RESEARCH ARTICLE

Reproductive isolation in temperate reef fishes

Maren Wellenreuther · Kendall D. Clements

Abstract Opportunity and recognition isolation can lead directly to reproductive isolation, the former via divergence in the location and timing of breeding, and the latter via differential mate preferences. We describe the potential significance of these factors in the maintenance of reproductive isolation in a clade of triplefin fishes that occur sympatrically around coastal New Zealand. Specifically, we investigate the roles of spawning time and nesting habitat in promoting opportunity isolation, and of interspecific variation in male body length and breeding colouration in promoting recognition isolation. The triplefin species investigated are reproductively active over several months and show high overlap in breeding times, thus rejecting temporal isolation as a mechanism. Differences in nesting habitats resulted in a reduced probability of encounter between some species, especially between sister-species pairs. Interspecific colour differences generally decreased during the reproductive period, and males of sister-species pairs showed no interspecific colour differences in the ultraviolet light spectrum, thus mate selection based on male colour patterns is unlikely to lead to premating isolation. Finally, males of closely related triplefin species differed in body length, a secondary sexual trait often involved in assortative mating. Thus, spatial differences in nesting habitats reduce the chances of encountering allospecific mates, which may facilitate opportunity isolation and differences in male length, possibly related to species-specific female selection on male body size, may lead to recognition isolation. The combination of limited spatial overlap in nesting habitat and differences in male body size may facilitate species assortative mating in sympatry or parapatry.

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Introduction

Reproductive isolation can occur when populations have unequal probabilities of encountering one another during the breeding season (opportunity isolation) or when they differ in mate preferences (recognition isolation, Palumbi 1998). Opportunity isolation occurs when populations breed at different times (temporal isolation), thereby reducing the frequency of encounters between individuals with different temporal breeding preferences (Dobzhansky 1937; Mayr 1970). Opportunity isolation also occurs when individuals have decreased opportunities to mate because they are separated by a geographic barrier, or have different preferences for breeding habitat (spatial isolation, Dobzhansky 1937; Mayr 1970). Several studies on fishes have demonstrated that both spatial (Quinn et al. 2000; Hendry et al. 2004; Munday et al. 2004) and temporal divergence (Palstra et al. 2004; Bekkevold et al. 2005; Hendry and Troy 2005) in breeding characteristics can lead to opportunity isolation.

Recognition isolation occurs when populations display divergent preferences for secondary sexual traits (Palumbi 1998), so that individuals from different populations no longer recognise each other as potential mates, even when they co-occur in a similar breeding habitat at the same time. In fishes, a wide variety of traits has been linked with recognition isolation and include preferences for acoustic (Ryan 2001) and chemical signals (Zimmer and Butman 2000), body size (Nagel and Schluter 1998) and colour patterns (Seehausen and van Alphen 1998). One of the traits that has received considerable recent attention is male body size, and

several studies have demonstrated that male body size is important in mate selection and thus can evolve under sexual selection (e.g. McKaye 1986; Lindström 1992; Nagel and Schluter 1998; Schliewen et al. 2001; McKinnon et al. 2004). Another well-studied mate choice trait is female preference for male body colouration in both the visible (Seehausen and van Alphen 1998; Allender et al. 2003) and ultraviolet (UV) light spectra (Smith et al. 2002; Jordan et al. 2004). In these cases, males of sister-species are generally highly divergent in colour pattern (Seehausen and van Alphen 1998), and selection on male colour pattern has been implicated in the divergence of some Mediterranean triplefin species (Wirtz 1978; De Jonge and Videler 1989; Geertjes and Videler 2002) and rift lake cichlids (e.g. Fryer and Iles 1972; Greenwood 1974; Allender et al. 2003; Seehausen 2006).

This paper aims to assess the potential significance of opportunity and recognition isolation in the maintenance of reproductive isolation in New Zealand triplefin fishes (Tripterygiidae). The New Zealand triplefin fauna consists of 26 endemic species, and thus represents the greatest diversity of triplefin species anywhere in the world (Clements 2003). At least 20 of these species form a monophyletic group and represent a local radiation (Clements et al. unpublished.) Most New Zealand triplefin species display overlapping, sympatric distributions and are found all around New Zealand's coastline (Paulin and Roberts 1992; Fricke 1994; Francis 2001; Clements 2003). Despite their broad and sympatric distributions, New Zealand triplefin fishes have diversified considerably in habitat use (Syms 1995; Feary and Clements 2006; Wellenreuther et al. 2007a), and selection of alternative habitat types has been invoked as a key mechanism to explain this divergence (Wellenreuther et al. 2007a). Unlike the marked partitioning along the habitat axis, there is little evidence for specialisation in jaw morphology or choice of diet (Feary 2001). This is in contrast to many freshwater fish radiations (e.g. Fryer and Iles 1972; Greenwood 1974; Lu and Bernatchez 1999; Streelmann and Danley 2003; Seehausen 2006), where trophic diversification has been implicated as one of the main components in the divergence between closely related species.

Although triplefins are easy to observe in the wild and maintain in the laboratory, little is known about the contribution of reproductive barriers to the maintenance of species boundaries in this group. Feary and Clements (2006) studied habitat associations in the New Zealand triplefin fauna, and briefly addressed the locations of nesting sites in this group. This paper expands on the work of Feary and Clements (2006) by including more species and habitat parameters in the description of nesting sites and reproductive behaviours, and describes the spawning time of these triplefin species. Furthermore, this paper is the first to explore the possibility of recognition isolation due to divergence in male body size and colouration in this group.

Materials and methods

Study species

Reproduction in New Zealand triplefin species takes place during the austral winter (Hickford and Schiel 2003) and is characterised by a fixed nest site that is selected and guarded by the male (Ruck 1973; Thompson 1986). Nests are typically built on different types of hard substrata (e.g. rocks), and females may choose several mates during the breeding season (Thompson 1986). Males care for the eggs until hatching, which generally takes between 12 and 20 days (Ruck 1973, 1980). Nest care includes defence against predators and the fanning of eggs for oxygenation and to prevent silt from accumulating (Thompson 1979). Triplefins are highly philopatric and occupy territories of 1–2 m^2 for their entire life (Thompson 1979), and the nest area represents a subset of this habitat (<0.25 m^2, M. Wellenreuther, unpublished data). During the reproductive season, particularly when guarding nests and during courtship, males adopt distinctive spawning colours and can be easily distinguished from females (Ayling and Cox 1987; Paulin and Roberts 1992; Francis 2001; Clements 2003). Hybrid crosses have only been found between *Forsterygion varium* and *Grahamina capito* (Hickey 2004), suggesting that prezygotic barriers to reproduction prevent hybridisation in the remaining species.

Data collection

Details of nesting and non-nesting triplefin habitat use were recorded over three consecutive spawning seasons from January 2002 to May 2005 in the Hauraki Gulf (36°36′S, 174°50′E) in northeastern New Zealand (Fig. 1) using underwater visual counts (UVC). The Hauraki Gulf area was chosen as a study site as it is a large area with a wide variety of accessible habitats, enabling the quantification of
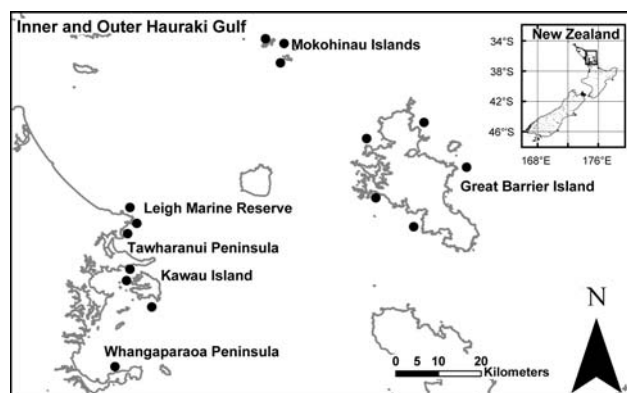
Fig. 1 Map of study sites in the Inner and Outer Hauraki Gulf in north-eastern New Zealand. Study sites are marked with a *black circle*

the breeding characteristics of a number of triplefin species from a diverse range of exposures and depths at coastal and offshore sites. At least three UVC (area sampled for each UVC 4 × 4 m) were conducted at each site (total number of sites 33, UVC 155), with the first UVC sampled at the deepest depth that could be safely reached (maximum depth dived 26 m), and the two subsequent UVC sampled at approximately 33 and 66% of the deepest depth. Any additional UVC were conducted in intermediate depths. This sampling design allowed flexibility across a wide variety of habitat types. 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 handheld Garmin® 12 global positioning system and from this information a physically derived exposure index could be estimated. The exposure index was calculated based on the total sum of fetch (radial distance 300 km) for each 20° sector on a compass rose using the program 'Fetch Effect Analysis' (Pickard 2000). The centre line of each UVC was marked with a leaded line, and a steel quadrat was used to outline each 1 m² within the UVC area. All transects were done by the same diver and consisted of a close and systematic searching pattern, spending at least 1 min on each 1 m², with all interstices and overhangs examined to ensure a complete census.

Nests were defined by the presence of eggs and a guarding male. For each nest the date, depth (m), exposure (km), on- and offshore location, degree of shelter (sheltered or exposed nest), substratum variables and nest microposition were recorded. The substratum variables were quantified by measuring the substratum coverage (rock [rocks > 7 cm], cobbles [rocks < 7 cm], gravel [rocks < 4 cm], sand, and mud) and algal coverage (*Ecklonia radiata*, *Carpophyllum* spp. and coralline and turfing algae) for each nest (15 × 15 cm area from around the centre of the eggs) and territory (1 × 1 m area from around the centre of the eggs). While the first five substratum variables always sum to 100%, the algal coverage could range from 0 to 100%. Lastly, the identity and total length (L_T [cm]) of the nesting male was visually estimated and the nest microposition was recorded. The nest micropositions included the 'top of rocks', 'side of rocks', 'under hard substratum', and in 'crack in vertical rock'. Micropositions were defined as the substratum upon which >50% of a fish's body rested (Feary and Clements 2006).

Whenever possible, male and female breeding behaviour was observed in the field and males were photographed in the field to quantify male body colouration during the reproductive season. Male breeding colouration was quantified by measuring the intensity of colouration from photographs of nesting and non-nesting individuals. No photographs of *Obliquichthys maryannae* in spawning colouration could be obtained, thus this species had to be excluded from the analysis. This is because unlike other species *O. maryannae* does not guard the demersal eggs (M. Wellenreuther, personal observation), presumably because of its semi-pelagic lifestyle. All photographs were taken in the natural environment of the species apart from a few aquarium shots of breeding *G. capito*, as the low water clarity in the habitat of this species made the use of an underwater camera difficult (for details on the habitat use of this species see Wellenreuther et al. 2007a).

Data analysis

Data analysis was conducted on all species for which more than ten nests could be identified, namely *F. flavonigrum* (n = 16), *F. lapillum* (n = 162), *F. varium* (n = 32), *F. malcolmi* (n = 81), *G. capito* (n = 31), *G. nigripenne* (n = 42), *O. maryannae* (n = 24), *Ruanoho whero* (n = 56) and *R. decemdigitatus* (n = 12). It should be noted that the genus *Obliquichthys* and the polyphyletic genus *Grahamina* are nested within *Forsterygion* (Hickey and Clements 2005).

First, temporal and spatial variation in triplefin breeding habitat was assessed to investigate the role of these factors in promoting opportunity isolation in this group. Temporal variation was assessed by comparing the length and timing of the reproductive season of triplefin species to calculate overlap during the breeding season. The reproductive season of a species was determined by the presence of nests. Spatial overlap between species in breeding habitat was measured by comparing the continuous (depth, exposure, substratum variables of the nest and territory) 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).

Second, body size and male body colouration were investigated to assess whether species have diverged in traits that are known to be important in sexual selection in

other fish species and thus may promote recognition isolation in this group. Interspecific differences in nesting male body length (as estimated from the UVC) were investigated using the non-parametric Kruskal–Wallis test in Statistica (version 7.1). To examine which species differed significantly from other species post-hoc tests for multiple comparisons were conducted. Male spawning colouration was 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). All photographs were converted into binary 8-bit grey scale images (grey scale range 0–256). The intensity of colouration was measured using a rectangular shape that was approximately the size of the eyes of the individual (the shape was created with the ROI plug-in tool), and measured along a transect from the area behind the eyes along the lateral line to the caudal peduncle. Colour intensity was measured at ten 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 using independent *t* tests in Statistica (version 7.1).

In addition, differences in UV colour patterning were photographically documented for the males of the sister-species pairs identified in Hickey and Clements (2005). Individuals that were presumed to be 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 killed 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 a 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 known to have underlying UV colour patterns.

Results

Spawning times

The spawning periods of all nine-triplefin species examined in this study showed considerable overlap (Table 1). The majority of species started spawning around April–June and spawned until September–November, with a peak in spawning activity around August (Table 1). *Forsterygion lapillum* was the only species that remained reproductively active almost all year round (all months except March, Table 1). Long spawning periods were also recorded for *F. varium* (8 months), *R. decemdigitatus* (6 months) and *R. whero* (7 months), and moderately long spawning periods for *F. flavonigrum* and *F. malcolmi* (5 months), and *O. maryannae*, *G. capito* and *G. nigripenne* (4 months) (Table 1).

Spawning habitats

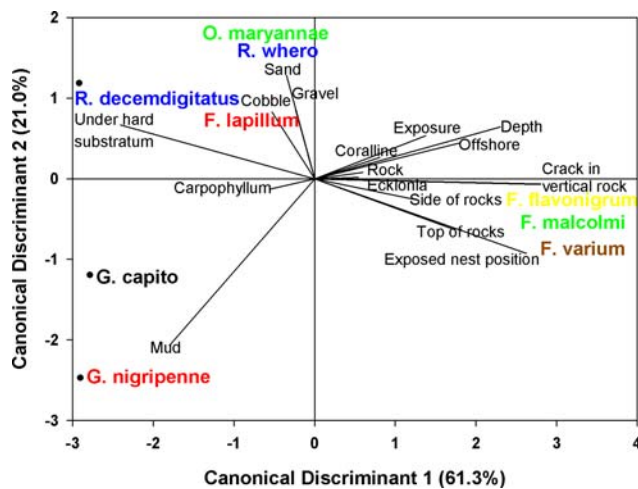All spawning males were found to occupy distinct nest habitats that were defended vigorously against all intra- and

Table 1 Spawning periods of triplefin species in New Zealand from the Inner and Outer Hauraki Gulf area

Species	Jan	Feb	Mar	Apr	May	Jun	Jul	Aug	Sept	Oct	Nov	Dec
Fosterygion flavonigrum												
Fosterygion lapillum[1]												
Fosterygion malcolmi[2]												
Fosterygion varium												
Grahamina capito												
Grahamina nigripenne[1]												
Obliquichthys maryannae[2]												
Ruanoho decemdigitatus[3]												
Ruanoho whero[3]												

The presence of nests was used as evidence for reproductive activity. Nesting intensity is represented by light grey (0–25% of all observed nests), grey (26–50%), dark grey (51–75%) and black (76–100%). No shading indicates no nests were observed for the month. Sister-species pairs are denoted with superscript numbers

interspecific intruders. No species showed evidence for opportunistic mating behaviour or reproductive brood parasitism. CDA showed considerable interspecific differences in nesting habitat. The relatively shallow and sheltered nest sites of *F. lapillum*, *G. nigripenne*, *G. capito*, *O. maryannae*, *R. decemdigitatus* and *R. whero* reduced overlap with the remaining three species studied (Fig. 2). These latter species, i.e. *F. flavonigrum*, *F. malcolmi* and *F. varium*, were generally associated with more exposed and deeper habitats (Fig. 2). 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 (Fig. 2). The majority of species built nests in microhabitats that provided high amounts of structural cover (i.e. under rocks) (Fig. 2). 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* (Fig. 2). The microposition in 'crack in vertical rock' was solely occupied by *F. flavonigrum* (Fig. 2). 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' (Fig. 2).

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*
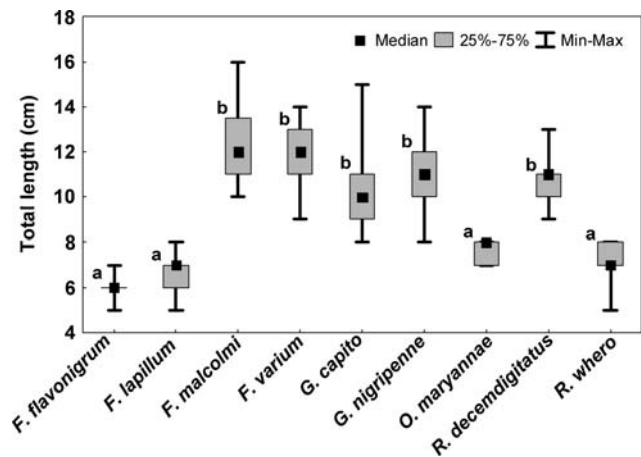
displayed no overlap in nesting habitats. The sister-species pair *F. lapillum* and *G. nigripenne* was also almost completely separated in nesting habitat, with *G. nigripenne* nests displaying no overlap and *F. lapillum* nests only minimal overlap, with 7% of all nests being misclassified as *G. nigripenne* 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%).

Male body length

The size of nesting males differed greatly between species (Fig. 3), and two statistically distinct species clusters were present (Kruskal–Wallis test H (8, $N = 462$) = 363.13, $P < 0.001$). The first cluster contained *F. flavonigrum*, *F. lapillum*, *O. maryannae* and *R. whero*, with a median nesting male body length of 6–8 cm (Fig. 3). The second cluster contained the species *F. malcolmi*, *F. varium*, *G. capito*, *G. nigripenne* and *R. decemdigitatus*, with nesting male body lengths between 10 and 12 cm (Fig. 3). Importantly, the three sister-species pairs of *R. decemdigitatus* and *R. whero*, *G. nigripenne* and *F. lapillum*, and *F. malcolmi* and *O. maryannae* (Hickey and Clements 2005) were all split across the two clusters.

Male colour patterns

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 ten points measured (Fig. 4). Greatest differences

Fig. 2 Canonical Discriminant Analysis of triplefin species and nesting habitats. Triplefin species are in colour with sister-species pairs sharing the same colour

Fig. 3 Median body lengths of spawning triplefin males of nine species. Species denoted with '*a*' are significantly different from species designated with '*b*'

Fig. 4 Male body colouration of eight triplefin species. 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
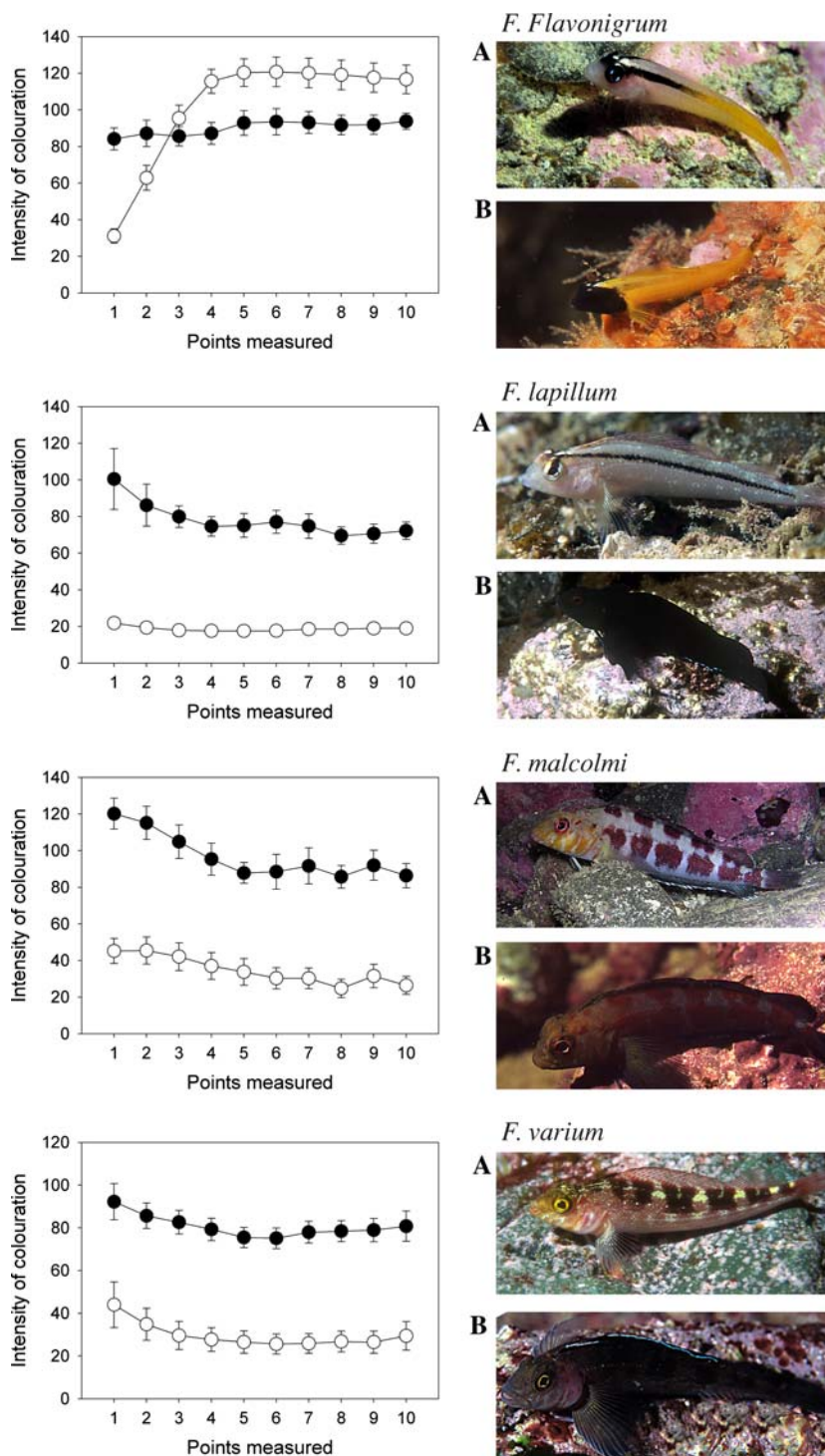
between spawning and non-spawning individuals were seen in *R. decemdigitatus* (mean intensity of colouration spawning 91, non-spawning 18, difference 73) and *F. lapillum* (mean intensity of colouration spawning 90, non-spawning 22, difference 68, Fig. 4). In three species, i.e. *G. capito*, *G. nigripenne* and *R. whero*, the difference between the spawning and non-spawning colouration was moderate (≈50, Fig. 4). 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 4–10 assumed a significantly brighter yellow spawning colouration, (Fig. 4). The aver-

Fig. 4 continued
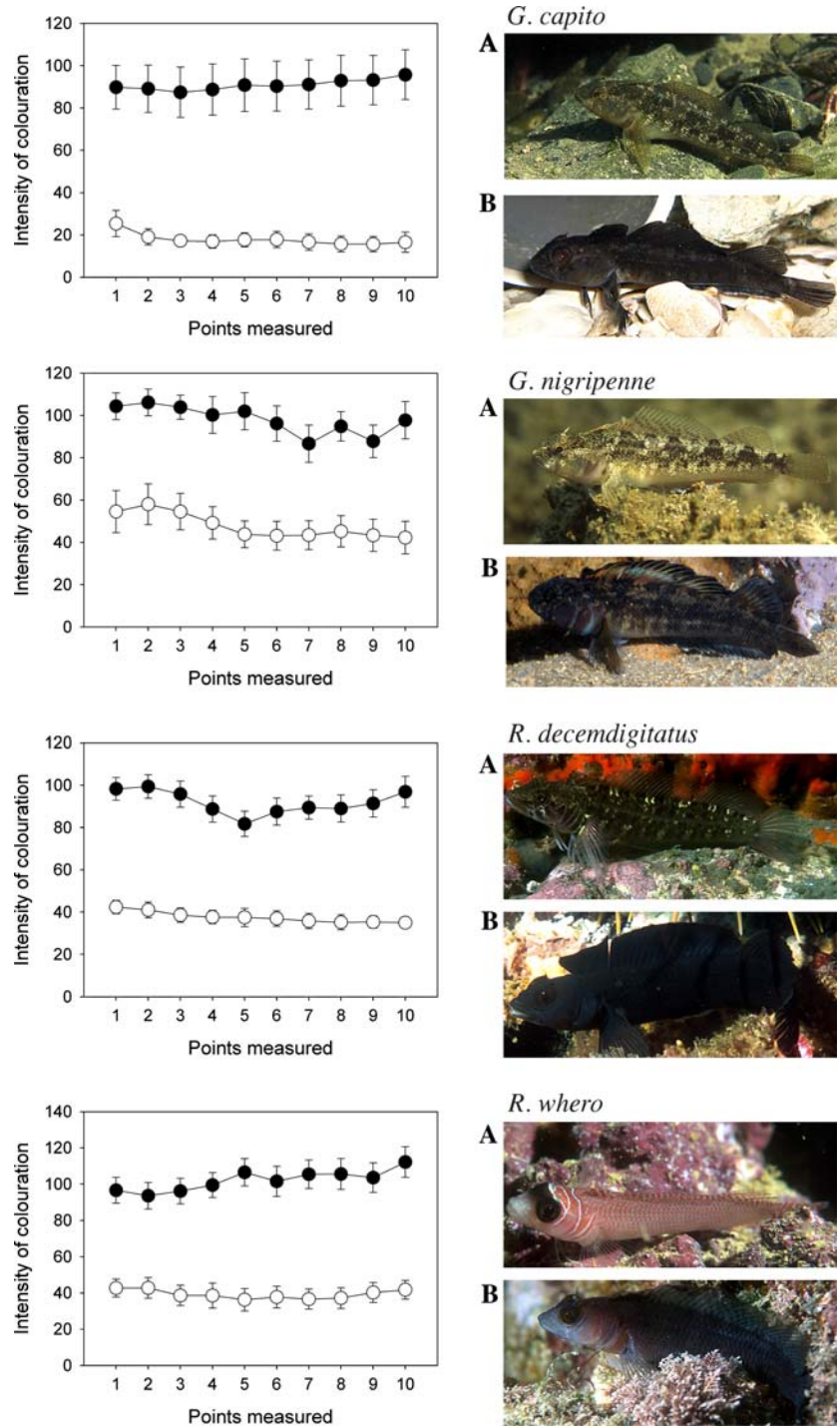
age difference in the intensity of colouration between breeding and non-breeding individuals for *F. flavonigrum* was 12, with the spawning individuals showing a higher intensity in colouration than the non-spawning individuals.

The photographs of the UV colour patterns of the three-triplefin sister-species pairs were not different from photographs taken under the visible light spectrum, indicating that males do not reflect light in the UV bandwidth (photo-graphs taken of *R. whero* and *R. decemdigitatus* can be seen in Fig. 5).

Discussion

The contribution of reproductive barriers to the maintenance of species boundaries is poorly understood in the New Zealand triplefin fauna. The present study is the first to

Fig. 5 Body colouration of **a** *R. decemdigitatus* and **b** *R. whero* under visible and UV light (*blue photographs*)
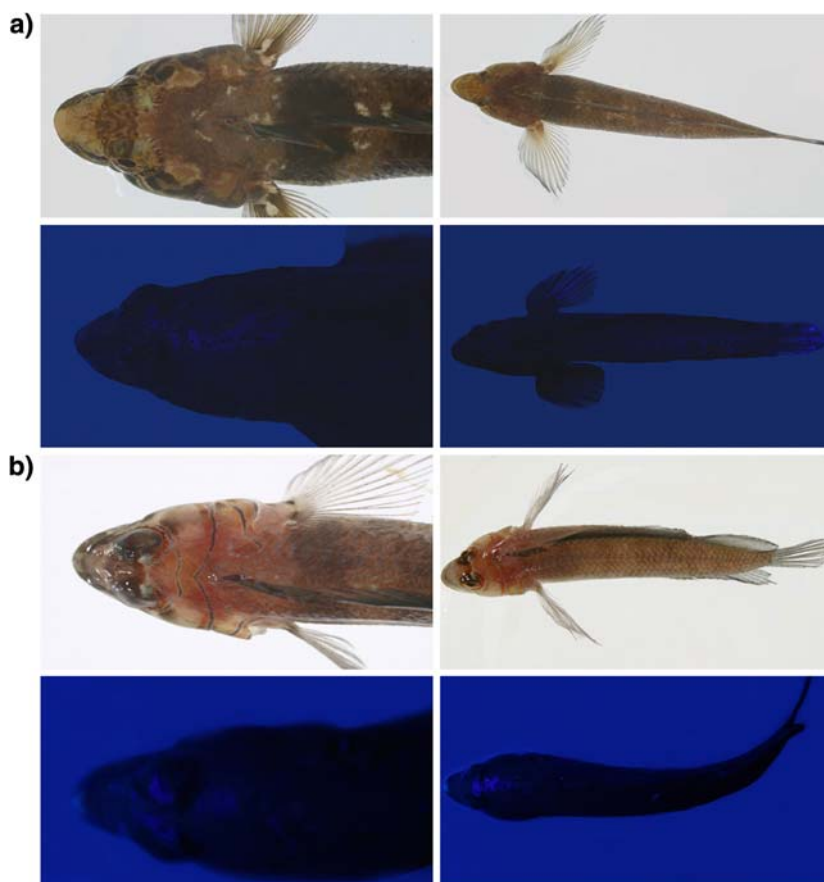
investigate the role of secondary sexual traits in the maintenance of reproductive isolation in this group. In addition, this study expands on previous work on spatial variation in spawning in this group (Feary and Clements 2006), and describes the degree of overlap between species in breeding times and nesting sites. Overall, the study found little evidence for either opportunity isolation through divergence in breeding season (temporal isolation) or recognition isolation through divergence in male body colouration. In contrast, opportunity isolation through diversification of nesting habitat (spatial isolation) was extensive, and recognition isolation could be facilitated by body size divergence between closely related species.

This study found strong evidence for spatial isolation through species-specific breeding habitat characteristics, especially between sister-species pairs. This is in contrast to an earlier study on breeding associations based on microposition characteristics alone, in which triplefin species were reported to show high interspecific overlap in nest sites (Feary and Clements 2006). The different findings indicate that the results and conclusions about the degree of spatial overlap can change dramatically when a wider range of habitat variables (e.g. substratum cover) are incorporated into the analysis. Isolation of breeding habitats was mainly achieved through different preferences for depth and exposure, for the substratum variables rock and mud, and for exposed versus sheltered nest micropositions. The finding that depth, exposure and substratum are the some of the main determinants of nesting habitat in New Zealand triplefin species is consistent with previous work on habitat associations of New Zealand triplefin fishes, and supports the suggestion that interspecific divergence in habitat characteristics may have been a key component in the evolution of this clade (Wellenreuther et al. 2007a). The degree of shelter of nest positions was another main 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 habitats 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 substratum). Similar interspecific differences in the degree of shelter of nesting habitats 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* aggressively defends eggs against predation by 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 have to protect the nest from higher physical exposure (e.g. waves) as well as potential predators. 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 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. whero* 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 (Wellenreuther et al. 2007a). 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. Indeed, a recent study on the mate choice and courtship behaviour of the *Ruanoho* species has shown that the species differ in preferences for male body size and courtship behaviour (Wellenreuther et al. 2007b). 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 species of 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.

In this study, males of all species were highly territorial and defended nest sites vigorously against intruders, and this is consistent with previous observations of male nesting behaviour (Thompson 1986; Paulin and Roberts 1992; Fricke 1994; Feary 2001; Francis 2001; Clements 2003). This uniform behaviour is in strong contrast with the male reproductive behaviour observed in many other triplefin species elsewhere around the world (De Jonge and Videler 1989; Neat 2001; Geertjes and Videler 2002), where different males of the same species can show a variety of reproductive strategies. The most common reproductive strategies observed in non-New Zealand triplefins include (1) males with a nest site and paternal brood care, (2) males with opportunistic mating behaviour, and (3) males with reproductive brood parasitism ('sneaking' behaviour) (e.g. Geertjes and Videler 2002). Sneaking can impose high costs on territorial males, both in terms of reduced reproductive output and caring for unrelated offspring (Alonzo and Warner 1999). Brood parasitism can also cause fitness trade-offs in females (Alonzo and Warner 2000), for example because mate selection based on paternal qualities or male characteristics is not possible. The general absence of sneak spawning behaviour observed in New Zealand triplefin species suggests that male and nest characteristics can be used as reliable indicators on which to base mate choice decisions, thereby helping females to decrease mate choice errors. This is important, because theoretical models predict that the use of highly divergent nest habitats in combination with female choice of nest characteristics would provide a powerful mechanism for speciation in this group, as long as the trait under selection (i.e. habitat choice) is under sufficiently strong disruptive selection and assortative mating is high (Gavrilets 2004). These theoretical models have also received experimental support (see Rice 1985; Rice and Salt 1988, 1990). Indeed, such a linkage between habitat choice and mate choice has been suggested to be highly conducive for speciation in sympatry and parapatry (Gavrilets 2004).

The mean body 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* in this study. 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 suggest that morphological

differentiation in body size may be an important component in the divergence of this clade. The differences in body size between the two *Ruanoho* species might explain why these species do not hybridise, despite extensive overlap in breeding habitats. Strong support for the role of body size as an important secondary sexual trait in New Zealand triplefin species comes from a study on the *Ruanoho* sister-species pair (Wellenreuther et al. 2007b). Female *R. whero* show a preference for smaller males, thereby minimising the chance of hybridisation with the larger *R. decemdigitatus* (Wellenreuther et al. 2007b). The tendency for assortative mating based on body size is known in 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), and has also been well documented in other taxa (e.g. Ratcliffe and Grant 1983).

The results of the current study show that although some triplefin species co-occur in the same nesting habitat and have a similar body size, they do not interbreed. Specifically, the two groups of species (i.e. *G. capito* and *G. nigripenne*; and *R. whero*, *F. lapillum* and *O. maryannae*) were each found to occupy similar nesting habitats and were of similar body size. This clearly suggests that there must be other factors that are responsible for the maintenance of reproductive isolation in these two groups of species, such as differences in courtship behaviour. Courtship differences are pronounced in the *Ruanoho* species (Wellenreuther et al. 2007b), but have not been investigated in any other New Zealand triplefin species. Examining the role of behavioural factors in the maintenance of reproductive isolation in these two groups of species would be an interesting future study.

Interspecific overlap of spawning periods was extensive, with most species spawning over several months between May and November. Thus temporal isolation is unlikely as a reproductive barrier. Similarly long spawning periods have also been found in tropical (Longenencker and Langston 2005) and Mediterranean triplefin species (Geertjes and Videler 2002), and thus may be a common characteristic of this family. The spawning periods for *F. flavonigrum*, *F. malcolmi* and *F. varium* were similar to the spawning periods reported by Francis (2001) and Ayling and Cox (1987), while the spawning period for *F. lapillum* and *G. capito* was slightly longer. The almost year-round reproductive activity of *F. lapillum* might explain the year-round presence of *Forsterygion* spp. larvae in ichthyoplankton surveys on the east coast of the South Island in New Zealand (Hickford and Schiel 2003). The spawning period of both *Ruanoho* species was similar, spanning from May/June–November, and is consistent with the spawning times reported previously (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 black spawning colouration seen in males of seven of the eight-triplefin species examined suggests that a darkening of the male body during the breeding season may be a plesiomorphic trait. The jet-black spawning colouration seen throughout the sister-species pairs *F. lapillum* and *G. nigripenne*, and *R. decemdigitatus* and *R. whero*, suggests that male spawning colouration is unlikely to have been important in female mate selection in these species (the lack of photographs of breeding *O. maryannae* prevented comparisons with its sister-species *F. malcomi*). The absence of UV reflection in the three sister-species pairs further supports this claim. In addition, because all New Zealand triplefin species spawn during the winter months and are substrate spawners light transmission levels would be relatively low, particularly in species (e.g. the estuarine *G. nigripenne*) that inhabit turbid environments (Wellenreuther et al. 2007a). Thus, given that low light levels have been shown to prohibit sexual selection on male nuptial colouration (Seehausen et al. 1997), and given the absence of divergent colour patterns between sister-species, it seems unlikely that sexual selection of male breeding colouration has been a dominant feature in the radiation of this group. Furthermore, theoretical models predict that populations with strong assortative mating based on ecological traits (e.g. habitat) are less likely to evolve a sexual dimorphism, because females cannot simultaneously prefer males more similar to themselves while still allowing the males to diverge (Bolnick and Doebeli 2003). For this reason, it seems unlikely that the sexual dimorphism in reproductive colour patterns in New Zealand triplefin fishes is related to mate choice. The conspicuous jet-black breeding colouration may, however, act as a signalling colour to indicate the position of breeding males and nests (Boughman 2001). Colour signals are most easily detected when they differ from the background noise against which they are emitted (Fuller 2002), hence it is likely that the jet-black body of reproductive triplefin males makes them more conspicuous against the spawning background, as occurs in stickleback fishes (Scott 2001). Stickleback males usually assume a red nuptial colouration, and males in tea-stained lakes assume a black nuptial colouration (Boughman 2001). The short wavelengths attenuate rapidly in these lakes, which creates a 'reddish' photic environment (Sargent et al. 1998), with the result that black males are more conspicuous to females than red males (Boughman 2001). The general absence of divergent breeding colours in many pairs of sister-species sets the New Zealand triplefin radiation apart from Mediterranean triplefin species (Geertjes and Videler 2002) and the radiation of rift lake cichlids (Fryer and Iles 1972; Greenwood 1974; Allender et al. 2003; Seehausen 2006), where sister-species

pairs usually exhibit highly divergent colour patterns (e.g. Seehausen and van Alphen 1998).

Conclusions

The results of this study 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 indication that spatial differences in nest characteristics and divergence in male body size may have facilitated reproductive isolation between species in this group. Specifically, spatial differences in breeding habitat and male body size were pronounced between sister-species, suggesting that divergence in these factors may have been involved in the build-up of reproductive isolation in these pairs. This indicates that habitat use and morphological differentiation in body size may be important components of divergence in this clade. Together, the results suggest that opportunity isolation leads to reduced encounter rates between heterospecifics during the reproductive season, and that recognition isolation based on male body size may facilitate assortative mating in sympatry or parapatry.

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