliable indicator of full-sibling relatedness. Thus, kin recognition by familiarity is expected. Conversely, the tributaries of the Paria River are characterized by a high degree of multiple mating and low
predation (this study, Reznick et al. 1996). Thus, consistent with my results, phenotype matching is expected to evolve. Together these studies provide the first within-species support for the kin recognition hypothesis that local ecology and mating system are associated with the evolution of kin recognition mechanisms. However, this hypothesis will be explained further in Chapter 5 of this thesis.

4.5 References


Chapter 5

5 Multiple mating predicts intensity but not mechanism of kin recognition\(^4\)

Understanding how animals recognize their kin has been a major challenge in biology. Most animals use one of two mechanisms: “familiarity” whereby kin are remembered from interactions early in life, such as in a nest, or “phenotype matching” whereby putative kin are compared to a template of what kin should look, smell, or sound like. Cross-species studies suggest that there is a link between which of these two mechanisms is used and the degree of female promiscuity (multiple-mating). Phenotype matching is more likely to be used by promiscuous species, because these species have lower average brood relatedness than monogamous species and familiarity is thus an unreliable cue of relatedness. However, it is unclear if this relationship holds within species, across populations that differ in their degree of promiscuity. Here I take advantage of variation in brood relatedness across populations of guppies (Poecilia reticulata) to examine the relationship between kin recognition mechanisms and multiple-mating within a single species. Contrary to the established hypothesis, I show that variation in recognition mechanism across populations is not governed by multiple mating. Instead, my data show that kin recognition, quantified as association preferences for shoalmates, is strongest when brood relatedness is high, consistent with Hamilton’s rule, but multiple mating does not otherwise influence the specific recognition mechanism used.

\(^4\) A version of this chapter has been published and is presented here with permission from *Behavioral Ecology*.

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5.1 Introduction

The formalization of kin selection theory (Hamilton 1964) has greatly increased the understanding of social behaviour. Kin selection has been used to explain behavioural phenomena such as cooperative breeding (Hatchwell et al. 2014), alarm calls (Sherman 1977), and gregarious association behaviour (Viblanc 2010). These behaviours appear to be costly to the individual performing the behaviour because of reduced reproductive output, increased vulnerability to predators, or increased competition for resources, but these behaviours can indirectly benefit the individual by increasing the reproductive output, survivorship, or growth of relatives. Kin-directed social behaviours can arise through passive mechanisms, such as directing the behaviours to conspecifics found in or close to a natal nest, but there are also many examples of direct discrimination between related and unrelated individuals (Greenberg 1979; Wu et al. 1980; Grosberg and Quinn 1986). The ways that animals recognize their kin has thus become a major area of research for psychologists and behavioural ecologists.

Direct kin recognition is typically done using one of two mechanisms: familiarity (also known as prior association) and phenotype matching (Mateo 2004). With familiarity, individuals remember other conspecifics encountered early in life, particularly in the vicinity of the natal area (e.g. the nest), and later treat those individuals as related. With phenotype matching, individuals instead use aspects of their own phenotype, or those of conspecifics encountered early in life, such as odour, appearance, or sound, to build a “kin template.” Later, putative kin are compared to the kin template and treated as related or unrelated based on the similarity to the template (Holmes and Sherman 1982; Mateo 2004). So, for familiarity, specific individuals are remembered as kin, whereas for phenotype matching, putative kin are not remembered but instead compared to a template of what kin should look, smell, or sound like. Some species, such as humans and some apes, can use both mechanisms (e.g. Olsson et al. 2006; Alvergne et al. 2009).

Generally, it is hypothesized that animals will use familiarity when it is a reliable form of kin recognition. When familiarity is unreliable, phenotype matching should be used instead (Holmes and Sherman 1982). Familiarity is most commonly an unreliable
mechanism when reproductive behaviours, such as brood parasitism or multiple mating, lead to unrelated individuals being born together, or when dispersal or overlapping generations lead to unfamiliar kin encountering each other later in life (Holmes and Sherman 1982; Hauber and Sherman 2001). This hypothesis implies that variation among species or populations in their ecology, life histories, or mating systems should lead to variation in recognition mechanisms. In particular, when multiple mating leads to a low average relatedness of broodmates, phenotype matching should evolve over familiarity as the mechanism of kin recognition. Indeed, several studies have revealed variation in recognition mechanism that relates to the degree of multiple mating. For example, bluegill (Lepomis macrochirus) is a fish with alternative mating tactics and high levels of multiple mating: 92% of broods studied were multiply mated, with 77% of the larvae in a nest sired by the “parental” male guarding that nest and 23% of the larvae sired by intruding “cuckolder” males (Neff 2001). Consequently, offspring of cuckolder males were less related to their nestmates than the offspring of parental males. As predicted, cuckolders’ offspring used phenotype matching to recognize and shoal with their kin, whereas parents’ offspring did not use phenotype matching (Hain and Neff 2006). In birds, Indian peafowl (Pavo cristatus) have high levels of multiple mating and use phenotype matching to recognize kin (Petrie et al. 1999). In contrast, other bird species characterized by low-multiple mating and relative monogamy, such as cliff swallows (Hirundo pyrrhonota) and barnacle geese (Branta leucopsis), use familiarity through prior association to recognize kin (Beecher et al. 1985; van der Jeugd et al. 2002). However, it is not clear if this pattern holds within a single species that is able to use both familiarity and phenotype matching. Here I provide the first such test by examining the link between multiple mating and the mechanism of kin recognition in the guppy (Poecilia reticulata), a species that is known to use both familiarity and phenotype matching (Griffiths and Magurran 1999; Hain and Neff 2007).

Guppies are a small freshwater fish that has emerged as a model system for the study of evolutionary ecology because populations have repeatedly experienced convergent evolution across river systems in response to similar selection pressures (Reznick et al. 1997). For example, differences in the intensity of predation affect the
frequency of forced copulations (Magurran and Seghers 1994), brood size (Reznick and Endler 1982), and male colouration (Endler 1980). Guppies from different rivers also vary in the degree of multiple mating, which ranges from 70% to 100% of broods in the rivers examined to date (Hain and Neff 2007; Neff et al. 2008; Elgee et al. 2012). Guppy populations are known to show variation in their kin recognition mechanisms (Griffiths and Magurran 1999; Hain and Neff 2007), but these differences in mechanism have never been related to population-level differences in ecology. Guppies thus offer an exceptional opportunity to examine if recognition mechanisms differ across populations, and if the pattern of recognition mechanisms among populations can be explained by differences in multiple mating or other ecological or life-history factors.

In this study, I selected six guppy populations from Trinidad that captured variation in mating system, predation pressure, and life history (Hain and Neff 2007; Neff et al. 2008). I tested if individuals from each population used familiarity to associate with familiar rather than unfamiliar individuals as shoaling partners, and phenotype matching to associate with related rather than unrelated individuals as shoaling partners. In fish, some potential benefits that arise from associating with kin include improved predator defence arising from group members having similar phenotypes, which enhances the confusion effect on predators (Ruxton et al. 2007; Croft et al. 2009), a more coordinated response against predators (Hain and Neff 2009), or reduced aggression among related shoalmates, leading to increased growth rates of individuals (Brown and Brown 1996). In order to experience these potential benefits, I expected juvenile guppies to prefer to associate with familiar or related individuals over unfamiliar or unrelated individuals. I then asked if the observed variation in kin recognition could be explained by multiple mating, predation regime, or brood size. I predicted that multiple mating would affect recognition mechanism because it reduces the reliability that being born together (familiarity) is a cue of full-sibling relatedness (Hauber and Sherman 2001). Second, I predicted that predation regime would affect recognition mechanism because guppies from low-predation populations tend to live longer than guppies from high-predation populations (Reznick et al. 1996), increasing the likelihood of low-predation guppies meeting relatives from earlier or later broods, in which case phenotype matching would be required for kin recognition. Finally,
I predicted that mean brood size in a population would be negatively correlated with the use or accuracy of familiarity. The recognition of individuals is thought to be cognitively demanding (Brosnan et al. 2010), and the ability to remember individuals from large broods may therefore be more difficult than remembering individuals from small broods.

5.2 Methods

5.2.1 Field collections and trials

I collected guppies from six populations in Trinidad that varied in mating system, predation pressure, and life history (Lower Oropouche: 10°40’ N, 61°08’ W; Tunapuna: 10°42’ N, 61°21’ W; Upper Yarra: 10°47’ N, 61°21’ W; Upper Aripo: 10°42’ N, 61°12’ W; Lower Guanapo: 10°39’ N, 61°12’ W; Paria: 10°45 N, 61°16’ W). As in other studies with guppies (e.g. Rodd and Reznick 1997), populations were classified as being from a high-predation regime if they were sympatric with the piscivore *Crenicichla sp.* (Lower Oropouche and Lower Guanapo) and from a low-predation regime if the rivers did not contain *Crenicichla sp.* (Tunapuna, Upper Yarra, Upper Aripo, Paria). All fish were collected with dip nets or a 2 m seine net and transported to the University of the West Indies within 3 h of capture (St. Augustine, Trinidad and Tobago). Guppies from two populations (Paria, Upper Yarra) were further transported to the University of Western Ontario (London, Ontario, Canada) before conducting the kin recognition trials. All captive guppies were maintained at 24-26°C on a 12 h:12 h light:dark cycle (Houde 1997) and fed *ad libitum* twice daily, once with brine shrimp (Brine Shrimp Direct, Ogden, UT, USA) and once with flakes (Tetra Werke, Melle, Germany).

Relatedness within broods was measured using females that were pregnant at the time of collection. Each female was placed in an individual tank that contained a small clipping of filamentous algae to act as a refuge for her offspring. Tanks were checked daily for the presence of newborn guppies, and 24 hours after the first juvenile was observed the mother and all her offspring were euthanized with an overdose of clove oil and preserved.
in 95% ethanol for genetic analysis. Broods of fewer than 3 juveniles were not analyzed for paternity.

DNA was extracted from a subset of females and each their offspring using a proteinase K digestion (Neff et al. 2000). Three microsatellite loci (previously described in Becher et al. 2002; Paterson et al. 2005) were then polymerase chain reaction (PCR) amplified for the broods from each population (Lower Oropouche, Tunapuna, Upper Aripo: Pre8, Pre9, Pre39; Paria: Pre1, Pre13, Pre15; Lower Guanapo, Upper Yarra: Pre13, Pre15, Pre80). The PCR products were visualized on a CEQ 8000 (Beckman Coulter) and the allele sizes determined using a reference size standard. Full and half-sibling relationships within broods were reconstructed using the maximum likelihood approach implemented in the program COLONY (Wang 2004). Brood relatedness was then calculated as the mean of all pairwise relatedness comparisons within a brood. The average brood relatedness value for guppies could theoretically range from 0.25 (all individuals in a brood sired by different fathers) to 0.50 (all individuals in a brood sired by the same father).

Next I conducted kin recognition trials to ascertain which, if either, mechanism was used by each population. For each population, approximately 60 juveniles were collected from the wild. These juveniles were checked daily for signs of male sexual dimorphism (appearance of a dark lateral spot or constriction of the anal fin, which typically occurs at about 5 weeks of age: Evans et al. 2002). The immature males were then isolated from females to prevent mating and ensure accurate pedigrees. Once fish had matured (after 7 weeks of age; Houde 1997), I mated each virgin female to a single male by placing the pair in a tank together for 7 days (Appendix D). The male was then removed from the tank and was not mated again. Females were monitored twice daily for births, and newborn guppies were visually and chemically isolated from their broodmates as soon as they were discovered, which was always within 24 hours of birth to prevent familiarity among broodmates (familiarity preferences develop after 12 days of association in guppies and are undeveloped at 8 days or less of association; Griffiths and Magurran 1997). This methodology of isolating or cross-fostering newborns within 24 hours of birth is commonly performed in kin recognition studies that use internally-brooded animals (Mateo and
Because the female guppies were virgins, each brood was composed entirely of full-siblings, and because each mature guppy was mated only once, all full-sibling families were unrelated to each other.

Familiarity among individuals was manipulated by rearing two full-sibling families from the same population that were unrelated to each other together in a single tank. Families were divided into up to three groups of three or four fish, anaesthetized with MS-222, and given tail clippings (a notch in either the top or bottom part of the caudal fin) to allow identification to the family level. Familiarity was then developed by taking two such groups from two different families (born within 7 days of each other) and placing them together in a single rearing tank for 12-15 days in a group size of 6-8 individuals, which is within the shoal size range observed in nature (Magurran and Seghers 1991). Thus, individuals in these tanks were reared with familiar full-siblings and familiar non-kin. Individuals reared in separate tanks were treated as unfamiliar to each other.

At the end of the familiarization period, dichotomous choice trials were used to measure association preferences in sexually immature juvenile guppies for familiar versus unfamiliar and related versus unrelated individuals. The test arena was a 34 cm long tank (total volume = 10 L) divided into three compartments by two transparent, porous barriers that allowed visual and chemical communication between compartments, as both visual and chemical cues have previously been shown to be used in kin recognition in guppies (Griffiths and Magurran 1999). The centre compartment was 18 cm in length and contained a focal fish. Each of the outer compartments was 8 cm in length and contained a single juvenile stimulus fish. Each trial presented focal fish with the choice of associating with either of two stimulus fish that differed from each other in either familiarity or relatedness (but not both). That is, in trials that manipulated familiarity, one stimulus fish was familiar and one stimulus fish was unfamiliar to the focal fish, with both stimulus fish sharing the same relatedness to the focal fish (both related or both unrelated). In trials that manipulated relatedness, one stimulus fish was related and one stimulus fish was unrelated to the focal fish, with both stimulus fish sharing the same familiarity to the focal fish (both familiar or both unfamiliar). The side of the tank occupied by the more familiar (or more related)
stimulus fish was assigned randomly and stimulus fish were matched for similarity in age and size.

For each trial, a focal fish was allowed 15 min to acclimate to the test arena, after which its behaviour was recorded for 15 min. The focal fish’s compartment was divided into three zones: two 5 cm “association zones” next to the barriers separating it from the stimulus fish, and one 8 cm “neutral zone” in the centre. I recorded the amount of time the focal fish spent in each association zone, counting both the time that the focal fish was entirely within the association zone and the time the focal fish had its gill slits in the association zone with its head oriented toward the stimulus fish on that side. Fish were used only once as focal fish, but were used up to four times as a stimulus fish across tests of familiarity and phenotype matching. Among fish used four times as stimuli, there was no tendency for stimulus fish to be preferred more or less as shoaling partners as they were used in multiple trials ($F_{3,176} = 2.0$, $p = 0.11$). Tanks were cleaned between trials and filled with fresh water to remove olfactory cues. A total of 402 trials were conducted. Five trials were excluded from analysis either because of inactivity of the focal fish ($n = 4$), or because a stimulus fish displayed courtship behaviour ($n = 1$). Variation in the number of broods produced by the six populations was associated with differences in sample size (25 to 119 trials per population).

5.2.2 Statistical analysis

For each of the six populations, the percentage of time spent associating with the full-sibling (or familiar) stimulus fish was compared to the percentage of time spent with the non-kin (or unfamiliar) stimulus fish using one-tailed paired-sample t-tests (i.e. the time spent in the centre “neutral zone” was excluded). I then combined these two types of association trials into a single analysis, in which I compared the percentage of time spent associating with putative kin (i.e. either familiar or related) to the percentage of time spent associating with putative non-kin (either unfamiliar or unrelated) using one-tailed paired-sample t-tests. I did this latter comparison to test if populations in general preferred to associate with putative kin over unrelated individuals, independent of mechanism. Next, I
used Pearson correlations to test if the time spent associating with the full-sibling (or familiar, or putative kin) stimulus was related to the average brood relatedness in the population. Average brood relatedness is inversely related to multiple mating but also captures reproductive skew among sires, where broods predominantly sired by one male have higher brood relatedness than broods equally sired by many males. Thus, it is average brood relatedness that is expected to affect the reliability of familiarity for kin recognition (Hain and Neff 2007). These correlations were based on population averages, so might underestimate the effect of within-population variance on the relationship between these factors. Thus, to better explore these relationships, I generated 10,000 simulated datasets by randomly drawing values for association time and brood relatedness for each population using the observed sample sizes and the total distribution of these parameters across populations. I then calculated the slope of the relationship between these factors for each simulated dataset to generate a null distribution of expected slopes. Finally, I compared the magnitude of the observed slopes to the random distributions to determine the probability of observing a slope of at least that magnitude given the underlying structure of my data.

I tested the effect of predation regime on time spent associating with the related (or familiar) stimulus using ANOVAs with population nested within predation regime, where predation was coded as either high or low. Finally, I used Pearson correlations to test if the time spent associating with the full-sibling (or familiar) stimulus was related to the average brood size in the population. For all tests, the significance level was set at $\alpha = 0.05$ and performed using SPSS (version 22.0).

5.3 Results

The relatedness figures calculated from the COLONY analysis and the proportion of broods that were multiply mated are presented in Table 5.1. Across the six populations, the average allelic richness was 8.4 (range = 6.3-17), average brood size was 10.4 (range = 3.8-14.7) average brood relatedness was 0.345 (range = 0.323-0.363), and an average of 95% of broods were multiply mated (range = 83-100%). There was no correlation between
allelic richness and brood relatedness across populations ($R = 0.26$, $n = 6$, $P = 0.62$), or between brood size and brood relatedness across populations ($R = 0.37$, $n = 6$, $P = 0.47$).

**Table 5.1** Summary of genetic and parentage analyses for six guppy populations. Data comprise predation regime (high, low), allelic richness (average and range across loci), number of broods analyzed, brood size (average and range), brood relatedness and the percentage of broods that had multiple sires.

<table>
<thead>
<tr>
<th>Population</th>
<th>Predation regime</th>
<th>Allelic richness</th>
<th>Number of broods</th>
<th>Brood size</th>
<th>Brood relatedness</th>
<th>Multiple mating</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lower Oropouche</td>
<td>High</td>
<td>17 (12-24)</td>
<td>10</td>
<td>14.3 (8-21)</td>
<td>0.323</td>
<td>100%</td>
</tr>
<tr>
<td>Tunapuna</td>
<td>Low</td>
<td>6.7 (6-7)</td>
<td>10</td>
<td>14.7 (4-27)</td>
<td>0.334</td>
<td>100%</td>
</tr>
<tr>
<td>Upper Yarra</td>
<td>Low</td>
<td>6.7 (4-10)</td>
<td>10</td>
<td>3.8 (3-5)</td>
<td>0.346</td>
<td>90%</td>
</tr>
<tr>
<td>Upper Aripo</td>
<td>Low</td>
<td>6.3 (3-9)</td>
<td>10</td>
<td>7.3 (4-11)</td>
<td>0.348</td>
<td>100%</td>
</tr>
<tr>
<td>Lower Guanapo</td>
<td>High</td>
<td>12.7 (7-20)</td>
<td>12</td>
<td>9.3 (4-25)</td>
<td>0.357</td>
<td>83%</td>
</tr>
<tr>
<td>Paria</td>
<td>Low</td>
<td>11 (10-13)</td>
<td>23</td>
<td>12.8 (3-40)</td>
<td>0.363</td>
<td>96%</td>
</tr>
</tbody>
</table>

* Data on multiple mating in the Lower Oropouche, Tunapuna, Upper Aripo and Paria populations were previously published (Hain and Neff 2007; Neff et al. 2008). One additional brood from the Paria population was included in the current study.
Among populations, guppies varied in their association preferences for both related and familiar individuals (Table 5.2). Specifically, guppies from the Tunapuna, Paria, Upper Yarra, and Lower Guanapo populations preferentially associated with related rather than unrelated individuals, indicating their ability to discriminate between individuals using phenotype matching (Fig. 5.1). Guppies from the Upper Aripo and Paria populations preferentially associated with familiar rather than unfamiliar individuals, showing their ability to discriminate between individuals using familiarity (Fig. 5.1). When all association trials were combined into a single analysis, every population except the Lower Oropouche preferred to associate with the putative kin stimulus rather than the non-kin stimulus (Table 5.2).

**Table 5.2** Significance of association preferences based on paired t-tests in six populations of guppies. Significant preferences for the full-sibling or familiar stimuli are indicated with boldface type and an asterisk (*).

<table>
<thead>
<tr>
<th>Population</th>
<th>Phenotype matching</th>
<th></th>
<th>Familiarity</th>
<th></th>
<th>Combined</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n</td>
<td>t</td>
<td>P</td>
<td>n</td>
<td>t</td>
<td>P</td>
</tr>
<tr>
<td>Lower Oropouche</td>
<td>12</td>
<td>0.94</td>
<td>0.18</td>
<td>13</td>
<td>0.27</td>
<td>0.39</td>
</tr>
<tr>
<td>Tunapuna</td>
<td>60</td>
<td>2.66</td>
<td><strong>0.005</strong>*</td>
<td>59</td>
<td>0.34</td>
<td>0.37</td>
</tr>
<tr>
<td>Upper Yarra</td>
<td>12</td>
<td>2.08</td>
<td><strong>0.031</strong>*</td>
<td>24</td>
<td>1.56</td>
<td>0.067</td>
</tr>
<tr>
<td>Upper Aripo</td>
<td>37</td>
<td>1.26</td>
<td>0.11</td>
<td>35</td>
<td>2.38</td>
<td><strong>0.012</strong>*</td>
</tr>
<tr>
<td>Lower Guanapo</td>
<td>40</td>
<td>3.60</td>
<td><strong>&lt;0.001</strong>*</td>
<td>43</td>
<td>1.16</td>
<td>0.13</td>
</tr>
<tr>
<td>Paria</td>
<td>24</td>
<td>2.85</td>
<td><strong>0.005</strong>*</td>
<td>38</td>
<td>2.82</td>
<td><strong>0.004</strong>*</td>
</tr>
</tbody>
</table>
Figure 5.1 Association preferences of juvenile guppies for related versus unrelated individuals (white bars) and familiar versus unfamiliar individuals (black bars). Data are from six populations in Trinidad: (a) Lower Oropouche; (b) Tunapuna; (c) Upper Yarra; (d) Upper Aripo; (e) Lower Guanapo; and (f) Paria. Each bar represents an individual presented in rank order. Percent association time was calculated as time spent associating with the related (or familiar) stimulus as a percentage of the total time spent associating with either stimulus in a 15-minute trial. The 50% line indicates fish that associated equally with both stimulus fish, with bars above the 50% line representing time spent with related (or familiar) individuals that was greater than expected by chance. The results of paired t-tests comparing association time for related versus unrelated stimuli are shown above each panel.
There was a significant positive correlation between association time with full-
sibling stimuli and brood relatedness (R = 0.86, n = 6, P = 0.028; Fig. 5.2a). The simulation
analysis confirmed that the slope of this regression was greater than expected by chance (P
= 0.025; for full distributions see Appendix E). There was no significant relationship
between association time with familiar fish and brood relatedness (R = 0.70, n = 6, P
= 0.12; Fig. 5.2b), but when association times with either familiar or related fish were
combined into a single analysis, I found a strong positive correlation between association
time with putative kin and brood relatedness (R = 0.90, n = 6, P = 0.014; Fig. 5.2c), and
the simulation analysis confirmed that the slope of this regression was significantly greater
than expected by chance (P < 0.001).

I found no significant effect of predation regime on association time with familiar
fish (F_{1,206} = 1.22, P = 0.27), and no difference among populations in association time with
familiar fish when population was nested within predation regime (F_{4,206} = 1.22, P = 0.19). There was no significant difference in association time with related fish between high- and
low-predation populations (F_{1,179} = 3.39, P = 0.067), but there was a significant difference
among populations in association time with related fish, and thus differences among
populations in their use of phenotype matching, when population was nested within
predation regime (F_{4,179} = 2.81, P = 0.027). Tukey’s post-hoc tests revealed that this
difference was driven by the Lower Oropouche population associating with related fish
less than the Lower Guanapo, Upper Yarra, and Paria populations (P < 0.05 for each).

Finally, I found no significant relationship between association time with related
fish and brood size (R = 0.45, n = 6, P = 0.37), or between association time with familiar
fish and brood size (R = 0.46, n = 6, P = 0.36).
Figure 5.2 Relationships between association time with related or familiar individuals and within-brood relatedness for six guppy populations in Trinidad. Averages ± SE are presented for each population, showing the relationships between average within-brood relatedness and association time with (a) related individuals; (b) familiar individuals; or (c) related and familiar individuals combined. P-values testing the significance of the relationships between these factors were generated by a Pearson correlation, and best fit lines are shown for significant correlations.
5.4 Discussion

Here I have provided the first data showing variation in kin recognition mechanisms across populations within a single species. Previous studies of guppies suggested the presence of population-level differences in kin recognition mechanisms, as the Lower Tacarigua River population used familiarity to recognize kin (Griffiths and Magurran 1999), whereas the Paria River population used both phenotype matching and familiarity (Hain and Neff 2007). However, these studies differed in methodology, so could not rule out effects of the experimental design. By examining six populations of Trinidadian guppies using the same methodology, I found that one population used familiarity, three populations used phenotype matching, one population used both familiarity and phenotype matching, and one population did not use either kin recognition mechanism to associate with relatives as shoalmates. I also confirmed the earlier finding that the Paria River population used both phenotype matching and familiarity. My data show that kin recognition mechanisms are variable within a species and that there is thus the potential for local conditions to influence the recognition mechanism used by a population.

One variable that has long been thought to influence kin recognition mechanisms is the level of multiple mating within a population (Hauber and Sherman 2001). Previous studies have found a positive association between the use of phenotype matching and multiple mating, both among species (e.g. Petrie et al. 1999; van der Jeugd et al. 2002) and among reproductive tactics within a species (Hain and Neff 2006). Here I examined the effect of natural variation in multiple mating among guppy populations on the expression of kin recognition behaviours. I found that the strength of kin recognition was related to brood relatedness, despite a relatively narrow range of brood relatedness values across populations. However, this relationship was in the opposite direction from the prevailing hypothesis based on cross-species studies, as populations were more likely to use phenotype matching when brood relatedness was relatively high (i.e. multiple mating was low). There was also a similar trend to use familiarity more when brood relatedness was high, albeit the relationship was marginally non-significant. Based on these data, I thus
reject the hypothesis that the degree of multiple mating affects the specific mechanism of kin recognition in guppies. Further within-species studies that examine the relationship between multiple mating and kin recognitions mechanism would help clarify the generality of this result.

The observed positive relationship between association behaviour and brood relatedness may instead reflect Hamilton’s rule whereby a social behaviour should be performed when the product of the relatedness coefficient of the individual being helped and the benefits of the behaviour exceed the costs (Hamilton 1964). If costs and benefits of associating as a shoal are equal across the populations I studied, then the probability of associating with broodmates, regardless of the specific mechanism used to recognize them, scales directly with relatedness. Indeed, even though the differences among populations in within-brood relatedness were relatively small, there was a strong positive linear relationship between association time with putative kin and the mean within-brood relatedness of the population (in the combined analysis). Hamilton’s rule can explain the observed relationship between brood relatedness and the strength of kin recognition, regardless of the actual mechanism used.

Hamilton’s rule also offers an alternative explanation for previously observed relationships between multiple mating and kin recognition mechanism. For example, in social insects promiscuous colonies have low within-colony relatedness and frequently show reduced preferences for kin and less aggression toward intruders than monogamous colonies that have high within-colony relatedness (e.g., Hogendoorn and Velthuis 1988; Pirk et al. 2001; Tsutsui et al. 2003; Adams et al. 2007). This trend has predominantly been interpreted as a breakdown in kin recognition, because the greater genetic diversity in colonies with multiple breeders could increase the likelihood of recognition errors. However, the reduced recognition by promiscuous colonies are explained equally well by Hamilton’s rule, as monogamous colonies have a greater within-colony relatedness coefficient and would thus gain greater benefits from kin discrimination. Indeed, in this study, I can rule out variation in recognition errors among populations arising from difference in brood relatedness during development because I raised test individuals from all populations with the same degree of mixed relatedness. Evolutionary effects driven by
Hamilton’s rule thus offer a robust explanation for differences in kin recognition across guppy populations and might also be important across a wide range of species.

The contributions of ecological or life-history factors other than multiple mating to the expression of kin recognition are not well understood. Perhaps the best example of a study that linked variation in recognition mechanism to ecological variation was an examination of three *Drosophila* species, which showed that recognition mechanisms could be explained by the species’ mating system, gregariousness, or diet (Lizé et al. 2014). Here, I investigated the effects of predation regime and brood size on recognition mechanism and found no effect of either factor. I hypothesized that predation regime might affect recognition mechanism because it explains much of the variation in other traits among guppy populations (e.g. Reznick et al. 1982). In particular, guppies from low-predation populations have significantly lower mortality than guppies from high-predation populations (Reznick et al. 1996; Reznick and Bryant 2007) and consequently also have an increased likelihood of encountering unfamiliar relatives from other broods. I thus expected that phenotype matching would evolve as a recognition mechanism in low-predation populations. However, I found that there was no difference in recognition mechanism between low- and high-predation populations. A previous study has shown that juvenile guppies from both low-predation and high-predation populations have a large number of siblings outside of their natal shoals (Piyapong et al. 2011). This high dispersal from natal shoals, independent of predation regime, may result in juveniles from both population types encountering unfamiliar kin and might explain the absence of any apparent effect of predation regime on kin recognition. Second, I hypothesized that populations with large broods might use phenotype matching because of the increased difficulty associated with remembering many individuals, as would be required with familiarity. Many species with large family sizes use phenotype matching to recognize relatives (e.g., fish: Olsén et al. 1998; Hinz et al. 2013; insects: Getz and Smith 1983; El-Showk et al. 2010). However, although there was almost four-fold variation in average brood sizes across populations, I did not observe a relationship between brood size and the use of phenotype matching. It is possible that the brood sizes seen across the populations studied here were too small and cognitively undemanding to have an effect on recognition
by familiarity. Indeed, guppies have relatively small broods compared to externally fertilizing fishes and insects known to use phenotype matching (e.g., Olsén et al. 1998; Power et al. 2005; Whitehouse and Jaffe 1995; Ferguson-Gow et al. 2014).

In conclusion, I have provided the first within-species test of the effect of multiple mating on the mechanism of kin recognition. Although I found variation in mechanism across populations, I did not find the expected relationship with multiple mating. Instead, I found that kin recognition, regardless of mechanism, is strongest when multiple mating is low, and hence brood relatedness is high. This result is consistent with Hamilton’s rule that the expression of a social behaviour is directly related to the relatedness of the individuals.

5.5 References


Chapter 6

6 Evolution of kin recognition mechanisms in a fish

The extent to which phylogenetic history influences current traits has interested biologists for decades. In particular, behavioural traits are thought to be among the most evolutionarily labile and thus may be key to survival in changing environments. Here I used recently-characterized variation in kin recognition mechanisms among six guppy populations to explore the phylogenetic history of this trait and its evolutionary lability. When a recognition mechanism is used by guppies, they can use phenotype matching, in which individuals are identified based on comparison to a recognition template, or familiarity, in which individuals are remembered based on previous interactions. Across the six populations I identified four transitions in recognition mechanism: phenotype matching evolved once and was subsequently lost in a single lineage, whereas familiarity evolved twice. Based on a molecular clock, these transitions occurred over timescales of hundreds of thousands of years, two orders of magnitude faster than previously documented changes in recognition mechanisms. A randomization test provided no evidence for phylogenetic signal, suggesting that kin recognition mechanisms are evolutionarily labile, though the specific selection pressures that may be contributing to variation in recognition mechanisms across the populations remains unknown.

6.1 Introduction

Biologists have long been interested in the degree to which traits reflect current selection pressures relative to evolutionary constraints (e.g., Wilson 1975). On one hand, selection can favour adaptations that best match individuals to their environments, while on the other, evolutionary constraints can limit adaptation if populations lack genetic variation on which selection can act, which may be especially important for complex traits. Consequently, phylogenetic signal, in which closely-related taxa are more similar than distantly-related taxa, is widespread across taxa and is present for many classes of traits (Blomberg et al. 2003). Interestingly, the strength of phylogenetic signal has been shown
to be lower for behavioural traits than for other traits (Blomberg et al. 2003), suggesting that behaviour may be particularly evolutionarily labile, and may thus be a key component to rapid adaptation in changing environments.

Kin recognition is an important aspect of social behaviour that allows individuals to respond adaptively to the presence of genetic relatives, with familiarity and phenotype matching being the most common mechanisms (Hamilton 1964; Mateo 2004). Familiarity is based on prior association among family members, and when it is used as a recognition mechanism, individuals remember conspecifics encountered early in life, particularly in the vicinity of the natal area (e.g., the nest), and later treat these individuals as related. When phenotype matching is used for kin recognition, individuals instead use aspects of the phenotype such as odour, sound, or appearance of conspecifics encountered early in life to build a “kin template”. Later, putative kin are compared to the kin template and treated as related or unrelated based on the degree of similarity (Holmes and Sherman 1982; Mateo 2004). Although the mechanisms of kin recognition have been widely studied, the questions of how these mechanisms evolved, and the degree to which variation in mechanisms reflects evolutionary constraints relative to current selection pressures remains unresolved.

Guppies (*Poecilia reticulata*) have emerged as a model system for the study of evolution because populations have repeatedly experienced convergent evolution in behavioural, morphological, and life history traits in response to differences in local predation pressure (Seghers 1974; Endler 1983; Reznick et al. 1997) and other ecological factors (e.g., Grether et al. 2001). Hain et al. (2016) recently characterized variation in kin recognition mechanisms across six guppy populations. The authors found that one population used both familiarity and phenotype matching, one used familiarity, three used phenotype matching, and one population did not use either mechanism. Here I take advantage of this variation in recognition mechanism to provide the first test of the relationship between kin recognition mechanisms and phylogeny. I use mitochondrial control region sequences to assess the phylogenetic relationships among the guppy populations and fit the observed recognition mechanisms to the resulting tree. I then use a
randomization routine to examine the evolutionary lability of recognition mechanisms by testing whether or not phylogenetic signal contributes to recognition mechanisms.

6.2 Methods

6.2.1 Genetic analysis

A combination of previously published sequences and field-collected samples were used to obtain mitochondrial control region sequences for each of the six Trinidadian populations of guppies for which data on kin recognition mechanisms were available (Appendix F). These populations encompass the three major drainage systems in Trinidad: the east-flowing Oropouche drainage (Lower Oropouche population: 10°40’ N, 61°08’ W; Appendix G), the Northern drainages (Upper Yarra: 10°47’ N, 61°21’ W; Paria: 10°45 N, 61°16’ W), and the west-flowing Caroni drainage (Tunapuna: 10°42’ N, 61°21’ W; Upper Aripo: 10°42’ N, 61°12’ W; Lower Guanapo: 10°39’ N, 61°12’ W). In total, mitochondrial control region sequences were obtained from 41 guppies, including 5 to 13 fish from each population.

The phylogenetic relationships among populations were determined by analyzing the mitochondrial control region sequences using MrBayes version 3.2.2. (Ronquist et al. 2012). This analysis used the program’s recommended settings, and included a sequence from the closely related Poecilia picta to root the tree (Genbank accession: AF033053). The resulting tree indicated that all sequences from the same population grouped together (Appendix H), so the relationships among populations were collapsed into a single consensus tree.

6.2.2 Transition analysis

I first mapped the observed kin recognition mechanisms for the six guppy populations onto the consensus tree to determine the minimum number of evolutionary transitions between recognition mechanisms. I then used a randomization routine to determine the expected
number of evolutionary transitions in the absence of phylogenetic signal. This routine incorporated the variance in the kin recognition observations (i.e. individual kin recognition trials) by resampling observations within each population with replacement, with one-tailed t-tests used to determine if there was evidence for each kin recognition mechanism (as in Hain et al. 2016). Phylogenetic signal was removed by randomizing the location of populations on the consensus tree, after which I calculated the number of evolutionary transitions needed to explain the simulated data. The randomization routine was repeated 10,000 times to produce a distribution of the expected number of evolutionary transitions in the absence of phylogenetic signal. The probability of obtaining the observed number of transitions in the absence of phylogenetic signal was then calculated as the proportion of the simulated data that were less than or equal to the observed value.

To estimate the timescale over which recognition mechanisms have changed among populations, I used a molecular clock based on the mitochondrial control region sequences. This analysis was performed in BEAST 1.7 (Drummond et al. 2012), with the mutation rate parameter set using a lognormal distribution based on nine previous estimates of mitochondrial control region mutation rate in fishes (Burridge et al. 2008). The time to most recent common ancestor was then estimated for each node in the population tree based on simulations with 10 million generations in which values were logged every 1000 generations. Median and 95% confidence intervals for the node ages are presented based on the distribution of the 10 000 logged values.

### 6.3 Results

The phylogenetic analysis showed that the guppies from the Oropouche drainage were the most genetically distinct, having diverged from the other populations 330 000 – 2 970 000 years ago (95% CI, Figure 6.1a). Guppies from the Northern drainage diverged next, with the Paria population diverging 110 000 – 1 010 000 years ago, followed by the U. Yarra population 80 000 – 760 000 years ago. Lastly, the three populations in the Caroni drainage (L. Guanapo, Tunapuna, U. Aripo) diverged from each other 30 000 – 350 000 years ago. Mapping the observed recognition mechanisms onto the consensus phylogenetic tree, the
most parsimonious explanation was that phenotype matching evolved once and was subsequently lost in one population, whereas familiarity evolved twice. The most rapid transitions in recognition mechanisms occurred in the Upper Aripo population, which both gained familiarity and lost phenotype matching within at most 350,000 years of diverging from the other Caroni drainage populations (Figure 6.1a). Overall, the four total transitions in recognition mechanisms that I observed did not differ from the number expected in the absence of a phylogenetic signal (p = 0.72; Figure 6.1b).
Figure 6.1 Analysis of evolutionary transitions between kin recognition mechanisms in six populations of guppies (*Poecilia reticulata*) from Trinidad. Panel (a) shows a phylogenetic tree based on the mitochondrial control region, as well as the kin recognition mechanism for each branch and node. The branch lengths of the tree are scaled based on a molecular clock, with the median age for each node indicated on the axis. Phenotype matching is represented with squares and familiarity with circles (present = filled, absent = open). Transitions between states are highlighted with a black outline. Panel (b) shows a histogram with the results of a simulation analysis that determined the expected number of transitions between kin recognition mechanisms assuming recognition mechanisms were independent of phylogeny. The observed number of transitions was not significantly different from the expected number in the absence of phylogenetic signal (p = 0.72).
6.4 Discussion

There is considerable interest in the evolution of recognition systems. Previously, the finest-scale that variation in recognition mechanisms had been observed was across species within a genus (Lizé et al. 2014), with associated divergence times conservatively estimated on the order of tens of millions of years (Gao et al. 2007). I now show that kin recognition mechanisms have repeatedly evolved across guppy populations, with associated divergence times on the order of hundreds of thousands of years. Thus, I have provided evidence for the fastest evolutionary divergence in recognition mechanisms to date. Interestingly, I found that recognition by phenotype matching had a single origin, whereas familiarity emerged in two separate lineages. Although based on a small number of populations, it is plausible that kin recognition by familiarity can evolve more rapidly than recognition by phenotype matching, as familiarity may be pre-adapted as an extension of existing social behaviours (Mateo 2004). Phenotype matching on the other hand requires the evolution of both a recognition template and the subsequent development of recognition behaviours (Holmes and Sherman 1982). The rate of evolution for familiarity versus phenotype matching deserves further investigation.

Behavioural traits have been shown to be more evolutionary labile than other biological traits (Blomberg et al. 2003), and there are numerous examples of rapid evolution of behavioural traits in response to changing selection pressures (e.g., Magurran et al. 1992; Singer et al. 1993). Therefore, it is not really surprising that the patterns of recognition mechanisms that I observed across populations did not show evidence of phylogenetic signal, suggesting that recognition mechanisms are also evolutionarily labile. However, the selection pressures that might be shaping variation in recognition mechanisms are not yet well-resolved. Recent studies comparing closely-related taxa suggest that diet and social behaviour (Lizé et al. 2014) or mating system (Hain et al. 2016) are important in determining recognition mechanisms. Ultimately, additional studies that examine recognition mechanisms within the context of phylogeny and ecology are needed to more fully understand the evolution of this important behavioural trait.
6.5 References


Chapter 7

General discussion

Altruistic behaviours that apparently have a cost to reproductive fitness are common in nature, but their very existence is puzzling because natural selection is expected to purge these behaviours from populations (Darwin 1859; Nowak et al. 2010). Kin selection theory has provided valuable insight into the benefits associated with these costly behaviours and has greatly enhanced our understanding of social behaviour (Hamilton 1964). Since the formalization of kin selection theory, researchers have been particularly interested in understanding the mechanisms that allow individuals to direct their helping behaviours towards kin (Hauber and Sherman 2001; Mateo 2004; Section 1.3). Several hypotheses have emerged to predict the ecological and life history conditions that favour the evolution of one recognition mechanism over another (Holmes and Sherman 1982; Hauber and Sherman 2001). However, there has been a shortage of data that test these kin recognition hypotheses empirically. In my thesis, I address this lack of data by examining the evolution of kin recognition in two fish species, bluegill and guppies. In doing so, my thesis makes four important contributions to our understanding of the environmental and historical factors that influence the evolution of kin recognition mechanisms.

Summary of findings

Benefits of recognizing kin

Kin selection theory was originally developed to explain behaviours with large fitness costs and benefits, such as non-reproductive castes in social insects (Hamilton 1964). However, preference for associating with kin has been observed in many animals where the benefits of associating with relatives are less clear (Ward and Hart 2003; Mateo 2004). In fishes, kin groups have been shown to have improved growth versus groups of mixed kinship (Brown and Brown 1996; Gerlach et al. 2007), and this improved growth may be because of reduced agonism between kin (Brown and Brown 1993). In Chapter 2, I show that kin groups show an enhanced innate response to a predator odour cue by shoaling more closely.
together than do mixed-kin groups. Cohesive shoals of bluegill are better at evading predators than dispersed shoals (Chipps et al. 2004), thus, my findings suggest that kin groups will have improved survivorship versus non-kin groups in bluegill. Interestingly, kin grouping behaviour in fishes may also explain the evolution of alarm cues emitted after a conspecific is depredated (Meuthen et al. 2012), but this possibility must be tested further. Regardless, my thesis has identified a new potential benefit of kin association behaviour in fish.

7.1.2 Self-referent phenotype matching

Although phenotype matching has been observed in many species (reviewed by Mateo 2004; Section 1.3), biologists have been challenged to show that species use self-referent phenotype matching. Previously, biologists have attempted to demonstrate self-referent phenotype matching by manipulating plumage colour, and thereby the phenotype of nestling cowbirds (Hauber et al. 2000), or by scrambling cues of kinship, either by mating peahens to multiple males and rearing eggs separately (Petrie et al. 1999), or by cross-fostering newborn hamsters (Mateo and Johnston 2000). However, alternative explanations for the results of these studies left some researchers unconvinced by the evidence for self-referent phenotype matching (reviewed by Hauber and Sherman 2001; Hare et al. 2003). To conclusively show self-referent phenotype matching, individuals must have no reliable cues of kinship at any point in their life, and must show discrimination between kin and non-kin even in the absence of locational cues (Hauber and Sherman 2003). In Chapter 3, I used bluegill to create broods of mixed relatedness from the moment of fertilization, and I showed that in spite of being raised in an environment with unreliable cues of relatedness, the offspring of cuckolder males could discriminate between the odours of full-siblings and unrelated individuals. Thus, my study is a strong demonstration of self-referent phenotype matching (Hauber and Safran 2006). Although self-referent phenotype matching is valuable for recognizing relatives, recognizing cues of one’s self is important in other contexts, such as recognizing previous mates (Ivy et al. 2005) or one’s own territory (Mykytowycz et al. 1976; Alberts 1992). Now that the research community appears to have
accepted the evidence for self-referent phenotype matching, biologists can explore new situations in which the mechanism has adaptive value.

### 7.1.3 Relatedness as a predictor of recognition mechanism

Many ecological and life history factors that might influence the evolution of recognition mechanisms have been identified theoretically (Holmes and Sherman 1982; Mateo 2004), but there have been few empirical tests of the theory. Instead, biologists often observe the recognition mechanism used by a species, then state if this mechanism is consistent with theory, or if it is not. The problem with this approach is that many ecological or life history factors may influence recognition mechanism, and authors may only identify the factor that best explains their results *post-hoc*. A better test would involve identifying the predictive variables in advance, and then compare species or populations that differ in these key ecological or life history factors. In my thesis, I tested the effect of brood relatedness on kin recognition mechanism in two fish species, bluegill (Chapter 3) and guppies (Chapter 5), in which individuals differ in their average level of brood relatedness depending on either their sire’s reproductive strategy or their population. The prevailing kin recognition hypothesis is that familiarity will be favoured as a recognition mechanism when being born together is a reliable indicator of kinship (*i.e.*, brood relatedness is high), and that phenotype matching will evolve when being born together does not reliably indicate kinship (*i.e.*, brood relatedness is low). In guppies, I also tested the effects of population-level predation regime and average brood size on recognition mechanism. Although I found no relationship between either predation regime or brood size on recognition mechanism, I did find that brood relatedness influenced recognition mechanism in both bluegill and guppies, albeit in different ways.

In bluegill, I found that average brood relatedness predicts recognition mechanism in a way consistent with the hypothesis. Specifically, I found that in comparison to the offspring of parental males, offspring of cuckold males had a low average level of relatedness to their broodmates, and these individuals used self-referent phenotype matching to recognize their relatives. In contrast, the offspring of parental males had a
higher average level of relatedness to their broodmates and did not use either familiarity or phenotype matching to recognize their relatives, suggesting that they may use an indirect means of associating with relatives. Specifically, in Chapter 2, I observed that the offspring of parental males associated closely with other parental-sired larvae regardless of whether they were in full-sibling groups or mixed-kinship groups. This suggests that parental-sired larvae have a cue identifying the life history of their sire. When combined with information about their nest of origin, perhaps through a passive process by remaining in a group with nestmates, this information could lead to a reliable mechanism for identifying relatives, and a novel example of context-based cues used as a kin recognition mechanism.

In guppies, I found that average brood relatedness was important in determining the intensity of kin recognition, but not the recognition mechanism. That is, I found that guppies had a stronger preference for both familiar and related individuals as shoaling partners when the population’s average brood relatedness was high versus when it was low, suggesting that kin recognition in general was used more often when brood relatedness was high. This result is not consistent with the kin recognition hypothesis, which predicts that phenotype matching would be used more often when average brood relatedness was low. Instead, the strength of preference for kin in guppies is best explained by Hamilton’s Rule, which predicts that the behaviour will evolve if the product of the relatedness coefficient of the individual being helped and the benefit of performing the behaviour is greater than the cost of performing the behaviour (Hamilton 1964). Thus, my data support the kin recognition hypothesis in bluegill but not in guppies.

Although brood relatedness had a relationship with recognition mechanism for both bluegill and guppies, the direction of effect was different between the two species. Specifically, I found that in bluegill, low brood relatedness values effected the evolution of self-referent phenotype matching, but in guppies, I saw the opposite result, with a greater preference for kin at high brood relatedness values. One possible explanation for this difference is that there may be an optimal relatedness value at which individuals are expected to discriminate kin from non-kin. At low relatedness values, the benefit of recognizing kin may be too low, and at high relatedness values, the cost of evolving a recognition mechanism may exceed the benefit. Consistent with this idea, Griffin and West
(2003) have shown that across species, kin-helping behaviour is strongest when the benefits are highest. Conversely, at high relatedness values, the likelihood of a group member being a genetic relative is high enough that the additional benefit of evolving a recognition mechanism may be too low to cover the developmental costs of the mechanism (Reeve 1989; Hauber and Sherman 2001). The optimal relatedness values favouring the evolution of recognition mechanism will differ across species depending on the relative costs and benefits of recognizing kin. By comparing the data on bluegill from Chapter 3 and on guppies from Chapters 4 and 5, we could infer that either the benefits of recognizing kin in guppies are higher than in bluegill, or the costs are lower.

Indeed, the difference between bluegill and guppies in their use of recognition mechanisms at high relatedness values (i.e., $R = 0.30 - 0.36$) could be explained by a difference in the cost of recognizing kin. Recognizing kin might have a cost for developing the mechanism (Hauber and Sherman 2001), or it could have a cost in searching for recipients (Reeve 1989). Given the small size of juvenile fish (Rettig and Mittelbach 2002; Auer et al. 2010), these costs could represent a large part of their energy budgets. This is especially true for larval bluegill, which have a body mass of approximately 1.5 mg (Rettig and Mittelbach 2002), which is more than one order of magnitude smaller than a juvenile guppy (Auer et al. 2010). For parental-sired bluegill larvae, the energy expended to actively seek relatives may be too large to have positive net benefits, but for juvenile guppies, this energetic cost may be a relatively small percentage of their energy budget. Thus, for guppies, there is a positive net benefit to actively seek broodmates with an average relatedness of 0.36, but not for larval bluegill.

### 7.1.4 Evolutionary history of recognition mechanisms

Phylogenetic comparisons allow biologists to make insightful conclusions about how character traits have evolved (Harvey and Pagel 1991). Although my literature review in Chapter 1 revealed that specific mechanisms are more common in some taxa than in others, phylogeny is rarely considered when explaining the observed patterns in recognition mechanisms. In Chapter 6, I showed that like many behavioural traits (e.g., Magurran et
al. 1992; Singer et al. 1993), kin recognition mechanism can evolve quickly, and is apparently not constrained by phylogenetic history. Thus, when closely-related species exhibit identical kin recognition mechanisms, this commonality is more likely explained by similar selection pressures and not by phylogenetic history.

7.2 Future directions

My thesis has advanced our knowledge of how kin recognition mechanisms have evolved by testing the kin recognition hypothesis in two fishes, and by using phylogenetic analysis to show that recognition mechanism is not constrained by evolutionary history. I recommend that biologists perform further phylogenetic studies to test the generality of my results, with the understanding that species can display different recognition mechanisms when tested in different contexts (Holmes and Sherman 1982; Chapters 1 and 5). In addition, I recommend further tests of the kin recognition hypothesis, as I found partial support for the hypothesis in bluegill and not in guppies. Although the hypothesis has been described in multiple theoretical papers (Holmes and Sherman 1982; Hauber and Sherman 2001; Mateo 2004), the predictive value of the hypothesis will only be known once more experimental work is done.

By examining the differences between familiarity and phenotype matching, I have also addressed some of the mechanistic questions of how kin recognition operates. However, future work in kin recognition should further investigate the cues of recognition. The broad sensory cues of recognition have already been studied in some taxa. In social insects in particular, the importance of hydrocarbon signatures on the body cuticle has been the topic of study for many years (reviewed by Tsutsui 2013). However, a similar level of understanding has yet to be developed in non-insects. For example, in birds, odour (Coffin et al. 2011; Bonadonna and Sanz-Aguilar 2012), and vocal cues (Hatchwell et al. 2001, Akçay et al. 2013) are often identified as being important for kin recognition. Although Sharp et al. (2005) identified that the “churr” element of the long-tailed tit call was important in kin recognition, in general, the particular elements in the vocalizations or volatile elements of the odour cues that are important for kin recognition are largely
unknown. Similarly, in most fish and mammals, experimental studies tend to focus on species’ ability to recognize kin, rather than the specific cues used in recognition. Some studies have begun to investigate the cues used for kin recognition by fish and mammals, and these studies have revealed the importance of the MHC (reviewed by Brown and Eklund 1994), and an individual’s phenotype at the MHC seems to be assessed by others based on peptide ligands associated with the MHC binding site (Milinski et al. 2005). Although studies have made progress towards identifying the cues important in recognition, we are still far from having a comprehensive understanding of the mechanisms of kin recognition. Indeed, there are potentially important applications to human health that can be developed once we have a stronger understanding of these recognition cues.

Ecoimmunology is an emerging field that studies how ecological factors influence the evolution of animals’ immune systems and how diseases are recognized (Schulenberg et al. 2009; Martin et al. 2011). The immune system is not infallible, and indeed, can cause autoimmune disease when an individual’s own tissue is treated as foreign (Witebsky et al. 1957), which has been shown to happen more often in mice with diverse (i.e., heterozygous) genotypes (Doherty and Zinkernagel 1975). In this way, autoimmune diseases are analogous to a kin recognition error in which kin are incorrectly treated as unrelated because the kin template is too restrictive to account for genetically-diverse relatives. In humans, autoimmune diseases are common in Europe and North America, affecting approximately 8% of the population (Cooper et al. 2009). One such autoimmune disease is ulcerative colitis, which affects the large intestine and causes bloody diarrhea and abdominal discomfort (Danese and Fiocchi 2011). Several autoimmune diseases, including ulcerative colitis, have been shown to have a latitudinal gradient, with higher incidences at high latitudes (Shapira et al. 2010), possibly due to geographic variation in gut microbial diversity. Indeed, a normal, healthy gut at low latitudes has a greater frequency of certain microbes, leading to a more diverse microbial community composition, than a healthy gut at high latitudes (Escobar et al. 2014). Because of this low diversity, the gut at high latitudes may have evolved a more stringent, or restrictive, immune system than the gut at low latitudes so that it can eliminate foreign microbes. Such a restrictive pathogen-recognition system is analogous to a stringent kin recognition
system, in that both lead to recognition errors where kin (or self) are incorrectly identified as non-kin (or as a pathogen; Reeve 1989). Kin recognition and the immune system have mechanistic similarities as well, as the MHC is involved in both recognizing relatives in some species (Brown and Eklund 1994) and in mediating the immune response (Horton et al. 2004). I believe that the similarities between kin recognition and the immune system are too strong to ignore. Thus, ecoimmunologists may benefit from a cross-population comparison approach, similar to the approach I used in my thesis, to correlate ecological factors such as microbiota community diversity with the strength of the immune system. In this way, biologists will gain insight into the evolution of immunity, particularly in the evolution of recognition errors across populations.

7.3 Conclusions

Overall, my thesis has provided a broad look at the evolution of kin recognition mechanisms, making contributions to the field by identifying a novel benefit of recognizing relatives, illustrating variation in recognition mechanism among populations and among species, identifying ecological factors that influence recognition mechanism, and showing that phylogeny does not constrain the evolution of recognition mechanism. In doing so, I have assisted in moving the field from the observation of recognition mechanism to asking questions about the ultimate causes that influence kin recognition. The future’s so bright, I gotta wear shades (Timbuk3 1986)

7.4 References


Appendices

Appendix A Fertilization and two-choice trial protocol for the mixed brood experiment reported in Chapter 3. P1 refers to a parental male, C1 refers to a cuckolder male, and F1 and F2 refer to two females. In the leftmost jar, eggs from F1 were fertilized using sperm from P1. In the rightmost jar, eggs from F2 were fertilized using sperm from C1. In the centre jar, eggs from F1 were fertilized with sperm from the P1 on one side of a barrier, and eggs from F2 were fertilized with sperm from the C1 on the other side of the barrier. Five minutes later, the barrier was removed and the eggs were mixed by gently swirling the jar. Larvae from the center jar were used as focal larvae and water from the leftmost and rightmost jars provided ‘pure’ referent odours.
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Pairwise relatedness values for 54 guppies from a tributary of the Paria River in Trinidad, as referenced in Chapter 4. Individuals with the same letter suffix were shoalmates.
Appendix C The number of individuals in 11 guppy shoals from a tributary of the Paria River in Trinidad with their within-shoal (along diagonal, in bold) and between-shoal mean relatedness as referenced in Chapter 4. The outside shoal relatedness, calculated as the average pairwise relatedness of individuals within a shoal to all other individuals in the population, is presented in italics.

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Appendix D Methodology for evaluating association preferences in juvenile guppies as referenced in Chapter 5. In step A, a single virgin female and male are placed into each tank and allowed to mate for 7 days, after which the male was removed. In step B, the female was removed within 24 hours of giving birth (a brood of 6 newborn guppies is shown) and newborns were isolated to prevent familiarity from developing within a brood. Broods of mixed relatedness were then created by applying family-specific tail clippings to each individual and cross-fostering individuals between broods (shown in step C). Familiarity was allowed to develop in these mixed broods for 12-15 days. After the familiarization period, behavioural trials were conducted to test the kin recognition mechanisms of juvenile guppies in step D. A focal fish was placed in a centre compartment, comprised of a central “neutral zone” and two peripheral “association zones”, which were adjacent to compartments that housed stimulus fish. To test for the use of familiarity by the focal fish, stimulus fish differed in familiarity but not relatedness (as shown in the figure). To test for the use of phenotype matching by the focal fish, stimulus fish differed in relatedness but not familiarity. Preferences were determined by the relative amount of time focal fish spent in the association zone of either stimulus fish.
Appendix E Distributions of slopes of regression lines generated by the simulation analysis across six populations for the relationship of brood relatedness and association time with putative kin using A) phenotype matching; B) familiarity; C) the combined analysis for all putative kin as referenced in Chapter 5. The observed slope is denoted with a dotted line. The observed slope was greater than expected based on the null distribution for phenotype matching ($p = 0.025$) and the combined mechanisms ($p < 0.001$).
Appendix F  Map of northern Trinidad with the location of collection sites indicated for six populations of guppies (*Poecilia reticulata*). TN = Tunapuna; UY = Upper Yarra; PA = Paria; LG = Lower Guanapo; UA = Upper Aripo; LO = Lower Oropouche, as referenced in Chapter 6. The Tunapuna, Lower Guanapo and Upper Aripo populations are part of the Caroni Drainage, the Upper Yarra and Paria populations are part of the Northern Drainages, and the Lower Oropouche population is part of the Oropouche Drainage.
**Appendix G** Source of mitochondrial control region sequences used in determining phylogenetic relationships of six guppy (*Poecilia reticulata*) populations from Trinidad.

<table>
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<th>Population</th>
<th>N</th>
<th>Source</th>
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<td>Lower Oropouche</td>
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<td>Upper Yarra</td>
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<td>Paria</td>
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Appendix H Tree indicating the relationships among control region sequences from six populations of guppies (*Poecilia reticulata*) as referenced in Chapter 6. The scale represents the number of nucleotide substitutions per site.
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Appendix J Ethics statement

All experiments followed ethical guidelines from the Canadian Council on Animal Care as reviewed and approved by the Animal Use Subcommittee at the University of Western Ontario. I have attached an example of the Animal Use Approval form on the following page. Later procedures were approved under Animal care protocol #2010-214.
May 30, 2006

"This is the Original Approval for this protocol"
"A Full Protocol submission will be required in 2010"

Dear Dr. Neff,

Your Animal Use Protocol form entitled:
Behavioural and Molecular Ecology of Fishes
Funding Agency: NSERC - Grant #R3244A04

has been approved by the University Council on Animal Care. This approval is valid from May 30, 2006 to May 31, 2007. The protocol number for this project is 2006-062-05.

1. This number must be indicated when ordering animals for this project.
2. Animals for other projects may not be ordered under this number.
3. If no number appears please contact this office when grant approval is received.
4. If the application for funding is not successful and you wish to proceed with the project, request that an internal scientific peer review be performed by the Animal Use Subcommittee office.

ANIMALS APPROVED FOR 1 YR.

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<td>Fish</td>
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<td>1-6 yrs. M/F</td>
<td>C</td>
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STANDARD OPERATING PROCEDURES

Procedures in this protocol should be carried out according to the following SOPs. Please contact the Animal Use Subcommittee office (681-2111 ext. 69770) in case of difficulties or if you require copies.

SOPs are also available at http://www.uwo.ca/animal/ACVS

310 Holding Period Post-Admission

REQUIREMENTS/COMMENTS

Please ensure that individual(s) performing procedures on live animals, as described in this protocol, are familiar with the contents of this document.

1. Please ensure that the number of fish utilized/observed in this protocol are reported to the AUS office (aus@uwo.ca) prior to December 31, 2006.

c.c. Approved Protocol: B. Neff, J. Wasylecko, D. Cheshuk
   Approval Letter: J. Wasylecko, D. Cheshuk

University Council on Animal Care • The University of Western Ontario
Animal Use Subcommittee • Health Sciences Centre • London, Ontario • N6A 5C1 • Canada
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Albert O. Jeffrey Scholarship
2003
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Publications:


