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Ecological factors associated with speciation in New Zealand triplefin fishes (Family Tripterygiidae)

by

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Abstract

Theoretical research has demonstrated that ecological interactions in sympatry or parapatry can generate disruptive selection that in concert with assortative mating can lead to speciation. However, empirical examples are few and restricted to terrestrial and lacustrine systems. New Zealand triplefin fishes (Family Tripterygiidae) are an ideal model system to study speciation in the sea, as they conform to the criteria of an adaptive radiation, being philopatric, speciose and abundant, and having largely sympatric distributions. This thesis investigates two key aspects of the New Zealand triplefin radiation: 1) which ecological traits are under selection?; and 2) which traits are potentially available for the development of assortative mating?

Habitat use was identified as a possible key trait for selection and investigated in detail in this thesis. Habitat use of the majority of New Zealand triplefin species was censused quantitatively throughout most of their latitudinal range and analysed using novel statistical methods. Analyses showed that habitat use was highly divergent between species and thus diversification in habitat may have been a major component in the evolution of this clade. The phylogenetic analysis of habitat characters confirmed that there has been rapid evolution in habitat use among species. Habitat selection at settlement was highly species-specific, indicating that interspecific differences in adult habitat use may be the outcome of active habitat choice established at settlement. These species-specific habitat associations showed no evidence for geographic variation in habitat use. Laboratory trials and field observations of the sister-species pair Ruanoho decemdigitatus and R. whero showed that competition was linked with body size, with R. decemdigitatus being the larger and consequently dominant species. The second part of this thesis investigated which traits may have contributed to prezygotic isolation, and thus to assortative mating. Little evidence was found for divergence in breeding season or male colour patterns. However, divergence in habitat affected breeding habitat choice, as triplefins court and mate in the same territory as that occupied year round. This suggests that assortative mating in New Zealand triplefin species could be the by-product of adaptation to habitat resources. Body size affected mate choice and time at first maturity in the Ruanoho sister-species pair, suggesting that size is important in the maintenance of reproductive isolation in these species. Differences in body size may have also lead to assortative mating in other New Zealand triplefin sister-species pairs, as all sister-species pairs differ in maximum body size. The findings of this thesis invoke a strong role for ecologically-based selection in speciation, and support the hypothesis that adaptation to habitat has been a major factor in speciation in this system.

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1 General Introduction

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1.1 THEORETICAL BACKGROUND

Recent years have seen a renaissance in studies of speciation. For a long time, allopatric speciation (i.e. speciation involving the geographic isolation of populations) has been considered the only plausible mode of speciation. This view has changed only recently, due largely to empirical (reviewed in: Schluter 2000b; Coyne and Orr 2004) and theoretical (reviewed in: Dieckmann et al. 2004a; Gavrilets 2004) evidence on the role of natural selection in the speciation process. This recent work suggests that selection can play an important role in both character divergence and reproductive isolation (Funk et al. 2006; Jiggins 2006). The following section provides an introduction to the spatial patterns of speciation (i.e. allopatry, sympatry and parapatry) and the processes (i.e. sexual, disruptive and divergent selection) that can act on populations in these different biogeographic settings.

Ever since Darwin (1859), evolutionary biologists such as Fisher (1930) and Muller (1942) suggested that natural selection plays an important role in the origin of new species. However, in the mid 20th century the focus shifted away from natural selection as the driving force of speciation towards the role of geography in limiting gene flow. From this time allopatric speciation, initially proposed by Dobzhansky (1937) and Mayr (1942), has been the dominant model used to explain the origin of species. In the allopatric speciation model, divergence is caused by a geographic barrier that divides a species' range into two or more isolated populations, so that gene flow is eliminated and (given enough time) genetic differences accumulate. In these geographically isolated populations, reproductive isolation typically evolves through the gradual accumulation of non-adaptive genetic differences (neutral genetic drift) (Via 2001). When the vicariant barrier collapses, previously isolated populations may be sufficiently differentiated to co-occur without interbreeding, or alternatively, reproductive isolation may be completed by reinforcement (Kirkpatrick 2000; Schluter 2000b). Reinforcement is an increase in pre-mating isolation between two divergent groups, resulting from selection against hybrid offspring because they are less viable or fertile than their parents (Treganza and Butlin 1999). If populations have not diverged enough to prevent interbreeding, then any population differences will be diminished by recombination, leading to introgression. The primary cause of speciation in the allopatric model is thus long-term geographic isolation. Even though allopatric speciation is an intuitively appealing idea, the mechanisms that lead to reproductive isolation are poorly understood, both theoretically and empirically (Dieckmann et al. 2004a). Furthermore, allopatric speciation is generally a slow

process (for an exception see Near and Bernard 2004) because it typically involves neither inherent selection for differentiation nor selection for reproductive isolating mechanisms.

In contrast to the allopatric model, species divergence in sympatric and parapatric speciation occurs in the presence of gene flow (Rosenzweig 1978), and typically involves divergent and/or disruptive natural selection. Specifically, divergence in the sympatric model occurs in the complete absence of any geographic barriers and leads either directly or indirectly to reproductive isolation (Orr and Smith 1998). One direct mechanism that can cause species to diverge in sympatry is when assortative mating evolves as a pleiotropic by-product of adaptation to resources (e.g. host choice in phytophagous insects). Such examples correspond to the 1-allele models that were originally developed by Felsenstein (1981). Assortative mating can also be indirectly favoured if, for example, a tight genetic association between mate preference and preference traits exists, thereby leading to the coordinated evolution of mate recognition (Kronforst et al. 2006). Speciation in sympatry also includes cases in which divergence occurs due to sexual selection (but see Van Doorn et al. 2004 for a critical reevaluation) or chance events such as polyploidization and hybridisation (reviewed in: Coyne and Orr 2004). Although speciation in sympatry has long remained a controversial idea, empirical and theoretical evidence that provides support for the concept has been growing (reviewed in: Howard and Berlocher 1998; Schluter 2000b; Dieckmann et al. 2004a).

In the parapatric model of speciation, divergence occurs between populations with partially overlapping distributions which consequently share moderate gene flow (Meszena et al. 1997; Gavrilets 2000; Gavrilets et al. 2000). This speciation mode thus represents a hybrid of the allopatric and the sympatric speciation models. Population differentiation occurs due to a combination of contrasting selection pressures in alternative environments and reduced gene flow among neighbouring populations (Gavrilets et al. 2000; Gavrilets 2004). In this scenario, natural selection in alternative environments at adjacent locations overrides gene flow sufficiently enough to set population size, large geographic range and low migration rates are usually positively correlated with the likelihood of parapatric speciation (Gavrilets et al. 2000; Gavrilets 2004). The difficulties with speciation in the face of gene flow (sympatry and parapatry) are well known: without a limitation to gene flow recombination breaks down associations between loci under selection and the loci that cause prezygotic isolation; thus impeding the accumulation of species differences (Felsenstein 1981; Maynard-Smith 1998).

It should be noted that the sympatric and allopatric geographic speciation modes represent extremes in a spatial continuum (from no geographic isolation to complete geographic isolation), while the parapatric mode describes an intermediate position between the two. It can be argued that speciation under fully allopatric or sympatric conditions are limiting cases which are probably encountered rarely in nature (Dieckmann et al. 2004a). Although most speciation processes may thus be parapatric (at least initially), they can differ greatly in the level of possible gene flow and ecological contact between the incipient species (Dieckmann et al. 2004a). When studying speciation, it is thus important to understand that the extent of geographic isolation between populations can be positioned at any one point along this continuum, and that this position can change dynamically over time.

The speciation mechanisms that can operate under the three biogeographic scenarios differ fundamentally in the extent to which ecological interactions or neutral processes, such as genetic drift, are involved. In allopatric populations, genetic drift is the main mechanism of divergence, while environment-species interactions are the main mechanisms that create divergent and disruptive selection in sympatric and parapatric populations (Treganza and Butlin 1999; Via 2001). Two concepts have been developed to explain how divergence is caused by species-environment interactions. The first concept has been termed 'ecological speciation', and is mainly driven by adaptation of species to different environments (Schluter 1996b; Schluter 1998; Schluter 2000b; Schluter 2001; Rundle and Nosil 2005). The second concept has been termed 'adaptive speciation', and is based on frequency-dependent selection caused by interspecific interactions as a response to resource use (Dieckmann et al. 2004a). The concept of ecological speciation encompasses various speciation scenarios in which divergent natural selection between environments or resources is ultimately responsible for the evolution of reproductive isolation (Rundle and Schluter 2004). Ecological speciation does not necessarily require gene flow, as even geographically separated species can diverge in phenotype if they inhabit different environments, though contact and thus some level of gene flow typically enhances the possibility of speciation (Schluter 2000b). An additional, though somewhat rare, scenario that falls under the term of ecological speciation includes sexual selection of mating traits that are under divergent selection between contrasting environments (e.g. sensory drive hypothesis Boughman 2002; Fuller et al. 2005).

Adaptive speciation is a subset of ecological speciation, since it only includes modes in which frequency-dependent ecological interactions lead to reproductive isolation (Dieckmann et al. 2004b). For adaptive speciation to take place in sexual populations, two processes must occur. First, frequency-dependent interactions must generate disruptive selection, and second, a

lineage split in sexual populations requires the evolution of assortative mating mechanisms, as evolutionary branching can only occur if assortative mating can latch on to the trait under disruptive selection (Dieckmann et al. 2004b; Dieckmann et al. 2005). In principle, this can happen in a number of different ways, either through direct selection for assortative mating or because assortativeness is linked to the diverging trait (see above). Adaptive speciation requires sympatry or parapatry and becomes increasingly unlikely when gene flow diminishes (i.e. allopatry), since frequency-dependent ecological contact must occur between the individuals involved. Theoretically, adaptive speciation could also occur in allopatry if a mobile second species ecologically interacts with two separated populations, though the likelihood of this occurring in nature is thought to be restricted (Dieckmann et al. 2004b). Although adaptive and ecological speciation have a large amount of overlap, there are two key points about the ways in which these concepts differ. First, adaptive speciation differs from ecological speciation in that it includes cases in which disruptive selection and subsequent divergence results entirely from sexual selection. Second, in adaptive speciation disruptive frequency-dependent selection is the crucial feature that leads to the splitting of a population. In the book 'Adaptive Speciation', Dieckmann et al. (2004b) state that the main differentiation between the two concepts is that in 'ecological speciation there is no explicit role of frequency-dependence in creating the disruptive selection', though it should be noted that there are many cases of ecological speciation in which frequency-dependent selection is involved at least at some stage.

Some of the adaptive speciation concepts have been criticised in the last years by population geneticists because of the parameters and conditions used in models (Waxman and Gavrilets 2005a). In particular, Waxman and Gavrilets (2005a) criticised Dieckmann and Doebeli (1999) because they did not include costs of assortativeness and used unrealistically high initial mutation rates and allele frequencies. More recent simulations (Doebeli 2005; Doebeli and Dieckmann 2005; Waxman and Gavrilets 2005b) which included a combination of different parameter settings showed that a lowering of the mutation rate, a decrease in the strength of assortativeness or an increase in the number of loci greatly increases the waiting time to speciation, and in some cases these costs can significantly delay or even prevent speciation. Interestingly, both sides in the debate agreed that sympatric speciation is most plausible when the following conditions are present: disruptive selection is strong; both viability and mating preferences are controlled by the same set of loci so that recombination does not come into effect; initial population variation and/or rates of mutation are very high; and there is no selection for mating success so that there are no costs for choosiness.

Speciation becomes even more feasible where gene flow between populations follows an isolation-by-distance model (i.e. parapatry), and when species inhabit spatially heterogeneous habitats. Having established that adaptive speciation is theoretically possible, the next challenge is to marry these findings with ecological data and generate testable predictions of the evolutionary pathway of speciation.

The development of theoretical models to explain sympatric or parapatric speciation in the past few years is correlated with a resurgence in the interest of speciation in the absence of biogeographic barriers, with the most convincing examples being speciation in the face of gene flow in bacteria (Friesen et al. 2004), plants (Savolainen et al. 2006), phytophagous insects (Bush 1969; Berlocher 1998; Feder 1998; Berlocher and Feder 2002) and lacustrine fishes (Schliewen et al. 1994; Wilson et al. 2000; Schliewen et al. 2001; Carlon and Budd 2002; Barluenga and Meyer 2004; Barluenga et al. 2006). This evidence has strengthened the view that speciation in the absence of biogeographic barriers is possible, although to date only a few convincing examples of non-allopatric speciation have been published for marine species (Syms 1995; Rolán-Alvarez et al. 2005). Thus, unlike the empirical support that has accumulated for the role of sympatric speciation in the divergence of terrestrial or lacustrine species, few studies have investigated whether the same processes play a role in the divergence of marine species.

Coyne and Orr (2004) proposed the following criteria that need to be met to corroborate that a species has evolved under sympatry: 1) a sympatric distribution of the most closely related sister-species; 2) genetic evidence for reproductive isolation; 3) lineage monophyly; and 4) an ecological setting in which historical allopatric differentiation in a biogeographical context is very unlikely. Recent work suggests that the first criterion, 'the sympatric distribution of the most closely related sister-species', may not always be the case (Tautz 2003; Dieckmann et al. 2004a). For example, Doebeli and Dieckmann (2003) have shown that when ecological interactions actively drive diversification, then species that diverge in sympatry may subsequently become allopatric (i.e. to reduce competition). In light of this, the sympatric distribution of sister-species may not always be a reliable indicator of historical sympatry (or vice versa). The fourth criterion is also problematic as it states that there must be an ecological setting in which allopatric differentiation is very unlikely e.g. remote oceanic islands, hosts for parasites or small crater lakes (Coyne and Orr 2004). These are all very geographically constrained and homogenous habitats and do not apply to most species, and in particular the vast majority of marine species. Why should sympatric speciation be any less

likely in heterogeneous systems? Recent theoretical research has suggested that it is not (for details see Doebeli and Dieckmann 2004 and references therein). Recent theoretical models of sympatric speciation have shown that speciation may crucially depend on spatial structure (Doebeli and Dieckmann 2004). In a single and homogenous population, stochastic fluctuations will only be sufficiently large when the population size is small. Yet, with spatial structure, fluctuations can be considerable, even in a large population. Therefore, a small spatial component may greatly enlarge the potential for speciation, as local adaptation along an environmental gradient has the potential to increase the strength of frequency-dependent selection (Doebeli and Dieckmann 2003; Doebeli and Dieckmann 2004). Furthermore, inferring the historical patterns of speciation events (sensu Barraclough and Vogler 2000) is problematic because geographic distributions change over time (Losos and Glor 2003). Thus, it becomes clear that the current distribution of a species is not necessarily a reliable guide to its historical geographical range, therefore, it is crucial to acknowledge that geographical ranges shift and that geographical signal decays over time.

Another general criticism of the Coyne and Orr (2004) criteria is that there is an underlying burden of proof that requires sympatric models to exclude historical geographic barriers to gene flow. Conversely, no studies of allopatric speciation to date have excluded the possibility of historical sympatric distributions (Berlocher 1998). Researchers seem to be expected to treat allopatric speciation as the null model, even though several empirical studies have convincingly demonstrated sympatric speciation (see above). Dieckmann et al. (2004a) proposed that the time has come to do away with the notion of allopatric speciation as the null model, a notion that prevails partly because of the deceptive simplicity of allopatric scenarios. Once the bias towards detecting allopatric speciation in empirical data is removed, the data may actually suggest speciation events. This dichotomy can partly explain why evidence for sympatric speciation in heterogeneous environments is sparse. Given that most species inhabit heterogeneous environments, case studies of speciation events on small islands or lakes tell us little about the processes that act on species in spatially variable environments (Jiggins 2006).

Ruling out that ancestral species may have once been allopatric is particularly problematic in marine fish species, as it is usually impossible to exclude the possibility that sympatric species were historically allopatric (Sponer and Roy 2002). Furthermore, speciation in the face of gene flow has been largely discounted in marine fishes because most species have a planktonic larval phase with the potential to disperse widely and generate genetic homogeneity over large spatial scales (Knowlton 1993; Palumbi 1994). However, there is

increasing evidence that larvae do not always disperse long distances and some return to their natal reefs (Jones et al. 1999; Swearer et al. 1999; Swearer et al. 2002). Furthermore, habitat selection at settlement (reviewed in: Montgomery et al. 2001) and assortative mating (e.g. McMillan et al. 1999) can produce reproductive isolation at very fine spatial scales. This evidence suggests that a pelagic larval phase need not preclude the formation of fine-scale genetic structure because behaviour can override the potential for genetic mixing (Taylor and Hellberg 2003).

Recent phylogenetic and phylogeographic studies suggest that the New Zealand triplefin fauna (Family: Tripterygiidae) may provide an excellent model system to study the mechanisms of speciation in the sea. Triplefins are small, blennioid fishes that reach their greatest level of endemism and disparity in New Zealand, where the fauna comprises nearly one sixth of the world's known species (Fricke 1994; Fricke 1997). Importantly, most New Zealand triplefin species have broad, sympatric distributions throughout the region (Fricke 1994). This lack of major barriers to gene flow around New Zealand probably also reflects the situation in the past. The relationship of oceanic fronts and currents was no more structured during Pliocene and Pleistocene glacial cycles than it is today (Beu 1990), and stronger currents during glacial maxima may have even enhanced larval dispersal and gene flow (Nelson et al. 2000). The sympatric distribution suggests that non-allopatric mechanisms may have been involved in their evolution. Molecular phylogenetic analysis on these fishes show that almost all of the New Zealand species belong to a single group that shares a common ancestor and is not closely related to triplefins from elsewhere (i.e. a clade), indicating that the New Zealand fauna represents a local evolutionary radiation (Clements et al. unpublished). These characteristics suggest that the New Zealand triplefin radiation provides an opportunity to test mechanisms of sympatric speciation in the marine environment. This thesis examines ecological factors associated with the evolution of this fish-group. The main goal throughout this thesis is to identify likely traits that have been under selection. The following section summarises what is known about New Zealand triplefins to date, and identifies potential characteristics that may have contributed to the diversity seen in this unique marine fish group.

1.2 TRIPLEFIN FISHES: AN INTRODUCTION

Worldwide there are at least 150 triplefin species in approximately 30 genera (Fricke 1997). New Zealand has with one sixth of the world's known species, the highest centre for endemism of triplefin species in the world (Fricke 1994), however, the mechanisms that have led to this high diversity remain unclear. New Zealand triplefin fishes are a useful model system to study speciation in the marine environment because they appear to conform to the criteria of an adaptive radiation (sensu Schluter 2000b). Broadly defined, an adaptive radiation is the evolution of ecological diversity within a rapidly multiplying lineage (Schluter 2000b). Radiations are characterised by a disproportionately high number of closely related species, which have evolved rapidly, are endemic to a geographically limited area, and are often still in the process of diverging from one another (Echelle and Kornfield 1984; Schluter 2000b). With approximately 25,000 extant species, fish are the most species rich group of vertebrates (Nelson 1994), and several fish radiations have been thoroughly investigated (Echelle and Kornfield 1984; Schluter 1993; Schluter 1996a; Eastman and McCune 2000; Schluter 2000b; Near et al. 2004; Carreras-Carbonell et al. 2005; Genner and Turner 2005). Classic examples of adaptive radiation in fish include the Antarctic notothenioid fishes (Eastman and McCune 2000; Near et al. 2004) and East African lake cichlids (Kosswig 1947; Fryer and Iles 1972; Greenwood 1974; McKaye 1991; Turner 1994; Streelmann and Danley 2003; Genner and Turner 2005; Seehausen 2006). A common feature of these fish radiations is the relationship of secondary divergence in morphological traits to trophic resource specialisation. This is best illustrated by East African cichlids of Lakes Malawi, Victoria and Tanganyika, which have evolved numerous trophic adaptations for food acquisition (Kocher 2004; Seehausen 2006). Each of these lakes is characterised by species flocks composed of hundreds of endemic species that have diverged extensively in trophic morphology (Kornfield and Smith 2000; Genner and Turner 2005). Divergence in trophic morphology facilitated by the functional decoupling of the oral and pharyngeal jaws is thought to have led to a fundamental shift in jaw function from food transport to food manipulation and preparation (Wilson et al. 2000). This has allowed an extensive partitioning of trophic niches and produced herbivorous, planktivorous, omnivorous, detritivorous and carnivorous species (Danley and Kocher 2001).

Schluter (2000b) lists four criteria of an adaptive radiation, namely common ancestry (monophyly), rapid speciation, phenotype-environment correlation, and trait utility. Recent phylogenetic work has identified 26 triplefin species in New Zealand (Hickey and Clements 2005), and apart from the species *Enneapterygius kermadecensis* and *Apopterygion oculus* and the genus *Notoclinus*, the remaining 22 species appear to form a monophyletic group that shares a common ancestor and has speciated in the last 27 - 30 million years (Clements et al. unpublished). All species are distinct biological species, although there is genetic evidence

that Grahamina capito and Forsterygion varium hybridise (Hickey 2004; Hannan 2005). The monophyly of most of the New Zealand triplefin fauna, and the apparently rapid speciation of this group (as indicated by the relatively short branch lengths between clades, in particular in the genus *Forsterygion*), together provide evidence of an adaptive radiation. Further evidence for an adaptive radiation in New Zealand triplefins comes from studies that have shown phenotype-environment correlations resulting from ecological (Anderson 1973; Handford 1979; Thompson 1979; Syms 1992; Syms 1995; Feary 2001; Clements 2003) and physiological (Brix et al. 1999; Hickey and Clements 2003) diversification. Ecological work has demonstrated that triplefins have diversified considerably in habitat use (Anderson 1973; Handford 1979; Thompson 1979; Syms 1992; Syms 1995; Fisher 1998; Feary 2001; Feary and Clements 2006). For example, New Zealand has the only estuarine, planktivorous and deep-water triplefins in the world (Clements 2003). Physiological work has shown that triplefin species differ in the types of isohaemoglobins expressed, and that these interspecific differences were related to functional trade-offs and thus represent evidence for trait utility (Brix et al. 1999). Furthermore, triplefin species in shallow and thermally unstable habitats possess a greater number of cathodally migrating isohaemoglobins, have higher oxygen affinity and are less sensitive to changes in pH than species in more thermally stable and deeper habitats (Brix et al. 1999). The authors suggested that these interspecific differences may be linked with species-specific habitat preferences and are therefore adaptive. In this sense, species differences in isohaemoglobins may partly explain why triplefin species differ in the depth of their habitat. Further evidence for trait utility comes from a study which found a positive relationship between metabolic potential and the effective water velocity at respective habitat depths (Hickey and Clements 2003). Given that the New Zealand triplefin fauna exhibits monophyly, apparent rapid speciation, phenotype-environment correlations and trait utility, the group therefore appears to have undergone an adaptive radiation sensu Schluter (2000b).

New Zealand's temperate reefs occur as far north as the Three Kings Islands (34°S latitude) and as far south as Campbell Island (52°S latitude), and therefore contain a diverse range of habitats. In between this area lies the North and South Island, to the south Stewart Island and to the east the Chatham Islands at 44°S latitude. Although much of New Zealand lies within the broadly defined temperate zone, the biotic characteristics of coastal habitats range from warm-temperate through to cool-temperate, reflecting the relatively long chain that the islands form in the South Pacific Ocean (Laing and Chiswell 2003). New Zealand waters are characterised by two types of oceanic waters: warm and higher salinity water in the north that

comes from the sub-tropics, and cold and lower salinity water in the south-west that comes from the Antarctic Circumpolar Current (Laing and Chiswell 2003). These currents interact with the topography and cause temperate and salinity to vary along New Zealand's coastline, imposing a gradient in environmental conditions (for details see Heath 1985). This gradient affects the distribution of many reef fish species, and indeed most species of coastal New Zealand fishes are either distinctly northern or distinctly southern, with few species equally abundant throughout New Zealand (Francis 1996; Francis and Nelson 2003). Although New Zealand covers a wide latitudinal extent 23 of the 26 New Zealand triplefin species are sympatric throughout coastal New Zealand, and no species are known to display latitudinal trends in abundance (Clements 2003). The three species for which the distribution is not sympatric with the rest of the New Zealand triplefin assemblage are Enneapterygius kermadecensis, Apopterygion oculus and Matanui bathytaton. Enneapterygius kermadecensis is endemic to the subtropical Kermadec Islands and is not related to the remaining New Zealand triplefin species (Fricke 1994; Fricke 2002). Apopterygion oculus is restricted to the southern half of the North Island and south-east of the South Island (Fricke 1994), but again this species is not part of the New Zealand clade (Clements, unpublished data). The congener of A. oculus is A. alta, which is endemic to southern Australia (Fricke 1994). Lastly, M. bathytaton has not been recorded north of the subtropical convergence but is fully sympatric throughout its range with it sister-species M. profundum (for details see Jawad and Clements 2004).

The remaining 23 New Zealand triplefin species are distributed circum-coastally over 13° of latitude where there is suitable habitat (Fricke 1994). *Forsterygion lapillum* and *Notoclinops segmentatus* are absent from the Three Kings Islands and the Chatham Islands (Paulin and Roberts 1992; Fricke 1994), even though congeners with similar habitat requirements are present in these locations. The absence of these species from offshore islands suggests that triplefin species differ in larval dispersal, and thus offshore islands may be beyond the dispersal capacity of larvae of some species. The distributions of eight other species appear to be relatively patchy, although this may be an artefact of incomplete sampling. Some species have been inadequately sampled because they are cryptic (e.g. *Notoclinus fenestratus, N. compressus* and *Cryptichthys jojettae*), or inhabit areas that are highly exposed (e.g. *Blennodon dorsale* and *Gilloblennius abditus*). Other species, such as *A. oculus* and the two species of the genus *Matanui* (e.g. *M. profundum* and *M. bathytaton*), have been poorly sampled because they are found predominantly in deep-water habitats (> 100 m, Fricke 1994; Jawad and Clements 2004). Three species are also found outside the New Zealand region.

Forsterygion varium and *Grahamina gymnota* have been recorded in Tasmania, and *F. lapillum* has been recorded from Port Phillip Bay, Victoria (Clements et al. 2000). Molecular (Hickey et al. 2004) and morphological analyses (Clements et al. 2000) demonstrate that these species have been introduced to Australia from New Zealand, and so these species should be considered New Zealand endemics.

Phylogeographic work on eight New Zealand triplefin species showed strong evidence for phylogeographic breaks between populations of *G. capito* and *G. nigripenne* (Hickey 2004; Hannan 2005). There was evidence for three reciprocally monophyletic lineages in *G. capito* (A, B and C), with type A found all around coastal New Zealand except Banks Peninsula, type B found at Banks Peninsula and the Auckland Islands, and type C only found at the Chatham Islands (Hickey 2004; Hannan 2005). If the three *G. capito* lineages were caused by historical barriers to gene flow then it would be expected that *G. nigripenne* would exhibit similar phylogeographic breaks. There is, however, only a single phylogeographic break in *G. nigripenne* between populations in the southern parts of the South Island and the rest of New Zealand (Hickey 2004). Gene flow in *B. lesleyae, B, medius, F. lapillum* and *F. varium* suggests isolation by distance, though there was a relatively strong reduction in gene flow between the Three Kings Islands and mainland New Zealand in the latter species (Hickey 2004). *Grahamina gymnota* and *R. whero* displayed almost complete panmixia (Hickey 2004). Overall, the phylogeographic barriers to dispersal).

Despite being the most abundant reef fishes in New Zealand, detailed studies on triplefin ecology are still in their infancy. Although several studies have investigated the habitat use of New Zealand triplefin species and have indicated that there is fine-scale partitioning of depth, exposure and substratum use (Darby 1966; Anderson 1973; Handford 1979; Thompson 1979; Thompson and Jones 1983; Syms 1992; Syms 1995; Fisher 1998; Vasques 1999; Feary 2001; Feary and Clements 2006), most of these investigations have been primarily descriptive and were conducted over small temporal (< 2 years) and spatial scales (e.g. at one site). In addition, all aforementioned studies summarised single environmental variables (e.g. rock, algae) into broader habitat categories (e.g. urchin barrens, *Ecklonia* forest), which increases observer bias and complicates the repeatability and objectivity of studies (Mumby and Harbourne 1999). Furthermore, all previous studies were conducted on rocky reefs or in the intertidal, while none have investigated the habitat use of triplefin species in other habitats (see Table 1).

Six species are not found on rocky reefs but rather inhabitant deep-water (*M. bathytaton* and *M. profundum*), shell rubble (*A. oculus*), estuaries (*G. nigripenne*) or the intertidal (*B. lesleyae* and *B. medius*) (Table 1, Clements 2003). Although *G. capito* can be found on rocky reefs, this species is also commonly seen on muddy and sandy substrates (harbours, sheltered bays) (Francis 2001). As a consequence of the limitations of the previous studies, detailed interspecific comparisons of the habitat use of New Zealand triplefin species are greatly lacking.

Table 1: Scientific, common names and habitat types of triplefins found from the Stewart Island to Cape Reinga. Depth data marked '*' denotes data from Francis (2001); '•' Fricke (1994), '•' Syms (1992); and '⁽⁾, Feary (2001).

Scientific name	Common name	Habitat types	Depth (m)
Apopterygion oculus	Ocellate triplefin	Shell rubble	12-256 [•]
Bellapiscis lesleyae	Mottled twister	Intertidal pools	0-5 [•] , <2 [■] , 0-5 [◊]
Bellapiscis medius	Twister	Intertidal pools	0-5 [*] , 0-5 [•]
Blennodon dorsale	Giant triplefin	Rocky reef	0-8•
Cryptichthys jojettae	Cryptic triplefin	Rocky reef	$0-5^*, <12^{\bullet}, 0-5^{\diamond}$
Forsterygion flavonigrum	Yellow-black triplefin	Rocky reef	$>10^*, 7-110^\bullet, >10^\bullet, >10^\circ$
Forsterygion lapillum	Common triplefin	Rocky reef	<5 [*] , <20 [●] , <40 [■] , 0-30 [◊]
Forsterygion malcomi	Banded/Mottled triplefin	Rocky reef	10-25 [*] , 2-35 [•] , >10 [■] , >10 [◊]
Forsterygion varium	Variable triplefin	Rocky reef	<10 [*] ,0-21 [•] , 0-30 [◊]
Gilloblennius abditus	Obscure triplefin	Rocky reef	<9•
Gilloblennius tripennis	Twister	Rocky reef	<20•
Grahamina capito	Mottled/Spotted triplefin	Rocky reef/Mud	0-12•
Grahamina gymnota	Robust triplefin	Rocky reef	0.3-5•
Grahamina nigripenne	Estuarine triplefin	Estuaries	0-15 [•]
Karalepis stewarti	Scaly-headed triplefin	Rocky reef	0-33 [•] , 5-10 [◊]
Matanui bathytaton	-	Deep water	80-550 [•]
Matanui profundum	-	Deep water	9-220 [•]
Notoclinops caerulepunctus	Blue-dot triplefin	Rocky reef	$>10^*, 0.40^{\bullet}, >10^{\bullet}, >10^{\circ}$
Notoclinops segmentatus	Blue-eyed triplefin	Rocky reef	$0-40^{\bullet}, 0-30^{\diamond}$
Notoclinops yaldwyni	Yaldwyn's triplefin	Rocky reef	<10 [*] , 0-20 [•] , <7 [■] , 5-10 [◊]
Notoclinus compressus	Brown topknot	Rocky reef	<10 [*] , 0-5 [•]
Notoclinus fenestratus	Topknot	Rocky reef	<5 [*] , 0-15 [•]
Obliquichthys maryannae	Oblique swimming triplefin	Rocky reef	<5 [*] , 1-50 [•]
Ruanoho decemdigitatus	Long-finned triplefin	Rocky reef	<10 [*] , <2 [•]
Ruanoho whero	Spectacled triplefin	Rocky reef	0-30 [◊]

Two studies to date have investigated diet partitioning in New Zealand triplefin species. Vasques (1999) investigated dietary overlap of three triplefin species in Fiordland (*F. flavonigrum*, *F. lapillum* and *N. segmentatus*) and found that the species were morphologically very similar and showed high overlap in diet. Feary (2001) examined diet

and trophic morphology in 15 triplefin species in the Hauraki Gulf, and found little evidence for interspecific variation. Only two species were found to have specialised diets, namely *Obliquichthys maryannae*, which is a zoo-planktivore, and *Blennodon dorsale*, which feeds solely on mussels (Feary 2001). All other species prey upon a wide variety of invertebrates, especially crustaceans, although some ontogenetic differences in diet occur (Feary 2001). A low level of diet partitioning is also characteristic of triplefins in other parts of the world (Kotrschal and Thomson 1986). Given that most New Zealand triplefin species lack dietary specialisations but appear to have diversified in habitat use, it appears that habitat is a likely resource dimension along which the New Zealand triplefin assemblage may have diversified.

New Zealand triplefins establish a small territory $(1 - 2 m^2)$ in which all breeding and feeding is carried out, and this area is defended year round (Handford 1979; Thompson 1983). Studies on *F. varium* (Thompson 1979; Fisher 1998) and *F. lapillum* (Fisher 1998) demonstrated that these species will home if displaced from their territory. Reproduction in triplefins occurs in the same small territory and takes place during the winter-spring season (Thompson 1986; Connell 1990; Francis 2001; Clements 2003). During the spawning season males of most species show a darkening of the whole body (e.g. *R. whero*, Paulin and Roberts 1992), which is unlike many other fish species where males are found to assume a brightly coloured nuptial colouration (Deutsch 1997; Seehausen and van Alphen 1998; Allender et al. 2003; Genner and Turner 2005). It therefore appears that male nuptial colouration is not significant in female mate selection, however, as with the habitat work detailed, descriptive studies of the male nuptial colouration of New Zealand triplefin species are greatly lacking. Unlike male colouration, female colouration is retained throughout the year, and is indistinguishable from the colouration of non-reproductive males (Thompson 1979).

The mating system of New Zealand triplefins is polygynandrous, with different clutches simultaneously present in a nest (Thompson 1979). Males build nests on different types of hard substrata, and when encountering a female the male displays intensively and tries to lead the female to the nest (Thompson 1986). During spawning the male remains close to the female and chases out any intruders (Thompson 1979). Females lay eggs in one dense layer by slowly moving the protrusible ovipositor over the rock surface (Thompson 1979). When laying eggs the papillose pad of the oviduct becomes enlarged and secretes adhesive substances to allow the attachment of the eggs (Anderson 1973). The male periodically moves close to the female takes frequent rests and may leave the nest for a few minutes until the male displays to her again (Thompson 1979). Thus, spawning may continue, with interruptions, for

up to 2-3 hours (Handford 1979). When the female stops spawning the male immediately starts guarding and caring for the eggs. Paternal care by the male includes defence against predators and fanning to oxygenate the eggs and prevent silt from accumulating (Thompson 1979). Parental males are highly territorial, and if challenged inter- or intraspecifically, perform a typical aggressive behaviour by spreading the dorsal fins and displaying the dark nuptial colouration (Thompson 1979). Males spend the majority of their time in close proximity to the nest until the larvae hatch, with the exception of occasional feeding forays (Handford 1979; Thompson 1979).

Laboratory experiments showed that hatching typically occurs after 16 days in F. varium and 21 days in R. decemdigitatus/R. whero, depending on water temperature (Ruck 1980). After hatching, the larvae have been observed to swim directly to the surface (Ruck 1980). In the wild, planktonic triplefin larvae are often associated with drift algae and are one of the most abundant larvae recorded from offshore nekton samples (Kingsford 1988; Kingsford and Choat 1989; Tricklebank et al. 1992; Hickford 2000; Hickford and Schiel 2003). Prior to settlement larvae move closer to the shore (Hickford and Schiel 2003), where reef sound appears to play a crucial cue in attracting larvae to the reef (Tolimieri et al. 2000). Triplefin recruitment of many species at northeastern locations commences in August and peaks between December and February (Syms 1995), suggesting a pelagic larval duration (PLD) of approximately 2 - 3 months (Kingsford and Choat 1989). At settlement larvae appear to establish territories in the immediate vicinity of adults and become extremely territorial (Thompson 1983). Movement after this phase is limited to gradual encroachment into neighbouring territories or slight habitat shifts into recently vacated territories (Thompson 1983; Connell and Jones 1991). Studies by Connell and Jones (1991) and Thompson (1979) on the settlement patterns of F. varium suggested that settlement occurs homogenously across habitat types ('blanket settlement'), and that survivorship of juveniles is predominately affected by the complexity of settlement habitat. Based on this, Connell (1991) proposed that the pattern of adult triplefin habitat use is the result of post-settlement mortality, rather than active habitat selection.

Otolith readings of *F. lapillum*, *B. lesleyae/B. medius* (Handford 1979) and *F. varium* (Thompson 1979) show that triplefin species in northeastern New Zealand have a maximum life span of 2-3 years. Juvenile *F. varium* grow rapidly during their first year and reach sexual maturity after approximately 6 - 12 months (Handford 1979; Thompson 1979).

1.3 OBJECTIVES OF THIS RESEARCH

The mechanisms that have led to the high diversity of New Zealand triplefin species are unknown, but ecological speciation is one of the modes that have been suggested (Hickey 2004). Given the characteristics of New Zealand triplefin species it is also possible that adaptive speciation in sympatry or parapatry has occurred. The fact that all species are substrate spawners and are highly philopatric following settlement suggests that assortative mating could be very closely linked to habitat choice. Theoretical modelling has shown that such conditions are favourable for speciation in sympatry to occur (Dieckmann and Doebeli 2004; Gavrilets 2004; Gavrilets 2005). Gavrilets (2005) refers to such a linkage of habitat and mate choice as a 'magic trait', a trait that is both subject to disruptive selection and simultaneously controls non-random mating. Empirical evidence has demonstrated that such a linkage can lead to speciation in situ (Bush 1969; Berlocher 1998; Feder 1998; Via et al. 2000; Munday et al. 2004). Given that the basic ecology of New Zealand triplefin species has only been recorded for some species and locations, it is necessary to describe habitat use and reproductive behaviour of the majority of species in detail. Chapters 2-6 of this thesis presents detailed data of the inter- and intraspecific habitat use for adults and juveniles of the majority of New Zealand triplefin species, and uses novel sampling and data analysis techniques to overcome the limitations of previous studies. In addition to establishing these patterns, Chapters 7-8 of this thesis use field observations and laboratory experiments to investigate which processes may explain the patterns observed. Manipulative field experiments could not be used as triplefins are extremely philopatric and will home if displaced (Thompson 1983). Together, the descriptive and the experimental parts of this thesis aim to answer two basic questions:

- 1. What are the traits under selection that may have led to the divergence in the New Zealand triplefin species?
- 2. What are the traits that are potentially available for the developments of assortative mating in triplefins, and which have been recognised to be important in other species?

The field and laboratory work used to address these questions are arranged into seven data Chapters. Chapter 2 investigates the pattern of interspecific habitat use of 17 triplefin species at seven locations to determine the extent of interspecific differences in habitat use, and the phylogenetic context of habitat diversification. This was done to investigate whether habitat use has been a likely resource dimension along which the New Zealand triplefin assemblage may have diversified. Chapter 3 examines intraspecific variation in habitat use between locations, to investigate whether triplefin populations show geographic variation in across biogeographic gradients. Data on this will elucidate the importance of gene flow between distant locations and the strength of local population adaptation in shaping triplefin habitat associations. Chapter 4 describes the degree of habitat specialisation of 15 triplefin species in the Inner and Outer Hauraki Gulf to assess the degree to which species are habitat generalists versus specialists. Chapter 5 compares the pattern of habitat chosen by newly settled triplefins with that of adults to investigate whether triplefin recruits show evidence of active habitat selection or, as has been previously suggested, exhibit 'blanket settlement' (Thompson 1979; Connell and Jones 1991). Such information will help establish whether adult habitat use is the passive result of post-settlement mortality in different habitats, or a result of active habitat choice exhibited by the larvae. Chapter 6 investigates habitat diversification in the intertidal sister-species pair B. lesleyae and B. medius. There is little data available on the habitat use of this sister-species pair, presumably because of the high morphological similarity of the two species and the associated difficulty in identifying them. Chapter 7 assesses the importance of several prezygotic isolating mechanisms using both field data and laboratory experiments. Specifically, temporal and spatial isolation in breeding habitats, and interspecific divergence in male nuptial colour pattern (in the visible and UV spectrum) and body length was investigated. Reproductive isolation in the Ruanoho sister-species pair was studied in detail as these species show the lowest amount of genetic divergence within New Zealand triplefin fishes (Hickey and Clements 2005), suggesting that the mechanisms that have led to their ecological divergence are still in evidence. Hybridisation trials in which the *Ruanoho* species were denied a conspecific mate were conducted to establish the degree of reproductive isolation between the species. Furthermore, mate choice and courtship trials were conducted to test whether females of this genus show a preference for male body size and whether the species show interspecific differences in courtship display. Chapter 8 experimentally assesses the degree of inter- and intraspecific competition between the sister-species R. whero and R. decemdigitatus, and aims to identify factors associated with competitive ability in this recently diverged sister-species pair. This was done to investigate if the ghost of past competition may have led to habitat divergence in these species. Chapter 9 presents a discussion of the findings from the entire thesis.

2 Habitat Use in Subtidal Triplefin Fishes

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2.1 INTRODUCTION

The role that resource partitioning plays in the coexistence of ecologically similar species and in adaptive diversification has been the subject of much debate. It has been suggested that specialisation resulting from ecological diversification is a key factor in the partitioning of resources and the coexistence of species, and recent research has focused on the evolutionary and ecological processes responsible for generating and maintaining this diversity (Malavasi et al. 2005; Rocha et al. 2005). Diversification in the use of ecological resources has been widely documented in coral reef fishes, and numerous studies have documented distinct patterns of habitat utilisation among closely related species (Bouchon-Navaro et al. 2005). Examples can be found in a range of coral reef fish families including blennies (Wilson 2001), damselfishes (Bay et al. 2001), parrotfishes (Gust et al. 2001), and gobies (Munday and Jones 1997).

While resource use of fish species has been extensively studied within coral reef fish assemblages, the range of habitats occupied and the use of particular habitat features by temperate reef fishes are generally not well understood. Like coral reefs, temperate environments are characterised by great spatial heterogeneity in substrate composition and complexity on a range of spatial scales (Syms 1995). Temperate fish assemblages have been found to vary along gradients of topographic complexity (Connell and Jones 1991; Schofield 2003), substrate type (Nemeth 1998), exposure (Thorman 1986), the degree of substratum relief (La Mesa and Vacchi 2005) and microhabitat structure (Ormond et al. 1996). However, not all temperate fish species respond to every type of habitat variable, and different species may partition some environmental variables and not others.

New Zealand triplefin fishes are an ideal model system to study ecological diversification of habitat use, as all species occupy small territories after settlement, are highly philopatric, and mate within their territory (Clements 2003). The high site fidelity is further exemplified by the ability of some species to home, even if displaced over several 100 m (Thompson 1983; Fisher 1998). In addition, there is some evidence to suggest that triplefins exert species-specific habitat choice at settlement (see Syms 1995). The combination of these characteristics have been shown both theoretically (Gavrilets and Vose 2005) and empirically (Via et al. 2000; Munday et al. 2004) to lead to rapid ecological diversification in other animals.

Although common in the diversification of other fish radiations, trophic resource partitioning (e.g. Lu and Bernatchez 1999) and sexual selection on male body colouration (e.g. Allender et al. 2003) are unlikely to have been important in the diversification of the New Zealand triplefin fauna (see General Introduction). Previous work has shown that New Zealand triplefins have diversified into a variety of habitats, ranging from estuaries and shallow rockpools to deep reefs (see for details: Handford 1979; Thompson 1979; Syms 1995; Feary and Clements 2006). All previous studies, however, are restricted to a few species in northeastern New Zealand and rocky reef habitats. Furthermore, no studies to date have assessed habitat use quantitatively, and as a result, lack detailed interspecific comparisons. This study sets out to investigate the role of habitat specialisation in ecological diversification of the New Zealand triplefin assemblage by recording habitat use data for 17 triplefin species. Study locations were selected all around New Zealand from 35°50'S to 46°70'S to encompass the latitudinal and environmental range of most triplefin species (Fricke 1994). Two complementary hypotheses were tested:

- 1. Triplefin species do not show interspecific differences in habitat use.
- 2. Sister-species are more similar in habitat use than more distantly related species.

Hypothesis 1 addresses the possibility that adaptation to different habitat types within the same general area has played a role in the evolution of the New Zealand triplefin assemblage. To investigate this hypothesis a novel multidimensional scaling techniques was used to plot the Euclidean habitat similarity of 17 species in ecological space. If ecological adaptation to different habitats has played a part in the evolution of the New Zealand triplefin clade then it would be expected that species show considerable interspecific differences in habitat use. Conversely, it was predicted that if species show low differentiation in habitat use that adaptation to different habitats has not occurred or has been weak.

Hypothesis 2 more directly addresses the role of habitat adaptation in the diversification using both a statistical and Bayesian phylogenetic comparative analysis, as it has been suggested that it is more robust to use both approaches (Gittleman and Luh 1994; Price 1997), particularly when there is no strong phylogenetic signal in the data (Garamszegi et al. 2004). It was predicted that if sister-species were more similar in habitat use than distantly related species, then ecological selection has been weak and was unlikely to have caused speciation (i.e. non-adaptive speciation). In contrast, if sister-species were more dissimilar in habitat use than distantly related species then this could be taken as evidence for selection on habitat use.

2.2 MATERIALS AND METHODS

2.2.1 Selection of species

A sampling method was designed to census the majority of triplefin species that occur in coastal waters around New Zealand (Figure 1), namely *A. oculus*, *B. lesleyae*, *C. jojettae*, *F. flavonigrum*, *F. lapillum*, *F. malcolmi*, *F. varium*, *G. capito*, *G. gymnota*, *G. nigripenne*, *K. stewarti*, *N. caerulepunctus*, *N. segmentatus*, *N. yaldwyni*, *O. maryannae*, *R. decemdigitatus* and *R. whero*.



Figure 1: Sampling locations around New Zealand. The study locations included: 1 Three Kings Islands, 2 Hauraki Gulf, 3 Coromandel Peninsula, 4 Napier, 5 Wellington, 6 Fiordland and 7 Stewart Island.

The highly cryptic species *G. tripennis*, *N. compressus* and *N. fenestratus*, which are found amongst seaweed (Clements 2003), were not censused. Other species not sampled included those that inhabited intertidal (i.e. *B. medius*), highly exposed (i.e. *B. dorsale* and *G. abditus*), and deep-water habitats (i.e. *M. bathytaton* and *M. profundum*) (Clements 2003).

2.2.2 Selection of locations and sites

Triplefin habitat use was documented from 2002 to 2005 at seven locations around New Zealand (Three Kings Islands, Coromandel Peninsula, Hauraki Gulf, Napier, Wellington, Fiordland and Stewart Island, Figure 1) using underwater visual census (UVC). Locations were selected to cover the latitudinal range and environmental gradients of the habitats used by the study species (Fricke 1994; Francis 1996; Francis and Nelson 2003). The Three Kings Islands (35°50'S, 172°10'E) are situated approximately 60 km northwest of the northern most tip of New Zealand, and represent a highly exposed, isolated location that is the northern distributional limit for coastal triplefin species (Brook 2003). Sampling at the Three Kings Islands, in particular at depths of less than 5 m, was constrained by the high exposure of some sites. The northern tip of the eastern coast of the Coromandel Peninsula $(36^{\circ}29'S, 175^{\circ}19'E)$ and Hauraki Gulf and associated offshore islands (36°36'S, 174°50'E) were selected to represent coastal and offshore island sites in an intermediate position between the most northern and southern locations of the North Island. The Napier area (39°29'S, 176°55'E) is situated in a large semi-circular bay on the east coast of the North Island, and is characterised by relatively sheltered, shallow and silty shores (Chiswell 2002). To census exposed sites in the Napier region several boat trips to the highly exposed Pania Reef (39°26'S, 176°58'E) were undertaken. Wellington (41°16'S, 174°51'E) lies at the southern end of the North Island (Booth 1974), and represents an intermediate latitudinal location. To cover a wide exposure gradient in the Wellington area, sites were selected at Titahi Bay (41°10'S, 174°53'E) and Island Bay (41°33'S, 174°78'E). Fiordland was chosen as it represents a unique environment of deep fjords located over a 200 km stretch of the southwestern coast of South Island (Heath 1985). Heavy rainfall in the Fiordland region causes a thick freshwater layer that is stained by organic matter (Wing 2003), and this can cause a salinity stratification and extremely low light levels even at shallow depths. The most southern location sampled was Stewart Island (46°60'S, 168°20'E), which is New Zealand's third largest, and most southern large island, separated from the mainland by the shallow Foveaux Strait (Heath 1985). The isolated Snares

Islands (48°00'S, 166°35'E), which represent the southern limit of most New Zealand triplefin species, were not sampled.

Within each location, $4 \ge 4 \le 100$ were conducted at sites that were randomly selected with the aim of sampling as much of the exposure gradient as was practically possible. While the sampling sites attempted to be representative of the shallow subtidal environments occupied by triplefin species (< 36 m), some highly exposed sites could not be sampled (e.g. some sites at the Three Kings Islands). This limitation meant that species whose range extends into highly exposed habitats (i.e. *B. lesleyae*) were only sampled at the lower exposure range.

2.2.3 Data collection

In this study, all UVC were done by the same diver and consisted of a close, rigorous and systematic searching pattern, spending at least 1 min on each quadrat $(1 \times 1 \text{ m})$, with all interstices and overhangs examined to ensure a complete census. Prior to sampling a location fix was taken for each site using handheld Garmin® 12 global positioning system (GPS) (accuracy ±15 m). From this GPS information, a physically derived exposure (fetch) index could be calculated based on the total sum of the fetch (maximum radial distance 300 km). Fetch is the distance of open water over which waves can be generated by winds and can thus be used as an approximation of wave exposure. The fetch calculations were performed with the program 'Fetch Effect Analysis' (version 1.01. Pickard R 2000), which works by measuring fetch distance for each 20 degree sector (18 sectors in total) on a compass rose from a given point (GIS fix) (Thomas 1986).

At least three UVC were undertaken at each site. The first UVC at each site was done at the deepest depth that could safely be sampled (maximum depth dived 36 m), and the two subsequent UVC were done at approximately 33% and 66% of the deepest depth. Any additional UVC were conducted in intermediate depths. This design was employed to allow sampling flexibility at any location around New Zealand. A minimum distance of approximately 50 m between UVC was maintained to eliminate the chance of obtaining dependent samples (Andrew and Mapstone 1987). The centre line of the each 4 x 4 m UVC was marked with a leaded line, and a 1 x 1 m steel quadrat was used to outline each 1 m² along the quadrat. The spatial scale at which small fishes such as triplefins perceive differences in habitat features should be small and comparable with the range of movements exhibited by the fish (i.e. the size of the home range, 2005). For this reason, triplefin habitat

use was recorded for each 1 x 1 m within the quadrat. For each 1 m² the depth was recorded and seven habitat variables were estimated visually as percent cover of the substratum. The habitat variables were categorised as rock (rocks > 7 cm), cobbles (rocks < 7 cm), gravel (rocks < 4 cm), sand, mud, macroalgae, and coralline and turfing algae. While the first five variables always sum to 100%, the algal coverage could range from 0 - 100%. The fish within each quadrat were identified, and the microposition of each fish (Table 2) on which it was first encountered was recorded. The microposition use provides evidence of the fine-scale habitat use of each species and thus indicates interspecific overlap on a finer scale than the habitat analysis. For example, species can occupy the same 1 x1 m patch, but one species can occupy the 'top and side of the rocks' (STB) while the other can occupy the horizontal and vertical cracks of the rocks (UCS).

Microposition	Abbreviations
Side and top of rock	STB
Under rock or in crack on horizontal/vertical slope	UCS
Under an overhang	UOV
Upside down on overhang	ROV
On top of cobbles	TCO
On sand or mud, without cover	SM
Free-swimming	FRE
On algae	ALG

Table 2: Microposition abbreviations modified from Feary (2001) and Syms (1995).

The use of UVC as a sampling method for triplefins has recently been criticised in a comparative methodological study (Willis 2001). Willis compared ichthyocide and UVC counts and found that the density and diversity estimates were considerably lower in the UVC method. However, this conclusion is questionable for several reasons. First, Willis failed to restrict the census target to triplefin species, but instead attempted to census a wide range of pelagic and demersal species at the same time. Second, Willis conducted all censuses 0.5 m above the substratum, and thus could not have searched the substratum accurately. Third, the area that was effectively searched for the ichthyocide counts was larger than the area censused for the UVC method, since fish affected by the ichthyocide were collected outside the UVC census area. For these reasons Willis (2001) fails to provide substantive argument against the use of UVC for New Zealand triplefin species. Furthermore, several studies have used UVC counts to census triplefin species and achieved robust results (i.e. Thompson 1979; Connell and Jones 1991; Syms 1995; Syms and Jones 1999; Feary and Clements 2006).
2.2.4 Data analysis

2.2.4.1 Interspecific overlap in habitat use

In total, 15488 individual fish observations were recorded at seven locations around New Zealand (Table 3).

Table 3: Number of observations of each species at each location. Observations were pooled at species level (Total) and analysed globally. Locations included the Hauraki Gulf (H), Coromandel (C), Napier (N), Three Kings (3K), Wellington (W), Stewart Island (S) and Fiordland (F).

Species	Total	Н	С	Ν	3K	W	S	F
A. oculus	37	0	0	0	0	0	37	0
B. lesleyae	176	99	0	0	0	18	0	59
C. jojettae	234	66	5	0	129	0	34	0
F. flavonigrum	941	247	1	1	3	0	157	532
F. lapillum	3803	2865	148	124	0	351	76	239
F. malcolmi	384	80	67	84	7	1	46	99
F. varium	2167	830	186	201	77	218	477	178
G. capito	879	178	0	1	0	28	1	671
G. gymnota	33	0	0	33	0	0	0	0
G. nigripenne	337	152	0	0	0	0	0	185
K. stewarti	75	35	3	0	18	6	12	1
N. caerulepunctus	245	158	0	0	0	0	0	87
N. segmentatus	2336	1894	26	15	0	69	229	103
N. yaldwyni	465	334	12	18	101	0	0	0
O. maryannae	1495	596	70	0	46	16	322	445
R. decemdigitatus	232	79	0	101	0	52	0	0
R. whero	1649	1371	70	25	92	10	72	9

The overarching goal of this Chapter was to examine overlap in habitat use by New Zealand triplefin species based on habitat similarity. A survey of the literature revealed that there are no common procedures to analyse and summarise multidimensional, quantitative habitat data without losing the distance relationships between the habitat variables. For this reason, a method was developed that used 'double-scaled' Euclidean (DSE) distances as a dissimilarity measure between habitat variables for a species (Barrett 2005b). The 'double-scaling' refers to the procedures used to transform a conventional Euclidean distance into a universal 0 (no distance between objects) to 1 (maximum possible discrepancy) range using a strictly linear methodology. Essentially, squared discrepancies between two cases are standardised by the respective maximum possible squared discrepancy for each paired comparison, then the 'maximum-discrepancy standardised' Euclidean distance is again re-standardised by the

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square root of the number of 'components' forming the Euclidean distance. In this particular version of the coefficient, the 'components' are the number of individuals per species observed using two variables for which the DSE estimate is being computed. This double scaling transformation avoids the usual problems with non-linear data standardisation-normalisation methods by using only linear scaling procedures, making the DSE coefficients comparable between themselves and between studies (see Appendix I section ii for formulas).

Initially, for each species, every variable was compared to every other variable, with the comparison indexed using DSE distances to represent the usage 'distance' between each variable. The habitat data consisted of depth, exposure (fetch) and seven substratum variables (rock, cobble, gravel, sand and mud, macroalgae and coralline and turfing algae) for each species. A Statistica (version 7.1) Visual Basic program 'Agreement Matrix Constructor' (Barrett 2005a) was written for this purpose. Where an individual failed to be observed using both habitat variables (joint absence), then that individual was excluded from the calculation of the DSE distance for that particular habitat variable pair (akin to the logic of a Jaccard measure of an agreement). This is because a joint absence would result in producing a spurious decrease in distance between two habitat variables. The rationale here is that if an individual failed to be observed using two habitat variables, then actually no information exists about the habitat use by that individual. Only cases that have an observation on either one, or both variables were included in the analyses.

To obtain an overall species similarity matrix the DSE distances between variables for each species were submitted to a non-metric Guttman-Lingoes MDS procedure in Statistica to derive habitat variable usage maps for each species in 2-dimensional space. Two dimensional MDS plots were chosen because the stress values for each species were less than 0.2 in each case (Clarke and Warwick 2000). In order to generate a species similarity map, each 2-dimensional MDS solution for each species was compared to every other species solution using the procrustes orthogonal matrix comparison routine ORTHOSIM2 (Barrett 2005c). The program works by configurally rotating a comparison matrix of MDS coordinates against a target matrix to minimise the sum of squared deviations between the comparison matrix and target matrix coordinate values. When submitting MDS coordinate dimensions for comparison, both matrices are initially centered, row-normalised, and reflected (arithmetic sign reversal) where necessary. Row normalization is the 'procrustes' transformation, (which expresses each matrix in a normalised unit metric space which preserves the distance relations). The entire procedure is more generally known as 'configural similarity' (Borg and Groenen 2005). The reason for these specific transformations is that MDS solutions are

arbitrary in terms of their location, scale, and orientation of variables in geometric space. It is the distance relations between variables which are critical in the MDS procedure, and these relations can be preserved whilst allowing the origin, scale, and coordinate reflection of the solutions to vary. Thus, when comparing the coordinates from MDS solutions, the coordinates need to be expressed within a common geometric space with common origin and coordinate signs.

The similarity of the two rotated MDS habitat variable coordinate matrices to one another was expressed as a DSE-similarity (DSE-S) coefficient (simply 1 – DSE Distance), where 0 now equals maximum possible discrepancy and 1 equals absolute identity. The DSE-S values of each species comparison were entered into a species comparison similarity matrix and visualised using the same MDS routine in Statistica as before. D-hat raw stress was used to evaluate how well the final MDS configuration reproduced the estimated similarities (see Appendix I section ii for the formula). The appropriate number of MDS dimensions were chosen according to the stress value threshold of 0.2 (Clarke and Warwick 2000), above which configurations are considered to be poor representations of the data.

A prerequisite to run these coordinate comparisons is that the same variables are compared in the target and the comparison MDS plots, and that the coordinates are orthogonal to one another. As a consequence, species which possessed no observations for some of the habitat variables could only be compared to all other species on the basis of the mutual habitat subsets. The species with missing observations for some habitat variables were *G. nigripenne* (gravel and macroalgae), *G. gymnota* (sand and coralline and turfing algae), *B. lesleyae* (sand and mud) and *A. oculus* (cobble, gravel and coralline and turfing algae).

Microposition use of all species was analysed and graphically summarised via the Correspondence Analysis routine in Statistica. Correspondence Analysis uses Chi-square transformation, meaning the proportions of species are considered rather than their absolute abundances. Correspondence Analysis is an exploratory technique designed to analyse some measure of correspondence between the rows (species) and columns (microposition categories). The resulting graph provides information about which species are similar to one another in terms of microposition use, with similar species being closely placed in multi-dimensional space.

2.2.4.2 Similarity in habitat use between sister and non sister-species

This part consisted of both a statistical and phylogenetic analysis of habitat use between sister and non sister-species. The statistical approach used an independent sample t-test to examine whether the mean DSE-S of the five sister-species differed from the mean DSE-S of the nonsister-species. Although there was a large disparity between the numbers of cases in the two groups (five sister-species versus 131 non-sister-species comparisons), the sample of five cases for the sister-species is acceptable (from a sampling perspective) as it represents > 80%of the total population of sister-species in the New Zealand triplefin clade. It was assumed that the variances of the two groups were not homogenous in order to be conservative. Therefore, the Welch-test was used as this test does not rely on the assumption of homogeneity of variances (Zimmermann 2004b; Zimmermann 2004a). Statistical significance in the Welch-test is assessed by using an adjusted degree of freedom estimate. In addition, power estimates were calculated using the harmonic mean of the joint number of cases as the estimate of the group sample size, following Howell (2002). The power estimate, however, is just an approximation as the variances of the two groups are assumed to be unequal. The Welch-test was performed using the program 'independent means t-test with effect size' (Barrett 2006).

The second approach used a phylogenetically controlled method utilising a generalised leastsquares (GLS) approach to examine the covariance of characters explained by the relationships between the species (Pagel 1997; Pagel 1999). The GLS is a phylogenetic regression method, in which the covariance among species attributable to phylogeny is expressed in the regression error term, allowing for control of phylogenetic non-independence in the data (Garland et al. 2005). This approach was chosen because related species may share characteristics due to common ancestry as well as through convergent adaptation. Analyses were performed using the program 'BayesContinuous' (Pagel and Meade 2004; Pagel et al. 2004, program available upon request from the author), which allows the use of continuous character traits and posterior probabilities. Bayesian inference has the advantage over both parsimony and maximum likelihood methods of trait reconstruction by taking phylogenetic uncertainty into account (Pagel et al. 2004). Non-independence among taxa is controlled for by specifying a variance-covariance matrix based upon the assumed phylogeny.

Hypotheses are tested with likelihood-ratio (LR) tests. The log-likelihood of the model corresponding to a null hypothesis (H₀) is compared over the model for an alternative hypothesis (H₁), where the likelihood-ratio = $-2 \log_e [H0 / H1]$. The likelihood-ratio statistic

is asymptotically distributed as a chi-square variate with degrees of freedom equal to the difference in the number of parameters between the two models. The threshold for assigning significant differences were when $\alpha < 0.05$ ($\chi^2_{0.05(1)} = 3.84$).

The 3-dimensional coordinate axes obtained from the habitat similarity plot (see section 2.2.4.1) were used as continuous ecological traits, in which each of the 1-, 2- and 3dimensional coordinates represent one trait (Table 4). The triplefin phylogeny and branch lengths were obtained using MrBayes (Huelsenbeck and Ronquist 2001; Ronquist and Huelsenbeck 2003), based on data of three mitochondrial genes (12S, 16S and control region) and a nuclear gene (ETS2) provided by Hickey and Clements (2005). The species *A. oculus* was specified as the outgroup (Figure 2). Trees were generated for 10 million generations, with sampling every 20,000 generations, and the first 20% were discarded as 'burn-in'.

Species	Dimension 1	Dimension2	Dimension 3	
A. oculus	-1.093	-1.021	0.554	
B. lesleyae	-0.411	-0.138	0.089	
C. jojettae	0.732	-0.232	-0.247	
F. flavonigrum	-0.057	0.983	0.0457	
F. lapillum	0.800	0.349	-0.099	
F. malcolmi	0.511	0.312	0.448	
F. varium	0.458	0.069	0.136	
G. capito	-0.971	1.025	-0.715	
G. gymnota	-0.388	0.108	-0.716	
G. nigripenne	-0.344	0.911	1.041	
K. stewarti	-0.215	0.021	0.562	
N. caerulepunctus	0.277	-0.754	-0.406	
N. segmentatus	0.362	-0.245	-0.530	
N. yaldwyni	0.552	0.045	-0.516	
O. maryannae	0.915	-0.354	0.187	
R. decemdigitatus	-1.261	-0.488	-0.370	
R. whero	0.134	-0.591	0.536	

Table 4: Ecological traits used in the comparative phylogenetic analysis. The three habitat dimensions were derived from the MDS scaling technique described in 2.2.4.1.

BayesContinuous incorporates the constant-variance random walk (Brownian motion) model of character evolution, but it can also accommodate a directional component for trait evolution (Pagel 1997; Pagel 1999). The random walk model has a single parameter, the instantaneous variance of evolution. This model would represent a pattern of evolution in which the evolution of habitat specialisation had been subjected to changes in both directions

(i.e. increase or decrease) across different paths in the phylogenetic tree. The directed random walk model assumes that habitat specialisation has followed a dominant direction of evolutionary change across the tree, i.e. either a steady increase or decrease of the trait over time. The directional model accounts for the same variance of evolution as the non-directional model, but in addition has a parameter describing the tendency for directional change. Likelihood ratio (LR) tests were performed to determine if a random walk model described the data significantly better than a directional random walk model. LR tests work by comparing the log-likelihood of the model corresponding to a null hypothesis (H_0) over the model for an alternative hypothesis (H₁), where the LR = $-2 \log_{e} [H_0/H_1]$. The LR statistic is asymptotically distributed as a chi-squared variate with degrees of freedom equal to the difference in the number of parameters between the two models. However, since the Bayes Continuous output provides log-likelihoods, the LR statistic is calculated as twice the difference between the independent and dependent log-likelihoods (i.e. $2 \cdot [H_1 - H_0]$). A LR value greater than the chi-square variable indicates that the null hypothesis can be rejected, i.e. that the model allowing the parameter to take the most likely value (maximum likelihood) describes the data significantly better than the model where the parameter equals the fixed constant.

In addition to estimating which model (random or directional) fits the habitat data best, the mode, tempo and phylogenetic association of trait evolution were assessed through three scaling parameters: lambda (λ), kappa (κ) and delta (δ) (Pagel 2002). The parameter lambda is a measure of phylogenetic dependence that assesses the amount of phylogenetic component in character variation (Pagel 1999). Values of lambda < 1 correspond to traits being less similar among species than expected from their phylogenetic relationship, whereas lambda = 1suggests the reverse. The parameter kappa defines the relationship between individual branch lengths and the probability that a character changes state and thus, allows one to stretch or compress individual branches. In the case of kappa = 0, trait evolution is independent of the branch lengths and indicates punctuational evolution. The opposite scenario occurs when kappa = 1 which indicates gradualism (the default). Finally, delta determines whether character change is concentrated at the root or toward the tips of a phylogeny. A delta value of < 1.0 suggests species-specific adaptation—that is, longer paths (i.e. paths from the root to the tips that contain greater numbers of nodes) contribute more to trait evolution than shorter ones. In contrast, a delta of > 1.0 indicates a greater rate of evolution in the earlier states followed by slower rates of evolution among related species. The contribution of the three scaling parameters was tested sequentially by estimating the maximum likelihood value of each parameter. Model fit was estimated by comparing the log-likelihood of a H_0 model containing default (= 1) values for the scaling parameters with the log-likelihood of an alternative H_1 model in which one parameter is permitted to take its maximum likelihood value. If a significant effect was found (p < 0.05), the estimated values were used in the final model, otherwise default settings were used.



Figure 2: Phylogram with posterior probabilities of the 17 triplefin species. Genetic data were provided by T. Hickey. The bar represents 0.1 substitutions per site.

2.3 RESULTS

The relative densities of triplefin species varied across locations. In particular, the species *F*. *flavonigrum*, *G. capito*, *G. nigripenne* and *R. decemdigitatus* (Figure 3) varied by more than one individual m^{-2} between locations (Figure 3). Other species, namely *C. jojettae*, *K. stewarti*, *N. segmentatus*, *N. yaldwyni* and *R. whero* showed less variation in density.



Figure 3: Relative densities estimates of triplefin species at seven locations around New Zealand (mean \pm SD). Only presence data were used for this histogram. Error bars show the \pm SD of the mean. Species names are abbreviated by the first letter of the genus followed by the first letter of the species name.

2.3.1 Interspecific overlap in habitat use

All species showed strong differentiation in depth and exposure (Figure 4). Three clusters of species were apparent. The first cluster consisted of species characterised by sheltered and extremely shallow habitats, namely *R. decemdigitatus*, *F. lapillum*, *G. capito* and *G. nigripenne* (Figure 4). The placement of *B. lesleyae* in this cluster is not an accurate reflection of the true exposure range due to the fact the intertidal zone could not effectively be sampled in high exposure areas. This species occurs both in the intertidal and subtidal, and is most often found in the surge zone (Paulin and Roberts 1992), and as a consequence, the habitat of this species could only be surveyed in relatively sheltered sites (i.e. Hauraki Gulf and Fiordland). It was impossible to census *B. lesleyae* at highly exposed sites (i.e. Three King Islands and Stewart Island), despite the species being abundant at these locations. The arrow in Figure 4 indicates that the mean exposure of this species is higher than suggested by the data in this study.



Figure 4: Mean depth and exposure of triplefin habitats. The arrow indicates the presumed exposure range of *B. lesleyae*. The x- and y-error bars represent \pm SE. Species names are abbreviated by the first letter of the genus followed by the first letter of the species.

The second cluster was characterised by species that occupy deep and relatively sheltered habitats, and included A. oculus, N. caerulepunctus and F. flavonigrum (Figure 4). The third and biggest cluster included all the remaining species, which commonly use habitats of medium depths and moderate to high exposure (Figure 4). There was also a degree of interspecific difference in the use of the substratum types (Figure 5), but overlap between species was much more pronounced relative to the strong differentiation shown in depth and exposure. Rock was the most frequently used habitat component by all species with the exception of A. oculus, G. nigripenne and G. capito (Figure 5). Specifically, A. oculus was the only species that was never found near rock, but instead was exclusively found in a habitat characterised by a mixture of sand and mud with red drift algae. However, the results for A. oculus have to be viewed with caution as this species was only recorded from one location (Stewart Island). Grahamina nigripenne and G. capito were also commonly found on muddy substrates, though were never far from rocky shelter (Figure 5). The two remaining variables, gravel and cobble, were used to a low degrees by all species (< 20%, Figure 5). Some species occupied habitats that contained macroalgae and/or coralline and turfing algae. For example, coralline and turfing algae played a dominant part in the habitat of C. jojettae, and also formed a consistent part of the habitats of all three Notoclinops species, K. stewarti and R.





Figure 5: Percentage use of substratum variables. Substratum types (rock, cobble, gravel, sand and mud) always add up to 100%, whereas algal coverage can vary between 0 - 100%.

Figure 6 shows all 17 species observed in this study in 3-dimensional ecological space based on similarity in depth and exposure and the substratum types of the habitat. A 3-dimensional MDS solution (stress = 0.148) was chosen for interpretation as the 2-dimensional solution had a stress value of 0.205 and stress values above 0.2 are considered to be poor representations of the data (Clarke and Warwick 2000). Further, the 2-dimensional solution was much more difficult to interpret because of the poor representation of distances between species. The inclusion of four species (*A. oculus*, *B. lesleyae*, *G. nigripenne* and *G. gymnota*) with only a subset of the habitat data (see methods) allowed for the analysis of all 17 species, though this involved an increase in the stress value from 0.047 to 0.148. The increase in stress value indicates that the position in ecological space for the four-subset species is somewhat number of observations for *A. oculus*, *B. lesleyae* and *G. gymnota* (33, 37 and 176, respectively). Additionally, all four subset species differ considerably in habitat use from the remaining 13 species, as indicated by the difference in the use of depth, exposure (Figure 4) and substratum

type (Figure 5). The strong habitat differentiation of the four subset species can also be seen in the lower mean species comparison DSE-S values for *A. oculus* (mean = 0.66), *G. gymnota* (mean = 0.7396) and *G. nigripenne* (mean = 0.6847) relative to the mean similarity value of the remaining 13 species (mean = 0.7543). Despite some uncertainty about the position of the four-subset species in ecological space, the presence of the four subset species only slightly affected the relative position of the remaining 13 species, as indicated by a high DSE-S coefficient. The overall wide spread of species in ecological space indicates that triplefins have diversified considerably in habitat use. The most obvious ecological pattern that emerged was that species that use a similar depth and exposure are in close proximity to one another on the MDS plots. This was because triplefin species show greater partitioning in depth and exposure (Figure 4) than in the use of substratum variables (Figure 5).



Figure 6: The 3-dimensional MDS solution (stress 0.148) used to represent the DSE-S between 17 triplefin species in multivariate ecological space. Species names are abbreviated by the first letter of the genus followed by the first letter of the species name, respectively.

Five species were not closely positioned to any of the other species in MDS space (A. oculus, F. flavonigrum, G. capito, G. nigripenne and R. decemdigitatus, Figure 6), indicating a high

degree of habitat divergence. The rotation of *A. oculus* and *G. nigripenne* was based on habitat subsets, thus cautious interpretation is necessary given that only some habitat variables were considered in the analysis. *Apopterygion oculus* was presumably distantly positioned from all other species because, unlike all other species, it was not associated with rocky substratum (Figure 6). *Forsterygion flavonigrum* was detached in ecological because it was the only deep-water species (Figure 4) that also occurred sometimes in partly muddy and silty habitats (Figure 5). *Grahamina capito* and *G. nigripenne* were presumably distantly placed to all other species because both species inhabit exclusively shallow, sheltered (Figure 4) and muddy habitats (Figure 5). In addition, *G. nigripenne* differs from *G. capito* in that it uses rock to lesser degree (Figure 5). Lastly, *R. decemdigitatus* is separated from all other species as it is predominantly found in shallow and sheltered habitats (Figure 4) that are characterised by high amounts of structural complexity, such as boulders and rocks (Figure 5). Microposition use also differed between species (Figure 7). The 3-dimensional plot explains 75.5% of the variance, which is considered to be a good ordination (Greenacre 1993).



Figure 7: Micropositions use by triplefins. The cluster of micropositions and species represents STB, TCO, SM and ALG, *A. oculus, B. lesleyae, C. jojettae, F. flavonigrum, F. lapillum, F. malcolmi, F. varium, G. capito, G. gymnota, G. nigripenne, K. stewarti, N.* segmentatus and *N. yaldwyni*, which could not be displayed individually due to high overlap. Species names are abbreviated by the first letter of the genus followed by the first letter of the species name.

Strong overlap in microposition use was apparent for the majority of species, with only five species being separated out by the Correspondence Analysis (Figure 7, Table 5). These five species (*N. caerulepunctus, K. stewarti, O. maryannae, R. decemdigitatus* and *R. whero*) were separated from the rest due to their exclusive or predominant use of a few micropositions. In particular, *N. caerulepunctus* was removed because of its almost exclusive use of the microposition 'under overhangs' (UOV). This microposition was also used, though only to a minor extent, by *N. yaldwyni, F. malcolmi, B. lesleyae, N. segmentatus* and *F. flavonigrum* (Figure 7). *Karalepis stewarti* was detached in space from all other species due to its use of the microposition 'upside down on roof or overhang' (ROV), a position also used infrequently by *C. jojettae* and *F. malcolmi* (Figure 7). *Obliquichthys maryannae was* the only species to use the microposition 'free swimming' (FRE), and was thus separated from all other species (Figure 7). Lastly, *R. decemdigitatus* and *R. whero* were separated from all other species because of their use of the microposition 'upsof the microposition 'under rocks or in cracks' (UCS, Figure 7).

Table 5: Percentage microposition use (on side and top of boulders (STB), under substratum
or in crack (UCS), on top of cobbles (TCO), on plants (ALG), under overhang (UOV), free
swimming (FRE), upside down on roof of overhang (ROV), and on mud or sand (SM)).
Micropositions > 10% are highlighted in bold

Species	STB	UCS	TCO	ALG	UOV	FRE	ROV	SM
A. oculus	0	0	0	0	0	0	0	100
B. lesleyae	99	0	0	0	1	0	0	0
C. jojettae	98	0	0	0	0	0	2	0
F. flavonigrum	84	1	0	7	4	0	0	4
F. lapillum	72	5	15	2	0	0	0	7
F. malcolmi	94	1	0	1	1	0	1	2
F. varium	87	2	9	0	0	0	0	2
G. capito	60	2	1	7	0	0	0	30
G. gymnota	67	9	24	0	0	0	0	0
G. nigripenne	64	0	0	0	0	0	0	35
K. stewarti	59	0	0	0	0	0	41	0
N. caerulepunctus	31	2	0	0	67	0	0	0
N. segmentatus	95	3	0	1	1	0	0	0
N. yaldwyni	90	6	0	0	4	0	0	0
O. maryannae	7	3	0	0	0	90	0	0
R. decemdigitatus	5	89	3	0	0	0	0	2
R. whero	40	50	4	0	0	0	0	6

The rest of the species were characterised by the use of several micropositions. The microposition 'on top or side or rocks' (STB) was the most frequently used by all species except *A. oculus*, which was exclusively found on a mixture of muddy and sandy substratum

types (SM, Figure 7). *Grahamina capito* and *G. nigripenne* also predominantly occupied muddy and sandy substratum types (SM), however, overlap with other species was high as several other species were also infrequently found in these habitats (Figure 7, Table 5). The position 'on top of cobbles' (TCO) was commonly occupied by *F. lapillum* and *G. gymnota,* and to a lesser extent by *R. decemdigitatus, R. whero, F. varium* and *G. capito* (Figure 7, Table 5).

2.3.2 Similarity between sister-species

The habitat comparison of the sister pair N. segmentatus and N. yaldwyni yielded the highest similarity of all sister-species pair comparisons (0.91), and this value is higher than 95% of all non-sister-species comparisons. This indicates that the habitat use of this sister pair is more similar than between most other non sister-species comparisons. The sister pair O. maryannae and F. malcolmi had a lower similarity coefficient than N. yaldwyni and N. segmentatus (0.84), but this similarity estimate is still higher than the 85% of all non sister-species comparisons. The similarity for the sister pair G. capito and G. gymnota was considerably lower (0.71) than the previous two pairs and only higher than 41% percent of all non sisterspecies comparisons. Likewise, the habitat comparison of the sister pair G. nigripenne and F. *lapillum* and the sister pair *R*. *decemdigitatus* and *R*. *whero* yielded lower habitat similarity coefficients of 0.70 and 0.69, respectively, and were only higher than around 35% of all other species comparisons. A Welch-test was used to determine whether the average DSE-S coefficients of the sister-species pairs (n = 5, mean = 0.75, SD = 0.11) were different from the average of all other non sister-species pair comparisons (n = 131, mean = 0.74, SD = 0.09). The result of the Welch-test was not significant (p = 0.85, $\eta^2 = 0.009$) and thus clearly fails to reject the null hypothesis. However, the power of the test to correctly reject the null hypothesis is just 6.3%. This is because there was only a trivial difference between the two sample means of similarity indices (a difference of 0.01), with the pooled sample SD (0.09) over six times as large as the mean difference. In order to implement a significance test with at least 80% power to detect a difference between means this close to one another, one would need a sample size of at least 300 distinct sister-species pairs and 10,000 non-sister-species pairs (yielding a harmonic mean group sample size of 600). However, it seems doubtful if such a hypothesis test would even be meaningful given the trivial difference in similarity coefficients between the two groups is only 0.01. Essentially, the results indicate that there is no statistical or even meaningful conceptual difference between these two groups (sisterspecies and non sister-species) of species' similarity coefficients.

The second step used a phylogenetic comparative approach, which allowed the investigation of the mode, tempo and phylogenetic association of habitat use of 17 triplefin species, while simultaneously allowing for control of phylogenetic non-independence in the data. The random model performed significantly better than the directional model of trait evolution (LR test = 1.65, p = 0.199). Thus, the null hypothesis that habitat use in New Zealand triplefin fishes proceeds as a random walk in time was accepted. The maximum likelihood value of the scaling parameter lambda fitted the data significantly better (LR test = 19.22, p < 0.0001) than when the default settings where used (= 1) and the maximum likelihood value of lambda was estimated to be 0.457. The model in which kappa was allowed to take its maximum likelihood value also fitted the model significantly better than the model with the default setting (= 1), and the maximum likelihood parameter of kappa was 0.008 (LR test = 15.71, p < 0.0001). Similarly, the maximum likelihood value of delta also described the model of habitat evolution significantly better (LR test = 21.34, p < 0.0001), with a maximum likelihood value of 5.855.

2.4 DISCUSSION

This study is the first to examine habitat use of New Zealand triplefins over most of their geographic range, and in sites other than rocky reefs. The results show that New Zealand triplefin species have diversified considerably in habitat use, with species utilising different patches within the same location. These differences were structured mainly in terms of gradients in depth and exposure, however, some species showed finer sub-partitioning of substratum types (rock and mud) and micropositions. Statistical and phylogenetic comparative analyses demonstrated that habitat divergence between sister-species was comparable to the amount of divergence observed for more distantly related species, indicating that divergence in habitat use is not related to the phylogenetic position of species.

The strong partitioning of the habitat by depth and exposure is the most significant component in structuring the New Zealand triplefin assemblage. The mean depth use of the 17 triplefin species ranged from the shallow subtidal (*B. lesleyae*) to a depth of around 18 m (*N. caerulepunctus*), and is broadly consistent with previous work on New Zealand triplefins (Handford 1979; Thompson 1979; Syms 1995; Feary and Clements 2006). The only

noteworthy exception to this is *A. oculus*, which has been recorded from depths in excess off 200 m (Fricke 1994). However, the use of UVC as a sampling method precludes the census of deep-water habitats > 40 m, and as a result, the mean depth estimates of *A. oculus* were severely biased. Interspecific differences in the exposure of habitats were also extensive, and ranged from sheltered (*G. nigripenne*) to highly exposed (*K. stewarti*) open-water coasts. This is supported by previous work that has shown that the level of wave exposure affects the composition and the relative densities of New Zealand triplefin assemblages (Syms 1995; Feary and Clements 2006), though the exposure estimates used in these studies were based on subjective scales making direct comparisons with the current study difficult. In this study, three clusters were apparent that classify triplefin species according to mean habitat depth and exposure. The dispersion of closely related species across the three distinct clusters indicates that interspecific divergence in depth and exposure is extensive in this assemblage (two out of the four of sister-species pairs were found in different clusters), and all three clusters were made up of species from different genera.

Species differed far less in substratum use compared to the pronounced differences seen in depth and exposure. Rock was the most important component of triplefin habitat, which has also been shown in previous studies on F. varium (Thompson 1979). The only species in which rock did not form the main habitat component was A. oculus, which is the only triplefin species in New Zealand coastal waters that does not belong to an endemic genus (Fricke 1994). The amount of rock cover is positively correlated with the physical complexity of the habitat (Connell and Jones 1991), and this is associated with higher abundance in both New Zealand triplefins (Handford 1979; Thompson 1979; Syms 1995) and Mediterranean blennioids (Macpherson 1994). Some authors have suggested that substratum complexity can positively affect fish density through a number of mechanisms, but in particular by increasing shelter opportunities (La Mesa and Vacchi 2005). Therefore, it seems likely that the use of rock as a habitat component is related to the degree of protection required by triplefins to shelter from waves and hide from predators. Furthermore, many species exclusively use rock as a nesting substrate during the reproductive season (Thompson 1986), making the presence of rock in the habitat a necessity during spawning. Cobble and gravel were used to a much lesser extent, indicating that these substratum types are less crucial as a habitat component. Sand and mud were used to a high degree by some species, in particular the habitat of A. oculus, G. nigripenne, and G. capito consisted of more than 50% sand and mud. Grahamina *nigripenne* is the only triplefin species that inhabits estuaries (Clements 2003), thus the high quantity of sand and mud in the habitat of this species appears to be correlated with the

estuarine environment. The high amount of sand and mud in the habitat of *G. capito* is presumably a result of living in shallow and sheltered areas, as the accumulation of sediments is enhanced in these areas (Thorman 1986).

The Correspondence Analysis showed that five species were clearly distinct in their pattern of microposition use, which is indicative of a reduction in spatial overlap between species. Obliquichthys maryannae were generally found swimming in the water column (FRE), N. caerulepunctus were mainly associated with the underneath of overhangs (UOV), K. stewarti were virtually the only species found to orientate upside down on overhangs (ROV), and R. where and R. decemdigitatus used cracks or the underside of rocks to a significant degree (UCS). Syms (1995) reported that R. whero adults commonly occupied the microposition 'on top of cobbles' and sometimes the microposition 'under cobbles', while this study found that R. where adults mainly occupied the 'horizontal and vertical cracks of rocks' (UCS). The remainder of the species in the current study used a combination of micropositions, though the top and side of rocks was a common microposition for virtually all of these species. Syms (1995) found that F. varium was mainly found on 'top of cobbles' (TCO), however, in this study F. varium was predominantly observed 'on the side and top of boulders'. Differences in microposition use between the studies may reflect a degree of intraspecific variation between populations at different locations, as the current study censused populations across a wide latitudinal range, whereas Syms (1995) work was solely conducted in the Hauraki Gulf. The partitioning of microhabitats between ecologically similar reef fish species has been well documented (Bean et al. 2002), with a large range of fishes either actively selecting specific microhabitats at settlement (Carr 1991) or emigrating into them at juvenile or adult stages (Lewis 1997). Such preferences for distinct microhabitats may represent a survival advantage to fishes, mediating the effects of predation or competition (Forrester and Steele 2000), providing greater food resources (Clarke 1992), or nesting habitats (Koppel 1988). In this sense, interspecific differences in triplefin microposition use are likely to represent adaptive strategies that allow these fishes to increase their fitness.

The broad spread of species in the MDS plot of overall habitat similarity is another indication that habitat divergence has occurred. Specifically, habitat similarity among closely related species was not particularly strong as there was no close association between sister-species pairs and position in multivariate ecological space. For example, although the sister-species pair *N. segmentatus* and *N. yaldwyni* were closely positioned in ecological space, they were no more ecologically similar than some distantly related species pairs such as *C. jojettae* and *N. yaldwyni*. Even the sister-species pair of *R. decemdigitatus* and *R. whero*, which has the

least amount of genetic divergence of any species in the New Zealand triplefin assemblage (Hickey and Clements 2005), showed significant divergence in habitat use, indicating that ecological divergence has occurred at a relatively rapid rate. Thus, the relationship between ecological similarity and phylogenetic relatedness is very weak. These findings have implications for ecological interactions between species. It has long been suggested that the strength of ecological interactions among species may be correlated with the degree of evolutionary relationship (Hutchinson 1965), but in this triplefin system interspecific interactions, such as competition for space, may be as intense between distantly related species as between closely related species. The large MDS distances in ecological space between closely related triplefin species parallels the pattern in Caribbean *Anolis* lizards, which display a similar tendency for ecological dissimilarity among closely related species (Losos et al. 1998).

The weak relationship between phylogeny and habitat use was further confirmed by the statistical analysis of habitat similarity between sister-species and non sister-species. The phylogenetic comparative approach was used to analyse the habitat similarity data in a phylogenetic context while simultaneously controlling for shared ancestry. The results of the phylogenetic comparative analysis demonstrated that the random model better explained the evolution of habitat use than the directional model. This indicates that habitat use in New Zealand triplefin fishes has evolved in no particular sequence or direction. Furthermore, all three scaling parameters (λ , κ and δ) were significantly different from their default values (= 1). Specifically, the maximum likelihood value of lambda was around 0.457, which indicates that phylogeny can only explain the evolution of habitat use to some degree, and this indicates that selection has contributed to the divergence in triplefin habitat use. This suggests that the habitat traits are to a large degree evolving among the species as if they were independent (i.e. as in star-like phylogenies). It has been suggested that virtually all traits associated with a species should in one way or another be reflected in the phylogenetic history, either through direct descent or because of the fact that closely related species have a tendency to resemble each other (Price 1997). However, numerous real and theoretical circumstances have been identified in which distantly related species resemble each other more than by chance alone. In particular, selection (natural or sexual) on certain traits may drive phenotypes away from the pattern expected by hierarchical relationships of the underlying tree topology (Price 1997). For example, convergent evolution on traits can cause apparent similarity in phenotypes that is not due to common ancestry (Wake 1991). Moreover, character displacement in sympatry has long been suggested to drive phenotypic norms of sister-species apart (Losos 2000),

resulting in closely-related species that differ more than expected on the basis of the phylogeny. Thus, it appears possible that selection has affected the evolution of triplefin habitat use, and as a consequence habitat parameters would be unlikely to reflect their phylogenetic trajectory. Kappa was estimated to be close to zero, thereby indicating that habitat use is independent of individual phylogenetic branch lengths (which is consistent with punctuated evolution) (Pagel 1997; Pagel 1999). This suggests that habitat evolution in New Zealand triplefin species has not proceeded in a gradual manner but in rapid bursts triggered by speciation (Bokma 2002). Considerable cladogenetic change may occur when, upon reproductive isolation, differential selection regimes dissipate the phenotypes of populations that were until then prevented from adaptation to local conditions by gene flow (Lande 1980). However, a similar scenario could be caused by disruptive selection on spatial resource use in sympatry, particularly, when the resource shift also affects other life history traits. For example, a shift in triplefin depth use would necessarily also affect many other ecological and physiological traits and thus may have the potential to lead to rapid evolutionary change over short time scales. As outlined in the Introduction of this Chapter, spatial resource shifts induced by disruptive selection could also easily lead to reproductive isolation in New Zealand triplefin fishes, as habitat and mate choice are coupled. Thus, it seems plausible that both scenarios, either allopatry or sympatry, have the potential to lead to a pattern of punctuated equilibrium in trait evolution. The phase of stasis in-between the rapid bursts has been explained as the inertia of natural populations to respond to differential selection (Bokma 2002), which can equally be applied to allopatric and sympatric populations. Lastly, the scaling parameter delta was estimated to be much greater than 1, which indicates that most speciation events have occurred towards the tips of the phylogenetic tree. This pattern indicates species-specific adaptations and is consistent with fine-scale habitat differences. However, it should be noted that an accelerated rate of speciation events near the root of the tree are thought to be indicative of adaptive radiations. The reason for the absence of such a pattern in the habitat use data is not clear and requires further investigation. It is possible that the calculation of delta itself is problematic, as has been suggested by Freckleton (2002).

In summary, the findings of this study suggest that New Zealand triplefins have diverged significantly in habitat use, with species occupying different habitat patches in the same general area. Even closely related species such as *F. lapillum* and *G. nigripenne* and *R. decemdigitatus* and *R. whero* showed considerable divergence in habitat use. Given that most New Zealand triplefin species occur sympatrically around New Zealand's coastline (Paulin and Roberts 1992; Fricke 1994; Clements 2003) and that there is no evidence of vicariant

barriers to gene flow (Hickey 2004), it is possible that selection of alternative habitats has been involved in the diversification of these fishes. Divergence in habitat use was not dependent on phylogeny and may be indicative of selection favouring habitat divergence. Together, this work supports recent studies that invoke a general role of ecology in the diversification and speciation of animals (Rundle and Nosil 2005; Funk et al. 2006).

3 Do Triplefin Fishes Show Geographic Variation in Habitat use?

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3.1 INTRODUCTION

Many species have wide ranging distributions and thus are subjected to strong biogeographic gradients, particularly at the limit of their distributional range (Rosenzweig 1995; Hubbell 2001). As a consequence, different populations of a species may experience selection in opposing directions, which creates an opportunity for the generation and maintenance of phenotypic variation between them (Endler 1977; Travis 1996). Phenotypic differences between populations have been shown in a wide variety of species (e.g. Gould and Johnston 1972; Trussell and Etter 2001; Cox and Moore 2005), and may over time lead to the splitting of a sub-divided species into reproductively isolated units in spite of migration (Endler 1977).

In marine species with high fecundity, the prevalence of planktonic larvae has caused local selection to be neglected as a potential source of variation. In particular, many marine fishes have pelagic larvae that can disperse over long distances, resulting in adult populations that are distributed across a wide variety of environments (Palumbi 1994; Warner 1997; Kinlan et al. 2005). This high dispersal may result in mild and uniform genetic differentiation between populations over large spatial scales, thus compromising their ability to adapt to local conditions (Palumbi 1994). However, growing evidence highlights that a pelagic larval phase does not necessarily result in even recruitment among sub-populations (Shulman and Bermingham 1995; Parsons 1996; Swearer et al. 1999; Riginos and Nachman 2001; Swearer et al. 2002; Taylor and Hellberg 2003). Larval exchange between populations depends on a number of factors, including physical oceanographic factors and the behavioural and physiological capabilities of larvae (Taylor and Hellberg 2003) as well as larval survival and the habitat availability at the locations (Bradbury and Snelgrove 2001; Kinlan et al. 2005).

The purpose of this Chapter is to investigate the habitat use of New Zealand triplefin populations across a latitudinal and biogeographical gradient to assess geographic variation in this endemic group. Previous work has shown that New Zealand triplefin species use species-specific habitats (Syms 1995; Feary and Clements 2006, Chapter 2), but data on the consistency of this pattern around coastal New Zealand are lacking. New Zealand's temperate reefs occur as far north as the Three Kings Islands (34°S latitude) and as far south as Campbell Island (52°S latitude), imposing a strong biogeographic gradient in environmental conditions, which is reflected in the abundance and distribution of many New Zealand reef fishes (Paulin and Roberts 1992; Francis 1996; Brook 2002). Most coastal New Zealand fishes are either distinctly northern or southern in distribution, with few species equally abundant throughout New Zealand (Paulin and Roberts 1992; Francis 1996; Francis and

Nelson 2003). Triplefin fishes form an exception, with 23 of the 26 New Zealand triplefin species being sympatric all around coastal New Zealand, and no species are known to display latitudinal trends in abundance (Paulin and Roberts 1992; Fricke 1994; Francis 2001; Clements 2003). The three species for which the distribution is not sympatric with the rest of the New Zealand triplefin assemblage are *E. kermadecensis, A. oculus* and *M. bathytaton* (Fricke 1994). The wide ranging distribution of all New Zealand triplefin species is presumably related to the wide (Hickford and Schiel 2003) and long larval dispersal phase, with estimates ranging between 2-3 months (Kingsford and Choat 1989; McDermott and Shima 2006). Given the wide distribution of triplefin fishes in New Zealand and the environmentally diverse coastline (Heath 1985; Brook 2002; Francis and Nelson 2003), it becomes apparent that triplefin populations are exposed to biogeographic gradients that provide the potential for geographic variation.

The overall aim of this Chapter is to determine if, and to what extent, habitat use of New Zealand triplefin fishes is consistent across biogeographical scales along a latitudinal gradient from 35°50'S to 46°70'S. The first objective was to describe the triplefin assemblage composition and habitat availability at different locations, and to examine the habitat associations of populations of the same species exposed to different biogeographic gradients. The second objective was to try to account for the effect of local habitat characteristics on triplefin habitat use by partialling out the habitat effects from the location. This approach identifies whether populations have diverged in habitat use, or whether the presence of habitat types at particular locations limits or enhances the relative abundance of species. Strongly divergent habitat characteristics between populations of the same species were interpreted as evidence for geographic gradients was seen as evidence for consistent habitat selection by a species.

3.2 MATERIALS AND METHODS

3.2.1 Data collection

The habitat use of triplefin species was quantified at nine locations around coastal New Zealand from 2002 to 2004 (Figure 8). The habitat measures ranged from large between-site (e.g. exposure) to intermediate within-site scale (e.g. depth) and to fine-scale microhabitat characteristics (substratum types and fish microposition).



Figure 8: Location of study areas in New Zealand. \Box Three Kings Islands; \triangle Coromandel Peninsula; \clubsuit Wellington; \bullet Exposed offshore Hauraki Gulf; \bullet Mainland Hauraki Gulf; \bigcirc Sheltered Hauraki Gulf; \bigtriangledown Napier; \diamondsuit Fiordland; \bigcirc Stewart Island.

The locations covered a geographic range from 35°50'S to 46°70'S and included the Three Kings Islands (35°50'S, 172°10'E), the Coromandel Peninsula (36°29'S, 175°19'E), exposed offshore islands in the Hauraki Gulf (36°94'S, 174°57'E), sheltered Hauraki Gulf (36°70'S, 175°68'E), mainland Hauraki Gulf (36°32'S, 174°51'E), Napier (39°29'S, 176°55'E), Wellington (41°16'S, 174°51'E), Fiordland (45°30'S, 167°00'E) and Stewart Island (46°70'S, 168°20'E). The characteristic features of the locations have been described in detail in Chapter 2.

Within each location 4 x 4 m UVC were conducted at randomly selected sites with the aim of sampling as much of the exposure gradient as was practically possible. All UVC were done by the same diver and consisted of a close, rigorous and systematic searching pattern, spending at least 1 min on each quadrat (1 x 1 m), with all interstices and overhangs examined to ensure a complete census. Because all sites were surveyed by the same observer, the data were comparable and could be used in the analyses. Prior to sampling, a location fix was taken for each site using a handheld Garmin® 12 global positioning system (accuracy \pm 15 m) and a physically derived exposure index was calculated based on the total sum of the fetch (maximum radial distance 300 km). Fetch calculations were performed with the program 'Fetch Effect Analysis' (version 1.01. Pickard R 2000), which measures fetch distance for each 20 degree sector on a compass rose from a given point (Thomas 1986).

At least three 4 x 4 m quadrats were laid out on each site. The first quadrat at each site was done at the deepest depth that could safely be sampled, and the two subsequent quadrats at approximately 33% and 66% of the deepest depth. Any additional quadrats were conducted in intermediate depths. This design was employed to allow sampling flexibility throughout sampling locations. A minimum distance of approximately 50 m between quadrats was maintained to avoid dependent samples (Andrew and Mapstone 1987). The centre line of each quadrat was marked with a leaded line, and a steel quadrat used to outline each 1 m² along the quadrat. For each 1 m² quadrat the depth was recorded and eleven habitat variables estimated visually as percent cover of the substratum: rock (rocks > 7 cm); horizontal rock face (rocks > 7 cm); vertical rock face (rocks > 7 cm); cobbles (rocks < 7 cm); gravel (rocks < 4 cm); sand; mud; *Ecklonia radiata; Carpophyllum* spp.; other macroalgae; and coralline and turfing algae. While the first seven variables always sum to 100%, algal coverage could range from 0 - 100%.

The fish within each quadrat were identified. Triplefin species that were found in at least two locations were analysed and included *B. lesleyae* (n = 176), *C. jojettae* (n = 235), *F.*

flavonigrum (n = 942), F. lapillum (n = 3886), F. malcolmi (n = 346), F. varium (n = 2102), G. capito (n = 879), G. nigripenne (n = 337), K. stewarti (n = 75), O. maryannae (n = 1495), N. segmentatus (n = 2328), N. yaldwyni (n = 453), N. caerulepunctus (n = 245), R. decemdigitatus (n = 232) and R. whero (n = 1644).

3.2.2 Data analysis

Fish and habitat variables were initially examined for excessive skew and bivariate nonlinearities. Square-root transformations were found to decouple variance-mean relationships and improve bivariate linearity for both fish and habitat measures, so this transformation was used across all habitat variables. Analysis of abiotic (e.g. substratum type, depth, exposure) and biotic habitat (e.g. macroalgal cover) was carried out by Canonical Discriminant Analysis (CDA) of the variables using the location as the classification variable, in combination with summary graphs of mean habitat types across localities. The appropriateness of the constrained (by location) analysis was checked by comparing CDA results with a Principal Components Analysis of the same data. The dominant signals in the data were associated with location differences, so the constrained CDA ordination was used to display habitat differences between locations.

Habitats differed between locations, which presented a problem in quantifying triplefin assemblages and habitat associations independent of habitat distributions at locations. Therefore, an approach was used based on Partial Canonical Correlation to independently measure the relative effects of continuous habitat variables and categorical location differences on triplefin assemblages (see for a related example Borcard et al. 1992). One computational difficulty with this approach was that location was a categorical variable, and could not be used either as a partial or a correlation variable in the software (SAS 9.1, PROC CANCORR). This problem was resolved by recoding location as a set of effects-coding variables, which replaced a single categorical variable with a set of n-1 variables. These variables were assigned a value of 1 if the sample came from the location associated with the new variable or zero otherwise - except for the last location category which was assigned a value of -1 for each variable. This was necessary to avoid linear dependency of the variables. This approach is implicitly used in most General Linear Model software (e.g. Littell et al. 2002), and a Canonical Correlation on variables coded in this way is indeed mathematically equivalent to a CDA. Two Canonical Correlations were carried out: (i) the correlation of triplefins with habitat variables after partialling location effects (standard partial Canonical

Correlation), and (ii) the correlation of triplefins with the multiple location variables, after partialling habitat effects (equivalent to a partial CDA).

3.3 RESULTS

Most locations contained mixed amounts of hard and mobile substrata such as rocks, cobbles and gravel, and soft sediments like sand and mud (Figure 9).



Figure 9: Abiotic habitat composition at different biogeographic locations in New Zealand. Hard substratum classes are depicted with black shading, mobile hard substratum with grey shading, and soft sediment classes with white shading.

Similarly, most locations contained varying amounts of coralline and turfing algae as well as brown macroalgal types (Figure 10). In combination, however, relative abundances of both biotic and abiotic habitat types were typical of particular locations with no clear correlation with latitude. For example, the Three Kings Islands, beyond the northern tip of mainland New Zealand (Figure 8), were similar in habitat structure to exposed sites of the Hauraki Gulf in northeastern and Stewart Island in southern New Zealand (Figure 10). These exposed locations were characterised by a combination of high density of *Ecklonia radiata*, and coralline and turfing algae (Figure 10), and hard substratum categories such as rock, vertical rock faces, and horizontal rock faces (Figure 9). Some deep and sheltered sites at Stewart Island, however, differed from the Three Kings Islands and exposed Hauraki Gulf sites, in being dominated by mud and sand (Figure 9).



Figure 10: Biotic habitat composition at different biogeographic locations in New Zealand. The coralline category includes encrusting and turfing forms.

In contrast, sheltered sites in the Hauraki Gulf were more similar to sheltered sites in Wellington and Napier and characterised by shallow depths, *Carpophyllum* spp. (Figure 10, Figure 11), and soft and mobile benthic substrata such as gravel, cobble and mud (Figure 9). Fiordland sites were unique in their habitat structure in that the inner fiords were characterised by deep and sheltered basins that were covered with mud and little *Ecklonia radiata* cover (Figure 11), although shallow vertical rock faces were present. Coralline and turfing algae were either numerically dominant or equally abundant to brown algal cover across most exposed sites, with sheltered sites such as Napier characterised by the brown algae

Carpophyllum spp., and the Coromandel sites dominated by the brown alga *Ecklonia radiata* (Figure 10).



Figure 11: Canonical Discriminant Analysis of habitat variables among biogeographic locations in New Zealand. \Box Three Kings Islands; \triangle Coromandel Peninsula; Wellington; Exposed offshore Hauraki Gulf; Mainland Hauraki Gulf; \bigcirc Sheltered Hauraki Gulf; \bigtriangledown Napier; Fiordland; \bigcirc Stewart Island. Habitat vectors are structure coefficients, multiplied by 2 to improve clarity.

Different locations contained different proportions of triplefin species (Figure 12). However, as proportions of biotic and abiotic variables differed between locations it was important to distinguish between differences in triplefin assemblage due to habitat versus other intrinsic and perhaps unmeasured location differences. When location effects were statistically partialled from habitat, characteristic species-habitat associations were evident (Figure 12). *Forsterygion lapillum* and *R. decemdigitatus* were associated with shallow cobble and *Carpophyllum* spp. habitats (Figure 12). In contrast, *N. segmentatus, R. whero*, and *N. yaldwyni* were associated with *Ecklonia radiata*-covered rocky and exposed habitats (Figure 12). *Notoclinops caerulepunctus* and *F. malcolmi* were associated with deep rocky habitats, and *F. flavonigrum* were associated with deep habitats with either rocky or soft substrata (Figure 12). *Grahamina nigripenne* and *G. capito* were primarily found on mud and sand in shallow and sheltered sites (Figure 12).



Figure 12: Partial Canonical Correlation of fish and habitat variables, after correcting for biogeographic variation. Vectors are structure coefficients of variables in the ordination space defined by the habitat variables.

Although strong associations were found between most species and habitat types independent of location, when habitat was statistically partialled from location, some locations showed differences in the abundance of triplefin species that could not be explained by habitat alone, indicating that habitat was not the sole predictor of the assemblage composition (Figure 13). Three Partial Canonical Discriminant Axes, each explaining approximately equal amounts of variation, identified three location-specific differences in triplefin assemblage composition that were not accounted for by habitat variables. On the first axis, Napier, Wellington, and some quadrats in the Coromandel locations had greater proportions of *F. varium*, *R. decemdigitatus*, and *F. malcolmi* than would be predicted by habitat alone compared to other locations (Figure 13a), however these differences were generally due to small changes in density of these species (Figure 12). The second axis reflected differences between the Three Kings Islands and the Offshore and mainland Hauraki Gulf locations (Figure 13a).



Figure 13: Partial Canonical Discriminant Analysis of triplefin species among biogeographic locations in New Zealand, after correcting for habitat differences. \Box Three Kings Islands; \triangle Coromandel Peninsula; \clubsuit Wellington; \bullet Exposed offshore Hauraki Gulf; \bullet Mainland Hauraki Gulf; \bigcirc Sheltered Hauraki Gulf; \bigtriangledown Napier; \diamondsuit Fiordland; \bigcirc Stewart Island. Species vectors are structure coefficients, multiplied by 3 to improve clarity.

The Three Kings Islands had a comparatively depauperate fauna, with relatively low fish densities and the notable absences of numerically dominant mainland Hauraki Gulf species such as *N. segmentatus* and *F. lapillum* and small but notably higher densities of *C. jojettae* (Figure 12). The third axis distinguished Fiordland from other sites due to higher densities of *G. capito*, *F. flavonigrum*, and *O. maryannae* than would be predicted by habitat alone (Figure 13b). In general, location differences that were independent of habitat were usually idiosyncratic, and occasionally due to differences in abundance of species that were relatively uncommon.

3.4 DISCUSSION

Species with wide ranging distributions frequently show geographic variation across biogeographic gradients, either as a result of local selection, variability in larval supply, or both (e.g. Endler 1977; Rosenzweig 1995; Shulman and Bermingham 1995; Parsons 1996; Riginos and Nachman 2001; Taylor and Hellberg 2003). New Zealand triplefin fishes offer an opportunity to test the effects of biogeographic gradients on habitat use patterns in a diverse group of fishes, as all species show species-specific habitat use (Syms 1995; Feary and Clements 2006) and have broad distributions around coastal New Zealand (Paulin and Roberts 1992; Fricke 1994; Francis 2001; Clements 2003). The results of this study show that the abundance of triplefin species was highly variable between locations, but that this pattern could, in most cases, be explained by local habitat availability. Therefore, the close relationship between habitat availability and triplefin abundance suggested that habitat use was consistent across locations, indicating that biogeographic gradients in a species' range do not necessarily lead to geographic variation in habitat use, in particular for species with high dispersal abilities.

Although habitat use appeared to be consistent across most locations, the abundances of a few triplefin species at some locations were lower or higher than expected given the habitat availability. Therefore, habitat availability alone did not determine abundance patterns in these species. Instead, differences in abundance were likely to be related to unmeasured characteristics of locations, such as the geographic isolation of the Three Kings Islands from the mainland and the high freshwater input in Fiordland. For example, although *F. lapillum* and *N. segmentatus* were numerically dominant species at onshore locations throughout New Zealand, both species are absent from some offshore islands such as the Three Kings Islands and the Chatham Islands (Paulin and Roberts 1992; Fricke 1994). The absence of these

species is interesting because congeners with similar habitat requirements are present at these islands (Clements 2003), suggesting that the absence was related to factors other than local habitat availability. One factor that could affect species composition of reef fishes at offshore sites is the physical isolation of, and the typically limited availability of shallow water habitat (< 30 m) around, offshore islands. These factors, either singly or in combination, can diminish the chances of larval input from coastal locations (Floeter et al. 2001). Support for this in New Zealand triplefins is provided by phylogeographic data revealing interspecific differences in levels of gene flow between mainland sites and the Three Kings Islands (Hickey 2004). It thus appears likely that the absence of some triplefin species from offshore islands is related to species-specific differences in larval dispersal and recruitment success at these locations.

The absence of some triplefin species from Fiordland may be related to the year-round surface water layer of low salinity that can be found in all inner fiords (Wing 2003). The low salinity layer is produced by the high annual rainfall (> 7000 mm yr⁻¹) in the Fiordland region, and has been identified as a dominant physical feature and important structuring factor for biological communities (Rutger and Wing 2006). The increased freshwater input leads to a decrease in invertebrate and vertebrate species diversity, which may explain the reduced recruitment success of many species in the fiord system (Smith and Witman 1999; Wing et al. 2003). In particular, the pycnocline layer, which is associated with the bottom of the low salinity layer, has been suggested to act as a physical barrier to larvae (Smith and Witman 1999). Entrainment into this layer has been considered as a significant source of mortality for larvae, propagules, or newly settled recruits that are not physiologically tolerant of lowsalinity conditions (Wing 2003). In addition, the direction and magnitude of flow in the low salinity layer may have an important influence on the flow regime just below the pycnocline, which could directly influence dispersal and recruitment at shallow depths. Larvae or propagules entrained or released within the low salinity layer would be transported towards the mouth of the fiords (Smith and Witman 1999), during which time the increased tidal stirring towards the mouth causes the freshwater to become well-mixed with the rest of the seawater (Proctor and Hadfield 1998). This suggests that some unmeasured variables, such as salinity and water flow, may have profound effects on the abundance and diversity of triplefin species in the inner fiords. It therefore appears that although habitat availability may modify the composition of fish assemblages at all locations, the offshore location of the Three Kings Islands and the freshwater layer in Fiordland generate dispersal barriers for some triplefin species, and thus are additional modifiers that contribute to assemblage composition.

Biogeographic barriers to dispersal are known to have a strong impact on the distribution of many marine species, with some of the best examples including the oceanographic barrier created by the Amazon freshwater and sediment outflow in Brazil (e.g. Rocha 2003) and the province boundary at Point Conception in California (e.g. Dawson 2001; Dawson et al. 2006). Both barriers are marked with an abrupt change in hydrology, dissolved oxygen, topography and temperature, which profoundly affects species distribution and survival. Studies on these oceanographic breaks have shown that intraspecific phylogenies are often shaped by these biogeographic barriers to gene flow, leading over time to divergent populations on different sides of the barrier (Burton and Lee 1994; Cassone et al. 2005; Rocha et al. 2005). Despite this, distinct triplefin ecotypes are absent even from the most distant offshore islands such as the Chatham Islands (Fricke 1994), although the remote location of these islands provide one of the most likely settings for such variants to evolve. This suggests that unlike the high interpopulation variability in habitat associations of many marine species with wide ranging distributions (Gross 1979; Floeter et al. 2001; Floeter et al. 2004; Bouchon-Navaro et al. 2005), gene flow in New Zealand triplefin species may be sufficient to prevent local adaptation in habitat use.

The mechanisms responsible for the general absence of geographic variation in triplefin habitat use may be related to the long pelagic dispersal phase and the settlement behaviour of larvae. Previous studies have demonstrated that larvae of some New Zealand triplefin species are capable of wide dispersal, with the pelagic larval duration being estimated to last between 2-3 months (Kingsford and Choat 1989; McDermott and Shima 2006). There is also increasing evidence that some pre-settlement reef fish larvae have the capability to actively swim towards suitable settlement habitat (reviewed in: Leis 2006). Specifically, studies on New Zealand triplefin fishes have shown that pre-settlement larvae are not randomly distributed in the surface waters (Tolimieri et al. 2000; Hickford and Schiel 2003), suggesting that larvae can actively influence their position during that time. In addition, work on postsettlement triplefin larvae showed that species select specific habitat types at settlement (McDermott and Shima 2006). Therefore, these studies indicate that the long dispersal phase enables gene flow between distant locations while the highly species-specific larval behaviour appears to determine the habitat associations in this group, thereby countering local adaptation in habitat use across biogeographic discontinuities.

Given that the assemblage structure of triplefin species was strongly related to the prevalent habitat types at a location, the availability of habitat may be an important determinant of spatial patterns of abundance in this group. It is noteworthy that all 15 triplefin species

examined in this study showed consistent habitat associations across locations, indicating that consistent habitat use is a general feature of this assemblage. The close association between habitat availability and triplefin assemblage composition thus indicates that the availability of suitable habitat at any given site is generally a good predictor of abundance patterns in this group. Strong species-specific and consistent habitat use across latitudinal gradients would be expected in species for which fitness trade-offs in alternative habitats are high (Rosenfeld and Boss 2001), so that particular habitats offer considerable fitness advantages for species and, therefore, individuals strongly prefer to occupy these habitats (MacArthur 1972; Munday 2001; Srinivasan 2003). In this sense, the strong species-habitat associations may be an evolved response to patterns of post-settlement mortality in sub-optimal habitats (Keough and Downes 1982). Availability of suitable habitat predicts spatial patterns in the damselfish Dascyllus aruanus at several spatial scales, and similar correlations were also found for four other species of reef-associated fish (Hoolbrook et al. 2004), suggesting that availability of suitable reef habitat can be used as a prevalent determinant of spatial patterns in some reef fishes. This underscores the importance of accurately identifying the resource requirements of species and considering the role of resource availability at a site when determining the factors that contribute to the spatial patterns of abundance in fish assemblages.

Consistent habitat use across large spatial scales suggests that the processes driving this pattern are general and relatively homogenous across the biogeographic range of the species (Morris 1987). Density-dependent processes have been suggested to be the primary mechanisms in producing consistent habitat use patterns, as density-dependence leads to predictable quantitative and qualitative differences in the abundance of species across habitats (Morris 1988). Thus, while the species-specific habitat use patterns of adult triplefins (Syms 1995; Feary and Clements 2006) may be largely due to active habitat preferences exhibited by individual fish, the overall abundance of triplefin species on reefs is presumably regulated by inter- and intraspecific density-dependent processes, such as competition and predation (e.g. Resetarits 2005; Lindberg et al. 2006). This is because although individual fish can maximise their reproductive success by choosing those habitats which convey the greatest fitness rewards, an individual's fitness is an overall function of population and resource density. Therefore, the abundance of individuals across habitats will reflect the habitat-dependent trade-off between fitness and density (Morris 1987; Morris 1988) so that as population density increases and resource abundance decreases, each habitat occupant will likely have a progressively negative effect on the available habitat space to other individuals in that habitat patch (Morris 1988). For example, Steele and Forrester (2005) found that localised habitat differences in refuge density can accurately be aggregated to describe larger-scale patterns in the bridled goby *Coryphopterus glaucofraenum*. A shortage of refuges from predation in this species causes density-dependent mortality (Forrester and Steele 2004a), and so the strength of density-dependence at small scales is sensitive to changes in the local availability of shelter sites (Steele and Forrester 2005).

The spatial consistency in triplefin habitat associations may be seen as a mechanism to reduce interspecific competition (MacArthur and Levin 1964; Wiens 1977; Schoener 1982), as geographically uniform and species-specific habitat use would reduce interspecific resource overlap between otherwise ecologically similar species. Most studies demonstrating interspecific competition among reef fishes have measured shifts in local distribution or abundance of fishes in response to competitor density (e.g. Robertson 1996; Munday 2004). For example, work on the territorial damselfish Stegastes planifrons has shown that the presence of this species limits the abundance of four ecologically similar congeners (Robertson 1996). Thus, if competition is affecting space use in New Zealand triplefin species, then it may be expected that ecologically similar species expand their habitat range in locations, such as the Three Kings Islands and Fiordland, that lack species that are common elsewhere. For example, the absence of the dominant mainland species F. lapillum and N. segmentatus from the Three Kings Islands potentially enables other triplefin species to settle into unoccupied habitat space. The results of the present study, however, showed no evidence for a significant habitat shift or increase in habitat breadth of other triplefin species at these locations, suggesting that triplefin habitat use is highly species-specific and not obviously influenced by the absence of other species. The finding that release from interspecific competition does not lead to a significant expansion into previously unoccupied habitats further strengthens the view that New Zealand triplefin species use highly species-specific habitats, and that fitness trade-offs in sub-optimal habitats are high.

In conclusion, the current study demonstrated that New Zealand triplefin species showed consistent habitat use across biogeographic gradients, but that this pattern was modified in some cases by differences in larval dispersal and recruitment success at some locations. This indicated that species composition at locations could not be explained by a single factor, but was due to the combined influences of availability of suitable habitat, geographical distance from the mainland and some unmeasured abiotic habitat variables (e.g. freshwater layer). The marked absence of geographic variation in species habitat use indicated that species select particular habitats and that dispersal is strong enough to lead to sufficient larval exchange among sub-populations, thereby preventing local adaptation in habitat use. Recognition that
behaviour can generate similar patterns of distribution and abundance at multiples scales implicates habitat selection as an important factor affecting local and regional patterns of biodiversity. Habitat selection as a process thus forms a fundamental link between the dynamics of populations at the local scale, and the regional dynamics of communities at larger scales.

4 Evolution of Habitat Specialisation in Triplefin Fishes

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4.1 INTRODUCTION

It is commonly hypothesised that adaptive radiations are characterised by a directional evolution from generalist to specialist species (Futuyma and Moreno 1988; see Schluter 2000b for a list of theoretical reasons), leading to a recognisable progression towards greater resource specialisation. However, recent studies suggests that exceptions are common in nature (e.g. Elliott et al. 1999; Nosil 2002; Morse and Farrell 2005; Nosil and Mooers 2005). Schluter (2000b) reviewed the phylogenetic evidence for resource specialisation in a broad range of taxonomic groups, and found little support for a trend towards increasing resource specialisation. Instead, the results suggested that the founders of radiations were frequently specialist species that gave rise to both specialist and generalist descendants (Schluter 2000b). Thus, although the generalist-to-specialist theory has a wide acceptance and strong theoretical basis (e.g. Futuyma and Moreno 1988; Berenbaum 1996; Kelley and Farell 1998), these recent studies question its generality.

Since the degree of specialisation is commonly believed to be the result of adaptive processes (Futuyma and Moreno 1988), it is important to take the phylogenetic relationships into account when investigating generalist-specialist theories (Harvey and Pagel 1991; Irschick et al. 2005). This allows one to discriminate between the relative effects of past (phylogenetic) and present-day (ecological) influences (Futuyma and Moreno 1988). Despite the frequent use of phylogenetic methods in the study of ecological specialisation, very few studies have considered the potential impact of the method with which species have been assigned a degree of specialisation. The vast majority of studies have coded character traits as discrete entities (i.e. specialist and generalist species) when investigating the evolution of habitat specialisation (e.g. Nosil 2002). Stephens and Wiens (2003) used discrete and continuous coding methods to analyse emydid turtle evolution and found that the use of different coding methods can have profound effects on the outcome of phylogenetic tests. Specifically, they suggested that the use of continuous coding methods may have lead to the recent exceptions to the generalist-to-specialist hypothesis (Stephens and Wiens 2003). This highlights that a classification of species into discrete groups can be problematic, as ecological traits typically vary continuously within and between species and thus a discrete coding method is likely to fail to detect fine scale differences between species.

To test the generalist-to-specialist hypothesis in a phylogenetic context, the ideal study group would have the following attributes: one that is speciose; one in which the species have detailed descriptive ecological studies; and one which has diversified along a resource axis. The New Zealand triplefin fish fauna was used as a model system to investigate the evolution of habitat breadth on a continuous scale, as this group of small marine fishes conforms to all of these criteria. New Zealand triplefin fishes have the greatest diversity and disparity in the world with 26 endemic species (Clements 2003). Most species are sympatric throughout coastal New Zealand, and occur from the North Cape to Stewart Island, showing no obvious latitudinal trends in abundance (Fricke 1994). Previous work has indicated that there has been little specialisation in diet taxa and jaw morphology (Feary 2001), but considerable diversification in physiology (Brix et al. 1999; Hickey and Clements 2003) and habitat (Syms 1995; Feary and Clements 2006). New Zealand triplefin species are ideal candidates to study specialisation in habitat use because new recruits occupy similar habitat to those occupied by conspecific adults, thus suggesting active habitat selection at settlement (McDermott and Shima 2006). Furthermore, triplefins are extremely philopatric and individuals occupy the same small ($\approx 1 - 2 \text{ m}^2$) habitat territory for their entire life (Thompson 1979; Clements 2003). The intense territoriality and site attachment of triplefins is further illustrated by homing behaviour; individuals of F. varium, F. lapillum and B. lesleyae will home up to 800 m if displaced (Thompson 1983; Fisher 1998). Given this extreme philopatry and temporal stability in habitat use, habitat use can be effectively estimated in a single encounter with an adult individual. Although New Zealand triplefin species are fairly similar in morphological traits, closely related species show considerable interspecific variation in body size (Paulin and Roberts 1992; Fricke 1994). Differences in body size affect many physiological and ecological processes, and subsequently several studies have linked body size to resource specialisation (Peters 1983). Given the large size differences between closely related triplefin species in New Zealand, it is possible that body size is related to resource use patterns.

A combination of ecological and phylogenetic information was used to analyse the evolution of habitat specialisation in the New Zealand triplefin fauna. First, it was aimed to test if species differ in habitat specialisation. Estimates of habitat specialisation were obtained with a novel statistical method that calculated the overlap between individuals in the quantitative use of habitat resources. Second, the evolution of habitat specialisation was investigated in detail using a comparative Bayesian phylogenetic method. Specifically, it was tested whether the evolution of habitat specialisation shows a directional trend (i.e. from generalised to more specialised species), the evolutionary mode, tempo, and phylogenetic association of the evolution of habitat specialisation are consistent with the Brownian motion model of trait evolution, and whether there is a correlation between body size and the degree of specialisation.

4.2 MATERIALS AND METHODS

4.2.1 Data collection

The habitat use of triplefin species was recorded quantitatively at several sites within the Hauraki Gulf (36°36'S, 174°50'E) in northeastern New Zealand from January 2002 to May 2005 using UVC (Figure 14). The habitat measures ranged from large between-site (e.g. exposure) to intermediate within-site scale (e.g. depth) and to fine-scale microhabitat characteristics (substratum types). Species for which sufficient habitat data (> 10 observations) could be recorded included B. lesleyae, C. jojettae, F. flavonigrum, F. lapillum, F. malcolmi, F. varium, G. capito, G. nigripenne, K. stewarti, O. maryannae, N. caerulepunctus, N. segmentatus, N. yaldwyni, R. decemdigitatus and R. whero. The Hauraki Gulf was chosen as a study site as it is a large area with a wide variety of accessible habitats, enabling the quantification of habitat characteristics of a number of triplefin species over a range of exposures and depths at coastal and offshore sites (Figure 14). At least three 4 x 4 m UVC were laid out at each site sampled (total number of sites 33, UVC 155), with the first UVC sampled at the deepest depth that could be safely reached (maximum depth dived 36 m), and the two subsequent UVC sampled at approximately 33% and 66% of the deepest depth. Any additional UVC were conducted at intermediate depths. This design allowed sampling flexibility across a wide variety of habitats. A minimum distance of approximately 50 m between UVC was maintained to eliminate the chance of obtaining dependent samples.

Prior to sampling a location fix was taken for each site using a handheld Garmin® 12 global positioning system (GPS, accuracy \pm 15 m). From this GPS information a physically derived exposure index (fetch) could be calculated based on the total sum of the fetch (radial distance 300 km) for each 20 degree sector on a compass rose using the program 'Fetch Effect Analysis' (version 1.01. Pickard R 2000).

The centre line of the 4 x4 m UVC was marked with a leaded line, and a steel quadrat was used to outline each 1 m² within the UVC sampling area (this sums to 16 x 1 m² quadrats for each UVC). For each 1 x 1 m quadrat the identity and number of triplefin fishes and the depth (m), exposure (km) and coverage of the substratum was recorded. Variables that were measured during the substratum quantification included rock [rocks > 7 cm], cobbles [rocks < 7 cm], gravel [rocks < 4 cm], sand, mud, macroalgae and coralline and turfing algae. While the first five substratum variables always sum to 100%, the algal coverage could range from 0 - 100%.



Figure 14: Sites surveyed at the Hauraki Gulf from 2002-2005. Each circle indicates a site.

4.2.2 Data analysis

4.2.2.1 Analysis of habitat specialisation

The habitat breadth was assessed using an approach which examined the similarity of habitat use in the 1×1 m quadrat area between all individuals of a species. The logic here is that the

habitat breadth of a species may be indexed by a measure of agreement taken over measures of habitat usage, and computed between all individuals of a species (see Table 6). Whilst the logic may be sound, there is an inherent problem encountered with such an approach, namely that the measurement range of habitat variables which are measured using different magnitude scales may distort the value of an agreement index. To eliminate the problem of differing variable ranges, and so that the distance measure might be expressed in a convenient unit (0 -1) metric and as an agreement measure, the double-scaled Euclidean distance measure was utilised.

Essentially, this method calculated the squared discrepancy between two individuals on a variable and then divided this value by the maximum possible squared discrepancy for that variable. Summing and taking the square root of these 'scaled' discrepancies across habitat variables yielded a scaled Euclidean distance. The metric of this scaled and cumulatively summed variable discrepancy distance varied between 0 and some value greater than 1. Therefore, a further scaling operation was carried out in order to scale this coefficient into a unit metric (0 to 1) by dividing the initially scaled Euclidean distance by the square-root of the number of variables comprising the distance computation. This second scaling produced a coefficient which always varied between 0 (no distance between variables) to 1 (maximum possible distance between variables given the designated maximum and minimum values for each variable). This dual scaling ensured that the coefficient was comparable as different variable magnitudes might otherwise distort a conventional Euclidean distance. Finally, in order to complete the process, the double-scaled Euclidean distance was expressed as a similarity index by subtracting it from 1, thus yielding the double-scaled Euclidean similarity (DSE-S) index which was used as a proxy of habitat specialisation. A value of 0 for this coefficient indicates that all individuals within a species use completely different habitat resources (i.e. generalist species), and 1 indicates that all individuals within a species use exactly the same magnitudes of habitat variables (i.e. specialist species).

The computer program "Habitat Diversity Analysis" was used to generate the necessary calculations, and construct the coefficient distribution percentiles to provide the mean and interquartile range of agreement within each species. It was decided to display 50% of all cases located around the mean quartile, which is the difference between the 75th and 25th quartiles. The quartiles were computed from the vector of comparison coefficients constructed by comparing each member of a species to every other member of that species (across all habitat variables). It should be noted that the DSE-S values cannot be used for statistical

significance testing, because the DSE-S values are derived by comparing each observation with all other observations and thus does not present a vector of independent observations.

The distinction between species with a wide (generalist species) and narrow habitat breadth (specialist species) was based on the mean habitat specialisation index, however, it should be noted that this distinction was done in relative terms. The degree of habitat specialisation was assessed by calculating the extent of habitat specialisation in depth and exposure, macrohabitat use $(1 \times 1 \text{ m})$ and lastly all habitat variables together. While the first two analyses were conducted to separately examine the degree of specialisation in the use of depth and exposure and the substratum of the habitat, the phylogenetic analysis was done using the combined specialisation index for all habitat variables, as the aim was to investigate the evolution of the overall degree of habitat specialisation in the fauna.

4.2.2.2 Evolution of habitat specialisation

Closely related species are more likely to share similar ecological features due to common ancestry, therefore data for related species cannot be considered as independent points in comparative studies (Harvey and Pagel 1991). For this reason, a phylogenetic comparative method was employed to assess the extent to which habitat specialisation is related interspecifically in 15 triplefin species (Figure 15).

The phylogenetic data were treated as explained in Chapter 2. The sister-species pair *R. whero* and *R. decemdigitatus* was specified as the outgroup following the topology of Hickey and Clements (2005). Trees were generated for 10 million generations, with sampling every 20,000 generations, and the first 20% were discarded as 'burn-in' (Figure 15).

The ecological habitat dataset consisted of the habitat specialisation index of the overall habitat use and maximum body length data (standard length) for each of the 15 triplefin species. Data on triplefin body length were taken from specimens at the Museum of New Zealand, Te Papa Tongarewa (Table 6). The evolution of habitat specialisation was analysed using the generalised least squares model implemented in the program BayesContinuous (Pagel and Meade 2004; Pagel et al. 2004), using the same framework as described in Chapter 2.



Figure 15: Phylogeny of the 15 triplefin species used for the comparative analyses of habitat specialisation. The scale for branch lengths is given in the bottom left corner (number of bp substitutions per site).

Table 6: Number of triplefin individuals per species analysed to compute habitat specialisation and the total length of species. The first column shows the species observed in the Hauraki Gulf, the second column the number of observations per species, the third column the number of comparisons that were run in the HDA-1 program to calculate the DSE-S coefficients and the fourth column the maximum body size (mm) of species. The maximum size estimates were obtained from the Museum of New Zealand, Te Papa Tongarewa (courtesy of Andrew Stewart). Superscripts denote sister-species pairs (Hickey and Clements 2005).

Species	Number of individuals	Number of comparisons	Maximum (SL)
F. lapillum ¹	99	4851	65
G. nigripenne ¹	66	2145	55
F. malcolmi ²	247	30381	53
O. maryannae ²	2865	4102680	71
R. decemdigitatus ³	80	3160	123
$R. whero^3$	830	344035	125
B. lesleyae	178	15753	91
C. jojettae	152	11476	88
F. flavonigrum	35	595	127
F. varium	158	12403	38
G. capito	1894	1792671	49
K. stewarti	334	55611	53
N. caerulepunctus	596	177310	60
N. segmentatus	79	3081	120
N. yaldwyni	1371	939135	81

The first hypothesise assessed whether there was a directional trend in the evolution of habitat specialisation (directional vs random model). It should be noted that under the random model the ancestral state estimate is predicted to fall somewhere within the range of observed values in the species data (Pagel 1999). In contrast, the ancestral character state estimate under a directional model can lie outside of the range of observed values in the data (Pagel 1999). A LR test was used to test whether the directional model fitted the data better than the simpler non-directional model. The second hypothesis assessed whether the three scaling parameters lambda (λ), kappa (κ) and delta (δ) assume a maximum likelihood value that fits the data significantly better than when the parameter equals the fixed constant predicted by the model of Brownian motion (default setting in BayesContinuous for each parameter = 1). Lastly, the third hypothesis assessed whether body size and habitat specialisation were phylogenetically related. LR tests were used to measure the correlation between body size and habitat specialisation by comparing the fit of a model where character covariance was set to 0, to that of a model where both characters were allowed to co-vary. In addition, body size divergence between sister-species pairs was calculated as the absolute value of the percentage size difference between the species ([size of species 1 - size of species 2]/[(size of species 1 + size

of species 2)/2]) following Funk et al. (2006). This was done to test whether divergence in body size between sister-species pairs has been associated with habitat specialisation.

4.3 RESULTS

4.3.1 Habitat specialisation

Triplefin species varied extensively in the amount of specialisation in depth and exposure, and this was reflected in a wide range of values for the degree of habitat specialisation between species (0.77 - 1.00, Figure 16).



Figure 16: Habitat specialisation index of 15 triplefin species in the Hauraki Gulf based on specialisation in depth and exposure. The black circles show the mean habitat specialisation index and the whiskers show the lower and upper quartile points (i.e. the lower 25th percentile and upper 75th percentile).

The high habitat specialisation index of *B. lesleyae*, *G. nigripenne*, *G. capito* and *R. decemdigitatus* (0.94 - 1.00) reflected the fact that these four species had pronounced

preferences for particular depths and exposures, and thus a narrow habitat breadth in these variables. This finding was further illustrated by the low quartile values for *B. lesleyae*, *G nigripenne* and *R. decemdigitatus* (Figure 16). *Cryptichthys jojettae* also showed small variation in depth and exposure, and had the fifth highest habitat specialisation index (0.91, Figure 16). All other species had a habitat specialisation index of under 0.9, with three species having a coefficient of under 0.8 (*F. malcolmi, O. maryannae* and *F. flavonigrum*), indicating that these species are relatively generalised in terms of depth and exposure (Figure 16). The degree of habitat specialisation in the use of substratum variables (1 x 1 m) did not differ as much between species as that found for depth and exposure (Figure 17).



Figure 17: Habitat specialisation index of 15 triplefin species in the Hauraki Gulf based on specialisation in substratum types $(1 \times 1 \text{ m})$. The black circles show the mean habitat specialisation index and the whiskers show the lower and upper quartile points (i.e. the lower 25^{th} percentile and upper 75^{th} percentile).

The habitat specialisation index of 14 triplefin species ranged from 0.72 - 0.83, with strongly overlapping quartile ranges (Figure 17). *Bellapiscis lesleyae* had the highest habitat specialisation index (0.96), indicating a narrow habitat breadth in the use of macrohabitat variables (Figure 17). This shows that triplefins have specialised predominately along the

depth and exposure axis, while specialisation for substratum types appears to be less pronounced.

Lastly, all variables (depth, exposure and substratum use) were analysed together to summarise the overall habitat breadth for each species (Figure 18). The habitat specialisation index for all habitat variables ranged from 0.72 - 0.96. Overall, *B. lesleyae* showed by far the narrowest habitat breadth (0.96, Figure 18), indicating that this species uses a highly specialised habitat. *Forsterygion lapillum* and *G. capito* showed the widest habitat breadth (Figure 18), suggesting that these species can be classified as the least specialised of the triplefin species examined.



Figure 18: Combined habitat specialisation index of 15 triplefin species in the Hauraki Gulf based on specialisation in depth, exposure and substratum types $(1 \times 1 \text{ m})$. The black circles show the mean habitat specialisation index and the whiskers show the lower and upper quartile points (i.e. the lower 25th percentile and upper 75th percentile).

4.3.2 Evolution of habitat specialisation

The directional model did not perform significantly better than the random walk model in describing phylogenetic differences in habitat specialisation (LR test = 1.83, p > 0.05). Thus,

the null hypothesis that habitat specialisation in New Zealand triplefin fishes proceeds as a random walk in time was accepted. The maximum likelihood parameter of lambda under the constant variable random walk model was not significantly different from 1 (H₀), indicating that the phylogeny correctly predicted habitat specialisation observed in the present taxa (LR test = 2.7, p > 0.05). Similarly, the maximum likelihood parameter of kappa did not perform significantly better then when the default settings were used (= 1), suggesting default gradualism (LR test = 0.17, df = 1, p > 0.05). Finally, the maximum log-likelihood estimates for delta was estimated to be 6.8 and differed significantly from the default settings of 1 (LR test = 18, p < 0.001), which is suggestive of accelerated trait evolution towards the tips of the phylogeny. This indicates that despite the lack of a general trend towards increase or decrease in habitat specialisation over time, most species-specific differences in the degree of habitat specialisation have evolved in later phases of evolution (i.e. towards the tips of the tree).

Lastly, it was tested whether the habitat specialisation index correlates with triplefin body size. The results showed no evidence for a trait correlation (LR test = 0.017, p > 0.05), suggesting that the evolution of the two traits has proceeded independently. However, there was a trend for sister-species pairs to differ in maximum body length (*F. lapillum* and *G. nigripenne* 21%, *F. malcolmi* and *O. maryannae* 69%, and *R. decemdigitatus* and *R. whero* 39%, see Table 6 for a list of triplefin body lengths), and in all cases the larger of the pair was the more specialised (Figure 18).

4.4 DISCUSSION

It is commonly hypothesised that ecological diversification proceeds from generalised ancestors to more specialised descendants, and that ecological specialisation will be largely irreversible once achieved (Mayr 1942; Simpson 1953; Futuyma and Moreno 1988). In this paper, it was tested whether New Zealand triplefin species show a sequence towards greater resource specialisation by measuring the habitat use of 15 species and calculating the degree of habitat specialisation on a continuous scale using a novel statistical method. The results indicated that specialisation does not show a recognisable progression towards greater resource specialisation. The analyses also showed that the common triplefin ancestor was within the range of observed values in the species data, therefore rejecting that the common triplefin ancestor was more generalised than present day species. These findings are in contrast to the general view that the founders of adaptive radiations are typically resource generalists that give rise to descendants that become more and more specialised as radiations age and species diversity builds. More detailed analyses showed that the degree of habitat specialisation showed a strong phylogenetic signal and that most interspecific changes in habitat specialisation have occurred towards the tips of the tree, independently of body size. This indicates that specialisation has proceeded at a faster rate with increasing species diversity and thus may be related to a filling of ecological niches.

Interspecific comparisons of habitat breadth demonstrated that triplefin species differ in the degree of habitat specialisation for the two habitat scales investigated (depth and exposure and the finer scale substratum types), thus, a study that only incorporates one of these habitat scales would come to different conclusions about the degree of habitat specialisation in this group. This finding highlights that a careful selection of habitat parameters is crucial in the assessment of resource specialisation. Triplefin species generally showed a higher degree of resource specialisation for the depth and exposure of the habitat, and indicates that triplefin species occupy habitats that are well-defined in terms of depth and exposure. This is consistent with previous work on the habitat partitioning in this group, which has shown that interspecific overlap in the New Zealand triplefin fauna is mainly reduced by differences in the exposure and depth of the habitat (Syms 1995; Feary and Clements 2006, Chapter 2). The combined analysis of all habitat variables demonstrated B. lesleyae yielded the highest habitat specialisation index in this group, as it was exclusively found in shallow and rocky areas. It should be noted that the variation in depth and exposure of this species may have been underestimated, as highly exposed intertidal habitats could not be sampled subtidally. However, given that not only the use of depth and exposure but also the use of substratum variables was indicative of specialisation, it is unlikely that the result was simply a sampling artefact. In comparison, F. lapillum and G. capito exhibited the widest habitat breadth and can thus be described as the least specialised of the species studied. The large habitat breadth may allow F. lapillum and G. capito to use whichever substratum type is unused or underutilised by other species, as long as it is within the species' preferred depth and exposure range. This flexibility is advantageous considering the heterogeneous New Zealand coastline, and may give these species increased environmental tolerance. The remainder of the triplefins surveyed may be considered as moderately specialised in terms of habitat use given the spectrum of habitat use between *B. lesleyae* and *F. lapillum/G. capito*.

Most natural assemblages are composed of ecologically similar, competing species that vary from those with very narrow habitat requirements to others with more or less ubiquitous habitat distributions (Morris 1996; Bonesi and Macdonald 2004). It is commonly thought that

habitat specialists competitively exclude less well-adapted phenotypes and species from their most preferred habitats. Brown (1996) demonstrated that widespread habitat generalists might coexist with competing habitat specialists if they exploit the shared environment at a larger spatial scale, because the habitat generalist will exploit whichever habitats are unused or underused by more specialized species. In this sense, it appears that the differing degrees of habitat specialisation observed in the New Zealand triplefin assemblage may facilitate coexistence in this assemblage, as it allows the more specialised species to occupy the most suitable habitats while the more generalised species possess greater habitat flexibility and thus are able to adjust their habitat use to the prevailing conditions. The results of this study suggest that the more generalised species are most likely to show a habitat shift in the use of substratum types, as the selection of depth and exposure was more specialised in the vast majority species (DSE-S was close to 1). Thus, coexistence between ecologically similar triplefin species may be achieved by slight substratum shifts of the more generalised species in the presence of a more specialised species, thereby allowing the mutual survival of both species in the same habitat patch. The more specialised species would gain fitness advantages as it can occupy the most suitable habitat type (e.g. rock), while the more generalised species benefits from the ability to access a much wider range of habitats, thus facilitating survival in a much broader range of habitats.

Bayesian analyses of the evolution of triplefin habitat specialisation showed that the directional model did not perform significantly better than a random walk model, indicating that there is no general trend towards increasing habitat specialisation. This indicates that the evolution of habitat breadth has proceeded in both directions, towards one that favours specialisation and another one that favours generalisation in habitat use. Furthermore, the finding that the random model explains the data significantly better than the directional model indicates that the ancestral state at the root of the phylogeny can only fall somewhere within the range of the observed values in the present day species data (Pagel 1999). Therefore, the degree of habitat specialisation of the common triplefin ancestor was somewhere between the least and most specialised species. This finding contradicts traditional models of adaptive radiation whereby a generalist ancestor is commonly assumed to yield specialist descendants (reviewed in: Schluter 2000b). The sequence from generalised to more and more specialised species has commonly been explained by the greater probability of a generalist species to utilises a wider range resource types (Futuyma and Moreno 1988), thus enabling the entry into novel environments. Over time, the diversification of the founding species is thought to result in more and more specialised species, and this process is presumed to continue until new resources become scarce or until specialisation leads to an ecological 'dead-end' (Futuyma and Moreno 1988; Kelley and Farell 1998). The results from the present study do not conform to this traditional view and indicate that both expansion to new resources and the sub-divisioning of old resources has been involved in the diversification of New Zealand triplefin fishes.

Maximum likelihood estimates showed that lambda and kappa were not significantly different from 1.0, which suggests that the evolution of habitat specialisation has a strong phylogenetic component and has been gradual over time. The parameter estimate of delta was significantly greater than 1.0, suggesting that evolution of habitat specialisation tended to accelerate towards the tips of the tree, indicative of species-specific adaptations. The results thus suggested a scenario where habitat specialisation in New Zealand triplefin species has occurred at a species-specific level where longer branches have contributed more to trait evolution. Further analysis demonstrated that the evolution of habitat specialisation between body size and habitat specialisation suggests that body size evolution in New Zealand triplefin species is not clearly linked with habitat breadth, however, it was interesting to note that the more specialised species of a sister pair generally had a greater standard length. This was particularly pronounced for the sister-species pairs *F. malcolmi* and *O. maryannae* and *R. decemdigitatus* and *R. whero* (following the topology of Hickey and Clements 2005).

A common characteristic of many other adaptive radiations is diversification in diet choice and trophic morphology as well as diversification in sensory communication (Streelmann and Danley 2003). In particular, the radiations of many other fish groups, such as the explosive radiation of crater lake cichlids, are characterised by diet partitioning and diversification in nuptial colouration (Fryer and Iles 1972; Van Alphen et al. 2004; Seehausen 2006). However, all previous studies the New Zealand triplefin fauna show little evidence for divergence along the trophic axis or in sensory communication (Handford 1979; Thompson 1986; Vasques 1999; Feary 2001; Clements 2003), while several lines of evidence support the view that divergence in habitat use has been a main component in the diversification of this clade (Syms 1995; Feary and Clements 2006). Habitat divergence in this clade is presumably a key trait because habitat use simultaneously codes for mate choice, as triplefins mate and court in the same area that they occupy at other times of the years (Thompson 1986). The linkage between habitat and mate choice has been identified as a powerful mechanisms of speciation in parapatry or sympatry and has been referred to as a 'magic trait' (Gavrilets 2004; Gavrilets 2005). In summary, habitat specialisation in New Zealand triplefin species shows no evidence in support of a directional trend towards increasing resource specialisation. Instead, species appear to have followed different evolutionary trajectories in habitat specialisation, one in which species appear to sub-partition available resources, while the other leads to an expansion in the use of resources. Thus, it appears that an evolutionary trend towards greater habitat specialisation is weak or nonexistent. This supports the notion of Schluter (2000b) that expansion into new resources and novel environments (rather than increased resource partitioning) is a common feature of adaptive radiations, and that ancestral specialisation does not appear to be an impediment to subsequent ecological diversification within lineages (Kelley and Farell 1998). The results of this chapter thus stand in contrast to the long standing hypothesis that ecological generalists give rise to specialists more often than the reverse (e.g. Mayr 1942; Simpson 1953). However, they are consistent with more recent studies on the evolution of resource specialisation in fishes (Elliott et al. 1999) and turtles (Stephens and Wiens 2003), and the host range evolution of phytophagous insects (Crespi and Sandoval 2000; Nosil 2002; Nosil and Mooers 2005; Sandoval and Nosil 2005).

5 Similarities in habitat use between triplefin recruits and adults

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5.1 INTRODUCTION

Many studies have investigated the settlement habitat of reef fishes (Wilson and Osenberg 2002; Srinivasan 2003; Bergenius et al. 2005), however, less attention has been given to whether this selection is the result of active or passive processes (reviewed in: Montgomery et al. 2001). Active habitat selection at settlement describes the process by which larvae non-randomly select specific areas in which to live. Such active habitat selection would be expected in species for which fitness trade-offs in alternative habitats are high (Rosenfeld and Boss 2001), and thus may be an evolved response to patterns of post-settlement mortality (Keough and Downes 1982). In contrast, if fitness trade-offs in alternative habitats are low, then species may be expected to settle at random with respect to habitat.

One factor that may have a strong influence on settlement patterns is the density of conspecifics already present in the community (Öhman et al. 1998). Numerous experimental manipulations of resident density have shown that established fishes can have negative, positive or negligible effects on settlement. Residents may negatively affect settler survival through predation or competition (Sweatman and St John 1990; Almany 2004), or residents may positively facilitate settlement (Sweatman 1983; Sweatman 1988; Booth 1992) by indicating suitable settlement territory (Levin 1993). Other studies have shown that adult density has no effect on settlement (Jones 1984; Levin 1993; Tolimieri 1995; Forrester 1999). The variability in adult-settler relationships is likely due to the wide range of taxa studied, and differences in the range of densities examined and the methods used to measure habitat selection at settlement. It is therefore difficult to make *a priori* predictions about adult-settler correlations.

New Zealand triplefin fishes exhibit extreme habitat specificity and fidelity as adults (Syms 1995; Feary and Clements 2006), and work on *F. varium* has shown that movement between habitats is extremely rare (Connell and Jones 1991). Tolimieri et al. (2000) demonstrated that light traps with reef noise attracted substantially more triplefin larvae than traps without reef noise, indicating that pre-settlement larvae use sound as a cue in the selection of habitat. Hickford and Schiel (2003) sampled triplefin larval abundance at various distances from the shore, and found several distinct species-specific larval distribution patterns. Furthermore, triplefin larvae have been observed to swim actively in the water column and clearly maintain their position, even in strong currents (Kingsford and Choat 1989). Together, these studies indicate that larval behaviour plays a role in determining settlement location and habitat.

While it is well established that adult New Zealand triplefins are associated with speciesspecific habitat types (Syms 1995; Feary and Clements 2006), it is unclear if this is the result of active processes at settlement or due to passive post-settlement processes. Work by Connell and Jones (1991) on *F. varium* found that newly settled larvae are found in a much wider range of habitats than adults, and concluded that higher post-settlement mortality in some habitat patches leads to the more narrow habitat use pattern in adults. Conversely, Syms (1995) found that newly settled triplefins were generally found in habitats (i.e. depth and biotic zonation) similar to those of adult conspecifics, and suggested that this pattern was likely to be determined by habitat preference exerted at settlement. Thus, it still remains unclear whether the species-specific habitat use in New Zealand triplefin species is the result of active choice by individual fish or the result of differential mortality in habitats.

This study aims to improve understanding of the causal factors affecting triplefin habitat associations by comparing the habitat use of new recruits with that of adult triplefin species. Two hypotheses concerning the distribution of newly settled recruits (< one week old) of five triplefin species were tested. The first hypothesis addressed whether triplefin recruits occupy habitats similar to those occupied by conspecific adults. If recruits occur in same habitats as adults, then this is suggestive that habitat associations are largely established at the time of settlement. In contrast, if recruits occur in a different and wider range of habitats to adults then this would appear to indicate that post-settlement modification is shaping speciesspecific habitat use. The second hypothesis investigated whether adult density has an influence on settlement patterns by comparing conspecific recruit and adult densities in habitat patches. Testing the second hypothesis will help to determine whether adult density facilitates, inhibits or has no effect on the habitat associations of new triplefin recruits. Knowledge about this relationship is necessary to evaluate the possibility of both densitydependent (Rosenzweig 1991) and density-independent (Caselle and Warner 1996) effects on habitat patterns of new recruits in these species, as these effects could further modify triplefinhabitat associations. Recruit and adult density can be accurately assessed in the field, as triplefins are highly philopatric (Clements 2003) and thus exhibit minimal movement after settlement (Connell and Jones 1991).

5.2 MATERIALS AND METHODS

5.2.1 Data collection

This study was conducted over three recruitment seasons from January 2002 to May 2005 in the Hauraki Gulf (36°36'S, 174°50'E, Figure 19) in northeastern New Zealand using UVC.



Figure 19: Map of study sites in the Inner and Outer Hauraki Gulf. Black circles indicate study areas.

Habitat associations of new recruits and adult triplefins were sampled as frequently as logistically possible, with most months being sampled. Sites were selected to sample as broad a range of habitats as practically possible. In each year, regular UVC were conducted at various onshore sites from the Whangaparaoa Peninsula to the Leigh Marine Reserve (Figure 19), and at selected offshore islands (Mokohinau Islands, Little Barrier and Great Barrier Island (Figure 19). Between 3 - 6 UVC (each measuring 4 x 4 m) were laid out at each site. The first UVC was conducted at the deepest depth that could safely be sampled (maximum depth dived 30 m), and the two subsequent UVC were done at approximately 33% and 66% of the deepest depth. Additional UVC were conducted in variable depths if time and logistic constraints permitted. This design was employed to allow sampling flexibility at a range of sites. A minimum distance of approximately 50 m between UVC was maintained to eliminate dependent samples (Andrew and Mapstone 1987). In total, 151 randomly placed UVC at 36 sites were sampled to examine the distribution patterns of newly settled recruits and adults.

Newly settled recruits could be distinguished from older individuals and adults by their small size (≈ 30 mm) (Connell and Jones 1991; McDermott and Shima 2006) and lack of fully developed pigmentation (Connell and Jones 1991). Pigmentation starts to develop 4 - 7 days after settlement (Connell and Jones 1991), and can thus be used as a reliable indicator of recent settlement (i.e. < one week old). Furthermore, repeated surveys by Connell and Jones (1991) and Syms and Jones (1999) indicated that the habitat distribution of newly settled recruits was stable over at least three days, suggesting that habitat surveys of new recruits are suitable to detect settlement signals.

Prior to sampling, a location fix was taken for each site using a handheld Garmin® 12 global positioning system, and a physically derived exposure index (fetch) was calculated based on the total sum of the fetch (maximum radial distance 300 km). Fetch calculations were performed with the program 'Fetch Effect Analysis' (version 1.01. Pickard R 2000), a measure that describes fetch distance from a given point (GIS fix) for each 20 degree compass sector (Thomas 1986). The centre line of each UVC was marked with a leaded line, and a steel quadrat was used to outline each 1 m². Habitat use of new recruits and adults was recorded for each 1 m² within the 4 x 4 sampling area. For each 1 m², the depth was recorded, and seven habitat variables were estimated visually as percent cover of the substratum: rock (rocks > 7 cm), cobbles (rocks < 7 cm), gravel (rocks < 4 cm), sand, mud, macroalgae, and coralline and turfing algae. The first five variables always summed to 100%, while algal coverage could range from 0 - 100%. Triplefins within each quadrat were identified and their micropositions recorded as follows: on the side or top of rocks, free swimming, under rocks

or in cracks, on top of cobbles, on algae, on mud or sand, and under overhang. Micropositions were defined as the substratum upon which >50% of a fish's body rested (Feary and Clements 2006). Comparisons of habitat associations of new recruits and adults were based on the microposition use, the substratum and depth of the 1 m² quadrat and the exposure of the 4 x 4 m UVC sampling area in which each individual fish was recorded.

5.2.2 Data analysis

5.2.2.1 Habitat use of new recruits and adults

The analysis included only species for which settlers were observed in each of the three recruitment seasons, and in which the total number of settlers exceeded 50 observations over the duration of the study. This ensured that observations spanned more than two recruitment years and that habitat use could be estimated with some certainty. Species that met these criteria were *F. lapillum* (adults: 2865; settlers: 577), *F. varium* (adults: 830; settlers: 353), *N. segmentatus* (adults: 1994; settlers: 291), *O. maryannae* (adults: 1371; settlers: 385) and *R. whero* (adults: 1894; settlers: 291).

Before testing whether habitat associations of new recruits were similar to those of conspecific adults, it was necessary to establish whether adults of the five species occupied distinct habitats. First, differences in habitat use were assessed by comparing the substratum, depth and exposure of the habitat occupied by adults of the five species. ANOVA could not be used as the majority of habitat variables demonstrated large heterogeneity of variance (using Levene's test), even after an arcsine (substratum variables) and log (depth and exposure) transformations. Therefore, habitat variables were examined using the Welch-Satterthwaite adjustment (Welch test) for degrees of freedom (Satterthwaite 1964; Welch 1983) within an independent means t-test (Zimmermann 2004b). A Bonferroni adjusted alpha of 0.0006 (for 90 multiple comparisons) was used in order to maintain an overall comparison two-tailed alpha of 0.05 (Howell 2002). Statistical significance is substantively influenced by sample size, meaning that an estimated test statistic is increasingly likely to be adjudged as indicative of a significant departure from a null-hypothesis population value (Kline 2004). For this reason, an effect size (eta squared, η^2) was computed for each Welch Test to obtain an estimate of the biological significance of a statistically significant test result. The key difference is that statistical significance testing evaluates the probability of obtaining the sampling outcome by chance alone, while the effect size provides some indication of the

result's explanatory power (Jennions and Møller 2003). When comparing two groups in a ttest, η^2 is the proportion of variance accounted for in the dependent variable by the independent variable of 'group' membership. An η^2 coefficient of 0.5 can be interpreted as indicating that 50% of the variation in this variable is accounted for by the group variable. Cohen (1992) provides the following magnitude guidelines for a correlation based (i.e. η^2) effect size. The small, medium and large effect label is applied to η^2 effect sizes between < 0.09 (1 - 9% of total variance explained), 0.09 - 0.25 (9 - 25% of total variance explained) and > 0.25 (more than 25% of total variance explained), respectively. All Welch Tests were calculated in the program 'independent means t-test with effect size' (available from http://www.pbmetrix.com). The relationships between new recruits and conspecific adult habitat preferences were investigated as above, though the Bonferroni adjusted alpha was 0.001 (for 45 multiple comparisons).

The second part of the analysis involved comparing the microposition use of adults and new recruits using the Correspondence Analysis routine implemented in Statistica (version 7.1). In addition, the degree of intraspecific variation in habitat use of new recruits and adults was calculated using the computer program 'Habitat Diversity Analysis' (available from: http://www.pbmetrix.com), to estimate the degree of variation in habitat use exhibited by new recruits and adults of a species. Prior to the data analysis, each species was divided into two groups, one which consisted of all recruits and another one which consisted of all adults. The program works by comparing each individual of a group (recruits or adults) to all other individuals within the group. Thus, the raw data of the within group comparison consists of $(N^2 - N) / 2$ comparison coefficients, where N equals the number of individuals within a group. The similarity between two individuals within a group is expressed as double-scaled Euclidean distance, that has been scaled between 0-1. Finally, the double-scaled distance is expressed as a similarity index by subtracting it from 1, thus yielding the double-scaled Euclidean similarity (DSE-S) measure, where 0 for this coefficient now indicates that all individuals within a group use completely different magnitudes of habitat variables, and 1 indicates that all individuals use exactly the same magnitudes of habitat variables. It was chosen to display the DSE-S coefficient distribution and median value of each group for the use of depth and exposure and for substratum use.

The variable mud was not used by new recruits of *N. segmentatus* and *O. maryannae*, and thus was excluded from the comparisons for these species.

5.2.2.2 Analysis of spatial associations between new recruits and adults

A correlative rather than a manipulative approach was used to investigate the effect of conspecific adult density on the habitat use patterns of new recruits. Two methods have been commonly used to investigate the relationship between adults and new recruits in habitat patches. The first method plots the density of new recruits against that of adults, while in the second method the per capita settlement rate is used. The use of the per capita rate (i.e. the density of settlers divided by the density of adults) has been criticised because the relationship between adults and recruits is mathematically constrained, that is, if the population gets larger (all else being equal) the per capita rate decreases (Caley et al. 1996; Sale and Tolimieri 2000). A conservative approach was taken in this study, and both methods were used to investigate the spatial relationship between new recruits and conspecific adults.

Only the three months with the highest densities of new recruits for each species were analysed, as the recruit-adult interactions were likely to be the strongest during this period. To estimate the per capita rate, the density of new recruits was divided by the density of conspecific adults, and this estimate of per capita rate was regressed against adult density. To examine the shape of the per capita relationship the regressions were fitted by exponential functions, using the non-linear regression module in the statistical software package Statistica (version 7.1).

5.3 RESULTS

New recruits were observed from October to April with a noticeable peak from November to February (see Figure 20, Figure 21, Figure 22, Figure 23, and Figure 24). Adult density was usually highest just before the start of the breeding season, and started to decline towards the end of the season. Two species, *F. varium* and *O. maryannae*, were almost exclusively found on offshore sites (Figure 22 and Figure 23), while the remaining species showed no preference.















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Figure 24: Density of new recruits and adults of R. whero. Circles denote mean density ±SE.

5.3.1 Habitat use of new recruits and adults

The five species used in the analysis all displayed distinct differences in adult habitat use. Many of the comparisons were statistically significant, though a large portion of these had small effect sizes of < 0.09 (i.e. explained less than 9% of the effect), and were therefore not further considered.

Comparisons for which there were significant differences and moderate effect sizes included the lower use of cobbles by *O. maryannae* compared to *F. lapillum*, *F. varium* and *R. whero* (*F. lapillum*: p > 0.001, $\eta^2 = 0.14$, *F. varium*: p < 0.001, $\eta^2 = 0.11$; *R. whero*: p < 0.001, $\eta^2 =$ 0.09, Figure 25). The habitat of *F. lapillum* differed significantly from adults of all other species (p < 0.001 for all comparisons) by generally being less deep and exposed (*F. varium*: depth $\eta^2 = 0.31$, exposure $\eta^2 = 0.21$; *N. segmentatus*: depth $\eta^2 = 0.49$, exposure $\eta^2 = 0.19$; *O. maryannae*: depth $\eta^2 = 0.54$, exposure $\eta^2 = 0.17$; *R. whero*: depth $\eta^2 = 0.38$ exposure $\eta^2 = 0.19$, Figure 26). Forsterygion lapillum also differed significantly in the use of rock from *N. segmentatus* and *O. maryannae* (p < 0.001, $\eta^2 = 0.12$ in both cases, Figure 25).



Figure 25: Substratum use of triplefin adults and recruits. The percentage cover of the substratum variables always adds up to 100%, whereas the algal coverage could vary between 0-100%.



Figure 26: Use of depth and exposure by triplefin adults and new recruits (\pm SE). Species names are abbreviated by the first letter of the genus followed by the first letter of the species name, respectively. The blue shading represents the increasing depth.

Furthermore, the Correspondence Analysis demonstrated that adult *R. whero* and *O. maryannae* were distinct in the use of the microposition UCS and FRE, respectively (Figure 27).



Figure 27: Microposition use of triplefin adults and new recruits. The circle shows the position of new recruits and adults of the species *Forsterygion lapillum*, *F. varium* and *Notoclinops segmentatus*, which could not be displayed individually due to high overlap.

Analysis of new recruit and conspecific adult habitat use showed that even though some of the habitat comparisons between groups were significantly different (Table 7), effect sizes were in all cases < 0.085, meaning that the magnitude of the difference between the groups was small. For example, although some statistical comparisons of settler and adult substratum use were significant (Table 7), new recruits were generally found to occupy the same range of substratum variables as conspecific adults (Figure 25).

Species	Variables	df	<i>p</i> -value (2 tailed)	η^2
F. varium	Depth	920.81	<0.001	0.026
Adult (n=2101)	Exposure	947.53	0.747	0.000
New recruits (n=515)	Rock	830.13	0.168	0.002
	Cobble	744.54	0.065	0.005
	Gravel	663.23	0.864	0.000
	Sand	827.63	<0.001	0.016
	Mud	742.04	0.325	0.001
	Macroalgae	758.00	0.465	0.001
	Coralline and turfing algae	728.53	<0.001	0.015
F. lapillum	Depth	847.29	<0.001	0.023
Adult (n=3884)	Exposure	834.05	<0.001	0.036
New recruits (n=690)	Rock	965.91	<0.001	0.035
	Cobble	899.47	0.955	0.000
	Gravel	1013.72	<0.001	0.011
	Sand	860.81	<0.001	0.078
	Mud	984.91	0.932	0.000
	Macroalgae	1020.40	0.021	0.005
	Coralline and turfing algae	1014.47	0.019	0.005
O. maryannae	Depth	468.32	<0.001	0.034
Adult (n=1495)	Exposure	319.33	0.008	0.022
New recruits (n=248)	Rock	303.13	0.962	0.000
	Cobble	331.65	0.039	0.013
	Gravel	312.78	0.496	0.001
	Sand	297.44	0.515	0.001
	Mud	298.13	0.876	0.020
	Macroalgae	308.96	0.134	0.007
	Coralline and turfing algae	308.30	0.043	0.013
N. segmentatus	Depth	513.59	0.118	0.005
Adult (n=2328)	Exposure	406.56	<0.001	0.085
New recruits (n=315)	Rock	477.86	<0.001	0.034
	Cobble	506.19	0.154	0.004
	Gravel	437.98	0.875	0.000
	Sand	497.33	<0.001	0.035
	Mud	476.33	0.866	0.010
	Macroalgae	420.69	<0.001	0.052
	Coralline and turfing algae	427.71	<0.001	0.042
R. whero	Depth	604.18	0.564	0.001
Adult (n=1643)	Exposure	535.13	<0.001	0.065
New recruits (n=389)	Rock	535.60	0.129	0.004
	Cobble	479.18	<0.001	0.031
	Gravel	537.07	0.495	0.001
	Sand	569.72	0.149	0.004
	Mud	1790.86	0.003	0.005
	Macroalgae	554.09	0.353	0.002
	Coralline and turfing algae	656.48	0.011	0.010

Table 7: Welch-test results of habitat comparisons between conspecific adults and new recruits. η^2 indicates the effect size. Significant effects (at a Bonferroni adjusted *p*-level of 0.001) are highlighted in bold.

Similarly, although new recruits and adults of some species (*F. lapillum*, *F. varium* and *O. maryannae*) differed in depth distribution, their mean depth use never varied by more than 1 m, (Figure 26). Adults of *F. lapillum* and *N. segmentatus* occupied slightly shallower habitats than new recruits, while adults of *F. varium* were found in marginally deeper habitats, as illustrated by the small effect size ($\eta^2 < 0.04$). The exposure of the habitat occupied by new recruits was also comparable to that of adults. Although new recruits and adults were similar in habitat exposure in most species, new recruits of all species except *F. varium* were found in slightly more exposed habitats than adults (Figure 26). These ontogenetic differences in habitat exposure, while statistically significant, were negligible in terms of effect size ($\eta^2 < 0.09$). The fact that these comparisons yielded significant differences despite low effect sizes can be attributed to the large sample sizes, which makes the detection of differences extremely likely, even if these differences are only trivial.

Microposition use was also very similar between conspecific new recruits and adults, with the exception of *F. lapillum*. New recruits of *F. lapillum* were more closely positioned to *F. varium* new recruits because of their mutual use of the microposition 'on the sides and tops of boulders' (STB) (Figure 27). *Forsterygion lapillum* adults used the micropositions 'sand and mud' (SM), 'on algae' (ALG), 'on top of cobbles' (TCO) and 'under overhangs' (UOV) more frequently than *F. varium* (Figure 27). Settler and adult *F. varium* and *N. segmentatus* were also both strongly associated with the microposition STB. The microposition use of *O. maryannae* new recruits and adults was weakly associated with the 'free swimming' (FRE) category, and *R. whero* new recruits and adults were associated with the microposition 'under rock and in cracks' (UCS) (Figure 27).

Analysis of intraspecific variation in the use of depth and exposure and substratum types showed that the frequency of DSE-S values for new recruits and conspecific adults were similar. While the median DSE-S values of adult *F. lapillum*, *F. varium*, *N. segmentatus* and *R. whero* were higher than those of new recruits, the difference was negligible (0.01 - 0.04), indicating similar levels of variation in adult and settler habitat use. New recruits of *O. maryannae* had an apparently higher variance in depth and exposure use than adults, although again this difference was small (0.03). A similar pattern was found for substratum type use, with *F. varium* (0.02), *N. segmentatus* (0.03) and *R. whero* (0.02) showing a negligible difference between new recruits and adults in the variation in substratum use. Similarly, compared to adults new recruits of *F. lapillum* showed no difference and new recruits of *O. maryannae* showed only slightly higher median value (0.02, Figure 28). A consistent pattern that emerged was that new recruits and adults both showed less variation in the use of depth

and exposure than variation in the use of substratum types. This was evident in the overall lower mean DSE-S value for depth and exposure (0.84) compared to substratum types (0.77).

5.3.2 Spatial correlations between new recruits and adults

The per capita settlement rate indicated a negative density-dependent relationship between adult and recruit density for all five triplefin species examined. However, evidence for density-dependent settlement was much weaker when the density of new recruits alone (i.e. rather than per capita rate) was regressed against adult density, with only *F. lapillum*, *R. whero* and *O. maryannae* showing weak evidence for density-dependent settlement (Figure 29). Nevertheless, although the relationship was weaker

Figure 29 clearly shows that the highest densities of new recruits tended to be at low to intermediate adult densities (around about 1 - 1.5 m^{-2} adults). This suggests that habitat patches may become saturated once a certain number of fish occupy the same patch.



Figure 28: Bar chart histogram of the DSE-S coefficient of triplefin new recruits (black bars) and adults (white bars) in the Hauraki Gulf based on intraspecific variation in the use of depth and exposure (fetch) and substratum variables (rock, cobble, gravel, mud, sand, macroalgae and coralline and turfing algae). The open circle shows the median DSE-S value for adults and closed circle the median DSE-S value for new recruits.



Figure 29: Recruit density (left) and per capita rate of new recruits (right) versus adult density (m^{-2}) of the four demersal triplefin species *F. lapillum*, *F. varium*, *N. segmentatus*, *R. whero* and the semi-pelagic *O. maryannae*. Each data point represents a UVC. Solid lines represents exponential fit to data.
5.4 DISCUSSION

This study investigated whether newly settled recruits of five triplefin species show habitat associations similar to those of conspecific adults. This was done in two parts. First, tests were conducted to determine whether (i) habitat use by newly settled recruits matched that of conspecific adults, or (ii) new recruits exhibited 'blanket settlement' (sensu Connell and Jones 1991), with post-settlement processes subsequently modifying habitat patterns. Second, it was tested whether the density of conspecific adults was correlated with recruit density within quadrats, as density-dependent relationships could further modify triplefin habitat use. These two parts will be discussed in turn.

Adults of the five species surveyed did not appear in all habitats types surveyed, but occupied distinct habitats. Forsterygion lapillum differed from other species in that it was found to occupy relatively shallow and sheltered habitats, *Obliquichthys maryannae* was typically found swimming in mid-water ('free swimming'), and Ruanoho whero was distinct in that it often was found in micropositions 'under rocks or in cracks'. Forsterygion varium and Notoclinops segmentatus were generally quite broad in their habitat distribution, although the latter tended to use more rock and algae as substrates. These habitat preferences are similar to those reported by Feary and Clements (2006). Examination of habitat associations of recruits indicated that none of these five species exhibited 'blanket settlement', but generally selected the range of habitats used by conspecific adults. Although the use of some of the habitat variables differed between conspecific new recruits and adults, effect sizes indicated that differences were not biologically meaningful, as there was often less than 10% variation between age classes. Thus, new recruits and adults of a given species differed only marginally in habitat use. Of all variables studied, the mean use of depth and exposure by recently settled and adult fish were particularly similar. It would appear, therefore, that habitat depth and exposure is of particular importance, while selection for substratum variables appeared to be less marked. Studies of other fish assemblages have documented a similarly strong role of depth (Bean et al. 2002) and exposure (Thorman 1986; La Mesa and Vacchi 2005) in accounting for most of the spatial variation apparent within fish assemblages. Similarity between new recruits and adults in the use of micropositions was also high, showing that habitat selection occurs even at a very fine spatial scale. Analysis of intraspecific variation in habitat depth, exposure and substratum type confirmed that new recruits occupy a similar range of habitats to conspecific adults. Are these patterns of habitat use determined actively (e.g. by larval behaviour) or passively (e.g. by predation), and are the methods sufficient to discriminate between these? These points will be discussed separately.

Many reef fishes sample habitat prior to settlement (Kauffman et al. 1992; Carr and Syms 2006), demonstrating that larvae are competent to make active choices about where to settle. New Zealand triplefin species exhibit differences in horizontal and vertical distributions as pelagic larvae (Kingsford and Choat 1989; Hickford and Schiel 2003), and pre-settlement larvae actively use reef sound as a settlement cue (Tolimieri et al. 2000). Furthermore, a recent study demonstrated that newly recruited *Forsterygion lapillum* exhibited habitat selection under both experimental and field conditions (McDermott and Shima 2006). Larval behaviour is thus likely to be involved in determining settlement habitat in all species of New Zealand triplefins.

Studies on both tropical reef fishes (Tupper and Boutilier 1997; Almany and Webster 2006) and New Zealand triplefins (Connell and Jones 1991) show that predation can have strong impacts on the density of newly settled recruits. However, the effects of predation must be highly species-specific to create distinct patterns of habitat use. The five triplefin species examined in this study are closely related (Hickey and Clements 2005). Although they differ markedly in habitat use (Syms 1995; Feary and Clements 2006), they share many morphological and ecological characters (Paulin and Roberts 1992; Fricke 1994; Francis 2001), suggesting that the susceptibility of recruits to predators of these species is similar. Furthermore, the local abundance of predators would have to be spatially and temporally consistent for predation to shape the habitat distribution of triplefin recruits in a species-specific manner. Data on the ecology of potential predatory species of New Zealand triplefin fishes (Jones 1988) suggest that this is very unlikely to be the case.

Finally, could high early post-settlement mortality influence the ability to differentiate between active habitat selection and post-settlement processes as determinants of habitat use patterns? Repeated surveys by Connell and Jones (1991) and Syms and Jones (1999) indicated that the habitat distribution of newly settled recruits was stable over at least three days, suggesting that habitat surveys of new recruits are suitable to detect settlement signals. Connell and Jones (1991) found that mortality was highest during the first week after settlement. Similar results have been obtained in tropical reef fishes, where some species suffer a mortality rate of over 50% in the first two days after settlement (Almany and Webster 2006). If mortality is highest immediately after settlement then cohort density is also highest immediately after settlement, and consequently the ability to observe individuals within a cohort (and their habitat associations) is highest during this period. Patterns of habitat association produced by post-settlement mortality by definition will involve a diminished proportion of each cohort. Thus, the probability of detecting patterns of habitat association

through ontogeny are a balance between cohort density and temporal stability. In other words, even if habitat distribution was heavily modified by mortality in the first few days after settlement, this study nevertheless had a good likelihood of being able to detect the original (i.e. pre-mortality) pattern of habitat association.

The ability to find a suitable habitat directly at settlement is critical to individual fitness, since growth and survivorship of juvenile fish is affected by habitat structure (Jones 1988; Hixon and Beets 1989). Habitat selection at settlement may therefore be an evolved response to fitness trade-offs and patterns of post-settlement mortality (Keough and Downes 1982). However, the finding that triplefin recruits have similar patterns of habitat use to conspecific adults conflicts with the conclusions of Connell and Jones (1991). This discrepancy may be attributable to differences in the spatial and temporal scales used to examine habitat selection in the respective studies. Connell and Jones (1991) sampled over two years at a single site, and their habitat comparisons between new recruits and adults were based on a single species (*Forsterygion varium*) at a single depth stratum (10 m). In the present study, habitat associations of new recruits and adult triplefins were compared in five species that were observed over three years in a wide range of habitat types at multiple sites, thereby allowing more comprehensive sampling of habitat associations.

The second part of this study addressed the question of whether adult density affects the density of new recruits. Because triplefin species are highly philopatric (Clements 2003) and show negligible movement (Connell and Jones 1991), density-dependent processes are likely to limit the number of fish that can settle on a reef. The analysis of the per capita settlement rate showed strong evidence that at high conspecific adult densities the per capita settlement rates are reduced in all species, indicating that settlement patterns are negatively densitydependent. However, the evidence for density-dependence was much weaker when just the density of new recruits was plotted against the density of conspecific adults, presumably because this method is mathematically less constrained. Three species still showed some evidence of density-dependence (Forsterygion lapillum, Ruanoho whero and Obliquichthys maryannae), while the pattern for F. varium and Notoclinops segmentatus was less clear. This suggests that while density-dependent settlement may affect the overall abundance of triplefins on reefs, the strength of this relationship appears to differ between species. The reduced density of new recruits in habitats with high adult density suggests that space may be limiting when habitats are already occupied by conspecifics, and that settlement of new recruits may be inhibited once a certain threshold of adult density is reached. Specifically, the analysis indicated that the density of recruits may be inhibited at an adult density of around 1 -

1.5 individuals m^{-2} . This density fits well with the estimated territory size of triplefins, which has been estimated to be around 1 - 2 m^{-2} (Thompson 1986). Detailed data on settlement patterns on experimental reefs that differ in adult density would be useful to investigate further the pattern in these species and to examine recruit-adult relationships in other triplefin species.

Density-dependent settlement rates can theoretically arise by two different mechanisms: new recruits may detect conspecifics and avoid settling in areas where they are abundant, or conspecifics may predate or displace recently settled fishes (Steele and Anderson 2006). While there is no knowledge whether triplefin recruits have the ability to detect and respond to conspecifics, numerous studies of marine organisms provide evidence that competition for shelter and predation are often major factors controlling the overall number of individuals on reefs (Caley et al. 1996; Hixon and Jones 2005). In a study of subtidal blennioids, Buchheim and Hixon (1992) found that individuals that were unable to secure suitable shelter became highly vulnerable to predation, so that the availability of shelters placed a limit on the density of local populations. Given the small territory size of New Zealand triplefin fishes it is possible that competition for suitable shelter space may lead to density-dependent settlement rates, and thus may be an important factor in the overall regulation of population density. Although competition appears to be a likely factor influencing density in triplefins, it should be noted that different size classes of reef fishes associate with different shelter sizes (Hixon and Beets 1989). This may reduce competition for shelter sites between triplefin size classes.

In addition to competition, predation appears to be a common cause of density-dependent mortality, and it is thought that shelter limitation may be a frequent cause of density-dependent predation (Forrester and Steele 2004b). Evidence that predation may be a potential mechanism that could lead to density-dependence in triplefin species comes from a study by Feary (2001), who found fish remains in the gut contents of 18 adults. While the species identity of these fish items could not be identified, it is likely that triplefin new recruits are easy prey items for adults due to their small size (Feary 2001). More direct evidence for cannibalism comes from observations of adult triplefins preying on newly settled larvae, for example, *Forsterygion varium* has been observed to predate newly settled conspecifics and *F. lapillum* (Clements pers. obs., Montgomery 2003). Therefore, it can be expected that density-dependent processes could have a strong effect on the overall population structure and this could potentially reduce a positive relationships between new recruits and conspecific adult density.

How do the results of this study results bear on the relative importance of pre- and postsettlement processes as determinants of habitat use patterns in New Zealand triplefins? These processes would appear to counteract each other to an extent, since (i) new recruits occupy the same habitats as conspecific adults, and (ii) in some species densities of new recruits are reduced in suitable habitats because of high adult densities. In long-lived species of reef fishes, suitable habitat space may be occupied by adults over long time periods, and hence storage effects are very important (Chesson 2000). In contrast, short-lived species such as triplefins (2 - 3 years, Thompson 1979) may encounter considerable spatial and temporal variability in adult densities, and thus in habitat availability. Adults of New Zealand triplefin species appear to suffer highest mortality around the end of the reproductive season, presumably due to the higher physiological stresses experienced during that time. This suggests that triplefin recruits may encounter a significant amount of unoccupied and suitable settlement habitat at settlement, and it is thus unlikely that many new recruits are forced to settle into marginal habitats.

New triplefin recruits displayed habitat use that was consistent with habitat associations of conspecific adults. This was particularly pronounced for habitat depth and exposure, and to a lesser extent for substratum type and microposition use. This consistency suggests that settlement patterns are species-specific, and that new recruits maintain the use of particular habitats throughout post-settlement life. This supports the view that diversification of habitat selection may have been involved in the evolution of this group of fishes. There was some evidence to suggest that density-dependent factors influenced the overall density of some species, indicating that post-settlement processes are also important in determining patterns of distribution and abundance of triplefin fishes on reefs. The finding that both pre-settlement (i.e. active habitat selection at settlement) and post-settlement (i.e. density-dependence) processes influence the distribution and abundance of triplefin species is likely to be generally applicable to other philopatric and short-lived reef fishes.

6 Ecological Diversification of Two Intertidal Triplefin Fishes, *B. lesleyae* and *B. medius*

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6.1 INTRODUCTION

The ecological relationships between sympatric species and the effect that habitat divergence has on the realised niche of a species have long been of major interest in ecology. Over time, the process of habitat divergence may result in the variation of morphological and/or behavioural traits, which in turn enable sympatric species to successfully exploit their respective habitats and presumably facilitate long term coexistence (Schluter 2000b). This Chapter investigates ecological divergence in an intertidal triplefin sister-species pair, *B. lesleyae* and *B. medius*, which are described as being sympatric in habitat distribution (Paulin and Roberts 1992).

There are several physical and biological factors unique to the rocky intertidal that may have contributed to the divergence of the *Bellapiscis* genus. The rocky intertidal environment is heterogeneous and subjected to extreme physical changes over very short time scales, i.e. tidal cycles. The behavioural patterns of intertidal animals, like fish, are often correlated with these physical variables. Fish are highly mobile organisms and can adjust their elevational distribution in response to environmental fluctuations in the intertidal zone (Gibson 1988), allowing them to move to habitats that are only tolerable during certain tidal, diel, lunar or seasonal cycles or habitats that exclude potential competitors or predators (Davis 2001).

In general, rockpools form a spatially restrictive habitat and act as a refuge for intertidal organisms during low tide, protecting them during periods of emersion. Rockpools also offer protection from subtidal predators and competitors during low tide but in turn expose organisms to predation by intertidal predators, such as birds (Yoshiyama et al. 1986), and restrict foraging area (Zander et al. 1999). Mechanisms of resource partitioning by marine animals in the rocky intertidal include differentiation of daily activity cycles (Davis 2001), seasonal cycles (Underwood and Jernakoff 1984; Berger and Mayr 1992; Davis 2000), shelter sites (Koppel 1988; Kotrschal 1988; Faria and Almada 2001a; Silberschneider and Booth 2001; Szabo 2002), tolerances to exposure (Underwood and Jernakoff 1984), dietary specialization (Szabo 2002; Pulgar et al. 2003), tolerances to chemical changes (Hernández et al. 2002; Somero 2002; Pulgar et al. 2003) and vertical zonation (Little and Kitching 1996; Raffaelli and Hawkins 1996; Zander et al. 1999; Hernández et al. 2002; Somero 2002; Griffiths et al. 2003). The study of the elevational distribution of organisms in the intertidal zone has received considerable attention and many studies have shown vertical zonation patterns at low tide among fishes (Zander et al. 1999). Several factors have been suggested as an explanation for this. In general, elevated rockpools are highly variable in physical factors

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such as temperature, salinity, oxygen, and exposure to wave action. Variability in temperature, in particular, has been shown to an important determinant of an organism's vertical distribution (Nakamura 1976; Davis 2001; Pulgar et al. 2003). In addition, fluctuations in factors such as salinity and oxygen also influence the abundance of species in the intertidal along a vertical gradient (Pulgar et al. 2003).

The height above chart datum (ACD) and the exposure gradient of the rockpool greatly influences the degree to which the tides affect emersion rates, and it has been proposed that a combination of these factors causes the elevational zonation of many intertidal species (Green 1971; Nakamura 1976). Generally, the degree of variation (e.g. temperature and salinity) increases with the isolation and height of the pool ACD, although the surface area, volume, and depth of the pool may modify variability in these factors (Mahon and Mahon 1994). This means that pools in the lower intertidal zone do not undergo the same chemical and thermal changes as rockpools in the upper intertidal, as they are frequently inundated by waves and have thus water properties approximating that of the surrounding seawater (Little and Kitching 1996; Raffaelli and Hawkins 1996; Griffiths et al. 2003).

Only those species that have evolved special behavioural mechanisms or physiological tolerances are able to cope with the variable environment in the upper intertidal (Raffaelli and Hawkins 1996). For example, high physiological tolerances to hypoxia occur in some intertidal organisms (Little and Kitching 1996; Raffaelli and Hawkins 1996; Somero 2002). Other studies suggest that mechanisms such as behaviour are likely to be the dominant factor affecting survival in elevation rockpools, rather than superior physiological tolerances (Fangue et al. 2001). While the vertical zonation in the rocky intertidal environment is clearly a response to the emersion gradient, it has been suggested that physical factors do not set the upper limits of the distribution of all species. For species inhabiting the mid- to low-intertidal zone, biological interactions, especially inter- and intraspecific competition for space and predation, can set the boundaries between many species, though their ultimate extension up the shore would be set by physical factors (Connell 1961; Little and Kitching 1996; Tomanek and Helmuth 2002). In other words, physical factors are thought to set the ultimate upper limits to organisms but interspecific interactions with other species often prevent this physiological barrier from being reached (Connell 1961; Little and Kitching 1996).

This study aims to determine the biological factors influencing the abundance of the *Bellapiscis* sister-species in order to assess how much resource overlap between the two species occurs. *Bellapiscis lesleyae* and *B. medius* were first described in 1987 (Hardy) and

differ from all other New Zealand triplefin species in that they are predominantly found in the intertidal zone throughout their life. Both species are distributed all around coastal New Zealand where there is suitable habitat, from the Three Kings Islands to Stewart Island, including the Chatham Islands (Fricke 1994). The species are morphologically very similar but differ in total length $(L_{\rm T})$, head shape, extent of the lateral line and colour pattern (Paulin and Roberts 1992). Gut content analysis of B. lesleyae and B. medius has shown that both species are microcarnivorous predators that prey on similar dietary spectra, ranging from small sessile to mobile benthic invertebrates (Feary 2001). This lack of dietary specialisation indicates that other factors, such as space, could potentially be an important factor in the partitioning of resources. Bellapiscis lesleyae demonstrates homing ability, with individuals repeatedly occupying the same rockpools or set of pools over relatively long time periods (Fisher 1998). The habitat of both *Bellapiscis* species is described as shallow, rocky, coastal habitats, especially rockpools (Hardy 1987). However, quantitative work on their distribution patterns and abundance has not been carried out, thus, it remains unclear how the species partition their resources. Subtidal transect work shows that B. lesleyae is distributed deeper than B. medius, thereby suggesting divergence in habitat use (Francis 2001). Moreover, there is observational evidence to suggest that only *B. medius* is found in the upper intertidal rockpools (A. Stewart, pers. comm.).

This study investigates if *B. lesleyae* and *B. medius* exhibit resource partitioning in the rocky intertidal by testing the following three hypotheses. First, given that there is evidence that the two species are distributed differently amongst rockpools, it was hypothesised that it was possible to predict the occurrence of both fish species in any rockpool based solely upon the variation in particular rockpool variables (e.g. rockpool height and substratum type). Second, an assessment was made as to whether the aggregation size of both species was similar. Lastly, it was investigated whether a correlation existed between the intertidal height of rockpools and fish size. Relationships of fish abundance and size were examined as mechanisms leading to variation in rockpool habitat use.

6.2 MATERIALS AND METHODS

6.2.1 Data collection

A total of 109 pools were sampled at five locations in the North Island, New Zealand (Figure 30) from May 2003 to June 2004.



Figure 30: Position of study locations in the North Island, New Zealand. Points indicate positions of the study sites.

Clove oil anaesthetic (clove oil 10% v/v in 70% ethanol, active ingredient eugenol, Griffiths 2000) diluted with water was added to rockpools prior to sampling to ensure that all fish were recorded. Sampling was only conducted in rockpools that were clearly separated from the sea, thus, rockpools in the surge zone were often unable to be sampled. The crevices and floors of the pools were extensively searched and all fish species found were collected with dipnets or by hand. Captured fish were retained in plastic buckets or bins filled with untainted seawater, until all fish in the rockpool were caught and identified.

Fish identifications were based on the following characteristics. Bellapiscis lesleyae was characterised by distinctive green and red patches on the upper body (especially on the front of the head), a thinner caudal peduncle, the head shape was more slender, and the caudal fin was marked with six colour bands (Figure 31). Bellapiscis medius was characterised by beige body colouration that was coarser, cream-like blotches and a regular checkerboard pattern on the ventral side of the body (Figure 31). Furthermore, lateral line scales in *B. medius* extend to the area between the second and third dorsal fin, whereas B. lesleyae lateral line scales go beyond that area. After identification, all fish were allowed to recover and returned unharmed to the sea. To estimate the size distribution of B. lesleyae and B. medius in the intertidal a random sample of both species was collected from rockpools at Matheson Bay, Takapuna Beach and Waheike Island (Figure 30) during the reproductive season (June - September) and measured to the nearest mm (total length, $L_{\rm T}$). Sampling during that time of the year ensured that fish individuals were not newly settled recruits, as the recruitment season commences approximately from October to March (see Chapter 5 and 7). The location of each rockpool was fixed using a handheld Garmin[®] 12 global positioning system (accuracy \pm 15 m). For each rockpool, a physically derived exposure index (Thomas 1986) was calculated using the program 'Fetch Effect Analysis' (version 1.01. Pickard R 2000, for details see Chapter 2).

Rockpool data were collected only during tides lower than 0.6 m above mean high water spring (MHWS). Parameters recorded for each pool included pool volume (cm³), pool surface area (cm²), exposure (estimated as maximum fetch in km), rockpool height ACD (cm), percentage cover of the substratum (rock [rocks > 7 cm], cobbles [rocks < 7 cm], gravel [rocks < 4 cm], sand) and percentage cover of biotic growth. Major algal species in the rockpools included the phaeophytes *Hormosira banksii*, *Carpophyllum maschalocarpum*, *Cystophora* spp., *Ecklonia radiata*, the chlorophytes *Ulva lactua* and *Caulerpa* spp. and the rhodophyte *Corallina officinalis* (nomenclature follows Adams 1994). The percentage cover of all abiotic substrate types and biotic growth was assessed visually.

The rockpools were measured with a plastic ruler to the nearest cm (length, width and depth). Rockpool surface area was calculated by multiplying mean length by mean width, and volume was calculated by multiplying surface area by mean depth. The tidal levels of each rockpool in relation to chart datum were calculated on days with minimal swell using a Leica-Geosystems dumpy surveyor's level (model number: Wild NA20, Heerbrugg, Switzerland). These measurements were later corrected for barometric pressure (mercury barometer corrected to mean sea level pressure recorded by J. Evans, Leigh Marine Laboratory, 2004).



Figure 31: (a) *Bellapiscis lesleyae* lateral view, (b) *B. medius* lateral view, (c) *B. lesleyae* dorsal view, (d) *B. medius* dorsal view (photographs taken by Iain MacDonald).

6.2.2 Data analysis

6.2.2.1 Investigation of rockpool resource partitioning

A Classification and Regression Tree analysis (CART, Breiman et al. 1984) was used to investigate which rockpools variables discriminate best between *B. lesleyae* and *B. medius*. The advantages of a CART analysis are that the same variable can be re-used in different parts of a tree, robustness to the effects of outliers, mixed data types can be used and surrogate variables can be used for missing values (Breiman et al. 1984). This means that that a greater capacity exists for extracting the maximum possible information from the dataset.

The FACT-style direct stopping method was selected as the stopping rule in which the desired minimum fraction was specified as the fraction of objects (FOB, see Appendix II for a detailed explanation of the FACT-style stopping procedure). 'Discriminant based univariate

splits for categorical and ordered splits' were chosen as the split selection method to determine the best terminal node to split the tree. This split selection method uses discriminant function analysis to determine which variables discriminate between two or more naturally occurring groups. Predictor variables used in the analysis were rock, cobbles, gravel, sand, algae, pool surface area, pool volume, rockpool height ACD and exposure (Table 8). The fully constructed tree was constrained (pruned) in depth afterwards to remove redundant nodes, and then evaluated using V-fold cross-validation (V = 5), global/stratified global crossvalidation (V = 5) and a relative ratio holdout training sample (cases: B. lesleyae n = 115, B. *medius* n = 85). The false positive, false negative and overall predictive accuracy for each cross-validation technique was graphed against the FOB to assess the effect of choosing the final stopping point or tree level. The program 'dichotomous relationship and decision table statistics' (DICHOT 3, available from http://www.pbmetrix.com/) was used to calculate-the false, positive, false negative and overall predictive accuracy outcomes (Barrett 1999). The holdout sample was constructed by randomly selecting cases throughout the entire data file, given that the ratio of codes/categories in the dependent variable is maintained as closely as possible. The holdout sample was created using a general purpose Statistica BASIC macro constructed for this purpose, available from http://www.pbmetrix.com/.

Conventional indices for the final model solution were calculated using the classification rates of the holdout sample validation, as this validation method is considered the most 'powerful' indicator of model fit (Barrett 2005d). The following indices were used: the false positive rate (the proportion of cases where a prediction for a positive outcome is made, but no outcome is observed), the true negative rate (the proportion of cases where a prediction for a negative outcome is made, but an outcome is observed), the classification accuracy, sensitivity (the probability that an actual observed event is predicted correctly), specificity (the probability that the actual non-occurrence of an event is predicted correctly) and 'Relative Improvement Over Chance' (RIOC, or the relative improvement of prediction over chance). The specificity rate is also known as the true negative and the sensitivity as the true positive rate (both terms are used synonymously in the literature). The indices were calculated using the program DICHOT 3. Linear regressions were used to further examine the relationships between intertidal height ACD and the other rockpool characteristics. The CART analyses were performed using Statistica (version 6.1).

A full explanation of the CART methodology and terminology can be found in Appendix II section i. Detailed information on cross-validation and pruning procedures can be found in Appendix II section ii and 0.

Variables	Mean	Minimum	Maximum	±SD
Height ACD (cm)	99.6	19	309	63.47
Rock (%)	67.3	20	100	21.57
Cobble (%)	16.3	0	80	18.2
Gravel (%)	8.3	0	50	9.41
Sand (%)	5.9	0	80	16.18
Algae (%)	28.7	0	95	20.79
Surface area (m ²)	9.8	0.4	67.0	10.8
Pool volume (l^3)	175.6	1.0	1184.1	222.0
Exposure (km)	299.7	29.4	3016.00	238.9

Table 8: Basic distributional statistics of the rockpool predictor variables for each species (*B. lesleyae* = 352, *B. medius* = 260) used in the CART analysis.

6.2.2.2 Mean number of fish per rockpool

Independent t-tests were used to analyse the abundance of each species in rockpools. Only pools that contained at least one individual per species were included in the analysis. Preliminary tests of equality of variances were not used in this study because it is inefficient to base a decision on preliminary tests of equality of variances (Zimmermann 2004a) and modern textbooks no longer recommend preliminary tests (Zimmermann 2004b). Instead, the Welch statistic was used as the primary indicator for statistical significance as this statistic is preferable to the F-statistic when the assumption of equal variances does not hold (Zimmermann 2004b, for more information see Chapter 5).

In general, the Welch-test assesses whether the means of two populations are equal when the variance of each population is different. It provides a t-statistic that asymptotically approaches a t-distribution, thus allowing for an approximate t-test to be calculated when the population variances are not equal (Zimmermann 2004b). All t-tests were run using SPSS, version 12.01 (SPSS 2003).

6.2.2.3 Fish size distribution in relation to rockpool height ACD

The body size of individual fish was correlated with the rockpool position ACD, to examine if there are intraspecific trends towards increasing or decreasing body size in the intertidal habitat. The linear regressions were performed using Statistica (version 6.1).

6.3 RESULTS

6.3.1 Spatial patterns

Species distribution was not random among rockpools. Only *B. medius* was found in rockpools greater than 246 cm ACD, with an upper distributional limit of 339 cm ACD and a lower limit of 31 cm ACD (Figure 32). In contrast, *B. lesleyae* was found predominantly in low intertidal rockpools with an upper distributional limit of 220 cm ACD (Figure 32). The partitioning of the vertical habitat is also reflected in the mean rockpool height for each species, with a mean of 71 cm ACD in *B. lesleyae* and 139 cm ACD in *B. medius*.



Figure 32: Vertical zonation of *B. medius* (n = 260) and *B. lesleyae* (n = 352). Black histograms denote observations for *B. lesleyae* and white histograms observations for *B. medius*.

Results of the CART analysis confirmed that the species differ in habitat use. The final tree solution was determined by cross-validation procedures. The initial tree with a FOB of 0.02 had eighteen splits and nineteen nodes and a classification accuracy of 95% for the V-fold, 91% for the global/stratified and 96% for the holdout sample (Figure 33). All three cross-validation figures (Figure 33) show a drop off in classification accuracy after a FOB of 0.09 and an increase in the misclassification rate, that is the false positive and false negative rates,

for both species. Consequently, the initial tree was pruned using a FOB of 0.09, which resulted in a tree with only seven splits and eight nodes (Figure 34). Cross validations showed that the classification accuracies of the much shorter and final tree dropped between 3 - 6% (89.7% for the V-fold, 88.4% for the global/stratified cross-validation and 89% for the holdout sample validation, Figure 33). Further pruning using a FOB of 0.13 would result in a tree with only four splits and five nodes. However, the trade-off of this very simple solution was an additional 7 - 8% reduction in classification accuracy (Figure 33). For this reason, the tree with a FOB of 0.09 was chosen as the optimal tree solution as there were many fewer nodes than the initial tree, whilst maximising the classification accuracy and ensuring some protection from 'overfitting' or capitalisation on random sampling errors.

Table 9 shows some conventional indices for the final model solution using the classification rates computed from the holdout sample validation. Both the false positive and false negative rates were relatively small (0.10 and 0.12, respectively), indicating that the overall species misclassification rate of the final model is minimal. This accuracy was reflected in the corresponding inverse values of sensitivity and specificity of 0.90 and 0.88, respectively. The RIOC value of the final model of 0.31 indexes the degree to which the final model increases predictive accuracy above chance levels (an RIOC of 0 would indicate that a test is no better than chance, whereas an RIOC of > 0 indicates increasing improvement over chance levels of prediction).

Classification indices for the final model			
False positive rate	0.10		
False negative rate	0.12		
Classification accuracy	0.89		
RIOC	3.10		
Sensitivity/True positive rate	0.90		
Specificity/True negative rate	0.88		

Table 9: Classification indices obtained from DICHOT 3 for the final model (FOB = 0.09) using the classification rates of the holdout sample validation.

Of nine potential predictors in the model specification, only four were selected by the algorithm to be used in the construction of the tree, indicating that four variables were sufficient to predict species membership. These variables were rockpool height ACD, pool surface area, pool volume, and algal coverage.



Figure 33: Results for the a) global/stratified cross validation, b) V-fold cross validation, and c) holdout sample variation. The graphs show the overall predictive accuracy (diamonds) for the CART analysis with different FOB values (0.02 - 0.13) and the false negative (squares) and false positive (circles) rates for each analysis. The red line indicates the FOB cut off point.

From these four variables, rockpool height ACD was found to be the most important variable in predicting fish group membership (Figure 34), confirming the observed vertical habitat partitioning among both species (Figure 32). This variable produced four splits in the tree at node 1, 2, 5 and 6. The successive splits on this variable were produced because both species overlapped in rockpools at a medium height ACD (Figure 32). In addition, the surface area of the rockpool was also found to be an important classifier and split the dataset at node 3. This split partitioned the dataset into rockpools with a small surface area (≤ 9202.7 cm²), containing mainly B. lesleyae, and rockpools with a larger area containing mainly B. medius (Figure 34). Pool volume was the third variable to be selected by the CART algorithm and produced a split on node 4 with B. lesleyae occurring more often in rockpools containing a water volume of > 239 1 (Figure 34). *Bellapiscis medius*, in contrast, was found to occupy pools with less water volume. The fourth variable selected by the algorithm was the variable 'algae'. This predictor produced a split at node 12 and separated the dataset into rockpools with a density of algae higher than 56% that contained mainly B. leslevae, and pools with density of less than 56% containing mainly B. medius (Figure 34). Together these results indicate that B. lesleyae was mostly found in shallow pools with a large surface area and little algae, whereas *B. medius* was more abundant in narrow, deep pools with abundant algae.

Further explorations of the rockpool habitat variables were done to test for correlations with intertidal height ACD. No significant correlations of intertidal height ACD with the variables cobble, sand, algae, pool surface, pool volume and exposure were found (Table 10). Rock

showed a significant positive (r = 0.42, p < 0.00) and gravel a significant negative (r = -0.31, p < 0.002) correlation with increasing vertical height ACD (Table 10). The magnitude of the correlation coefficients for both regressions were, however, relatively low (Table 10), suggesting that the biological significance is minimal (Daniel 1998).



Figure 34: Final pruned tree (FOB = 0.09) with the dependent variable (*B. medius*: n = 352 and *B. lesleyae* n = 260) and nine predictor variables (rock (%), cobbles (%), gravel (%), sand (%), algae (%), pool surface area (cm²), pool volume 1), pool height ACD (cm), and exposure (m)). Sample sizes are shown above each node. The decision block constituting the classifier for the final tree with a FOB of 0.09 can be found in Appendix II.

Table 10: r^2 -square values, correlation coefficients (r), and *p*-values for the linear regressions of the eight rockpool habitat variables with intertidal height ACD (cm).

Variables	r^2	r	<i>p</i> -value
Rock (%)	0.1408	0.3752	0.00006
Cobble (%)	0.0147	-0.1212	0.2204
Gravel (%)	0.0948	-0.3079	0.0028
Sand (%)	0.0156	-0.1251	0.195
Algae (%)	0.0098	-0.0989	0.3061
Pool surface (cm ²)	0.0267	-0.1635	0.0893
Pool Volume (cm ³)	0.0044	-0.0661	0.4949
Exposure (m)	0.0019	0.0433	0.6551

6.3.2 Fish size distribution

The *Bellapiscis* sister-species pair differed in body size, and overlap was minimal. *Bellapiscis lesleyae* has a mean body size of 3.64 cm (SD \pm 0.65 cm) and *B. medius* has a mean body size of 6.19 cm (SD \pm 0.14 cm) (Figure 35). Individuals of *B. medius* ranged from 4.0 - 8.5 cm L_T , whereas *B. lesleyae* ranged only from 2.1 - 5.0 cm L_T (Figure 35). Although the species size ranges were large, no intraspecific effect of L_T on the position in the intertidal was detected for either species (*B. lesleyae*: r = -0.07; p = 0.51; *B. medius*: r = 0.19, p = 0.132, Figure 36), suggesting that small and large individuals are evenly distributed in the species' preferred habitat space. This indicates that the occupation of intertidal rockpools was primarily affected by species identity, while the actual size of the individuals appeared to be less important.



Figure 35: Frequency of body lengths (L_T) of the intertidal sister-species pair *B. lesleyae* and *B. medius*. Black histograms denote observations for *B. lesleyae* and white histograms observations for *B. medius*. The mean body size of *B. lesleyae* 3.64 cm (SD ± 0.65 cm) and mean body size of *B. medius* is 6.19 cm (SD ± 0.14 cm)



Figure 36: Graph showing the L_T (mm) of *B. lesleyae* (n = 94) and *B. medius* (n = 54) on the y-axis and intertidal height ACD on the x-axis. Confidence intervals are shown in red.

6.3.3 Number of individuals in rockpools

Species differed significantly in the mean number of individuals per rockpools (mean for *B. lesleyae* 8.0 and for *B. medius* 3.4, $t_{54,42} = 2.53$, p < 0.005, Welch p = 0.013, Figure 37). Number of pools that contained at least one individual per species were 47 and 65 for *B. lesleyae* and *B. medius*, respectively. The number of individuals per aggregation ranged from 1 - 22 for *B. medius* and 1 - 75 for *B. lesleyae*. Importantly, aggregations of *B. medius* individuals of more than nine were only found in three instances in rockpools lower than 110 cm ACD. Despite the interspecific differences in aggregation sizes there was no evidence for a relationship between aggregation size and intertidal height ACD (*B. medius*: r = -0.01; p = 0.94; *B. lesleyae*: r = -0.28; p < 0.02). Linear regressions were carried out to investigate whether aggregation size of relates to pool dimensions, i.e. pool volume and surface area. There was no statistically significant correlation between pool volume and pool surface area for *B. lesleyae* (pool volume: r = -0.1, p < 0.561; surface area: r = -0.04, p = 0.830). For *B. medius*, however, there was a significant relationship between the size of the aggregation and the pool volume and surface area (pool volume: r = 0.51, p < 0.001; surface area: r = 0.48, p < 0.001).



Figure 37: Mean number of *B. lesleyae* (Bl) and *B. medius* (Bm) found per rockpool. The small square denotes the mean, the big square \pm SD and the error bars $\pm 1.96 \times$ SD.

The triplefin species *F. lapillum*, *G. capito* and *R. decemdigitatus* were also caught during the sampling procedure in relatively low and sheltered intertidal rockpools (Figure 38). Out of these three species, *F. lapillum* was by far most commonly caught in rockpools.



Figure 38: Species other than *Bellapiscis* spp. found in rockpools during the study (*F. lapillum* n = 66; *G. capito* n = 5; *R. decemdigitatus* n=5).

6.4 DISCUSSION

This study found clear evidence for resource partitioning in *B. lesleyae* and *B. medius*. Four rockpool variables, namely rockpool height ACD, pool surface area, pool volume and macroalgae, mainly influenced the species distribution in the intertidal zone. By far the strongest predictor for species occurrence was intertidal height ACD, with *B. lesleyae* predominately occupying low to mid intertidal pools, and *B. medius* mid to high intertidal pools, thereby producing a vertical zonation of both species. In addition, the aggregation size differed largely among species. *Bellapiscis lesleyae* mean aggregation size was more than double that of the mean aggregation size of *B. medius*. There was also a great difference in the mean $L_{\rm T}$ of the two species, however, no intraspecific trend between intertidal height ACD and intraspecific fish size was found.

The results of this study show that *B. lesleyae* and *B. medius* differed in the use of rockpools. The differing preference for rockpool height ACD was the strongest factor affecting the distribution pattern of both species, resulting in a marked vertical habitat segregation. Although there was some overlap at medium heights, *B. lesleyae* was predominantly found in rockpools at a lower tidal level than *B. medius*. In addition, the majority of *B. medius* were found in rockpools that exceeded the mean distributional range of *B. lesleyae*. This finding is consistent with many other studies that have found tidal height zonation patterns among intertidal fish species (Nakamura 1976; Yoshiyama et al. 1986; Zander et al. 1999; Davis 2000; Hernández et al. 2002; Szabo 2002), suggesting that divergence along a vertical gradient may be a common characteristic of fish species in the intertidal zone.

Because of the difference in vertical distribution, *B. medius* are exposed to physically harsher conditions than *B. lesleyae*, such as extreme fluctuations in temperature and dissolved oxygen (Stillman and Somero 1996; Hochachka and Somero 2002). Strong fluctuations in environmental variables can only be tolerated if the species possesses physiological tolerances to these fluctuations. Because *B. medius* most commonly inhabits elevated rockpools that are more likely to become hypoxic and experience temperature fluctuations, this species is likely to have evolved physiological tolerance and/or behavioural mechanisms to counteract the variability in physico-chemical factors, thereby allowing them to live higher in the intertidal region than their congener. Physiological tolerances for pronounced changes in temperature (Nakamura 1976; Crawshaw 1980; Fangue et al. 2001; Hernández et al. 2002; Hochachka and Somero 2002; Somero 2002) and dissolved oxygen (Zander 1972b; Fangue et al. 2001) have been found for a number of intertidal fish species. Moreover, many intertidal fish species are

also capable of behavioural response mechanisms, for example by migrating to areas within the rockpool where conditions remain relatively stable (Zander 1972a). Some intertidal fish species have been shown to tolerate low oxygen conditions by decreasing gill ventilation, heart rate (Crawshaw 1980), locomotor activity (Watters and Cech Jr. 2003) and by using aquatic surface respiration (Luck and Martin 1999; Hochachka and Somero 2002; Watters and Cech Jr. 2003).

Conversely, *B. lesleyae* is accustomed to relatively high-oxygen and temperature stable environments and would thus be forced to deal with hypoxia and temperature fluctuations less often. Hence, differences in the ability to cope with variation in these factors, be they physiological or behavioural, could explain the different vertical distribution in the intertidal habitat. A study by Zander (1972b) found a similar pattern in the blennioid fish *Alticus kirki* from the Red Sea. This amphibious fish has highly developed physiological and behavioural mechanisms for surviving in the conditions experienced in the upper intertidal and terrestrial environments, whereas similar mechanisms were less well developed in species with a lower vertical distribution (Zander 1972b).

The results of the present study showed that rockpool surface area affects species distribution, with B. medius occurring predominantly in rockpools with greater surface area. The greater surface area of elevated pools certainly counteracts the low oxygen content experienced by such pools, making them more stable during isolation from the subtidal zone, than pools with smaller surface areas at identical heights (Fangue et al. 2001). The preference by B. medius for rockpools with a high surface area highlights again that behavioural preferences for particular rockpool types can potentially affect their survival in the upper intertidal zone. The volume of the rockpool also significantly affected the occurrence of B. lesleyae, with more individuals occurring in rockpools that contained greater volumes of water. This is consistent with several other studies that have found that pool size is an important abiotic factor in structuring fish assemblages (Nieder 1993; Davis 2000; Magoulick 2000). Nieder (1993) suggested that large rockpools provide a refuge for intertidal fishes because high water volumes act as a buffer against varying levels of salinity, temperature and oxygen, providing the inhabitants of these pools with a relatively stable environment similar to the sea. Furthermore, greater water volumes lower the concentration of metabolic end-products like ammonium and carbon dioxide. Thus, this preference indicates that *B. lesleyae* preferentially selects rockpools that have relatively stable water properties that are similar to the seawater. Lastly, rockpools with greater algal coverage positively affected the abundance of *B. lesleyae*. An explanation for this could be that greater algal cover leads to more shelter and hiding places within the pool. Many studies have shown that high algal densities are related to high fish abundance and this was mainly explained by the increased shelter provided by the plants (Green 1971; Choat and Ayling 1987; Carr 1991; Nieder 1993; Davis 2000; Silberschneider and Booth 2001). Since both *Bellapiscis* species are known to forage mainly on small encrusting invertebrates, notably small chitons and the cirri of barnacles, it is unlikely, that micro-invertebrates in seaweeds are affecting the abundance of *B. lesleyae* (Feary 2001).

Regression analysis of the rockpool characteristics with intertidal height ACD found a weak negative relationship with gravel. This relationship is not surprising as gravel is likely to accumulate in the lower intertidal because the higher turbulence and abrasive action imposed by the breaking waves causes boulders to break. However, the mean gravel coverage of rockpools was very low (< 9%), indicating that gravel does not form a major part of the triplefin habitat. Moreover, gravel in the lower intertidal can get easily overturned and washed away by wave action and is hence not a suitable shelter for small intertidal organisms (Raffaelli and Hawkins 1996). The regression analysis also found a weak positive relationship with rock. Rocks accounted for over half (> 65%) of the rockpool substrate, and thus rock forms a substantial proportion of the rockpool habitat occupied by the Bellapiscis species. Rocks are also not easily overturned by the waves and hence form a relatively stable and safe shelter habitat for rockpool fishes. Previous studies have found that rocks are suitable shelter places for a variety fish species (Gibson 1972; Silberschneider and Booth 2001). Gibson (1972), for example, suggested that a rocky substratum is necessary for most fish species as it provides cover in the form of crevices, boulders, and a surface for algal growth. Interstitial microhabitats also reduce the risk of temperature stress as temperatures in these microhabitats are more stable than at, for example, exposed rock faces (Stillman and Somero 1996; Monteiro et al. 2002). Moreover, although B. lesleyae and B. medius are largely inconspicuous as a result of their cryptic colouration, shelter provided by rocks likely affords individuals further protection from predation, especially by terrestrial predators in the upper intertidal zone.

The presence of the three other tripterygiid species in rockpools is not surprising as they can commonly be found in the shallow subtidal. These species are usually found in subtidal habitats in depths between 2 - 10 m (see Chapter 2) (Francis 2001). In particular, *R. decemdigitatus* and *G. capito* are most commonly found in sheltered habitats not deeper than 4 m, whereas *F. lapillum* occupies a much wider range of habitats, ranging from exposed to sheltered areas and depths between 2 - 20 m (Chapter 2), and sometimes even down to 40 m (Syms 1992). *Fosterygion lapillum* has been reported previously from the intertidal zone, and

recruits have been observed to settle frequently in rockpools (Fisher 1998). Relocation experiments by Fisher (1998) found that F. *lapillum* homes to a particular rockpool or to a set of rockpools. This suggests that this species does not get accidentally caught in the intertidal habitat but instead chooses this environment actively during the settlement phase. This study shows that the intertidal environment can be utilised by these species, however, it is unknown if they stay for their entire life span or whether they only occur on a temporal basis in the rocky intertidal.

The abundance of each species found in rockpools differed between species. Unlike B. *lesleyae*, *B. medius* was seldom found in large aggregations (> 23). Importantly, large aggregations of *B. medius* were positively correlated with pool volume and pool surface area. This indicates that aggregations of *B. medius* need significantly more space as well as more stable conditions in physico-chemical variables. Furthermore, the largest B. medius aggregations (> 9 individuals) were exclusively found in rockpools in the lower intertidal (< 110 cm ACD), where emersion times are reduced. This may highlight that large aggregations of B. medius are in need of more stable conditions. Because the occurrence of B. medius is intimately linked with the upper intertidal, individual fish and small aggregations of B. medius would be beneficial as they use less of the oxygen, thereby maximising the respiration time for the fish in hypoxic conditions. Therefore, it is not surprising that the mean aggregation size of *B. medius* is less than half of the size of *B. lesleyae*. Moreover, it is known that species abundance of invertebrates decreases towards the higher sectors of rocky intertidal areas (Raffaelli and Hawkins 1996). As a consequence, the abundance and quality of food available for mobile predators, such as fishes, also decreases along a vertical gradient in the intertidal zone (Raffaelli and Hawkins 1996). Thus, it would be beneficial for fish in the upper intertidal to co-occur with relatively few food competitors. For this reason, the low aggregation size of B. medius could also be a response to food limitation in the upper intertidal zone.

In contrast, *B. lesleyae* was often found to occur in large aggregations. For example, a single rockpool at Matheson's Bay contained 75 individuals. In addition, the aggregation size of *B. lesleyae* was not correlated with pool surface area or pool volume. However, *B. lesleyae* preference for low intertidal pools probably buffers against the negative effects of large aggregations, e.g. decrease in oxygen, because lower pools are more frequently inundated with fresh seawater.

This study found very large interspecific differences in adult size, with the largest individuals of *B. medius* being 4 - 5 times longer than the smallest *B. lesleyae* individuals. Hence,

elevated pools were almost exclusively occupied by the much larger species and low pools by the comparatively small species. This finding is not consistent with most other studies investigating fish size distribution in the intertidal zone, which found that larger fish tended to occur in the lower and smaller fish in the upper intertidal zone (Connell 1961; Prochazka and Griffiths 1992; Davis 2000). Several theories have been proposed to explain this size-based vertical partitioning. Prochazka and Griffiths (1992) suggested that this pattern is the result of territoriality of larger fish occupying lower, more environmentally stable pools. According to this view, low intertidal pools might be favourable as they are more stable from changes in physico-chemical factors because of the shorter duration of emergence time compared to pools in the upper intertidal. Thus, because territoriality is common among intertidal fishes (Mayr and Berger 1992), and because larger fish are usually more successful in inter- or intraspecific contests (Jones 1984; Thompson 1986; Mayr and Berger 1992; Szabo 2002), larger fish should be able to inhabit the most favourable pools. However, organisms with a large body size could have several advantages when living in the upper intertidal zone. First, protein damage occurs when temperatures reach the upper limit of an organism's physiological temperature range (Somero 1995; Tomanek and Helmuth 2002), and heat death when the upper temperature limit is exceeded (Somero 2002). Second, large fish of some species have been found to tolerate longer emersion times than small fish (Faria and Almada 2001b), making them more tolerant to the fluctuations in physico-chemical factors experienced in the upper intertidal zone. Alternatively, fish growth in the upper intertidal may simply be enhanced due to the higher temperature in elevated rockpools (Hochachka and Somero 2002; Pulgar et al. 2003), resulting in larger animals in the upper intertidal zone. Additionally, a smaller body size by individuals inhabiting the lower intertidal would be beneficial in this highly wave exposed habitat (Little and Kitching 1996; Cruz et al. 2004; La Mesa and Vacchi 2005). The rapid movement of water in the low intertidal increases drag forces as well as acceleration forces of the water, hence, fish with a greater surface area are more likely to dislodge from the substrate (Little and Kitching 1996).

Unlike the large interspecific size differences along the vertical gradient, there was little evidence for an intraspecific correlation between fish size and intertidal height ACD. It is noteworthy though that only *B. lesleyae* individuals longer than 3.5 cm were found in rockpools above 90 cm ACD. This lack of intraspecific vertical size partitioning in the intertidal stands in contrast with other studies that found clear ontogenetic differences in size distribution of fish (Faria and Almada 2001b; Szabo 2002). It has been suggested that intraspecific competition for space should be important in species with no obvious

ontogenetic shifts in morphology and behaviour (Szabo 2002), and several studies on intertidal fish have reported different habitat use by different age classes of the same species (Green 1971; Nakamura 1976; Faria and Almada 2001b). The morphology of *B. lesleyae* and *B. medius* juveniles does not change after settlement (Hardy 1987), and *B. lesleyae* juveniles have been reported to settle in the same rockpools as adults (Fisher 1998). However, competition for space only occurs when resources are limited. In this sense, habitat resources may not be limiting, thereby allowing the coexistence of individuals with similar habitat requirements. Alternatively, resources may be limiting and competition for habitat space may result in density-dependent settlement of new triplefin recruits (see Chapter 5), thus compensating for limited habitat space.

Differences in vertical distribution between intertidal species have been explained by two factors (Connell 1961). The first factor encompasses the environmental tolerance of a species, such as tolerance to thermal stress. Interspecific differences in stress tolerance allows the species with the higher upper bound to inhabit areas in which the other species cannot survive. The second factor relates to the competitive ability of the two species. The species with the higher environmental tolerance is typically the poorer competitor, and is limited at its lower bound by competition with the second species. In this sense, the vertical partitioning of rockpool resources seen in the Bellapiscis sister-species may be the results of the ghost of competition past. Intraspecific competition for limiting resources (e.g. habitat space) can be a potent force in the evolution of species (Schluter 2000a; Munday 2001; Munday et al. 2004; Nosil and Crespi 2006), and commonly leads to a fitness disadvantage of intermediate phenotypes. Extreme phenotypes, on the other hand, are favoured in this process as they are capable to use resource types that are only accessible by a few other individuals in the populations. Over time, this fitness trade-off can result in evolutionary branching (Dieckmann and Doebeli 2000), and if this branching event is simultaneously linked with assortative mating then this may produce two distinct species. Assortative mating in triplefins could easily evolve as a result of diverging habitat preferences, as differences in habitat preferences reduce spatial overlap in nesting sites. In the Bellapiscis pair, individuals with higher tolerance for fluctuating rockpool environments may experience a fitness advantage, as they can occupy the upper intertidal, thereby reducing competition with the rest of the population for habitat space. In this sense, differences in spatial resource use in the Bellapiscis sisterspecies pair are fully consistent with speciation caused by density-dependent interactions, and thus with speciation caused by ecological contact in sympatry.

In summary, this study demonstrates that *B. lesleyae* and *B. medius* diverged in resource use, with the rockpool variables surface height ACD, area, volume and macroalgae coverage being the main predictors of species distribution. Of these variables, vertical height ACD was the strongest factor affecting resources partitioning, resulting in an elevational species gradient, with *B. medius* occupying the upper and *B. lesleyae* the lower intertidal zone. This study also found evidence for species-specific behavioural mechanisms that appeared to be specialisations for living in their respective habitats, such as differences in aggregation sizes. It is likely that *B. medius* also possess physiological tolerances to the harsh conditions experienced in the upper intertidal zone, and that the lack of these characteristics may play a role in restricting *B. lesleyae* to the lower intertidal and subtidal zones. Density-dependent competition for mutually shared resources is consistent with the resource use pattern in the *Bellapiscis* sister-species pair, and thus may have been an important component in the divergence of these species.

7 Reproductive Isolation in Triplefin Fishes

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7.1 INTRODUCTION

Reproductive isolation is caused by any barrier that prevents successful reproduction between individuals. It includes prezygotic barriers such as spatial, temporal and sexual isolation, and postzygotic barriers such as hybrid inviability, hybrid sterility and F2 breakdown (Dobzhansky 1937; Mayr 1942). Both prezygotic and postzygotic isolating barriers may be active at the same time, and both types of barriers have generally been given equal importance (Coyne and Orr 2004). However, recent studies suggest that prezygotic isolation may be more important than postzygotic barriers in permitting speciation to take place readily and rapidly (Boake et al. 2000).

Spatial isolation is one prezygotic barrier which arises from divergent habitat preferences, and results in the spatial separation between ecologically divergent forms (Rice and Salt 1988; Rice and Hostert 1993). Probably the best examples of breeding habitat isolation involve studies of insect species colonising novel plant hosts (Bush 1969; Feder 1998; Via et al. 2000; Via 2001), such as the frugivorous flies of the genus *Rhagoletis* (Bush 1969). These insects court and mate on the larval host plant, thus, there is a direct correlation between mate and breeding habitat selection (Bush 1969) which prevents all contact during the reproductive season (Coyne and Orr 2004). There is also evidence for breeding habitat isolation in fishes. For example, two sympatric stickleback populations are almost entirely reproductively separated due to differences in their breeding microhabitat. The white stickleback nests above the substrate in filamentous algae while the threespine stickleback nests on substrate away from the algae (Blouw and Hagen 1990; Jamieson et al. 1992).

Two other prezygotic isolating factors that have been discussed extensively in the literature are temporal (e.g. spawning periods) and sexual isolation (e.g. mate choice based on body size or body colouration) (Nosil et al. 2005). Temporal isolation of breeding occurs when two populations are not physically isolated, but instead breed at different times, thereby reducing the frequency of inter-population matings (Quinn et al. 2000). It can occur on a diurnal, seasonal or annual scale and evolves via genetic correlations and/or genetic hitchhiking of other life history traits (Rice and Hostert 1993). A study by Knowlton et al. (1997) in the Caribbean showed that differences in spawning times lead to the reproductive isolation of sympatric sibling species of the coral genus *Montastraea*, suggesting that these differences might act as a temporal barrier to avoid hybridisation. Temporal isolation has also been shown to play a major role in the reproductive isolation of Chinook salmon populations, and can evolve over very short time frames (Quinn et al. 2000).

Sexual isolation occurs when potential mates meet in a reproductive context but do not reproduce because of different mate preferences (Nosil et al. 2005). A wide variety of traits have been linked with mate preferences, such as body size, colour patterns and courtship behaviour. Body size plays an important role in mate choice in many groups of vertebrate and invertebrate taxa, in particular snails (Rolán-Alvarez et al. 1999; Cruz et al. 2004), birds (Jouventin and Bried 2001; Podos 2001), and fishes (Lambrecht and Rebhan 1997; Nagel and Schluter 1998; Triefenbach and Itzkowitz 1998; Schliewen et al. 2001; Redenbach and Taylor 2003; McKinnon et al. 2004). In fish, body size is a trait that has been found to diverge in response to differences in habitat (Faria and Almada 2001b; Schliewen et al. 2001; McKinnon et al. 2004). For example, a study by McKinnon et al. (2004) showed that two stickleback ecotypes, known as the small 'limnetic' and the large 'benthic' ecotype, diverged in body size due to differences in their respective limnetic and benthic lifestyles. McKinnon et al. (2004) further showed that the ecotypes are reproductively isolated from one another, with levels of isolation being directly related to differences in body size.

Body colouration is another trait that has been strongly linked to mate preferences and is probably the best studied mate choice trait in fish to date. Most examples are based on female choice of male colouration in the visible spectrum (Seehausen et al. 1997; Seehausen and Van Alphen 1999; Couldridge and Alexander 2001). For instance, female choice on male nuptial colour patterns has been implicated as a major force in promoting the reproductive isolation and consequent explosive speciation of cichlid flocks in the East African lakes (Seehausen et al. 1997; Allender et al. 2003; Jordan et al. 2003; Pauers et al. 2004). These studies have shown that males of sister-species are generally very divergent in colour pattern.

Unlike the extensive interest that has surrounded the topic of female selection on male colouration in the visible spectrum, less attention has been given to female choice on male colouration in the non-visible spectrum, specifically ultraviolet (UV) wavelengths. Ultraviolet light is present in the sea at biologically detectable levels to around 100 m depth in clear tropical environments (Losey *et al.* 1999), and to a lesser extent in temperate areas. Work by Marshall (1996) showed that the bodies of 60% of the coral reef fish exhibited spectral reflectance of UV body colours, and other studies have shown that some fish species are sensitive to UV light (Siebeck and Marshall 2001). This has led to an increasing interest in the role of UV vision on mate choice in fish (Losey et al. 1999; Fritsches et al. 2000; Marshall 2001; Smith et al. 2002). Support for the importance of UV colour pattern in mate choice in fishes comes from a study by Smith et al. (2002) on guppies, where the visual appearance of potential mates was manipulated using either UV transmitting

or UV blocking filters. The results of Smith et al. (2002) clearly demonstrate that female guppies significantly prefer males without the UV filters, indicating that male UV body patterns are crucial in guppy mate selection. Lastly, interspecific differences in male courtship behaviour have also been implicated to cause behavioural isolation between closely related species (e.g. Boake *et al.* 2000). The cause of behavioural isolation is usually assumed to be female discrimination, because there should be very strong selection against females choosing a male of the wrong species (Andersson 1994).

Because New Zealand triplefin species have sympatric distributions, they provide an ideal system to examine the role of prezygotic barriers on reproductive isolation. Given some of the characteristics of New Zealand triplefins, there are several possible mechanisms of prezygotic mating isolation. For example, because several closely related species show divergence in breeding microhabitat (Feary 2001) and differences in annual reproductive periods (Handford 1979; Thompson 1979; Warren 1990; Feary 2001), both habitat and temporal isolation could have contributed to the New Zealand triplefin radiation. Sexual selection on secondary traits is also possible, given that there is some evidence that species differ in secondary sexual traits (such as body size, Francis 2001), however, little is known about the behavioural aspects that influence sexual selection in triplefins. This Chapter aims to assess the potential significance of spatial, temporal and sexual isolation as prezygotic isolating factors that may have been important in the evolution of the New Zealand triplefin fishes. Seven hypotheses were tested, which together cover most of the prezygotic isolating mechanisms reported for animals in the literature to date. The hypotheses tested are as follows:

- 1. Species do not show spatial isolation,
- 2. Species do not show temporal isolation,
- 3. Closely related species interbreed if denied a conspecific mate,
- 4. Sister-species do not differ in colour in the visible or UV bandwidth,
- 5. Body length of nesting males does not differ between species,
- 6. Mate choice in the *Ruanoho* species is not affected by male size,
- 7. The *Ruanoho* species do not differ in courtship display.

Hypotheses 1 - 2 relate to spatial and temporal isolation in a variety of species. Hypothesis 3 relates to the extent to which habitat-assortative mating drives reproductive isolation between

triplefin species. Hypotheses 4 - 7 relate to specific traits in triplefins that have been associated with sexual selection in other species (see above), and thus may have been of evolutionary importance in the built-up of reproductive isolation in this group. Particular emphasis in this Chapter is given to the *Ruanoho* species because they form the sister-species pair with the least amount of genetic divergence in the New Zealand triplefin radiation (Hickey and Clements 2005), and thus the factors that have led to their divergence should be the clearest (Schluter 2000b).

Testing the first hypothesis will establish whether triplefins exhibit divergence in nesting sites, while testing the second hypothesis will quantify the extent of interspecific overlap in spawning periods. To date, little descriptive work is available on nest site choice and reproductive timing in New Zealand triplefins. Most studies have focused on only one or two species (Anderson 1973; Handford 1979; Thompson 1986), with the exception of a recent study by Feary (2001) which looked at the nest sites of seven species. These studies found considerable overlap in spawning habitat on a broad scale, though nest site preferences on a finer scale suggested that some process of nest site selection is in place (Handford 1979; Thompson 1979; Thompson 1976; Feary 2001). All of these studies, however, lack detailed quantitative and comparable data on the nest site characteristics of the majority of species, thereby failing to assess how much overlap in space and time exists between co-occurring species.

The third hypothesis will experimentally assess the degree of opportunity isolation versus recognition isolation (i.e. the extent to which isolation is driven by probability of encounter versus mate choice, Palumbi 1994; Palumbi 1998) in the sympatric triplefin species pairs (i) *G. capito* and *F. varium*, and (ii) *R. whero* and *R. decemdigitatus*. Each species pair was selected for a different reason. *Grahamina capito* and *F. varium* were selected for the hybridisation trials because they are the only species for which hybrids have been found in the wild. Although there is genetic and morphological evidence for hybridisation between *G. capito* and *F. varium* (Hickey 2004; Hannan 2005), there are no observations of interbreeding in the wild. Hybrids were found in all locations sampled in southern New Zealand by Hickey (2004) and Hannan (2005), namely Doubtful Sound (Fiordland), the Catlins coast (Southland), Portobello (Otago Harbour) and Bluff. The hybrids morphologically resemble *G. capito* but have *F. varium* mitochondrial DNA, and thus represent an *F. varium* female lineage. The *Ruanoho* sister-species pair were selected as they have been found to have the least amount of genetic divergence in the New Zealand radiation, and hence may be more likely to hybridise than other species. Fricke (1994) synonymised the *Ruanoho* species and

treated them as ecological forms of the same species, however, recent work by Hickey and Clements (2005) clearly shows that *R. whero* and *R. decemdigitatus* are genetically distinct. The *Ruanoho* species differ in morphology (Hardy 1986), maximum size (Hardy 1986), colour pattern (Francis 2001), mitochondrial DNA, nuclear DNA and genome size (Hickey and Clements 2005). Hybrids of *R. whero* and *R. decemdigitatus* have never been found in the wild, suggesting that hybrids are not viable, that hybrid larvae could not be identified or because these species do not hybridise.

Testing the fourth hypothesis will establish whether there is sexual selection on colour patterns by comparing male spawning colouration in the UV and visible light spectrum with the colouration of non-breeding males. Unlike cichlids, in which males of closely related species show conspicuous differences in colouration, male nuptial colouration in triplefins may not be a particularly important reproductive cue. There is evidence that males of some triplefin species assume a darker body colouration during the spawning period (Francis 2001), though descriptive studies are lacking, hence the reason for investigating colouration in the visible spectrum. Ultraviolet colour patterns were also investigated because a significant proportion of fish are known to visualise UV light (Siebeck and Marshall 2001), therefore it is possible that there may be sexual selection based on UV colour patterning (e.g. Smith et al. 2002; Jordan et al. 2004). Three elements are essential for mate choice selection based on UV colour reflectance: (i) UV reflectance of the fish body (Jordan et al. 2004); (ii) evidence for UV visual pigments in fish cone telereceptors (Siebeck and Marshall 2001; Smith et al. 2002); and, (iii) female selection on these UV colour patterns (Smith et al. 2002). In this study only the reflectance colour pattern of the fish body was measured, as this would provide a prerequisite for mate selection based on male UV colour pattern.

Testing the fifth hypothesis will investigate whether nesting male triplefins differ interspecifically in body length (L_T) to evaluate the possibility of reproductive isolation caused by differences in female preferences for male body length (e.g. Beaugrad and Zayan 1985; Meyer et al. 1994; Nagel and Schluter 1998; Oliveira et al. 2000). The effect that different body lengths might have on mate choice in the *Ruanoho* species was experimentally assessed in the sixth hypothesis using mate choice trials in the laboratory. The *Ruanoho* sister pair was chosen because this pair differs considerably in body size, with *R. decemdigitatus* reaching a total length of over 12 cm and *R. whero* just over 9 cm (Francis 2001). Finally, testing the seventh hypothesis will examine differences in the courtship displays of *R. whero* and *R. decemdigitatus*, as preliminary observations have indicated differences in male sexual behaviour.

7.2 MATERIALS AND METHODS

7.2.1 Spatial and temporal isolation: data collection and analysis

Details of triplefin nesting sites were recorded at five locations around New Zealand using UVC (Hauraki Gulf, Coromandel, Napier, Dunedin and Fiordland, see Figure 1). The site selection and transect design was identical to that described in Chapter 2. Data collection at the Hauraki Gulf was done over two consecutive years from June 2003 to December 2004, while the other four locations were visited only once during this study (Figure 39). The Hauraki Gulf was chosen as the main study site as it is a large area with a wide variety of accessible habitats, enabling the quantification of the breeding characteristics of a number of triplefin species from a range of exposures and depths at coastal and offshore sites. For this reason, the spatial and temporal data from the Hauraki Gulf was analysed in detail, while the data for the remaining four locations was only used to investigate geographic variation in breeding habitat characteristics. The temporal data from the four other locations was not any analysed further, as the water temperatures and day light hours differ greatly along the New Zealand coastline (Francis and Nelson 2003) and potentially adds variation that is not solely due to interspecific differences in the spawning cycle.



Figure 39: Map of study sites in the Inner and Outer Hauraki Gulf in northeastern New Zealand. Study sites are marked with a black circle.

For each 1 x 1 m quadrat the identity and number of triplefin fishes, and the depth (m) and exposure (km) of the habitat was recorded (see Chapter 2 for details). In addition, during the spawning season the date and microhabitat characteristics were recorded for each nest by measuring the 15 x 15 cm area around the centre of each triplefin nest. Triplefin territories (1 x 1 m) and nests (15 x 15 cm) were quantified according to substratum coverage (rock [rocks > 7 cm], cobbles [rocks < 7 cm], gravel [rocks < 4 cm], sand, and mud) and algal coverage (macroalgae and coralline and turfing algae). While the first five substratum variables always sum to 100%, the algal coverage could range from 0 - 100%. Lastly, the total length (L_T [cm]) of the nesting males were visually estimated and the nest microposition was recorded. The nest micropositions included the 'top of rock' (TOP), 'side of rock' (SID), 'under rock or in crack of rock' (UCS), and in 'vertical crack in rock' (CVR). To identify nests of the *F. varium/G. capito*-hybrids eight nesting males that visually resembled *G. capito* were caught with hand nets in Bradshaw Sound, Fiordland, and their identity resolved by sequencing the D-loop of the mitochondrial DNA by D. Hannan at the University of Auckland (Hannan 2005).

Data analysis proceeded in the following way. The first hypothesises of this Chapter investigated whether species could spatially encounter each other during the spawning season. Only species that had more than 10 nest observations were included in the spatial analysis, which resulted in nine species being selected (*F. flavonigrum n*= 16, *F. lapillum n* = 162, *F. malcolmi n* = 32, *F. varium n* = 81, *G. capito n* = 31, *G. nigripenne n* = 42, *O. maryannae n* = 24, *R. decemdigitatus n* = 12 and *R. whero n* = 56). The breeding habitat characteristics for the hybrid triplefin were also included in the summary table (see Appendix III, section i), though only three individuals could be positively identified. This was done because this is the first data on the hybrid's ecology and may help to understand the processes leading to hybridisation.

Spatial overlap between species in breeding habitat was measured by comparing the continuous (depth, exposure, substratum variables of the macro- (1 x 1) and microhabitat (15 x 15 cm)) and categorical nest variables (microposition, on- and offshore location, and degree of shelter) of species nesting habitats with one another. The two binary categorical variables (on/offshore and exposed/sheltered) were re-coded as binary variables while the microposition variable was re-coded as a dummy binary variable, using the effects coding technique. The remaining continuous variables were checked for linearity, and a square root transformation was applied to improve linearity. An initial Principal Components Analysis of the independent variables indicated no strong collinearity between the habitat variables. A
Canonical Discriminant Analysis (CDA) was used to produce a bi-plot of species and nest variables. To determine the predictive accuracy of the solution, the data were cross-validated using a Linear Discriminant Function. The resulting classification and misclassification rates were used as an indication of overlap in breeding habitats between species. All spatial analyses were done in SAS (version 9.1).

In addition, geographical comparisons (Hauraki Gulf, Coromandel, Napier, Dunedin and Fiordland) of nests characteristics were conducted for seven out of the nine species, namely *F*. *flavonigrum* (nests: Hauraki n = 14, Fiordland n = 2), *F. lapillum* (nests: Hauraki n = 144, Fiordland n = 8, Napier n = 10), *F. malcolmi* (nests: Hauraki n = 27, Fiordland n = 5), *F. varium* (nests: Hauraki n = 75, Napier n = 6), *G. capito* (nests: Hauraki n = 13, Dunedin n = 17, Fiordland n = 8), *O. maryannae* (nests: Hauraki n = 3, Fiordland n = 21) and *R. whero* (nests: Hauraki n = 51, Fiordland n = 2, Napier n = 3). For the geographical comparisons, all species observations were used because of the scarcity of observations in many locations. Species nest characteristics for each location were visualised with box and whisker plots. Location comparisons of the use of micropositions and nest cover were done using Chi-square tests in Statistica (version 7.1).

The second hypothesis of this Chapter investigated whether triplefin species show temporal overlap of spawning times in the Hauraki Gulf (Figure 39). Temporal isolation was assessed by comparing the length and timing of the reproductive season between all species for which \geq 10 nests could be identified, namely *F. flavonigrum*, *F. lapillum*, *F. malcolmi*, *F. varium*, *G. capito*, *G. nigripenne*, *O. maryannae*, *R. decemdigitatus* and *R. whero*.

7.2.2 Hybridisation experiments: data collection and analysis

The third hypothesis investigated if the *Ruanoho* species hybridise if denied a conspecific mate. Specimens for the hybridisation trials were collected from northeastern New Zealand during the triplefin breeding season in 2003 and 2004 (Figure 39). Individuals of *R. whero* and *R. decemdigitatus* were obtained using slurp guns from the Whangaparaoa ($36^{\circ}36'S$, $174^{\circ}50'E$) and Tawharanui Peninsulas ($36^{\circ}22'S$, $174^{\circ}48'E$, Figure 39). *Grahamina capito* individuals were caught at Big Omaha Wharf, Whangateau Estuary, using bait catchers, and *F. varium* individuals were collected from the Whangaparaoa Peninsula using hand nets. After capture fish were immediately transported back to the laboratory and maintained in holding aquaria for at least four days prior to the commencement of the trials. Males were

sexed visually on capture by the dark spawning colouration (Francis 2001; Clements 2003). Fish were kept in holding aquaria that were provided with sand, gravel and different-sized stones to simulate their natural habitat. Each aquarium received fresh seawater at ambient temperature (approximately 15 - 17°C), salinity (34 - 34.7‰) and photoperiod. Fish were fed daily *ad libitum*, with a variety of different prey types including *Artemia* spp. nauplii, frozen bloodworms (*Chironomid* spp.), frozen adult brine shrimp (*Artemia* spp.) and New Zealand green lip mussels (*Perna canaliculus*).

Hybridisation trials of (i) *R. whero* and *R. decemdigitatus* and (ii) *G. capito* and *F. varium* were carried out during the reproductive season at the Leigh Marine Laboratory, University of Auckland. Hybridisation trials in 2003 were conducted using individuals of *R. whero* and *R. decemdigitatus*, and a second set of hybridisation trials in 2004 involved individuals of *G. capito* and *F. varium*. Specimens for the mixed species trials were selected on the basis that they had spawned previously in a same species trial to ensure that individuals were reproductively active. The limitation of this approach, is however, that prior experiences may lead to behavioural imprinting in mate choice and this could lower the chance of hybridisation (Jennions and Petrie 1997). This could be avoided by using naïve pre-settlement individuals from the wild, however, it is not possible to sex triplefin fishes accurately or to distinguish between mature and immature individuals without observing reproductive behaviour. On this basis it was reasoned that it was more important to avoid getting spurious negative results due to the individuals in the hybridisation trials being immature.

Mature males were placed individually in aquaria and allowed to establish a nest. A female was then introduced and monitored for 10 days to determine if mating took place. Polystyrene tiles were placed between all experimental aquaria to prevent visual contact between fish. Behaviour in the mixed and same species trials was observed on a daily basis and, in the case of a spawning event, the nest microposition recorded (STB or UCS). Any eggs produced in the *Ruanoho* trials in 2003 were assessed for viability, namely embryo development and prehatch duration. This was done to compare the developmental characteristics of the *Ruanoho* species with that of possible hybrids to determine if and how hybrids develop. The mixed trials of the *Ruanoho* species consisted of 11 pairs of female *R. whero* and male *R. decemdigitatus*, and nine pairs of male *R. decemdigitatus* and female *R. whero* pairs. The mixed species trials for *G. capito* and *F. varium* consisted of seven pairs of female *F. varium*, while the homogenous trials were run with nine pairs of *F. varium* and 10 pairs of *G. capito* (see

Appendix III section iii). The $L_{\rm T}$ of all fish used in the hybridisation trials was recorded to estimate the size at which *R. whero* and *R. decemdigitatus* show reproductive activity. Additionally, to verify the behavioural observations of reproductive behaviour, specimens of both species were dissected during the spawning season to determine gonad maturity. Maturity was determined by visual inspection of the gross anatomy using characteristics for triplefins employed by previous researchers (Handford 1979; Neat 2001).

7.2.3 Male body colouration: data collection and analysis

The fourth hypothesis assessed if interspecific differences in colour pattern in the visible and UV spectra could lead to reproductive isolation in triplefin species. Photographs of males in spawning colouration were taken for eight species (*F. flavonigrum*, *F. lapillum*, *F. malcolmi*, *F. varium*, *G. capito*, *G. nigripenne*, *R. decemdigitatus* and *R. whero*) and quantitatively compared to the colouration of individuals outside the spawning season (male and female are indistinguishable outside the spawning season).

Male spawning colouration was quantitatively examined by using lateral photographs of breeding and non-breeding individuals and then comparing the intensity of colouration of the fish bodies using ImageJ (1.36b, W. Rasband, USA). This method was chosen instead of the commonly used colour assessment method using Adobe PhotoshopTM (e.g. Alexander and Breden 2004) because it is not possible to take photographs under standard conditions in the field. This is because New Zealand triplefin species show highly divergent habitat use (Feary and Clements 2006), and thus habitats of nesting males will not only differ in exposure and depth, but also in light transmission levels due to differences in water clarity (e.g. estuarine versus open water habitats). It was not logistically possible to catch several individuals of each species (individuals can only be sexed during the breeding season) and to keep them in aquaria over several months and photograph males in the breeding season under standardised conditions. In addition, observations suggest that the colour intensity decreases in reproductively active males (Paulin and Roberts 1992; Francis 2001; Clements 2003), and for this reason a colour intensity analysis between breeding and non-breeding males was deemed adequate. All photographs were converted into binary 8-bit grey scale images (grey scale range 0 - 256) by selecting the function 'Type' and the '8-bit option' from the 'Image' menu. The intensity of colouration was measured using a rectangular shape that was approximately the size of the eyes of the individual measured (the shape was created with the ROI plug-in tool) along a transect from the area behind the eyes along the lateral line to the caudal

peduncle. Colour intensity was measured at 10 evenly spaced points along this transect. For intraspecific comparisons between individuals in normal and spawning colouration, the mean intensity estimate of each point was displayed for each species. With the exception of *O. maryannae* the comparisons were run for all species that were used in the spatial and temporal analysis, namely *F. flavonigrum* (non-spawning 12, spawning 10), *F. lapillum* (non-spawning 11, spawning 9), *F. malcolmi* (non-spawning 11, spawning 6), *F. varium* (non-spawning 13, spawning 8), *G. capito* (non-spawning 9, spawning 9), *G. nigripenne* (non-spawning 9, spawning 6), *R. decemdigitatus* (non-spawning 10, spawning 2), and *R. whero* (non-spawning 11, spawning 5). Statistical comparisons of the colour intensity between individuals without and with spawning colouration were conducted for each of the 10 points and for each species using independent t-tests in Statistica (version 7.1).

Male body colouration in the UV spectrum was investigated in three sister-species pairs triplefin, namely *F. lapillum* and *G. nigripenne*, *F. malcolmi* and *O. maryannae*, and lastly *R. decemdigitatus* and *R. whero*. Individuals that were presumed to be nesting males of these species (as indicated by the presence of spawning colouration and a nest) were captured in the wild and transported to the laboratory. Fish were dispatched by an overdose of clove oil and the UV colour pattern immediately visualised using a Mineralight multiband UV 254/366-nm lamp (UVP, California, USA). Photographs were taken using a Canon G1 camera with both a visible bandpass filter (No. 59875, Oriel Co., Connecticut, USA) and an UV transmitting bandpass filter (No. 7-60, Turner Designs, California, USA). The individuals were positively identified as males by macroscopic inspection of the gonads after the photographs had been taken. The method was validated with photographs of UV reflectance patterns of objects that are known to have underlying UV colour patterns (see results).

7.2.4 Male body length: data collection and analysis

The fifth hypothesis investigated if nesting males show interspecific differences in body length to assess whether female selection on male body size could lead to reproductive isolation in this group. The non-parametric Kruskal-Wallis test was used to investigate whether median nesting male body length differs between species (as estimated from the UVC in section 7.2.1) and post-hoc tests for multiple comparisons were conducted to examine which groups are significantly different (following Siegel and Castellan 1988) using Statistica (version 7.1).

7.2.5 Mate choice: data collection and analysis

The sixth hypothesis was that male body size affects female mate choice in the *Ruanoho* species. Mate choice trials were conducted to test if females of the *Ruanoho* species show a preference for either smaller- or larger-sized males. A further test examined whether females show a preference for males that were more similar or dissimilar in body size to the female. Specimens for the mate choice trials were collected from the Whangaparaoa and Tawharanui Peninsulas during the triplefin breeding season from late June to the end of August 2004 (Figure 39). Only fully developed sexually mature males and females (*R. decemdigitatus* > 8.5 cm and *R. whero* > 5 cm) were used in the experiments.

The design of the mate choice apparatus followed LaFleur et al. (1997) except for modifications in the aquarium dimensions because the species used in this experiment were considerably larger (Figure 40). The main apparatus consisted of a 45 l glass aquarium (50 cm x 30 cm x 30 cm) situated between two smaller chambers (8 cm x 30 cm x 30 cm) (Figure 40). Two transparent and removable Plexiglas sliding walls, placed 16 cm from each end, divided the main aquarium into three compartments. Several small perforations were made in the Plexiglas walls to allow water-flow between the compartments. Black plastic sheets were placed on the back and distal sides of the end chambers to minimise any visual interference. Water in the aquaria was aerated between trials. A digital Sony video camera (model number: DCR-PC10E) was placed 1.5 m in front of the apparatus to record the movement and behaviour of the fish.

At the start of each trial, two homospecific and differently-sized males were selected from the holding aquaria and randomly allocated to each end compartment. The holding aquaria contained at least 10 males of each species in varying sizes. A female of the same species was then placed in the inner compartment between the two Plexiglas sliding walls. Before the commencement of the trial all fish were allowed to acclimate and observe each other for 10 min. After the acclimation period the Plexiglas partitions were slowly lifted simultaneously and the female was allowed to move freely. Movement of the female (20 trials per species) was recorded for 10 min with a video camera. Data recording commenced after the female started to move. 10 minutes of videotape were analysed and the time spent in each zone was quantified for each trial. Females encountered each male only once during the experiments. To quantify the strength of the female choice, the aquarium was divided into four zones (for details see Figure 40), broadly based on those defined by Lafleur et al. (1997). The first zone was called the 'weak zone' (as it was assumed to represent a weak mate preference response)

and consisted of the half of the aquarium to the side of the centre line. The second zone, called the 'moderate zone', consisted of the area 17 cm from the end of the aquarium to the third zone, the 'strong zone'. The 'strong zone' started 10 cm from the end of the aquarium and was bordered by the final zone, called the 'very strong zone', which consisted of the area 2.5 cm from the end of the aquarium. Two complementary tests of size-assortative mating were investigated, namely (i) the initial mate size choice and (ii) the overall mate size choice. Initial choice (i) quantified the number of times that 'small' or 'large' males were chosen by females based on where the female spent the first 15 consecutive seconds in the 'very strong zone', and was analysed with a Chi-square analysis. The same analysis was used to test whether females spent more time with males that were more similar or dissimilar in body size to themselves. The overall mate choice (ii) was analysed using the percentages of summed time counts for each zone during a trial. Preference was assessed using paired t-tests on the difference of the time spent on the side of the 'small' male and the time spent on the side of the 'large' male. Again, the same analysis was conducted to investigate whether females prefer to spend more time with the male that is more similar or dissimilar in body size to themselves. All Chi-square and t-tests were computed using the statistical package Statistica (version 7.1). The level of significance for all tests was accepted at p < 0.05. Two females of each species showed no movement during the trials, hence, those trials were unable to be analysed and had to be repeated. Females in all other trials moved actively around and courted either one or both males. The average length of the males and females in the R. *decemdigitatus* trials were $L_{\rm T}$ 9.4 cm (± 0.8 SD) and 9.5 cm (± 0.8 SD), respectively, and in the *R*. whero trials 6.9 cm (\pm 0.6 SD) and 6.2 cm (\pm 0.7 SD), respectively.



Figure 40: Experimental apparatus for the mate choice trials showing the four different zones. Apparatus modified from LaFleur (1997). All measurements are shown in cm.

7.2.6 Courtship behaviour: data collection and analysis

The last hypothesis examined interspecific differences in the courtship behaviour of the *Ruanoho* sister-species pair. Individuals for the courtship comparisons were obtained from the Whangaparaoa and Tawharanui Peninsulas (Figure 39). After capture the L_T of all fish was measured, and only fish that were sexually mature (established from 7.3.3) were chosen for the trials (n = 6). Holding procedures were exactly as described in section 7.3.3, except that female fish were kept in a separate holding aquaria and each male was allocated to a single aquarium (50 cm x 30 cm). This was done so that the males could establish a territory and nest site. Each male was only used once. After at least two days a single female was then added to the aquarium of the male and any courtship behaviour was recorded for 10 min using a Sony video camera (model number: DCR-PC10E).

The presence of the following male behaviour patterns were recorded during each trial: (i) darkening of breeding colours and erection of anal, caudal and all dorsal fins, (ii) increase in opercular movements, (iii) movement towards the female, (iv) flicking of the first dorsal fin, (v) opercular spread displays, in which the male widely opens and closes the operculum, (vi) lateral displays, in which the male swims close to the female with erect dorsal and pectoral fins, shivering his body, (vii) pectoral fin waving, (viii) lead displays, in which the male swims from the female to the nest with exaggerated, undulating fin and body movements, (viii) biting of the female and swimming into her, (x) male swims to the nest and female follows, and (xi) lateral shivers of the male in the nest.

7.3 RESULTS

7.3.1 Spatial isolation

All spawning males were found to occupy distinct nest habitats that were defended vigorously against intruders. Canonical Discriminant Analysis showed considerably interspecific differences in nesting habitat. In particular, the relatively shallow and sheltered nest sites of *F*. *lapillum*, *G. nigripenne*, *G. capito*, *O. maryannae*, *R. decemdigitatus* and *R. whero* displayed reduced overlap with the remaining three species studied (Figure 41). These latter species, i.e. *F. flavonigrum*, *F. malcolmi* and *F. varium*, were generally associated with more exposed and deeper habitats (Figure 41). In addition to the differences in depth and exposure of the nesting sites, the variable mud was a strong component of the nest habitats of *G. nigripenne* and *G.*

capito, while the nests of all other species were not associated with mud (Figure 41). The majority of species built nests in microhabitats that provided high amounts of structural cover (i.e. under rocks), presumably to minimise predation and physical disturbance (Figure 41). For example, the well-protected microposition 'under hard substratum' was used by several species, i.e. *G. capito*, *G. nigripenne*, *R. decemdigitatus*, *R. whero*, and *F. lapillum* (Figure 41). The microposition in 'crack in vertical rock' was solely occupied by *F. flavonigrum* (Figure 41). Nests of this species were typically in crevices on vertical rock walls, and thus also have a high amount of structural cover. The large species *F. varium* and *F. malcolmi*, however, were found to use nesting sites that provided only a moderate to low degree of structural cover, such as the 'side of rocks' and 'top of rocks' (Figure 41).

The cross-validation results indicated that the majority (< 50%) of nests of *F. flavonigrum* (75%), *F. malcolmi* (66%), *F. varium* (72%), *G. nigripenne* (88%), *O. maryannae* (83%), and *R. decemdigitatus* (58%) could be correctly assigned to the species, while only a smaller proportion (>50%) could be assigned to *F. lapillum* (33%), *G. capito* (34%) and *R. whero* (39%). More detailed results showed that the sister-species pair *F. malcolmi* and *O. maryannae* displayed no overlap in nesting habitats.



Figure 41: Canonical Discriminant Analysis of triplefin species and nesting habitats. Triplefin species are in bold and colour and habitat variables in black. The two Canonical Discriminant axes explain > 80% of the data.

The sister-species pair *F. lapillum* and *G. nigripenne* was also almost completely separated in nesting habitat, with *G. nigripenne* nests displayed no overlap and *F. lapillum* nests only minimal overlap, with 7% of all nests being misclassified as *G. nigripenne* nests. Lastly, *R. decemdigitatus* displayed negligible overlap in nesting habitats with *R. whero* (misclassification rate of 8%), while *R. whero* displayed considerable overlap in the choice of nest habitats with *R. decemdigitatus* (29%). Differences in nest characteristics between locations were assessed for the species in which nests were observed in more than one location. Seven species were observed in more than one location and included *F. lapillum*, *F. flavonigrum*, *F. malcolmi*, *F. varium*, *R. whero*, *G. capito* and *O. maryannae* (Figure 42). Most species showed very little variation in nest microhabitat except for slight variations in algal coverage in *F. varium* and *G. capito*. In addition, no significant variation among nest sites in the use of microposition and the amount of shelter was found in the seven species (p < 0.05, Figure 42).

7.3.2 Temporal isolation

Spawning periods all nine triplefin species showed considerable overlap (Table 11). The majority of species started spawning around April-June and spawned until September-November, with a peak in spawning activity around August (Table 11). *Forsterygion lapillum* was the only species that remained reproductively active almost all year round (Table 11). Long spawning periods were recorded for *F. varium* (8 months), *R. decemdigitatus* (6 months) and *R. whero* (7 months), and moderately long spawning periods for *F. flavonigrum* (5 months) and *F. malcolmi*, *O. maryannae* and *G. nigripenne* (4 months) (Table 11).

Table 11: Spawning periods of triplefins in New Zealand. The presence of nests was used as evidence for reproductive activity. More intense nesting months are indicated by increasing darkness of the squares. Sister-species pairs are denoted with superscript numbers.

Species	Jan	Feb	Mar	Apr	May	Jun	Jul	Aug	Sept	Oct	Nov	Dec
F. flavonigrum												
F. lapillum ¹												
F. malcolmi ²												
F. varium												
G. capito												
G. nigripenne ¹												
O. maryannae ²												
<i>R. decemdigitatus</i> ³												
$R. whero^3$												



Figure 42: Percentage coverage of nests at different locations. Locations included the Hauraki Gulf, Dunedin, Fiordland and Napier.

7.3.3 Hybridisation experiments

Males in homospecific trials showed increased activity and territoriality, assumed a jet-black spawning colouration, and displayed frequently to the female. Homospecific pairs of R. decemdigitatus spawned 12 times and R. whero 11 times. Both Ruanoho species selected the microposition 'under rock' for all nests. The egg development in both species was recorded with photographs taken every 24 - 48 hours (see Appendix III, section iv for photographs of the embryonic development and section iii for the developmental times). In contrast, no evidence of nest building, courtship behaviour or increase in territoriality was observed in heterospecific *Ruanoho* pairs. One *R. decemdigitatus* female in a heterospecific trial laid eggs on the side of a rock, but this was not preceded by courtship nor did the nest site correspond with the heterospecific male's territorial area. Additionally, the *R. whero* male involved did not change colouration after the eggs were laid (all other males assumed a lighter colouration after egg fertilisation) and did not guard or fan the eggs. The eggs started to decompose after three days and were completely disintegrated after five days. For these reasons it is unlikely that the eggs were fertilised by the heterospecific male. Because no spawning took place in the mixed species trials the comparison of development times of eggs from the same and mixed species trials was not possible.

The trials also indicated that individuals of *R. decemdigitatus* less than 8.5 cm L_T were not reproductively active (Figure 43). Males below this size did not become territorial, were less aggressive and did not establish a nest site. Female *R. decemdigitatus* below 8.5 cm L_T did not produce any eggs. In contrast, *R. whero* individuals started to become reproductively active slightly below 5 cm L_T . Visual inspection of gonad maturity in 18 individuals of each species confirmed that *R. whero* mature at a smaller size than *R. decemdigitatus* (Figure 44), however, the species' size ranges show slightly more overlap compared to the behavioural indications of maturity (Figure 43). Mature ovaries were tubular and yellow while mature testes were flat, white and their ventral edges had a wave-like outline. Immature gonads were small and pink.

Same species pairs of *G. capito* and *F. varium* of varying lengths spawned successfully 10 and 9 times, respectively. In all spawning events, *G. capito* used the microposition 'under rock' and attached the eggs to the glass bottom of the aquarium. In contrast, *F. varium* selected the microposition 'top of boulder' for all nest sites. Males of both species in the same species trials assumed black spawning colouration and became extremely territorial. Frequently, males were seen to display to females and perform a courtship dance, leading from the female to the nest site and back. Similar interactions between individuals in the

mixed species trials were absent. The males in the heterospecific trials did not assume spawning colouration or increase in territoriality. *Grahamina capito* mainly hid under rocks and *F. varium* frequently sat either on top of a rock or on top of gravel or cobbles.



Figure 43: Based on observations of reproductively active males. L_T of reproductively active *R. whero* (males: n = 9, females: n = 8), *R. decemdigitatus* (males: n = 15, females: n = 15) and *R. decemdigitatus* that were not reproductively active (n = 10).



Figure 44: Based on examination of gonads. L_T of *R*. where (males: n = 5, females: n = 13), *R*. *decemdigitatus* (males: n = 14, females: n = 4) and *R*. *decemdigitatus* that were not mature (n = 4).

7.3.4 Male body colouration

Males of *F. lapillum*, *F. malcolmi*, *F. varium*, *G. capito*, *G. nigripenne*, *R. decemdigitatus* and *R. whero* assumed a completely black spawning colouration, and the darkening of spawning males was significant (p < 0.05) for all the 10 points measured (Figure 45, Figure 46). Greatest differences between spawning and non-spawning individuals were seen in *R. decemdigitatus* (mean intensity of colouration spawning 91, non-spawning 18, difference 73) and *F. lapillum* (mean intensity of colouration spawning 90, non-spawning 22, difference 68). In three species, i.e. *G. capito*, *G. nigripenne* and *R. whero*, the difference between the spawning and non-spawning colouration was moderate (≈ 50).

Unlike the uniform jet-black spawning colouration seen in seven of the eight triplefin species examined in this study, spawning male *F. flavonigrum* showed only darkening around the head region (the first two points were significantly darker in spawning males), while the points four to 10 assumed a significantly brighter yellow spawning colouration (Figure 45). The average difference in the intensity of colouration between breeding and non-breeding individuals for *F. flavonigrum* was 12, with the spawning individuals showing a higher intensity in colouration than the non-spawning individuals.

The method to detect UV colour pattern was first validated by using objects that are known to exhibit UV reflectance and triplefin species that do show UV colour patterns (*N. segmentatus* and *K. stewarti*). Figure 47 shows the head of *K. stewarti* under visible (Figure 47a) and UV light (Figure 47b). The photographs of the UV colour patterns of the three triplefin sisterspecies pairs were not different from photographs taken under the visible light spectrum, indicating that males of these species do not reflect light in the UV bandwidth. For example, the body colouration of the sister-species pair *R. decemdigitatus* (Figure 48) and *R. whero* (Figure 49) did not show any differences.



Figure 45: The graphs show the mean intensity of colouration for each of the 10 points that were measured along the lateral line of each species (black dots denote non-spawning and clear dots spawning males). Photographs denoted with A) show non-spawning individuals and photographs denoted with B) show spawning individuals of that species.



Figure 46: The graphs show the mean intensity of colouration for each of the 10 points that were measured along the lateral line of each species (black dots denote non-spawning and clear dots spawning males). Photographs denoted with A) show non-spawning individuals and photographs denoted with B) show spawning individuals of that species.



Figure 47: The head of *K. stewarti* under visible and UV light (blue photograph).



Figure 48: Body colouration of *R. decemdigitatus* under visible and UV light (blue photographs).



Figure 49: Body colouration of *R. whero* under visible and UV light (blue photographs).

7.3.5 Male body length

The size of nesting males differed greatly between species (Figure 50), and two statistically distinct species clusters were present (Kruskal–Wallis test H = 8.462; p < 0.001). The first cluster contained *F. flavonigrum*, *F. lapillum*, *O. maryannae* and *R. whero*, with nesting male body lengths between 5 - 8 cm (Figure 50). The second cluster contained the species *F. malcolmi*, *F. varium*, *G. capito*, *G. nigripenne* and *R. decemdigitatus*, with nesting male body lengths of approximately 10 - 13 cm (Figure 50). Importantly, the three sister-species pairs *R. decemdigitatus* and *R. whero*, *G. nigripenne* and *F. lapillum*, and *F. malcolmi* and *O. maryannae* were split across the two clusters.



Figure 50: Median body lengths of spawning triplefin males of nine species. Species names are abbreviated by the first letter of the genus followed by the first letter of the species name. Oval shapes indicate the two clusters. Error bars show \pm SE.

7.3.6 Mate choice

The results of the initial choice strongly indicated that females of *R. whero* chose smallersized and similar-sized males significantly more often than larger and dissimilar-sized males, respectively (p < 0.05 in both cases). Females of *R. whero* chose to be in the 'very strong zone' next to the smaller-sized males in 17 / 20 times (the zone closest to the male), indicating a strong response. Similarly, *R. whero* females selected similar sized males in 15 / 20 times, again indicating a strong response. In contrast, *R decemdigitatus* females showed no initial preference for larger or smaller-sized males, but females showed a statistical significant preference for males that were similar in size to themselves (p < 0.05). Specifically, *R. decemdigitatus* females selected to be near the males that were most similar in size to themselves in 19 / 20 trials (Figure 51).



Figure 51: Graph showing the results of the initial female mate choice (n = 20 for each *R*. *whero* and *R*. *decemdigitatus*). The upper graphs show female mate choice based on the size of the male relative to the size of the female. The lower graphs show female mate choice based on the size of the male relative to the size of the other male.

In the overall choice test *R. whero* females again showed a clear preference for smaller-sized males and males that were similar in size in most of the four choice zones (Table 12). In contrast, *R. decemdigitatus* showed no clear preference for either larger or smaller males, and although females showed a weak preference for similar sized males in the extreme zone, there was no evidence for this preference in any of the other experimental mate choice zones (Table 12). It should be noted that most of the *R. whero* females were smaller in size than the two experimental males (smaller = 13), while only a few females were larger (larger = 2) or intermediate in size to the males (intermediate = 3). This means that the smaller males were in most cases also the males that were most similar in body size to the female, thus confounding the results. This indicates that although it is not possible to determine whether female *R. whero* selected a smaller or similar sized male, the experiment showed no evidence that *R. whero* females show a preference for the larger of the two males. The interpretation of results is more straightforward in *R. decemdigitatus*, as most females were smaller (smaller = 3) or larger in size to the males (larger = 5).

Table 12: Results of paired t-tests of the overall choice test (*R. whero* n = 20; *R. decemdigitatus*, n = 20). The four zones were (ordered according to the distance to the male, with 1 being the zone closest to the male): the 'very strong zone' (1), the 'strong zone' (2), the 'moderate zone' (3) and the 'weak zone' (4). Degrees of freedom is 19 for all tests.

		R	. decema	ligitatus	5	R. whero					
Zone	Male size	Mean	SD	t	р	Mean	SD	t	р		
		Larger vs smaller									
1	Larger	18.77	27.51	-1.43	0.17	6.14	18.8	-2.9	0.009		
1	Smaller	37.05	38.28			37.23	38.7				
2	Larger	8.02	14.73	-0.16	0.877	2.56	3.35	-2.23	0.038		
2	Smaller	9.05	22.86			12.78	19.66				
3	Larger	6.33	14.94	0.95	0.355	9.29	20.19	-0.37	0.712		
3	Smaller	3.13	3.78			11.41	12.45				
4	Larger	13.8	26.29	1.61	0.125	8.42	9.52	-2.15	0.045		
4	Smaller	3.86	4.07			12.17	12.12				
		Similar vs dissimilar									
1	Similar	87.75	155.68	1.92	0.07	78.58	74.02	2.70	0.015		
1	Dissimilar	18.20	23.82			45.21	48.93				
2	Similar	28.70	30.54	0.03	0.98	90.00	130.09	1.98	0.063		
2	Dissimilar	28.05	88.38			27.74	37.80				
3	Similar	89.80	151.29	2.19	0.04	88.95	118.56	2.71	0.014		
3	Dissimilar	12.60	24.84			12.74	17.75				
4	Similar	237.20	208.45	1.88	0.08	247.42	222.77	4.50	<0.001		
4	Dissimilar	97.70	180.65			9.37	15.79				

7.3.7 Courtship behaviour

Courtship displays of *R. decemdigitatus* consisted of 11 steps: (i) darkening of breeding colours and erection of anal, caudal and all dorsal fins (Figure 52), (ii) increase in opercular movements, (iii) movement towards the female, (iv) flicking of the first dorsal fin (Figure 53), (v) opercular spread displays, in which the male widely opens and closes the operculum, (vi) lateral displays, in which the male swims close to the female with erect dorsal and pectoral fins, shivering his body, (vii) pectoral fin waving, (viii) lead displays, in which the male swims from the female to the nest with exaggerated, undulating fin and body movements, (viiii) biting of the female and swimming into her, (x) male swims to the nest and female follows, and (xi) lateral shivers of the male in the nest (Figure 54).

An Ethogram of the courtship behaviour of *R. decemdigitatus* can be seen in Figure 55 (for details see Table 13).



Figure 52: Picture showing *R. decemdigitatus* with all three dorsal fins erect.



Figure 53: Pictures showing the flicking of the first dorsal fin by *R. decemdigitatus*.



Figure 54: Pictures showing the lateral movement by *R. decemdigitatus*.

The courtship of *R. whero* consisted of a subset of the display in *R. decendigitatus* (see Figure 56). The courtship included the steps i-iii and viii-x, with steps iv-v and vii entirely absent and step vi was only present in two of the six individuals examined. An Ethogram of the courtship behaviour of *R. whero* can be seen in Figure 56 (for details see Table 13).

Table 13: Courtship display (n = 6) of *R. whero* and *R. decendigitatus*. The presence of courtship behaviour is indicated by grey cells. Latin numbers are used to mark individual fish. Greek numbers refer to the courtship steps.

Courtship behaviour: R. decemdigitatus (RD)								Courtship behaviour: R. whero (RW)					
	Rd	Rd	Rd	Rd	Rd	Rd	Rw	Rw	Rw	Rw	Rw	Rw	
Steps	1	2	3	4	5	6	1	2	3	4	5	6	
i													
ii													
iii													
iv							-	-	-	-	-	-	
v							-	-	-	-	-	-	
vi									-	-	-	-	
vii							-	-	-	-	-	-	
viii													
viiii													
x													
xi													



Figure 55: Ethogram of the male courtship display in *R. decemdigitatus*.



Figure 56: Ethogram of the male courtship display in *R. whero*.

7.4 DISCUSSION

The contribution of reproductive barriers to the maintenance of species boundaries is poorly understood in the New Zealand triplefin fauna. Overall, the results of this Chapter show little evidence for temporal isolation and divergence in male body colouration. In contrast, spatial isolation was found to be extensive and closely related species differed in body size, suggesting that spatial factors and selection on male body size may facilitate reproductive isolation in this group.

Overlap of breeding habitats was mainly achieved by different preferences for depth and exposure, by the substratum variables rock and mud, and exposed versus sheltered nest micropositions. The finding that depth, exposure, rock and mud are one of the main determinants of species-specific habitat use is consistent with previous work on habitat associations of New Zealand triplefin fishes, and supports the suggestion that interspecific divergence in these habitat characteristics may have been a major component in the evolution of this clade (Chapter 2). The degree of shelter of nest positions was another key factor in reducing overlap between species, with some species showing a clear preference for exposed and other species for sheltered nesting sites. In particular, F. varium and F. malcolmi used nest sites that were predominantly in the open (tops and sides of rocks), while several other species (e.g. Ruanoho spp.) were associated with more sheltered nest positions (e.g. under hard substrata). Similar differences in species-specific nesting sites were documented by Feary and Clements (2006), and may be related to the ability of the species to defend and hide the nest from egg predators. For example, F. varium has been shown to aggressively defend eggs from other fish species (Thompson 1986), and thus may be able to offer protection to the eggs despite the open placement of the nest in the habitat. All of the species that were associated with the highly sheltered nest microposition 'under hard substratum' (e.g. Ruanoho spp. and F. lapillum) were exclusively found in shallow habitats. Therefore, these species do not only have to protect the nest from potential predators, they also need to shelter the nest from higher physical exposure (e.g. waves). In this sense, the placement of triplefin nests appears to be affected by factors that help the nest gain protection from predation as well as physical disturbance. Another factor that may affect the placement of nests is the ability of males to attract females to the nest. By using the top and sides of rocks for nesting sites, males of F. varium and F. malcomi are more visible for potential mates, and thus may be able to attract a larger numbers of females. Experimental manipulations have indicated that specific nest characteristics, such as the amount of spawning substratum and the presence of large boulders adjacent to the nest site, increases male spawning success in F. varium (Thompson

1986). Furthermore, higher nest detectability in triplefin fishes has been suggested to increase the number of egg clutches in nests, and consequently the number of offspring (Feary and Clements 2006).

Nest habitats showed low misclassification rates for the majority of species, in particular for the sister-species pairs F. lapillum and G. nigripenne and F. malcolmi and O. maryannae. In contrast, the sister-species pair R. where and R. decemdigitatus showed considerable overlap in nest site choice. The pattern of overlap was highly asymmetric, with the nest sites of R. decemdigitatus forming a subset of R. whero, and this is consistent with previous work on the habitat characteristics of this pair (Chapter 2). Given this overlap, it appears that R. decemdigitatus encounters individuals of R. whero on a frequent basis, although the absence of hybrids from the wild suggests that mate recognition factors prevent interbreeding between species. This highlights that although spatial divergence in breeding habitats may be an important component in maintaining reproductive isolation in some triplefin species, additional mechanisms appear to be involved in the maintenance of reproductive isolation in other species. Divergence in habitat choice has also facilitated reproductive isolation between recently diverged cichlids (Schliewen et al. 2001; Palstra et al. 2004) and sticklebacks (Blouw and Hagen 1990; Jamieson et al. 1992; Rundle et al. 2000), suggesting that divergence in habitat is a wider phenomenon that may have also played a key role in the build-up and maintenance of reproductive isolation in other fish groups.

Interspecific overlap of spawning periods was found to be extensive, with most species spawning over several months between May and November, thus rejecting temporal isolation as a reproductive barrier. Similar long spawning periods have also been found for tropical (Longenencker and Langston 2005) and Mediterranean triplefin species (Geertjes and Videler 2002), and thus may be a common characteristic of this family. Hickford and Schiel (2003) conducted ichthyoplankton surveys on the east coast of the South Island in New Zealand and found *Forsterygion* spp. larvae throughout the year. This study suggests that the year-round abundance of *Forsterygion* spp. larvae was presumably caused by the extended spawning period of *F. lapillum*. The spawning periods for *F. malcolmi* and *F. flavonigrum* reported by Francis (2001) exceeded the spawning periods observed in this study by several months. It should be noted, however, that the current study was limited to northeastern New Zealand, where water temperatures are generally warmer than in the more southern parts of the country (Heath 1985). Therefore, these differences in spawning duration might be due to latitudinal variation in temperature, which influence the timing of breeding in many fishes (Webb and McLay 1996). *Grahamina capito* is reported to have a long spawning period from June-

January (Ayling and Cox 1982; Francis 2001), and the larval abundance patterns are consistent with this spawning period (Hickford and Schiel 2003). The results from this study show a much shorter spawning period which may be related to the smaller spatial scale of this study. The spawning period of both *Ruanoho* species was similar, spanning from May/June-November, and is consistent with the spawning times reported previously (Ayling and Cox 1982; Francis 2001). *Ruanoho decemdigitatus* larvae have been found between September and April (Hickford and Schiel 2003), suggesting that the spawning time of this species might start later in the south of New Zealand.

The absence of any spawning behaviour in the laboratory crossbreeding trials of G. capito and F. varium suggests that specific environmental conditions may have caused these species to hybridise in the wild. For example, reduced visibility due to high water turbidity may cause mate recognition systems to break down, thus increasing the possibility of mating between heterospecific pairs (e.g. Seehausen et al. 1997). Alternatively, it is also possible that G. *capito* males deliberately fertilised freshly laid *F. varium* eggs by sneak fertilisations as fish of many species commonly reproduce by ejecting sperm close to a spawning pair (Taborsky 1998; Wirtz 1999). However, the absence of any such behaviour in the hybridisation trials suggests that this is unlikely to have occurred in the wild. Numerical imbalance (i.e. heavily skewed abundance distributions) between hybridising species has also been invoked as one of the potent factors conducive to hybridisation in fishes (Wirtz 1999). For instance, Avise and Saunders (1984) proposed that the absence of conspecific partners and stimuli for females of a rarer species may be an important factor in increasing the likelihood of interspecific hybridisation (Wirtz 1999). Furthermore, studies of population structure and hybrid zones have also indicated that natural hybridisation is often found in tracts of intermediate habitats, or where conditions favouring both taxa are found in proximity and at the ecological limits of their distribution range (Huxel 1999). This idea is supported in the present study as the hybrids breeding habitats in southern New Zealand largely resembles the breeding habitat of G. capito, but in contrast have little in common with the breeding habitat of F. varium. The hybrid breeding habitats are found along shallow and sheltered coastlines and consist mainly of rocks on muddy substratum. Thus, if the present hybrid distribution can be used as an indicator of where interbreeding between G. capito males and F. varium females has occurred, then it can be assumed that F. varium was reproducing in a marginal habitat at the ecological limit of their distribution range, in which homospecific males were likely to be rare. Thus, heterospecific matings between female F. varium and male G. capito were probably facilitated because F. varium females were having difficulty finding homospecific

males in these marginal habitats. Furthermore, the results of the hybridisation trials indicate that the homospecific density of males is not the sole factor in determining whether females will hybridise, as homospecific males were absent from all trials. Therefore, it seems likely that a combination of factors, specifically a lack of suitable homospecific males combined with a change in environmental conditions (i.e. reduced visibility), might have facilitated hybridisation between *G. capito* and *F. varium*.

Both *Ruanoho* species spawned several times during the homospecific trials. The hybridisation trials showed, however, no evidence for interspecific interactions or any crossbreeding attempts in the *Ruanoho* species, indicating that mate recognition processes in these species are sufficient to prevent interspecific matings (Andersson 1994). These results, however, have to be interpreted cautiously because individuals may have been behaviourally imprinted in the preceding homospecific trials (Irwin and Price 1999; Hebets 2003). It was necessary to run homospecific breeding trials prior to the heterospecific hybridisation trials because it was impossible to unambiguously sex and to distinguish mature from immature individuals. A way to circumvent the possibility that mate preferences were affected by sexual imprinting would be to use naïve individuals. Triplefins are short-lived and consequently mature within the first year (Thompson 1979), therefore, individual fish would have to be caught within the first few months of their life and then subsequently kept in aquaria. The problem is, however, that it is impossible to assess the sex and reproductive maturity of an individual with absolute certainty without first positively identifying these attributes in homospecific trials, thus, there is a chance that hybridisation does take place because individuals were of the same sex or immature. For these reasons, a considered decision was taken to run homospecific trials first, as this was seen as the only way to prevent spurious negative results. Additional evidence for the role of mate recognition processes in the maintenance of reproductive isolation in R. whero and R. decemdigitatus comes from the fact that that hybrids of these species have never been recorded from the wild. In addition, results of the crossbreeding trials indicated that the Ruanoho species differ greatly in size at first maturity, with *R. whero* maturing at a much smaller size than *R. decemdigitatus*. Furthermore, the mate choice trials showed that *R. whero* females have a preference for smaller-sized males as well as for males that are most similar in size to themselves, while R. decemdigitatus females showed no clear and consistent preference for male size. Although it remains unclear whether mate choice by *R. whero* is primarily driven by selection for males that are most similar in size or smaller in size relative to females, this preference would result, in both instances, in the selection of males that are smaller than males of R. decemdigitatus.

Therefore, the preference of *R. whero* females for male body size may partly explain why the two species do not hybridise, as they differ greatly in maximum body size, with *R. decemdigitatus* being the larger species. Several studies on recently diverged species of fishes have found strong assortative mating based on body size (Nagel and Schluter 1998; Jones et al. 2003; McKinnon et al. 2004). For instance, McKinnon and co-workers (2004) found strong size-assortative mating between stickleback ecotypes (limnetic and benthic forms) that are specialised to alternative trophic niches. Benthic forms tended to be larger than limnetic forms in all lakes, and mating in sticklebacks is strongly size-assortative (Nagel and Schluter 1998).

This finding is consistent with one of the main predictions of the ecological theory, that is, that prezygotic isolation should be based on traits related to differential resource use between closely related species (Schluter 2000b). Research on sticklebacks has shown that differences in body size between benthic and limnetic ecotypes are adaptive for their respective lifestyles, thereby demonstrating a link between divergence in body size and mating incompatibilities (McKinnon et al. 2004). Several other studies have also demonstrated that body size is primary an evolutionary response of species to different environments (e.g. Bryant 1977). For example, work by Munday and Caley (2003) on coral gobies showed that habitat specialists grew faster on average compared to habitat generalists, but the former showed a reduced ability to maintain high growth rates on other resources. In contrast, habitat generalists were able to use a wider range of habitats, but this was associated with an overall reduced growth rate across all habitats (Caley and Munday 2003). As in the stickleback system, the Ruanoho species occupy different but overlapping habitats, with *R. decemdigitatus* being confined to a much smaller range of depth and exposure than R. whero (Chapter 2). Consequently, R. whero will be exposed to a much greater variety of habitats than R. decemdigitatus, which might have affected growth rates of both species. In this sense, the large body size of R. decemdigitatus may be the result of being specialised to a specific depth and exposure, whereas the smaller body of *R. whero* may be a trade-off in order to use a wider habitat range. Thus, it appears that there is also a link between ecological divergence in habitat use and the built up of reproductive isolation in the *Ruanoho* sister-species. The preference of *R. whero* females for smaller and similar sized males may partly explain prezygotic isolation between the *Ruanoho* species, and this preference has probably also accelerated body size divergence between the two species. It remains unclear, however, if R. decemdigitatus females had an ancestral preference for larger males and then lost it over time, or alternatively whether females never had a preference for larger males. Interestingly, the males of all other New

Zealand triplefin sister-species show considerable interspecific differences in the length of nesting male, indicating that body size may be an important component of divergence in this clade. Direct evidence for interspecific differences between the *Ruanoho* species were found in the courtship displays. Courtship behaviour of *R. whero* males was less complex and consisted of a subset of that shown by *R. decemdigitatus* males. This suggests that behavioural differences also contribute to assortative mating in the *Ruanoho* species. Differences in male courtship behaviour have been shown to play a crucial role in prezygotic isolation in several fish species because such ethological differences affect the probability of heterospecific matings (e.g. Ishikawa and Mori 2000). Thus, interspecific overlap of reproduction in the *Ruanoho* species appears to be prevented by a combination of factors including distinctions in ethology, size at first maturation, and female mate choice of male size.

Males of the species F. varium, F. malcolmi, F. lapillum, G. capito, G. nigripenne, R. whero and R. decemdigitatus were found to turn completely dark when spawning, while F. *flavonigrum* assumed a partially darker colouration. Although differences in colour between reproductively active and non-active males suggest some mate recognition process, black spawning colouration in males is common even in sister-species such as R. whero and R. decemdigitatus and F. lapillum and G. nigripenne, indicating that colour pattern in the visible spectrum plays little or no role in species recognition in these species. The darkening of male colouration (either of the whole or partial body) during the spawning season leads to a decrease in interspecific colour differences between males and thus suggests that male spawning colouration is not important in female mate selection. The uniformly dark and conspicuous colouration of these triplefin males may, however, act as signalling colours to indicate the position of a spawning male. It is thought that colour signals are most easily detected when they differ from the background noise against which they are emitted (Fuller 2002). It is possible that the black body of some reproductive triplefin males may make them more conspicuous against the spawning background, such as that observed in stickleback fishes. Stickleback males usually assume a red nuptial colouration, however, a study by Boughman (2005) has shown that males in tea-stained lakes assume a black nuptial colouration and this has been explained by the greater contrast in tannin-rich waters. In this sense, it may be that a black breeding colouration is favourable in triplefin fishes, and this will be particularly important for species that inhabit turbid environments, such as G. nigripenne. As with visible light colouration, there was no evidence for male UV colouration in the three sister-species pairs. There was, however, evidence for some UV colouration in K. stewarti and *N. segmentatus.* However, the UV colouration showed the same pattern that can be seen under visible light of spawning and non-spawning triplefins. Thus, it does not appear that the UV colouration in these species increases interspecific differences, which would be expected if sexual selection on colour pattern plays a crucial role in these species. This general lack of interspecific differences in male colouration between closely related species sets New Zealand triplefins apart from other triplefins species (De Jonge and Videler 1989) and the majority of other fish radiations (reviewed in: Schluter 2000b; Streelmann and Danley 2003).

The mean length of guarding triplefin males was relatively similar to the species lengths recorded by Francis (2001), with the exception of lower recorded lengths for F. varium. This difference could be due to the fact that the majority of observations in this study were done in northeastern New Zealand, where fish are generally smaller. Triplefin size tends to increase with increasing latitude (e.g. Gilligan 1991; Fricke 1994), and this could have led to a skewed result for F. varium. Analysis of nesting male body lengths demonstrated that a number of species differed in body length. In particular, there were interspecific differences in male body size in each of the three sister-species pairs (F. lapillum and G. nigripenne, F. malcolmi and O. maryannae, and R. decemdigitatus and R. whero). The differences in male body size between sister-species are unlikely to be related to diet differences, as all species have generalist microcarnivore diets (Feary 2001). Body size differences among closely related triplefin species may facilitate size assortative mating, and suggests that morphological differentiation in body size may be an important component in the divergence of this clade. The tendency for assortative mating based on body size is known for several fish species such as cichlids (Schliewen et al. 2001; Palstra et al. 2004), salmonids (Hendry et al. 2001) and closely related species pairs of sticklebacks (Nagel and Schluter 1998; Boughman et al. 2005), but has also been well documented in other taxa (e.g. Ratcliffe and Grant 1983).

In summary, the results from this Chapter show that the radiation of New Zealand triplefin fishes differs in many important aspects from other fish radiations. While there was little evidence for interspecific divergence in breeding time and male breeding colouration, there was strong evidence that spatial differences in nest characteristics and interspecific divergence in male body size may have facilitated reproductive isolation in this group. Specifically, spatial differences in breeding habitat and male body size were pronounced between sisterspecies, suggesting that divergence in these factors may have been involved in the built-up of reproductive isolation in sister-species pairs. This indicates that habitat use and morphological differentiation in body size may be an important component of divergence in this clade. Hybridisation trials demonstrated that R. where matures at a smaller size than R.

decemdigitatus, and the courtship trials showed that the *Ruanoho* species differ in courtship signals. Furthermore, mate choice trials indicated that female *R. whero* prefer smaller and similar-sized males over larger and dissimilar sized males. Such assortative mating is likely to maintain reproductive isolation of the two *Ruanoho* species, as they differ greatly in size at first maturation, which highlights the role of behavioural factors as prezygotic isolating mechanisms. Similar processes could also be important in the isolation of other sister-species, since males of many species show large differences in body length.

8 Competition for Resources between

Ruanoho whero and R. decemdigitatus

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8.1 INTRODUCTION

Resource competition is one of the most fundamental phenomena in ecology, affecting not only the distribution and success of species, but also their evolution. The ecological theory of adaptive radiation places resource competition as the main cause of divergence. Competition operates by forcing populations to exploit new resources, where they experience new selection pressures (Schluter 2000b). Naturalists in the 1940s and 1950s (e.g. Mayr 1942; Lack 1947) were strong proponents of competition-induced divergence. However scepticism about the role of resource competition became commonplace from the mid 1970's to the late 1990's, when numerous critical studies appeared (reviewed in Schluter 2000b). The debate has settled somewhat over the last decade, mainly because new evidence in favour of competitive divergence has accumulated (Schluter 2000b).

Competitive divergence arises from antagonistic interactions between similar phenotypes due to the depletion of shared resources, and was first suggested by Darwin (1859). When species are negatively affected by the presence of another species, natural selection favours the evolution of traits that either reduce co-occurrence (e.g. habitat separation) or the impact of competition (e.g. character shifts) between two species (Wotton 1994). Thus, over evolutionary time scales competitive interactions between closely related species are expected to lead to the evolution of ecological diversification due to divergence in resource use. A well-known historical example is Lack's study of Galapagos finches in the 1940's. Lack (1947) provided evidence that competition for seeds led to population divergence, thereby resulting in two coexisting species from a single ancestor. The repetition of this process is thought to have produced the adaptive radiation of Galapagos finches (Schluter 2000b). Since then, numerous studies have provided support for the role of competition in character divergence (e.g. Werner and Hall 1977; Dieckmann and Doebeli 1999; Bolnick 2001; Després and Mehdi 2004).

Indirect evidence for the role of past competition in ecological diversification of populations may be obtained from the study of resource use and competitive interactions of recently diverged species. Such studies have the potential to shed light on the processes that may have led to their ecological divergence, as these processes are likely to be still ongoing in newly speciated sister pairs. The extent of competition induced character divergence in sister-species depends on the degree of overlap of resource use and the competitive ability of each species. For example, where sister-species use similar habitats and have similar competitive abilities, strong character shifts are expected for both species (Munday et al. 2001). In this scenario, the resulting habitat use by the two species is symmetrically displaced from the pre-competitive condition. However, in many closely related species, competitive ability is often unequal between species (reviews by: Lawton and Hassell 1981; Connell 1983; Schoener 1983), resulting in the asymmetrical displacement of one species compared to the other. By measuring the symmetry/asymmetry of character shifts between species, competitive ability can be estimated and ranked. For instance, in cases with strong asymmetry, the species that is less affected by competition should be competitively superior to the one displaced. Among the many attributes that determine competitive superiority, size has been suggested by several authors as the most important, with larger individuals usually being superior (Connell 1983; Schoener 1983; Mayr and Berger 1992; Faria and Almada 2001a). These studies have shown that larger individuals are usually more successful in gaining access to preferred habitat, shelter and food (Connell, 1983). As a consequence, size differences between closely related species may lead to strong competitive asymmetry, with the larger species usually being competitively superior to the smaller species.

Although most competition studies have focused on food, competition for habitat space also fundamentally affects distribution and abundance. Unlike other resources, space is an absolute requirement that each organism must have at any time (Schoener 1983). Habitat space differs from food as a resource in that it is not renewable and only becomes available to other animals through competition or if the occupying animal dies (Schoener 1983). Therefore, competition is expected to be intense if favourable habitat space is limiting. Demonstrating that habitat is a limited resource with field data has proven problematical because patterns of habitat use observed in the field are not necessarily the sole result of competitive interactions between species (Munday et al. 2001). For example, species may exhibit low habitat overlap because of different preferences for resources, rather than competitive displacement of one species from a mutually preferred habitat (Wiens 1977). In addition, coexisting species will only compete for shared resources when resources are limited, but not when there is an oversupply (Munday et al. 2001). Thus, observational field studies require an even and similar resource distribution, a requirement that is usually difficult to measure and control. Furthermore, establishing that the preferred habitat is limiting and that competition is occurring in the wild requires careful control of species density (Tilman 1987) and habitat availability.

While it is possible to manipulate the availability of habitat to triplefins in the field, it is extremely difficult to manipulate species density in this study because triplefins exhibit strong site fidelity, and will home if displaced, even over long distances (Thompson 1983). Thus,

triplefin densities in the field can be reduced but not increased. However, a decrease in natural triplefin density would lead to an increase in available resources for each individual, thereby minimising any potential competitive interactions between individuals (Connell 1983). For these reasons, laboratory experiments are more suitable for testing competitive interactions between triplefin species because they allow the measurement of interspecific interactions in a common setting, thus avoiding the problem of inconsistent resource distribution.

This present Chapter investigates whether past competition may have led to divergence in habitat use between the recently diverged sister-species R. whero and R. decemdigitatus (Hickey 2004). The *Ruanoho* pair occurs sympatrically throughout coastal New Zealand where there is suitable habitat (Francis 2001) and it is common to find individuals of each species in close proximity (< 10 cm) to each other. Chapter 2 showed that the species have diverged in habitat use, but knowledge about the degree of spatial overlap between the species is lacking. Therefore, it was first necessary to examine the extent of spatial overlap in the field. To investigate the competitive ability of both species in detail, multiple choice laboratory experiments were carried out using single and mixed species aquaria with varying fish densities. Size is an important determinant of competitive ability and may be a key factor affecting competitive interactions between the species pair (see Chapter 7 for details on size differences). As triplefins have small home ranges between 1 - 1.5 m² (Thompson 1979) and rarely move between territories (Connell and Jones 1991), confinement in aquaria is unlikely to affect their behaviour. One factor that cannot be controlled is the lack of a resident effect, however, there is no reason to assume that dominance relationships would be reversed under laboratory conditions. Given that the behaviour of the fish is likely to represent the situation in the wild, the results from the laboratory trials can be used to investigate competitive abilities of both species. Six hypotheses were tested based.

- 1. The *Ruanoho* species occupy the same habitat types in the wild.
- 2. The *Ruanoho* species prefer different substrata types in the absence of a competitor.
- 3. Substrata preferences of each species are not affected by the presence of a conspecific.
- 4. Substrata preferences of each species are not affected by the presence of a heterospecific.
- 5. Substrata preferences of each species does not change in response to an increase in intra- and interspecific density.

6. Divergence in resource use between the *Ruanoho* species is symmetrical.

In addition, given that the two species differ in maximum body lengths, relationships between fish length and resource use were examined as mechanisms affecting competitive interactions in hypotheses 1 - 4.

8.2 MATERIALS AND METHODS

8.2.1 Data collection: Habitat use in the wild

Overlap in habitat use was investigated using UVC (4 x 4 m) conducted in the Inner Hauraki Gulf ($36^{\circ}36^{\circ}S$, $174^{\circ}50^{\circ}E$) in northeastern New Zealand.



Figure 57: Map of the study sites. Circles indicate the position of study sites.

UVC were undertaken at 49 sites between the Whangaparaoa Peninsula and the Leigh Marine Reserve to a depth of 30 m (Figure 57). The geographic position of each UVC was noted using a handheld Garmin® 12 global positioning system (GPS) (accuracy \pm 15 m). Exposure of each UVC was calculated based on the total sum of the fetch using the program 'Fetch

Effect Analysis' (version 1.01. Pickard R 2000), which measures fetch distance (i.e. distance to land up to a maximum of 300 km) for each 20 degree sector on a compass rose from a given point (Thomas 1986). For each UVC the depth and microposition of all *R. whero* and *R. decemdigitatus* were recorded. Based on the depth distribution of the *Ruanoho* species (see Chapter 2), the microposition use of *R. whero* was investigated in the absence (deeper than 5 m) and in the presence (shallower than 5 m) of *R. decemdigitatus*. Differences in microposition use were analysed using Chi-square analysis, and the densities of both species were calculated as the number of individuals m⁻².

8.2.2 Data collection: Experimental subjects and competition trials

Individuals of *R. whero* and *R. decemdigitatus* were collected from the Whangaparaoa (36°36'S, 174°50'E) and Tawharanui (36°22'S, 174°48'E) Peninsulas (Figure 57) during the triplefin spawning season from late June to the end of August using slurp guns and hand nets. Fish were immediately transported to the Leigh Marine Laboratory (University of Auckland) and maintained in holding aquaria. The total length (L_T) of all fish was measured with vernier calipers and males were sexed visually on capture by the jet-black spawning colouration. Holding aquaria contained sand, gravel and different-sized stones to simulate the natural habitat. Each aquarium received seawater from a flow-through circulation system at ambient temperature (approximately 15 - 17°C), salinity (34 - 34.7‰) and photoperiod. Fish were fed daily *ad libitum* with a variety of different prey types including *Artemia* spp. nauplii, frozen bloodworms (*Chironomid* spp.), frozen adult brine shrimp (*Artemia* spp.) and New Zealand green lip mussels (*Perna canaliculus*).

All fish were maintained in holding aquaria for at least two days prior to trials. Aquaria for the experiments were of identical dimensions (50 cm x 40 cm x 35 cm) and differed only in their placement within the room. Four substratum types (rocks (rocks > 7 cm), cobbles (rocks < 7 cm), gravel (rocks < 4 cm), sand) were placed in equal amounts in discrete sections of each aquaria to a depth of approximately 7 cm. These substratum types were chosen because they were frequently found in the habitats of both species in the wild.

The first experiment (no competition) was designed to determine substratum use of individual fish of each species per aquarium in the absence of a competitor. The second experiment (intraspecific competition) consisted of two individuals of each species to estimate the extent
of intraspecific competitive interaction by each species. The third experiment (interspecific competition) consisted of one individual of each species to test the substratum use of each species in the presence of an interspecific competitor. The fourth experiment (high density competition) consisted of four individuals of each species per aquarium to test substratum use of each individual in a high density sympatric situation. The no competition, intraspecific competition and interspecific competition experiments were conducted with eight replicate aquaria, however, the high density competition experiment was run with five aquaria because fish availability limited the number of trials at the higher density. Before every trial, each aquarium was assigned a designated number of fish. Prior to introduction into the centre of the aquarium, each fish was measured (L_T) to the nearest mm. The size distribution of each species did not differ between experiments (*R. whero* [F_{3,40} = 1.83, *p* = 0.16] and *R. decemdigitatus* [F_{3,40} = 0.79, *p* = 0.51], Figure 58), and individuals were never used more than once in each experiment.



Figure 58: Frequency histogram of the total lengths of fish used for experimentation. The mean of length for *R. whero* was 6.9 cm (\pm 0.9 SD, white bars) and for *R. decemdigitatus* 8.7 cm (\pm 1.4 SD, black histograms).

All experiments started at 0800 hours and observations of substratum and microposition use were made after 24 hours. Individual fish were easily recognised by length, colouration and

markings. Each observation consisted of the substratum type that each individual fish selected and the microposition on rock (if rock was selected as a substratum type). Two different rock micropositions were recorded, namely the 'side or top of rock' and 'under rock'.

8.2.3 Data analysis

Differences in habitat use in the wild were investigated with simple t-tests using the statistical software package Statistica (version 7.1). A categorical linear model was used to analyse the (i) substratum and (ii) microposition use data of all four experiments:

Habitat = Treatment Species Treatment*Species Size

There was a strong potential for body size effects, in addition to treatment and species effects, so' Treatment' and 'Species' were included as categorical factors, with a simple covariate of 'Size'. The habitat variable was a multinomial variable corresponding to either the substratum or microposition categories respectively, weighted by the number of individuals in each category. The generalised logit was used as the link function. The displacement of each species was calculated as the natural log of the ratio of its trait mean in interspecific competition and its mean in the absence of a heterospecific competitor, the larger divided by the smaller. Symmetry of displacement was computed as the ratio of the displacements for each species, the smaller divided by the larger (Schluter 2000b). Symmetry ranged from 0 (only one of two species shifted in habitat use in response to heterospecific competition) to 1 (both species shifted equally). The categorical linear model was run using SAS (version 9.1).

8.3 RESULTS

8.3.1 Habitat use in the wild

In the field, *R. decemdigitatus* used a subset of the habitat of *R. whero* (Figure 59). *Ruanoho decemdigitatus* occurred in shallow (0 - 6 m) and sheltered (0 - 119 m) fetch) habitats, whereas *R. whero* was found in a range of depths (0 - 21 m) and exposures (0 - 247 m) fetch).

A comparison of the mean fetch of sites containing *R. decemdigitatus* with the mean fetch of sites containing *R. whero* showed that the *Ruanoho* species differ in fetch use (t (60) = -2.15, p = 0.04). Depth differences between species were also significant (t (556) = -9.70; p < 0.001).



Figure 59: Depth and exposure (measured in fetch) use of *R. decemdigitatus* and *R. whero* in the Hauraki Gulf. Black square shows the median, the box around the mean shows 25 - 75% percentiles and the whiskers show the non-outlier range.

Four micropositions were used by both species, namely 'under rocks', 'side or top of rocks', 'sand' and 'on cobbles/gravel'. *Ruanoho decemdigitatus* was almost exclusively (95%) found 'under rocks' (Figure 60). *Ruanoho whero* preferred the microposition 'under rocks' (70%) and the 'side or top of rocks' (22%) in areas deeper than 5 m (Figure 60).

The use of the microposition 'under rocks' was lower (58%) and 'side or top of rocks' higher (33%) in areas shallower than 5 m, which were areas where *R. decemdigitatus* co-occurred (Figure 60). These differences in microposition use were significant between species ($\chi^2 = 37.4$, df = 6, p < 0.0001). Relative densities of *R. whero* were similar in habitats deeper and shallower than 5 m (0.68 m⁻² and 0.63 m⁻², respectively), although both densities were higher than that of *R. decemdigitatus* (0.25 m⁻²).



Figure 60: Percentage microposition use of *R. decemdigitatus* (< 5 m), *R. whero* (< 5 m) and *R. whero* (> 5 m) in the field.

8.3.2 Competition trials

The results of the 'no competition' experiment showed that both species exhibit almost identical substratum preferences. Both species preferred rock as a substratum, and only R. *whero* selected cobbles. Neither of the species selected gravel or sand (rock>>cobble>sand=gravel, Figure 61).

In the 'intraspecific competition' experiment *R. whero* occupied all four substratum types, of which rock was used predominantly (Figure 61). Similarly, *R. decemdigitatus* used a wider range of substratum types when in the presence of conspecifics, and rock was again the preferred substratum type (Figure 61). In the 'interspecific competition' experiment, the use of the preferred substrate by *R. whero* (i.e. rock) decreased greatly in the presence of *R. decemdigitatus*, while the substratum use by *R. decemdigitatus* remained almost unchanged, with 100% of the *R. decemdigitatus* individuals using rock (Figure 61). The mean use of cobble, gravel and sand in the 'high density competition' experiment was much higher by both species, however, rock was still the most preferred substratum type (rock>cobble>gravel>sand, Figure 61). The categorical linear model showed that body size was significant (p = 0.01), while both 'species' and 'treatment' were not significant (Table 14), in habitat use. This finding demonstrates that habitat use is driven primarily by the body size of an individual, rather than by species identity or experiment type.



Figure 61: Percentage use of gravel, rock, sand and cobble by *R*. *decemdigitatus* and *R*. *whero* in the four competition experiments.

Table	14:	Categorical	linear mo	odel ana	lysis o	of the	use o	f subst	ratum	(rock,	cobble	e, gravel	and
sand)	and	micropositi	on types	('under	rock'	and	'side	or top	of roc	k') by	R. de	cemdigit	atus
and R.	wh	ero in the co	ompetition	n trials.									

Substratum types							
Effect	DF	Wald chi-square	p-value				
Species	3	0.000	1.000				
Treatment	9	3.393	0.947				
Species*Treatment	9	0.025	1.000				
Body size	3	11.310	0.010				
Microposition use							
Effect	DF	Wald chi-square	p-value				
Species	1	0.000	0.999				
Treatment	3	3.678	0.298				
Species*Treatment	3	0.282	0.963				

The preference for the micropositions was equally clear. When rock was selected as a substratum type both species preferably selected the microposition 'under rock' in the absence of a competitor, though *R. whero* also appeared to use the side and tops of rocks (Figure 62). In the 'intraspecific competition' experiment the use of the microposition 'under rock' decreased in both species, while the use of 'side and top of rock' increased (Figure 62). In the presence of a heterospecific competitor, fewer *R. whero* used the microposition 'under rock' and no individuals were observed to use the microposition 'side or top of rock', whereas the use of micropositions by *R. decemdigitatus* remained virtually unchanged from the 'no competition' experiment (Figure 62).

Microposition use by both species in the 'high density competition' experiment differed considerably from that in the three other experiments, in that more individuals of both species used the microposition 'side or top of rocks' (Figure 62). The categorical linear model analysis of the 'side/top of rock' versus 'under rock' categories showed again that body length is the main determinant of habitat use in *R. decemdigitatus* and *R. whero* (p < 0.05), while species and treatment were not significant (Figure 62).



Figure 62: Percentage use of the microposition 'under rock' and 'on the side or top of rock' by *R. decemdigitatus* and *R. whero* in the four competition experiments. Note that the percentages only sum to 1 if a species was found in all experimental replicates on rock.

Interspecific character shifts from no competition to high density interspecific competition were strongly asymmetric for the most preferred substratum type rock (symmetry of character shift: 0.76), since *R. decemdigitatus* was competitively superior to *R. whero*. The shifts in resource use for sand, gravel and cobble could not be calculated as either one or both species did not use these substratum types at all in the no competition, interspecific competition or the high density competition experiments.

8.4 DISCUSSION

The results of this study show that body size differences between the *Ruanoho* species affect both habitat use and reproductive isolation. Field data demonstrated that the species differ in habitat use and that *R. whero* shifts in substratum use in the presence of *R. decemdigitatus*. Laboratory trials showed that the use of substratum types was related to body size. Despite having similar preferences for substratum type, inter- and intraspecific competition for the mutually-preferred rock habitat resulted in the displacement of small individuals of both species because the larger sized *R. decemdigitatus* had greater competitive ability in interspecific contests, a pattern that has been shown to apply in other species (Munday et al. 2001; Young 2004). The competitive dominance of *R. decemdigitatus* was illustrated by the highly asymmetric displacement ratio between the two species. The larger body size and consequent competitive superiority in interspecific contests is undoubtedly the main factor allowing *R. decemdigitatus* to exploit the preferred rock habitat when the species co-occur.

In the laboratory competition experiments, small *R. whero* had to compete with larger *R. decemdigitatus* and with large conspecifics for suitable habitat space. The displacement of *R. whero* into habitats devoid of rocks is likely to increase predation risk because these habitats are less complex and offer less shelter (Forrester and Steele 2004a; Gratwicke and Speight 2005). These negative effects are presumably magnified during the spawning season as both *Ruanoho* species usually use rocks as nesting substrata (Chapter 7). Substrata other than rock may not have enough area for the attachment of the eggs and are less stable, therefore nests would have a greater risk of physical damage (Piller and Burr 1999). In many fish species that exhibit paternal care, male body size is positively correlated with the size of the nesting substratum (Konishi and Takata 2004), and therefore body size is directly related to fitness (Andersson 1994). When body size is an important factor in interspecific competition between ecologically similar species, the larger species is usually a superior competitor and is more successful in establishing territories (Robertson 1996) and attracting females (Oliveira et al.

2000). In contrast, a smaller body size gives individuals access to a higher quantity of shelter places (Hixon and Beets 1989), and thus may offer increased protection from predation. Competition for resources has been implicated both theoretically (Rosenzweig 1978; Doebeli and Dieckmann 2003) and empirically (Bolnick 2004; Friesen et al. 2004; Munday et al. 2004; Bernardi 2005) in the divergence of ecological traits and reproductive isolation. The finding that the *Ruanoho* species have similar substratum preferences but differ in competitive ability is consistent with ecological competition being a factor in the evolution of these species.

The intrinsic habitat and microposition preferences in the absence of a competitor were almost identical for both species, irrespective of body size, with rock being by far the most preferred substratum type. The habitat preference for rocks is probably related to the access to shelter, as the other substrata types, gravel, cobble and sand, provide much smaller or no shelter interstices. Both *Ruanoho* species also preferred the same microposition, the underside of rocks (UCS), suggesting that potential habitat overlap is extremely high, even on a microscale level. This microposition preference is also found in the field, further indicating that shelter is an important environmental variable that affects the abundance of these species. Thus, because the two species broadly co-occur around New Zealand, the potential for competitive interactions, particularly at sites with a limited amount of rocks, is likely to be high.

When there is intraspecific competition for habitat, some individuals of both species are displaced onto less preferred substrata types (i.e. other than rock). In the case of *R*. *decemdigitatus*, larger individuals were competitively superior and displaced smaller individuals onto the less preferred substrata types. Intraspecific length differences did not appear to influence substrata use in *R. whero*, probably due to the smaller size range of this species. In this study the size variation of *R. whero* (4.5 - 8.5 cm L_T) was smaller than that of *R. decemdigitatus* (5.5 – 11 cm L_T). Thus, intraspecific size differences in *R. decemdigitatus* will be more pronounced and the potential for size dependent displacement greater.

The substrata preferences of *R. whero* were considerably influenced by the presence of an interspecific competitor, with almost half of the individuals being displaced to substrata types other than rock. In contrast, the substrata selection of *R. decemdigitatus* was unaffected by the presence of *R. whero*, suggesting that the former is the dominant species. This pattern holds true irrespective of the size of either species. The reliance of *R. decemdigitatus* on rocks (i.e. defined as larger than 7 cm in methods) as a shelter site presumably reflects the fact that this species attains a much larger body size than *R. whero*, and thus requires larger shelter sites to

protect it from predators. Under intense competition fish length was more important than species in determining competitive outcomes, suggesting that when resources are scare competitive dominance largely depends on fish size. Thus, this study adds to the growing body of evidence that competitive ability is strongly correlated with body size in a variety of fish species (Wilson 1975; Peters 1983; LaBarbera 1989; Mayr and Berger 1992; Robertson 1998; Davey et al. 2005). This experiment also showed that more individuals of both species were displaced onto the less preferred substrata types due to the increase of intra- and interspecific competition for suitable space. Only the largest individuals of *R. decemdigitatus* were able to occupy the preferred rock substrata type. Smaller individuals were forced to move onto less preferred substrata types, such as gravel and sand. This effect, known as a size-specific dominance hierarchy (Persson 1985), has been found in a variety of different animals (e.g. Cutts et al. 1999; Szabo 2002; Hesthagen and Heggenes 2003), including fish (Munday 2001; Hesthagen and Heggenes 2003). Most often larger individuals are stronger, and therefore superior competitors (Persson 1985). As a result, smaller individuals will experience fitness trade-offs due to their limited access to suitable shelter, food or mates (Tilman 1987; Munday 2001). Competitive interactions may be enhanced by stronger territorial behaviour of adult males during the winter breeding season. In the *Ruanoho* species, the fitness trade-offs experienced by smaller R. whero would be particularly high during the reproductive season, because both species exclusively use large rocks as spawning substrate (see Chapter 7). The failure of smaller R. whero males to obtain territories with rock means that these males would be unable to establish a suitable nest site and attract mates. Since competition for shelter rocks is strongly size-specific, large males of R. decemdigitatus are likely to monopolise spawning, and receive a disproportionate share of matings at the expense of smaller conspecific-and heterospecific males. A more general interpretation of the findings from this particular experiment is that the outcome of competitive interactions is partly dependent on the density of conspecific- and heterospecific competitors.

Overall, *Ruanoho whero* was greatly influenced by inter- and intraspecific competition for the mutually preferred resource rock, whereas *R. decemdigitatus* was not nearly so influenced. Such asymmetric competition is not uncommon among vertebrate and invertebrate species (reviewed by: Lawton and Hassell 1981; Connell 1983; Schoener 1983). The highly asymmetric character shift for rock appears to result from the great length differences of the two species, and the resulting differences in competitive ability. Dominance rank in the social hierarchy is often positively associated with fish size (Wilson 1975; Schoener 1983; Webster and Hixon 2000). For example, a study by Whiteman and Côté (2004) on gobies found that

dominance was directly related to fish length, with the larger, more competitively dominant individuals being able to monopolise areas with the highest food density and achieve the highest foraging rates. Furthermore, aggressive interactions, including threats, chases and bites, maintained a dominance hierarchy within each group that was strongly correlated with fish length (Whiteman and Côté 2004). Thus, size differences between competing species is likely to result in asymmetric competition, either through exploitation or some form of aggressive interference competition, or both (Wilson 1975).

In summary, the results of the present study provide evidence that interspecific and intraspecific competition for habitat space (i.e. rocks to shelter under) exist between the Ruanoho species, with the net result being a considerable degree of interactive segregation. Both inter- and intraspecific competition affected substrata use by R. where, though the former had an overall stronger effect. In contrast, R. decemdigitatus was only influenced by intraspecific competition. These results can explain the finding that R. decendigitatus are more frequently found under rocks than R. whero when both species co-occur. The multiplechoice experiments showed that small R. where are displaced from the mutually preferred rock habitat because larger R. decemdigitatus have greater competitive ability in interspecific contests. Furthermore, small R. where also have to compete intraspecifically with large R. where individuals for suitable habitat space, thereby further reducing the proportion of individuals that are able to occupy rocky habitats. This displacement into habitats devoid of rocks is likely to negatively affect R. where when there is a risk of predation because they provide less shelter space. Additionally, displaced R. where have to use nesting substrates other than rock, which might affect their success in attracting mates as females of many species are known to choose nesting males according to male attributes as well as nest characteristics (Oliveira et al. 2000). Breeding substrata other than rock (i.e. cobbles) may also affect egg survival because of the greater chance of abrasion or insufficient space for egg adhesion. Consequently, fitness trade-offs are likely to be greater for *R. whero*, as this species has to compete with conspecifics and heterospecifics for suitable habitat space, and as a result will get forced more often into less preferred habitats than R. decemdigitatus. Because competition is known to be a major cause of resource divergence in animals (Schluter 2000b) it appears likely that resource competition between R. whero and R. decemdigitatus has facilitated divergent habitat use in these species. Displacement of R. whero by the larger R. decemdigitatus might have caused R. where to occupy novel habitats, thereby experiencing new selection pressures and shaping its ability to survive under a variety of different conditions. In contrast, displacement of *R. decemdigitatus* into less preferred habitats devoid of rocks is extremely unlikely, thus, this species would have to deal to a lesser extent with different environmental selection pressures. The pattern of habitat use in the wild combined with the results of the competition trials suggests that R. decemdigitatus are habitat specialists that have evolved to utilise efficiently some habitat subsets (i.e. rocks). Ruanoho whero, in contrast, appears to be a small habitat generalist that uses a wider range of habitat types. Interspecific contests in these species appear to be closely linked to body size, with the specialist being larger in size than the generalist. The larger body size gives the habitat specialist an advantage over the smaller habitat generalists in areas of habitat overlap, as it can out-compete the smaller species. Similar patterns of body size divergence have been found in crater lake cichlids (Schliewen et al. 1994; Schliewen et al. 2001) and the largebenthic and small-limnetic species pairs of the threespine stickleback (McKinnon et al. 2004). Benthic sticklebacks are confined to the littoral zone and deeper areas of open sediment or attached vegetation (Schluter and McPhail 1992), while the limnetics are mainly planktivorous and forage in the open water (Nagel and Schluter 1998). Experimental tests of competition and selection suggests that the divergent characteristics of limnetics and benthics are partly the outcome of ecological character displacement (Schluter and McPhail 1992). This suggests that differences in body size between this species pair is thought to be the result of divergent natural selection between habitats (littoral zone vs. open water) (Nagel and Schluter 1998). Thus, the linkage of morphological and ecological phenotypic diversification appears to be a common feature in the divergence of fish species.

9 General Discussion

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9.1 SUMMARY OF RESULTS

This thesis investigates two key questions that will help understand the extraordinary diversity of New Zealand triplefin fishes, namely: 1) which ecological traits are under selection?; and 2) which traits are potentially available for the development of assortative mating?

9.1.1 Which traits are under selection?

Given that trophic partitioning is unlikely to have been a major factor in the diversification of New Zealand triplefin fishes, habitat use was identified as a possible key trait for selection and subsequently investigated in detail. This thesis was the first comprehensive study to look at habitat use of the majority of New Zealand triplefin species at a range of locations. It was necessary to first describe the pattern of habitat use in the field before further work could be carried out to establish the processes that have led to the habitat pattern. The results of this thesis suggest that New Zealand triplefins have diverged significantly in habitat use, with species occupying different habitat patches in the same general area. It is noteworthy that this pattern was pronounced between closely related species (i.e. sister-species). Diversification was most marked in the exposure and depth of the habitat, however, fine-scale differences in the use of substratum and microposition types resulted in even smaller sub-partitioning of the habitat (Chapter 2, 7 and 8). The high overlap in substratum and microposition types suggests that these habitat types are of particular importance to all New Zealand triplefin species (i.e. as breeding or shelter sites), hence species divergence in substratum and microposition use is inhibited. This in turn may explain why species partition the reef primarily by depth and exposure. The high divergence between species was particularly evident in Chapter 2 by the absence of any phylogenetic signal in habitat use, which suggests that habitat diversification may have been associated with the mechanisms of species divergence. Even sister-species differed strikingly in habitat use, implying a rapid rate of habitat diversification. Chapter 3 demonstrated that triplefin populations exhibit consistent habitat use across biogeographic gradients, indicating that habitat availability is the main driver of triplefin assemblage structure around New Zealand. The marked absence of geographic variation in species habitat use indicates that species may actively select particular habitats over others, and that larval dispersal is strong enough to lead to sufficient larval exchange among sub-populations to overcome local adaptation. Recognition that behaviour can generate similar patterns of distribution and abundance at multiples scales implicates habitat selection as an important

factor affecting local and regional patterns of biodiversity in this group. Habitat selection as a process thus forms a critical link between the dynamics of populations and communities at the local scale, and the regional dynamics of meta-communities at the landscape scale.

Chapter 5 demonstrated that habitat use by new recruits was highly similar to that of conspecific adults, indicating that larvae might exert species-specific habitat choice at settlement. This result is important as it suggests that habitat selection in New Zealand triplefin fishes may not be the passive outcome of differential mortality between habitat types following settlement (cf. Thompson 1979; Connell and Jones 1991). Together these results indicate that the New Zealand triplefin assemblage has diversified extensively in habitat use, and also that habitat selection is a species-specific process that might be driven by active habitat choice at settlement. In interpreting the results of the habitat survey, it was important to recognise that the use of SCUBA limited the range of habitats that could be effectively sampled. For example, highly exposed areas could only be sampled when sea conditions were extremely calm, and bottom time restrictions precluded effective sampling of deep habitats (> 30 m). In order to sample more completely deep-water habitats underwater video could be used in future studies. Although the range of habitats reported in this thesis may not be fully representative of some species, more extensive habitat sampling was likely to have resulted in greater, rather than fewer interspecific differences in habitat use. Further research should aim to marry differences in habitat use within species with fitness trade-offs. In particular, it would be interesting to investigate fitness trade-offs in habitat-generalist species such as F. *lapillum.* Fitness differences of F. *lapillum* individuals could be assessed by measuring the growth rate and reproductive output of individuals occupying different environments

Triplefin species were found to differ in the breadth of habitat use (Chapter 4). Some species occupied a wide range of habitats (e.g. *F. lapillum*), while individuals of other species were fairly uniform in terms of habitat choice (e.g. *B. lesleyae*), suggesting that species follow different ecological strategies (i.e. generalist versus specialist lifestyles). Phylogenetic comparative analyses indicated that the ancestor was probably intermediate between generalised and specialised species, which contrasts with the long held assumption that generalists are the founders of a radiation and specialisation results in subsequent sub-partitioning of resources (reviewed in: Futuyma and Moreno 1988; Schluter 2000b).

Because the majority of New Zealand triplefin species coexist spatially, and are therefore able to interact, interspecific divergence in habitat use is possibly the result of frequency- and density-dependent ecological interactions between species. Habitat diversification has also been an important component of divergence in lizards (Losos et al. 1997; Losos et al. 2003; Losos 2004), birds (Grant and Grant 2002; Tonnis et al. 2004), turtles (Stephens and Wiens 2003), and lacustrine (Schliewen et al. 1994; Schliewen et al. 2001; Barluenga and Meyer 2004; Genner and Turner 2005; Barluenga et al. 2006) and marine fish species (La Mesa et al. 2004; La Mesa and Vacchi 2005; Malavasi et al. 2005; Rocha et al. 2005), suggesting that habitat use may be an important component of vertebrate diversification (Streelmann and Danley 2003).

One process that might explain the degree of diversification in habitat use among closely related species is intra- and interspecific competition for shared habitat resources (Schoener 1982; Connell 1983; Schluter 1996a; Schluter 2000b; Munday 2004; Bernardi 2005). Laboratory experiments on the *Ruanoho* sister-species pair were conducted in Chapter 8 to explore if competition may have facilitated diversification in habitat use. The multiple-choice experiments provided evidence to suggest that both intra- and interspecific competition might be central to ecological divergence in the *Ruanoho* species. Specifically, the trials demonstrated that large R. decemdigitatus were competitively superior to R. whero and smaller conspecifics, and consequently most often occupied the mutually preferred rock substratum. The laboratory trials may explain the field observation that R. where are less often found under large rocks in the presence of *R. decemdigitatus*. Based on the results, it seems likely that competition for habitat resources has been a factor in the divergence of the Ruanoho sister-species pair. Given that other species have also diverged considerably in habitat use, competition for habitat resources could have also been involved in the divergence of other New Zealand triplefin species. Therefore, it would be useful to investigate the effect of competition on habitat use in other species, particularly other sister-species pairs. While it would be ideal to use manipulative field experiments to validate the laboratory observations, the strong philopatry and homing ability of triplefins makes the use of such experiments extremely labour intensive (Thompson 1983). The optimal way to measure the effects of competition on habitat use would be to remove triplefin species from patches and then to subsequently monitor the response of other species. Despite this limitation, the small body and territory size of triplefin species means that laboratory conditions are likely to be a good representation of the situation in the wild.

9.1.2 Which traits are available for assortative mating?

The second key question of this thesis related to the traits that are potentially available for the developments of assortative mating. Chapter 7 investigated whether triplefin species differed in traits that are known to affect mate choice in other organisms, and could thus potentially lead to prezygotic isolation in New Zealand triplefin fishes. Specifically, variation in breeding habitat, breeding period, male colour pattern and male body length were assessed. All species investigated showed strong temporal overlap in breeding times, thus temporal isolation can be rejected as an isolating barrier. Differences in breeding habitats were strong, and resulted in considerable spatial segregation, even among closely related species. This was particularly pronounced for the sister-species pair *O. maryannae* and *F. malcolmi* as well as *F. lapillum* and *G. nigripenne*, as indicated by the extremely low misclassification rates of each species with its sister-species. These differences are presumably a by-product of differences in general habitat use, as triplefins court and mate in the same territory as that occupied year round

The finding that habitat divergence in New Zealand triplefins ultimately affects breeding habitat choice shows some parallels to studies on host choice in phytophagous insects (e.g. Bush 1969; Feder 1998; Via et al. 2000; Nosil et al. 2005; Nosil 2007) and coral dwelling gobies (Munday 2004). Assortative mating in New Zealand triplefin species could also be the by-product of adaptation to habitat resources, which would be consistent with existing theoretical models. The evolution of assortative mating due to density- and frequency-dependent selection of habitat use would have gradually increased as a side effect of selection against common phenotypes. This is because common phenotypes are thought to have reduced fitness because of increased competition, while extreme phenotypes enjoy less competition (Dieckmann et al. 2004c). Therefore, it can be expected that any tendency for assortative mating will be favoured by natural selection as this will decrease the production of maladaptive genotypes (Dieckmann and Doebeli 1999; Kondrashov and Kondrashov 1999).

Little evidence was found for divergence in male colour pattern during the reproductive season in both the visible and UV spectra (Chapter 7). In particular, males of most species assumed a darker colouration during the spawning season, thereby decreasing interspecific colour differences. This suggests that male nuptial colouration does not affect sexual selection in these species. Therefore, sexual selection of male body colour cannot be discounted in these species. Conversely, the sister-species pairs *F. lapillum* and *G. nigripenne*, and *R. decemdigitatus* and *R. whero* (Hickey and Clements 2005) turn completely dark during the

reproductive season and show no evidence of colouration in the UV spectrum, and thus the possibility that sexual selection on male body colouration has led to reproductive isolation in these species is highly unlikely.

In addition to surveying several species for traits that may contribute to prezygotic isolation, detailed behavioural investigations were carried out on the *Ruanoho* sister-species pair. These species show the least amount of genetic difference of all New Zealand triplefins (Hickey and Clements 2005), suggesting the mechanisms that have lead to their divergence may still be evident (Schluter 2000b). Maximum body size was highly divergent between within the Ruanoho genus, and mate choice trials showed that R. whero females preferred smaller and similar-sized whereas female *R. decemdigitatus* had no clear preference for male body size. Similar patterns of body size divergence have been found in crater lake cichlids (Schliewen et al. 1994; Schliewen et al. 2001) and the large-benthic and small-limnetic species pairs of threespine stickleback (Nagel and Schluter 1998; McKinnon et al. 2004). Observations of reproductive behaviour and visual inspection of gonad maturity showed that R. where matured at a size at which R. decemdigitatus was immature. Homospecific mating trials showed that R. whero exhibited a subset of the courtship behaviour recorded for R. *decemdigitatus*, and the absence of any reproductive behaviour in the heterospecific breeding trials provided evidence that the *Ruanoho* species are reproductively isolated. Therefore, it appears that hybridisation was prevented by a combination of differences in size at first maturity, courtship behaviour and preferences for male body size. Together, the behavioural experiments suggest that the evolution of body size differences in the *Ruanoho* sister pair may be driven not simply by adaptation to habitat, but by subtle interactions between resource competition and sexual selection. The effects of competition and mate selection on body size divergence described here have wider implications for our understanding of adaptive radiation in New Zealand triplefins as a whole. Other sister-species pairs of New Zealand triplefins, e.g. N. segmentatus and N. yaldwyni, B. lesleyae and B. medius, O. maryannae and F. malcolmi, and F. lapillum and G. nigripenne (Hickey and Clements 2005) also show considerable interspecific differences in body length and habitat use (Francis 2001; Clements 2003). This indicates that habitat and morphological differentiation in body size may be an important component of divergence in this clade. These findings invoke a strong role for ecologicallybased selection in speciation (Funk et al. 2006), and support the hypothesis that adaptation to habitat is a major factor in speciation in this system. Future work should focus on the role of body size in mate choice and size at first maturity in other sister-species pairs, in particular in species that differ considerably in maximum body size (e.g. F. lapillum and G. nigripenne).

The *Ruanoho* work was also important in providing information concerning the taxonomic status of these species. Fricke (1994) experienced difficulty in differentiating the two species using preserved material, and subsequently treated *Ruanoho* as synonymous ecological forms. Other authors (e.g. Francis 2001) continued to recognise two species. Recent work has shown that the species differ in genome size and form distinct monophyletic lineages in both nuclear and mitochondrial DNA (Hickey 2004). This thesis has provided ecological information to demonstrate that the *Ruanoho* species represent distinct biological entities.

9.2 SPECIATION IN NEW ZEALAND TRIPLEFIN FISHES

Based on the ecological characteristics established in previous work and in this thesis, it is valuable to discuss what factors may have been important in the adaptive radiation of New Zealand triplefins. Given that most New Zealand triplefin species have a broad sympatric distribution (Clements 2003) it appears highly likely that speciation has occurred in the face of gene flow, and that ecological contact has been involved in the speciation process. Recent theoretical research (Dieckmann and Doebeli 2004; Gavrilets 2005; Gavrilets and Vose 2005) has identified three criteria that facilitate speciation in sexual populations in the absence of biogeographic barriers to gene flow: 1) the presence of a trait that is subject to disruptive selection and simultaneously controls non-random mating (so that recombination does not prevent splitting of the population); 2) high levels of initial genetic variation; and 3) no selection for mating success so that choosy organisms pay no costs. This thesis, in combination with other studies, provides evidence that triplefin species conform to some extent to all three criteria. Chapter 7 has shown evidence that in some species divergence in habitat use automatically leads to differences in spatial breeding habitat, thus satisfying the first criterion. Such a linkage between a trait that is under disruptive selection which in turn controls non-random mating has been referred to as a 'magic trait' (Gavrilets 2004; Gavrilets 2005), as it presents a simple mechanism for speciation in sympatry or parapatry. Theory predicts that, in this case, mating assortativeness should increase as long as the trait is under disruptive selection, so that given sufficient time, trait divergence is accompanied by reproductive isolation (Beltman and Metz 2005). A similar linkage of habitat preference and non-random mating has also been found in phytophagous insects (Bush 1969; Berlocher 1998; Feder 1998; Nosil et al. 2005; Nosil 2007) and in other fishes (Munday et al. 2004).

The second criterion of high initial genetic diversity is difficult to quantify given that the most recent speciation events in New Zealand triplefin species occurred at around 0.8 million years

ago, according to 12S/16S transversion distance clock estimates (Clements et al., unpublished). However, some inference of the genetic diversity of traits associated with habitat use can be made given that triplefins appear to have dominated the New Zealand rocky reef fauna for some time (Clements 2003). Studies have shown that such a situation can lead to relaxed selection in novel environments and can thus facilitate diversification (McCune 1990; Rüber and Zardoyaa 2005). It therefore appears that the absence of ecologically similar competitors such as blennies and gobies would have lead to relaxed selection, which may have facilitated the diversification of habitat use in triplefin species.

The third criterion appears satisfied given that triplefins reach a high density of up to 4 individuals m^{-2} in New Zealand (Chapter 2), therefore it seems likely that the costs of finding the right mate is relatively low. However, given their small size, triplefins would have a high risk of predation, and so the more they move about looking for the right mate, the greater the chances of predation. In this sense, the risk of predation would have to be balanced against the fitness benefits of finding the right mate, which in turn will be strongly dependent on the density of potential mates and predators. Further studies could investigate the costs of choosiness in more detail by collecting data on the movement rates of males and females during the reproduction season. This would help to establish the potential number of reproductive encounters as well as some measure of predation risk for triplefin species.

Future studies would benefit from predictions about the spatial patterns of genetic variation and linkage disequilibria between ecological and mating characters expected from adaptive speciation (Dieckmann et al. 2004c). These patterns are likely to depend on the speciation process, therefore, mechanisms should be grouped according to the patterns they engender. It would be particularly valuable to distinguish between patterns of sympatric and parapatric speciation and those resulting from two forms of allopatric diversification, namely (i) adaptation to geographically-separate environments, and (ii) neutral divergence in allopatric populations followed by character displacement upon secondary contact. While it is clear that this thesis cannot rule out that historical allopatry has been involved in evolution of the New Zealand triplefin clade, it is possible to examine the likelihood of different scenarios. At present, the majority of triplefin species are distributed sympatrically around New Zealand's coastline, and there is no evidence to suggests that the distribution of species has been different in the past (see General Introduction). This lack of allopatric distributions suggests the diversification in habitat use and the divergence in mating habitat is unlikely to have been the result of character displacement after secondary contact or from adaptation to geographically-separated environments. What seems much more likely is that frequency- and density-dependent selection has led to the high diversity in New Zealand triplefin fishes in sympatry or parapatry.

Although all evidence points to a sympatric distribution in New Zealand triplefin species, it is also important to consider the extent of population connectivity between distant locations. In the extreme case of complete gene flow (i.e. sympatry), it has been shown that if there is an environmental gradient across the spatial area, strong frequency-dependent selection due to competition for limiting resources can result in speciation (Doebeli and Dieckmann 2003). Modelling work has established that even partial restriction of gene flow (i.e. parapatry) increases the likelihood of speciation (Gavrilets and Vose 2005). Recent work by Hickey (2004) suggests that a parapatric model may be most suitable to explain gene flow in a number New Zealand triplefin species. In summary, not only is there a biogeographic and genetic evidence to suggest that the New Zealand triplefin clade has evolved under non-allopatric conditions, this thesis provides traits and mechanisms that help explain how this unique group of fishes has radiated.

The adaptive radiation of lake cichlids is probably the best known example of non-allopatric speciation in vertebrates (Fryer and Iles 1972; Greenwood 1974; Schliewen et al. 1994; Turner 1994; Johnson et al. 1996; Seehausen et al. 1997; Seehausen and van Alphen 1998; Seehausen et al. 1998; Seehausen and Van Alphen 1999; Kornfield and Smith 2000; Schliewen et al. 2001; Bouton et al. 2002; Streelmann and Danley 2003). Several authors have proposed a set of general rules to explain ecological and evolutionary patterns of vertebrate radiations based on the studies of cichlids and other taxa (see Schluter 2000b for a review; Stephens and Wiens 2003; Streelmann and Danley 2003). For example, Streelmann and Danley (2003) compared the stages of vertebrate evolution and found that different radiations follow similar evolutionary trajectories. The authors suggested that vertebrate radiations follow a three step process: 1) differentiation in habitat; 2) differentiation in diet, and; 3) differentiation in sexual selection (Streelmann and Danley 2003). The same sequence is not seen in the New Zealand triplefin radiation where the second stage, differentiation in diet, appears relatively unimportant. Despite extensive ecological specialisation in habitat use, the majority of New Zealand triplefins are generalist carnivores (Vasques 1999; Feary 2001). Two species display more specialised diets, namely the mussel-feeding *B. dorsale* and the planktivorous O. maryannae (Feary 2001), although these dietary specialisations are associated with an extreme shift in habitat (Francis 2001). Furthermore, the majority of species show little evidence for specialisation in jaw or tooth morphology (Feary 2001). This lack of specialisation in diet and tropic morphology stands in contrast to other fish radiations

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in which extreme trophic partitioning has occurred. For instance, the radiation of reef fishes of the family Labridae is marked by extreme differentiation in trophic morphology and diet (Westneat et al. 2005). There is indication that some triplefin species have diversified in the third stage, namely sexual selection. Breeding experiments demonstrated that the *Ruanoho* species differ in courtship display, and mate choice experiments indicated that *R. whero* has a preference for smaller and similar-sized males, while no preference was found for *R. decemdigitatus* (see Chapter 7). Taken together, this indicates that New Zealand triplefins have radiated along the habitat axis and also partly along the communication axis, while trophic diversification seems to be limited. Triplefins do not appear to conform to the model of vertebrate radiation suggested by Streelmann and Danley (2003), highlighting the need to investigate more vertebrate radiations to clarify whether such patterns have generality.

9.3 CONCLUSIONS

This thesis provides strong evidence that habitat use is a key ecological trait that has allowed New Zealand triplefin species to diversify and co-occur despite sympatric distributions. Density- and frequency-dependent competition for habitat resources appears to have been a potent process in the divergence of the *Ruanoho* sister-species pair and may also be an important process in the divergence of other species in this radiation. The finding that New Zealand triplefin species exhibit extensive habitat partitioning without significant geographic or genetic isolation invokes a strong role for ecologically based selection in speciation in this system. This is consistent with recent theoretical work that has shown that strong ecologically-based, spatially heterogeneous selection, coupled with limited migration and genetically based habitat choice that simultaneously codes for mate choice, can indeed result in rapid phenotypic and ecological diversification and the emergence of multiple species (Dieckmann and Doebeli 2004; Doebeli and Dieckmann 2004; Gavrilets and Vose 2005). The results presented in this thesis add to the mounting body of evidence indicating that densityand frequency-dependent selection can lead to phenotypic divergence in marine fish species (e.g. Rocha 2003; Munday et al. 2004; Rocha et al. 2005), and therefore suggest that speciation in heterogeneous marine systems is not necessarily associated with allopatry. This indicates that the use of allopatric divergence as the null model in marine systems is hard to justify. The findings in this thesis are consistent with recent work that has suggested that a renewed emphasis on the importance of selection in species formation is warranted.

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I. APPENDIX (Chapter 2)

i Calculation of the DSE distances

Step 1: Determine the maximum possible squared discrepancy for each MDS coordinate dimension using the minimum and maximum values observed for these MDS coordinate axes. Call these values md. All MDS loadings are standardized between -1.0 and + 1.0, hence the maximum possible discrepancy for any two variable loadings is calculated as:

$$md = (min-max)^2$$

 $md = ((-1)-(+1))^2$
 $md = 4$

Step 2: Compute the sum of squared discrepancies per observation using

$$d = \sqrt{\sum_{i=1}^{N} (c_i - t_i)^2}$$

and divide through the squared discrepancy for each pair of observations by the maximum possible discrepancy observable given these two variables. Then take the square root of the sum to produce the scaled variable Euclidean distance as follows

$$d_1 = \sqrt{\sum_{i=1}^{N} \left(\frac{(c_i - t_i)^2}{md} \right)},$$

where d1 = the "scaled variable' Euclidean distance and md = the maximum possible squared discrepancy between these two variables (which is always 4 for each variable, see Step 1).

Step 3: Compute the scaled value from Step 2 by dividing it by the square root of N, where N = the number of paired MDS coordinate values, as follows

$$d_2 = \frac{\sqrt{\sum\limits_{i=1}^{N} \left(\frac{(c_i - t_i)^2}{md}\right)}}{\sqrt{N}} = \frac{d_1}{\sqrt{N}}$$

This scaled distance (which varies between 0 = absolute identity to 1.0 = maximum possible dissimilarity) is then expressed as a DSE similarity by subtracting it from 1.0. The formula above then becomes

$$s_2 = 1.0 - \left(\frac{\sqrt{\sum_{i=1}^{N} \left(\frac{(c_i - t_i)^2}{md} \right)}}{\sqrt{N}} \right) = 1.0 - \left(\frac{d_1}{\sqrt{N}} \right)$$

ii Formula for the d-hat raw stress (for the evaluation of the MDS plot)

Phi =
$$\Sigma [d_{ij} - f(\delta_{ij})]^2$$

II. APPENDIX (Chapter 6)

i Explanation of the CART methodology and terminology

The major role of CART is to produce an accurate set of data classifiers by uncovering the predictive structure of a dataset. The concept and major algorithms for constructing a decision tree were introduced by Breiman et al. (1984), including the commonly used Classification and Regression Tree algorithm (CART). Quinlan (1986; 1993) introduced further decision trees through the creation of the ID3 and C.4.5 classifier algorithms (Barrett 2005d). A decision tree is a structure built from a series of decisions that aim to maximize classification accuracy of two or more outcome classes, levels, or measures (Barrett 2005d). The analogy with the form of a tree is what gives this analysis its name. Even though CART is an ideally suited technique for both exploring and modelling complex ecological data it has seldom been used in ecology (Rejwan 1999; De'ath and Fabricius 2000). This is especially surprising because the CART algorithm has been shown to outperform many of the conventional noncross-validated regression methods (Rejwan 1999; De'ath and Fabricius 2000; Barrett 2005d). The following example uses a fictitious dataset to illustrate the methodology and terminology used in CART analysis by attempting to classify fish species 1 and 2 using two attributes: $L_{\rm T}$ and opercular ventilation rate (ventilation min⁻¹). The data table for this problem is shown in Table 15, with information from 10 individuals of each species.

Fish species	Opercular rate	$L_{\mathrm{T}}\left(\mathrm{cm}\right)$		
Fish1	8	5		
Fish1	7	5		
Fish1	6	3		
Fish1	3	4		
Fish1	8	7		
Fish1	7	5		
Fish1	5	5		
Fish1	7	3		
Fish1	6	6		
Fish1	7	5		
Fish2	2	6		
Fish2	5	7		
Fish2	4	8		
Fish2	5	6		
Fish2	3	6		
Fish2	4	7		
Fish2	3	7		
Fish2	5	9		
Fish2	3	7		
Fish2	2	8		

Table 15: Data table for the first CART examp

Analyzing the data using CART results in the structure displayed in Figure 63. The first box in this figure, labelled 'Root Node', represents the undivided data at the top – with the Fish1 and Fish2 frequencies shown as histograms within the box. The CART algorithm searches all the independent variables for the one variable that provides the best separation of the cases of the two groups into their respective classes (Barrett 2005d).



Figure 63: CART analysis for the data in Table 15, classifying fish species on the basis of the opercular rate and L_{T} . Fish1 is shown as a solid black bar, and for Fish2 as a dotted bar.

For this example the CART algorithm found 'opercular rate' to be the variable that best separates the two species. As there are only quantitative variables in this example, all splits can be made at that value which maximally separates the two species. For the first split the algorithm splits at a value of 5.5 ventilations min⁻¹ (Figure 63). This means that above this value, cases are classified as Fish1 members, below this value, they are classed as Fish2 members. Figure 63 shows that eight cases are classed as Fish1, and 12 as Fish2. The decision tree produces what are called nodes – each node 'branching' from the root node at the top. The eight-case Fish1 node is also referred to as a terminal node, which in this particular example means no more classified into one class within the node. Indeed eight Fish1 cases have been correctly classified by the decision statement. However, the other node includes two Fish1 cases which have been incorrectly classified by this single decision as Fish2 cases,

along with the 10 'actual' Fish2 cases. The algorithm searches for the best variable which will accurately discriminate or 'split' the cases at this node. The algorithm finds that scoring <= 5.5 cm on the variable 'fish length' discriminates perfectly between Fish1 and Fish2 at this node. Two more nodes are created which contain the two Fish1 cases and the 10 Fish2 cases. These nodes are also referred to as terminal nodes because no more classification is possible here. However, a node may be 'terminal' even when cases from two classes are required to be split within the node, but where a constraint on how 'deep' the tree may extend has forced termination of the tree at that node. The classification accuracy of this simple tree is 100%. That is, from the two decisions made, all cases can be accurately classified into Fish1 and Fish2. The final step in decision tree analysis is to create the decision statement-block which forms the basis for future case allocation. For the current tree, the decision block constituting the classifier profile is outlined in Table 16.

If opercular rate > 5.5 then	#1 Decision
Membership Class = Fish1	
Else if $L_{\rm T} \leq 5.5$ then	#2 Decision
Membership Class = Fish1	
Else	
Membership Class = Fish2	

Table 16: Decision blocks constituting the classifier profile for example 1.

ii Determining tree size: pruning

Whilst the first example helps to explain the basic terminology and logic of a CART analysis, actual field studies often employ larger datasets with more variables, resulting in more complex CART trees. To illustrate how to this a second sample dataset contains 'fish species' as an outcome variable with 24 independent variables (variable names A-X), each with a value between 0 - 100. Before each analysis, certain decisions regarding how to construct the tree have to be selected. In particular, decisions on how to select the optimal variable upon which to make a split and form a node, and when to stop splitting or growing the tree have to be made. For example, a splitting method was chosen based upon finding optimal values on a single variable that best discriminates between the species. A way to control splitting is to use the FACT style stopping method in which the desired minimum fraction can be specified as the Fraction of Objects (FOB). This technique allows splitting to continue until all terminal nodes are pure or contain no more cases than a specified minimum FOB of one or more classes. A FOB with of 0.02 was applied which indicates that if fewer than 2% of the FOB of

the observations are in one or the other species at a node, then no further splits will take place from that node. The resultant tree is shown in Figure 64. The discriminant based univariate splits algorithm works by computing the *p*-levels for ANOVAs of the relationship of the classes to the values of the ordered predictor that are present at the node. The predictor variable producing that smallest *p*-level is chosen to split at the corresponding node. The resulting tree (Figure 64) possesses 48 splits and 49 nodes.



Figure 64: CART analysis for classifying two species of fish (Fish1 and Fish2) on the basis of 24 variables. A 2% FOB stopping rule is employed, with a univariate optimal split algorithm criterion. Number of splits = 48; Number of terminal nodes = 49.

The early partitions near the root node have a high chance to reflect the relationships that actually exist in the data set ,however, as the tree grows the precision of each split diminishes (Rejwan 1999). This mainly occurs due to two causes. Firstly, the data set may contain noise, meaning that although one could classify all individuals nothing is learnt about the patterns that distinguish species. Secondly, the example data set may not be a good representation of the two species. In either of these cases, the terminal branches of the tree are considered too imprecise to be generaliseable beyond the data set (Rejwan 1999). The most commonly used

approach to estimate what part of the regression tree quantifies the more general principles is to stop generating new splits when subsequent splits only result in very little overall improvement of the prediction (Clark and Pregibon 1992). For example, if an analysis predicts 85% of all cases correctly from 10 splits, and 86% of all cases from eleven splits, then it obviously makes little sense to add that eleventh split to the tree. Generally, crossvalidation methods are employed to determine the likelihood of capitalisation on chance of a tree solution calculated on just a single sample of data. Cross validation refers to the process of assessing the predictive accuracy of a model in a test sample relative to its predictive accuracy in the learning sample from which the model was developed.

iii Determining tree size: cross-validation

Three cross-validation methods are most commonly used, namely 1) holdout sample validation, 2) V-fold cross-validation, and 3) global stratified cross-validation.

1) Holdout Sample Validation

Here the data set gets partitioned into one or more 'training' and 'holdout' samples. The training sample is used to construct the classifier function and the holdout sample is used to determine whether the classifier functions as expected on a new set of data (Barrett 2005d).

2) V-fold Cross-Validation

This technique is usually used when the data set is too small to have a holdout sample. The V-fold cross-validation re-samples the data from the total data set, taking each time V sub-samples, all of equal size. The classifier function is computed V times, each time leaving out one of the sub samples and using the sub sample-as a hold out sample. This means that each sub sample is used V - 1 times in the training sample and just once as the holdout sample (Barrett 2005d);

3) Global/Stratified Cross-Validation

This method simply maximises the size of the training sample relative to the holdout sample, but in turn increases the number of folds, V, to compensate for the smaller holdout samples. For example, if a 10 fold cross-validation was employed, nine tenths of the data would be used each time to construct the classifier and one tenth of the data would be used as a holdout sample. This method has been recommended for use with V = 10 whenever insufficient data exists for a large holdout sample (Witten and Frank 1999). If a V-fold cross-validation was employed with V = 3 folds on the example dataset from above, the result is a classification accuracy of 95%, which is an excellent characterisation of the pattern of variables that differentiate species. However, given the large number of splits the classification is probably not useful in differentiating at the species level, and this is confirmed by the use of a more sensitive cross-validation method – the global/stratified cross-validation procedure. With V =10 folds it becomes evident that this tree is actually more likely to possess just 48%classification accuracy on new cases, with balanced false positive and false negative rates of 52%. In short it is important to 'prune' the tree to avoid 'over-fitting' of the data. A model 'overfits' when the good fit of the training data is not replicated when the model is applied to a different sample. The idea of pruning is that one may sacrifice some of the correctly classified patterns in the training data for a more generaliseable tree, in turn making interpretation easier. In general, if not stopped, the tree algorithm will ultimately 'extract' all information from the data leading to noise or random variation. This can be avoided by changing the FOB to 0.25, which states that if fewer than 25% of the observations are in one or the other class at a node, then no further splits will take place from that node. With such a qualifier in place the resultant tree is shown in Figure 65.



Figure 65: A 0.25 (25%) FOB stopping rule is employed, with a univariate optimal split algorithm criterion. Fish 1 is represented by a dark line and fish 2 by a dotted line.

This tree has far fewer splits than that shown in Figure 64. The V-fold (= 3) cross-validation classification accuracy is reduced to 62% (from 95%), whilst the Global V-fold (= 10) classification accuracy is 55% (up from 48%).

iv CART decision block

Table 17 shows the decision block for the final CART tree (Chapter 5).

If Height ACD <=117.16 cm then	Decision #1		
If Height ADC <= 76.521cm then	Decision #2		
If Pool Volume <=2394E2 cm ³ then	Decision #3		
$Fish = B. \ lesleyae$			
Else			
Fish = B. medius			
Endif			
Else			
If Height ACD <=86.448 cm then	Decision #4		
$Fish = B. \ lesleyae$			
Else			
Fish = B. medius			
Endif			
Endif			
Else			
If Surface Area <=9202.7 cm ² then	Decision #5		
If Height ACD <= 182.99 cm then	Decision #6		
If Algae <=56.422 % then	Decision # 7		
$Fish = B. \ lesleyae$			
Else			
Fish = B. medius			
Endif			
Else			
Fish = B. medius			
Endif			
Else			
Fish = B. medius			
Endif			
Endif			

Table 17: Decision block constituting the classifier for the final tree with a FOB of 0.09.

III. APPENDIX (Chapter 7)

i Nest microhabitats of nine triplefin species

Table 18 shows the nest microhabitat characteristics of nine triplefin species and the hybrid triplefin (Chapter 6).

Table 18: Table showing the range and mean \pm SE of the microhabitat (15 x 15 cm) variables of nesting *F. flavonigrum* (Ff, n=16), *F. lapillum* (Fl, n=162), *F. malcolmi* (Fm, n=32), *F. varium* (Fv, n=81), *G. capito* (Gc, n=38), *G. nigripenne* (Gn, n=42), the hybrid triplefin (n=3), *O. maryannae* (Om, n=24), *R. decemdigitatus* (Rd, n=12)) and *R. whero* (Rw, n=56). Depth shown in m, exposure (fetch) in km and all other variables are shown as percentage coverage of the nest microhabitat area.

Habitat	Ff	Fl	Fm	Fv	Gc	Gn	Hybrid	Om	Rd	Rw
STB	87.5	28	97	97	7	7		4	-	4
UCS	12.5	72	3	3	93	93	100	96	100	96
Rock	80-100 95.6 (±1.6)	0-100 73.8 (±2.5)	80-100 97.8 (±0.9)	0-100 84.3 (±3.7)	20-100 68.7 (±3.8)	30-100 63.8 (±2.7)	60-80 70 (±5.8)	50-100 90 (±3)	50-100 74.2 (±5.8)	0-100 71.3 (±3.1)
Cobble	-	0-60 6.2 (±1)	-	0-20 0.3 (±0.2)	0-30 3.9 (±1.6)	-	-	0-50 3.3 (±2.2)	0-50 4.2 (±4.2)	0-50 6.3 (±2)
Gravel	-	0-100 8.1 (±1.7)	0-10 0.9 (±0.5)	0-60 1.7 (±1)	0-10 0.3 (±0.3)	-	-	0-40 2.5 (±1.8)	0-40 3.3 (±3.3)	0-50 6.7 (±1.7)
Sand	0-20 4.4 (±1.6)	0-80 6.6 (±1)	0-20 1.6 (±0.8)	0-50 1.6 (±0.8)	0-10 1.3 (±0.4)	-	-	0-20 3.3 (±1.3)	0-40 10.4 (±3.3)	0-50 9.2 (±1.9)
Mud	-	0-50 3.9 (±0.9)	-	0-20 1.5 (±0.6)	0-50 25.8 (±3.1)	0-70 36.2 (±2.7)	20-40 30 (±5.8)	-	0-30 7.9 (±3.1)	0-40 3.5 (±1)
СТА	0-70 16.9 (±6.2)	0-20 0.7 (±0.3)	0-40 2.7 (±1.5)	0-20 3.3 (±0.6)	-	0-10 0.36 (±0.3)	-	0-20 8.3 (±1.9)	0-10 0.8 (±0.8)	0-60 3.2 (1.2)
MAC	-	0-80 3.8 (±0.9)	0-20 0.9 (±0.7)	0-50 6.0 (±1.4)	0-40 9.5 (±2.8)	-	-	-	0-20 3.3 (±2.2)	0-50 3.6 (±1.4)
Depth	9-21 15.9 (±1)	0.2-16 4.1 (±0.3)	5-23 12.9 (±0.9)	1.8-19 7.9 (±0.4)	0.2-4 2.7 (±0.2)	0.2-1 0.7 (±0.03)	0.5-4 2.9 (±0.9)	4.5-10 5.2 (±0.3)	1.2-3 2.2 (±0.2)	1-27 6.3 (±0.8)
Fetch	0.9-35 29 (±0.4)	0.1-35 32 (±0.6)	0.5-35 11 (±0.4)	0.6-49 20 (±0.2)	0.5-2 0.9 (±0.02)	0.1-0.3 0.3 (±0.01)	0.4-0.5 0.3 (0.01)	0.02-22 15 (±15)	0.1-2 0.5 (±753)	0.2-13 5.5 (±549)

ii Pictures of triplefin nests

The following pictures show the nest habitats of seven triplefin species, namely *F. lapillum* (Figure 66), *F. malcolmi* (Figure 67), *F. varium* (Figure 68), *G nigripenne* (Figure 69), *G. capito* (Figure 70), *O. maryannae* (Figure 71), and *R. whero* (Figure 72).



Figure 66: Nest of *F. lapillum*. The make is in black spawning colouration. Freshly laid eggs have no colouration. After a few days the eggs assume a yellow-pink colouration. Nests of *F. lapillum* are commonly found under (UCS) and on the side of rocks (STB).



Figure 67: Nest of *F. malcolmi*. The red circle indicates the rock area to which the eggs are attached to. The egg colour is yellow. Nest of this species are often fairly visible to divers as they are typically positioned on the side and top of large rocks (STB).



Figure 68: Nest of *F. varium*. The male is in black spawning colouration. The flat rock surface is a typical spawning substrate for this species (similar to *F. malcolmi*, STB). The egg colour of *F. malcolmi* is white during the first days but then turn yellow with time. The eggs in this picture (in the red circle) are only a few minutes old and thus white.



Figure 69: Nest of *G. nigripenne*. Nests of this species were always found on the underside of boulders in otherwise muddy habitats. Egg colour is pink and clutches are large.


Figure 70: Nest of *G. capito*. The male is in black spawning colouration. The different egg colour shows the different developmental stages of the two egg clutches. The colour of eggs in this species ranges is initially yellow but the colour turns more orange-pink with time.



Figure 71: Nest of *O. maryannae*. The red egg colouration is unique to this species. Nests can be found on the underside of boulders (UCS).



Figure 72: Nest of *R. whero*. Only *R. whero* and *R. decemdigitatus* eggs have white filaments (see section iv for more details).

iii Details of the homospecific spawning trials

Table 19 shows the details of the homospecific spawning trials for *R. decemdigitatus*, *R. whero*, *F. varium*, and *G. capito*.

Table 19: Table showing the details of the homospecific spawning trials for (i) R. *decemdigitatus* and R. *whero* and (ii) F. *varium* and G. *capito*. Number of hatched clutches are shown as 'n'. Embryo development was classified as: 1) pre-neurulation, 2) postneurulation, 3) presence of eyes, 4) presence of heartbeat; 5) fully pigmented eyes and 6) visible blood circulation around the heart. Embryological development was checked every 24 - 48 hours, until hatching, using a stereomicroscope.

Species	R. decemdigitatus (n = 14)		<i>R. whero</i> (n = 11)		<i>F. varium</i> (n = 9)		<i>G. capito</i> (n = 10)	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Re-spawn duration	17.83	2.44	13.5	1.77	12.11	2.44	10.11	2.7
Embryo development	17.07	0.31	15.57	0.81	12.4	1.67	12.21	1.3
Started breeding	25/6/03	-	12/7/03	-	12/8/04	-	14/8/04	-
Ended breeding	26/8/03	-	24/10/04	-	20/9/04	-	20/9/04	-
Pre-neurulation	1-3	-	1-3	-	-	-	-	-
Post-neurulation	4-5	-	4-5	-	-	-	-	-
Presence of proto-eyes	6	-	6-7	-	-	-	-	-
Presence of heartbeat	7-8	-	8-11	-	-	-	-	-
Fully pigmented eyes	9-10	-	12-13	-	-	-	-	-
Blood circulation	>11	-	>14	-	-	-	-	-
Mean nest area (cm ²)	24.67	7.16	13.79	4.98	50.4	6.8	24.5	4.5

iv Pictures of the embryo development of R. whero and R. decemdigitatus

The following photographs show the embryo development of *R. whero* and *R. decemdigitatus* (Figure 73).



Figure 73: Developmental stages of (a) *R. decemdigitatus* eggs and (b) *R. whero* eggs. Eggs of both *Ruanoho* species were about 1 mm in diameter and were covered with white, long sticky filaments that are presumably used for adhesion to the nest substrate.

a)

b)