Behaviour and Morphology of the Zebrafish, *Danio rerio*

Victoria Louise Pritchard

Submitted in accordance with the requirements for the degree of Doctor of Philosophy

The University of Leeds
School of Biology
January 2001

The candidate confirms that all the work submitted is her own and that the appropriate credit has been given where reference has been made to the work of others.

Abstract

Speciation remains a process that is poorly understood. In particular, little progress has been made in elucidating the mechanisms underlying prezygotic isolation. A full understanding requires a knowledge of underlying genetics. To date the only real progress has been made using *Drosophila*: more model organisms are needed.

It is proposed that the zebrafish, *Danio rerio*, a species well suited to the laboratory environment and with much information available about its genetics, has potential in this area. However, currently little is know about mate choice in this species and whether its behaviour and morphology exhibit any genetic variation in the wild that may be amenable to further analysis. This thesis addresses these questions by investigating the behaviour and morphology of zebrafish deriving from several wild populations in Nepal and Bangladesh.

Mate choice trials in the laboratory revealed several features of the zebrafish that make it less amenable to such studies than several alternative teleost species. These include a lack of strong sexual dimorphism and the absence of behaviours that reliably indicate mate choice. Nevertheless observations in the course of this study suggest that male carotenoid coloration, longitudinal melanophore stripes and symmetry of caudal fin pattern may have behavioural roles in this species.

Investigations into the shoaling behaviour of the zebrafish revealed no evidence for association preferences for familiar individuals or kin. The ubiquity of a preference for familiars in shoaling fish may have been over emphasized. This study did, however, find evidence for inter-population variation in grouping behaviour that may provide another avenue for the investigation of the genetics of behavioural adaptations.

Documentation of morphological variation revealed heritable inter-population variability, in particular in body stripe pattern, as well as consistent sexual dimorphisms in body shape and anal fin morphology. Further investigation of the quantitative genetic basis of these traits revealed that several features of body stripe pattern were not heritable under laboratory conditions. This may indicate stabilising selection on body stripe pattern. In contrast, elements of anal fin morphology exhibited more underlying genetic variation.

Contents

Acknowledgements

Many people have contributed their help and advice during the course of this PhD. Firstly I am grateful to my supervisor, Roger Butlin, who had the zebrafish idea in the first place and gave me the opportunity to pursue it. I have particularly appreciated his statistical reassurance and his ability to come up with solutions to whatever problem I might bring to his office. I would also like to thank Jens Krause, in particular for inviting me to contribute to publications.

This work could not have gone ahead without the many people in Nepal and Bangladesh who helped me to collect zebrafish. The trip to Nepal was facilitated by Dr. Bhola H. Pradhan and Ash Kumar Rai. I am grateful to Hari Rama and Yam Limbu for their assistance and in particular to Mrs. Sushila K.C. for her help and hospitality. Lufthansa airlines were very accommodating when I needed to take the fish back to the U.K. In Bangladesh the people at BAFRU including Chris Price, Don Griffiths and Dr. P. K. Barua went out of their way to help me after I turned up on their doorstep (virtually) unannounced. I am especially grateful to Sattar and Samsu for putting up with three weeks of driving around and wading in paddyfields.

Lots of people provided ideas or equipment that helped me cross the numerous hurdles encountered along the way. Kai-Eric Witte's fish breeding advice was nothing short of miraculous, and he was also the source of much information about zebrafish phylogeny and distribution. Stuart McLelland helped me write the data-recording programs and I am grateful to him for much else besides. On the morphometrics front, Professeur Dennis Webb suggested the cold water technique, Paolo Viscardi and Laurence Couldrick suggested the negative scanner, Iain Couzin showed me Image Tool and Rob Wilson let me use his CD re-writer. Many people fed my fish for me while I was jaunting off elsewhere - I would like to thank in particular the CEU staff and especially Stuart Pickersgill who could always be relied on to get ancient pieces of machinery going at short notice. Members of the Butlin research group have frequently provided fruitful discussion and ideas - Tom Tregenza, Richard Bailey and Rosie Sharpe could usually be relied on for entertainment, biological and otherwise.

Production of this thesis is, of course, ultimately due to my parents who brought me up in the countryside with goats and chickens, built me moth cages and sent me on birdwatching holidays, not to mention looking after various animals when I got bored.

Special thanks go to Dan Hoare, without whom I might not have made it. He also drew Figure 2.1.

Abbreviations, acronyms and symbols

δ	minimum detectable difference
χ^2	chi-square
°C	degrees Centigrade
%	percentage
±	plus or minus
ANOVA	Analysis of Variance
B	Blue
cm	centimetres
dpi	dots per inch
Edn.	edition
eds.	editors
F1	first generation hybrid
Fig.	Figure
G	Gold
GLM	General Linear Model
h	hours
km	kilometres
$\log_{10}$	logarithm to base 10
MAFF	Ministry of Agriculture, Fisheries and Food
m	metres
max	maximum
MHC	Major Histocompatibility Complex
ml	millilitres
mm	millimetres
n	number / sample size
n.s.	not significant
p	probability
PC	Principal Component
P stripe	primary stripe
REML	Restricted Maximum Likelihood Method
s	seconds
SLR	Single Lens Reflex
spp.	species
U.K.	United Kingdom
UV	ultraviolet

List of Figures

List of Tables

Chapter 1
Introduction

1.1. The challenge of speciation

Speciation, the origin of new species via the splitting of lineages, is a process that remains poorly understood. Central to the process is the establishment of barriers to gene flow between populations, that is, the evolution of characters that prevent successful interbreeding. These barriers may be 'postzygotic', coming in to play only after fertilization has occurred, for example by reducing the viability of hybrid offspring. Alternatively, they may be 'prezygotic', acting to prevent hybrid fertilization in the first place. Such prezygotic barriers include sperm selection (e.g. Howard et al., 1998), temporal and ecological isolation in mating behaviour (e.g. Schluter, 1998) and divergence in mate choice signal-response systems so that members of the alternative population are no longer recognised as suitable mates. Of these mechanisms, perhaps the latter, often termed 'ethological isolation' has received most attention. The relative importance of pre-and postzygotic isolation to speciation remains under debate, as do the conditions required for each to arise and the relationship between them (e.g. Coyne & Orr, 1989, 1997; Otte & Endler, 1989; Howard & Berlocher, 1998).

Since speciation fundamentally involves barriers to gene flow, it is clear that a full understanding of the process requires, amongst other things, a full understanding of the genetic basis of the mechanisms involved. Some recent advances have been made in documenting the genetic basis of postzygotic isolation (Wu & Palopoli, 1994; Wu et al., 1996). However, genetic analysis of the changes in behaviour or ecology that lead to prezygotic isolation has seen much slower progress. Studies in this area have concentrated on the genetic basis of assortative mating between closely related taxa. Although this can provide insights into how prezygotic isolation may occur, it is often difficult using this approach to distinguish changes that originally generated reproductive barriers from those that have occurred since. In addition, such studies have concentrated almost exclusively on arthropods, with the great majority of them involving *Drosophila* (see Ritchie & Phillips 1998 for a review). There are good reasons why studies on *Drosophila* are so prevalent. Members of this genus are extremely easy to keep in captivity, with a high per-individual reproductive output and short generation. The genus is widespread, with much interspecific variability in mate choice signals. The recent identification of intraspecific assortative mating raises the possibility of study of speciation in progress

(Hollocher et al., 1997). The major reason why analysis of mate choice genes in *Drosophila* has made such good progress is, however, the vast amount of information available about its genetics and the great array of tools available for genetic analysis in this genus (e.g. Carradedo & Casares, 1995).

It is clear that a full understanding of the genetics of prezygotic isolation cannot be obtained from a body of research concentrating on just a single genus or order. Other model organisms are required, ideally from diverse phyla. For example, the teleost fish are by far the most species rich of the vertebrate orders and studies on a number of teleost taxa have contributed to our knowledge of the evolutionary process (e.g. Seehausen et al., 1999; Rundle et al., 2000), but no genetic analysis of assortative mating within this group has yet been performed. Potential models for the genetics of prezygotic isolation should ideally bear the features that have rendered *Drosophila* so useful: they should be easy to keep and breed in the laboratory and have a mate choice system easily amenable to genetic analysis. Most importantly, there should be plenty of information about their genome readily available. A species with many of these attributes is the zebrafish, *Danio rerio*.

1.2. The zebrafish as a model

Since it was first described by Francis Hamilton from the Ganges Delta in 1822, the small striped fish commonly known as the 'zebrafish' or 'zebra danio' (Figure 1.1) has become variously a popular aquarium fish (Talwar & Jhingran, 1992) an important bioassay (Von Hertell et al., 1990) and in the last few years a major model in vertebrate physiology and developmental genetics (Vascotto et al., 1997). Several attributes of the species explain its popularity in all these contexts: it reaches a length of only 30mm, is relatively easy to rear and breed in captivity, and produces large numbers of offspring with a generation time as short as four months.

As a result of the use of the zebrafish as a developmental genetic model, a dense microsatellite map is available, which will greatly facilitate future mapping of loci (e.g. Shimoda et al. 1999, Postlethwait et al. 1998). Within two years, the complete zebrafish genome sequence is expected to be known (Duyk & Schmitt, 2001). Much progress has been made in elucidating the genetic basis of the zebrafish colour pattern (e.g. Parichy et al., 2000), a trait which appears to be important in speciation via reproductive isolation in other teleost fish (e.g. Seehausen & van Alphen, 1998). Additionally, the recent interest in zebrafish genetics has

Figure 1.1. The zebrafish, *Danio rerio*.

resulted in molecular phylogenies of the *Danio* genus (Meyer et al., 1993, 1995) and the taxonomy of *Danio* is receiving renewed attention (e.g. Fang, 1998).

Despite these advantages, there are reasons why the zebrafish might not prove to be a good model for studying the genetic basis of prezygotic isolation. Primary amongst these is the current lack of knowledge about mate choice in this species. Behavioural work on the zebrafish has primarily concentrated on its shoaling behaviour (e.g. Krause et al., 1999) and its response to aquatic pollutants (e.g. Vogl et al., 1999). Although some work has been done investigating the role of olfactory cues in mediating reproduction (e.g. van den Hurk & Lambert, 1983), other aspects of sexual behaviour have been largely neglected. Studies of the genetics of prezygotic isolation require an organism whose mate choice can be easily quantified (Hollocher et al., 1997). It is not yet known whether this is the case with zebrafish.

Secondly, little is known about intraspecific phenotypic and behavioural variation in the zebrafish, its ecology and its distributional relationship to other members of the genus. An understanding of the processes which have contributed to species diversity within a taxa requires knowledge of how these species are isolated from each other today and the ecological processes which may have given rise to this isolation. In addition, most advances in the understanding of prezygotic isolation are likely to come not from the study of species which are already isolated but from conspecific populations which are beginning to diverge in their mate choice system and therefore may represent taxa on the way to speciation. The potential for such

intraspecific divergence in turn depends largely upon the genetic variation present in the ancestral population.

The purpose of this thesis is to address some of these issues in the zebrafish, by investigating behaviour, phenotypic variation and the genetic basis of this variation in zebrafish deriving from different areas of the species range.

1.3. Structure of the thesis

The thesis is largely structured so that each chapter represents a stand-alone piece of work, however chapters are cross-referenced where appropriate. A single chapter (Chapter 3) is devoted to methods common to all parts of this work in order to avoid unnecessary repetition.

Chapter 2 describes collection of zebrafish from populations in Nepal and Bangladesh. I include an overview of the current knowledge about zebrafish phylogeny and its distribution relative to other members of the genus. Some possible facets of the species' life history are discussed with reference to observations at the collection sites.

In Chapter 3, I present details of general methods used throughout this study. Two techniques were developed in the course of this research which may be of use to others in the field: a low-input breeding and rearing protocol and a photographic method for recording the morphology of small tropical fish.

Chapter 4 describes sexual and antagonistic behaviour in the zebrafish and presents work to assess the amenability of the species to investigations of mate choice. This work is presented in the context of research and techniques involving other fish species.

Chapter 5 presents work on the shoaling behaviour of the zebrafish. Although not directly connected with its utility as a model for ethological isolation, the shoaling habit of the zebrafish may have a role in mediating gene flow in this species. Additionally, shoaling tendency is another behaviour potentially amenable to genetic analysis. The introduction to this section includes a review of current knowledge about the importance of population, kinship and familiarity in shoal structure.

In Chapter 6, I describe investigations of morphological difference between zebrafish derived from different localities, together with some discussion of sexual dimorphism in this

species. Documentation of inter-population variability in traits with possible behavioural roles is an important first step in understanding the selective forces that may be acting on such traits and the potential for population divergence.

Chapter 7 investigates the quantitative genetic basis of such trait variability in one of the zebrafish populations, using fish generated in a North Carolina II breeding scheme.

Chapter 8 provides a brief final discussion of results and suggestions for future work.

Chapter 2
Zebrafish Distribution and Collection

2.1. The Genus *Danio*

The genus *Danio* belongs to the subfamily Rasborinae of the widespread fish family Cyprinidae and is native to South and South-east Asia. Members of the genus are all small fish (less than 120mm in length), exhibiting commonly striking coloration in the form of longitudinal stripes or vertical barring (Fang, 2000). The name *Danio* derives from the collective Bengali noun for such fishes, 'dhani' or 'of the paddyfield', referring to the typical habitat of many of these species (Talwar & Jhingran, 1992).

Weber and de Beaufort (1916) suggested that the genus be split into two sub-genera: *Brachydanio,* including smaller, narrow-bodied species such as the zebrafish which have an incomplete lateral line and seven or less branched rays in the dorsal fin, and *Danio*, comprising rather larger, deeper bodied species with a complete lateral line and more dorsal fin rays. Later authors have raised these sub-genera to full generic status (e.g. Myers, 1924), however the monophyly of these two groups is in doubt, and the diagnostic features given by Weber and de Beaufort do not reliably separate them. Meyer et al. (1993, 1995) have suggested on the basis of limited molecular studies that the *Danio:Brachydanio* distinction may indeed be phylogenetically robust. Fang (2000), however, proposes an alternative phylogenetic classification, on the basis of morphological characteristics, which is also supported by the molecular phylogeny. Under this classification the zebrafish is assigned to a monophyletic '*D. danglia* species group' which includes two species previously classified as '*Danio*' and four previously named *'Brachydanio'*. A number of other species historically attributed to *Brachydanio* are placed together in a second monophyletic group, the 'barred *Danio* species group*'*.

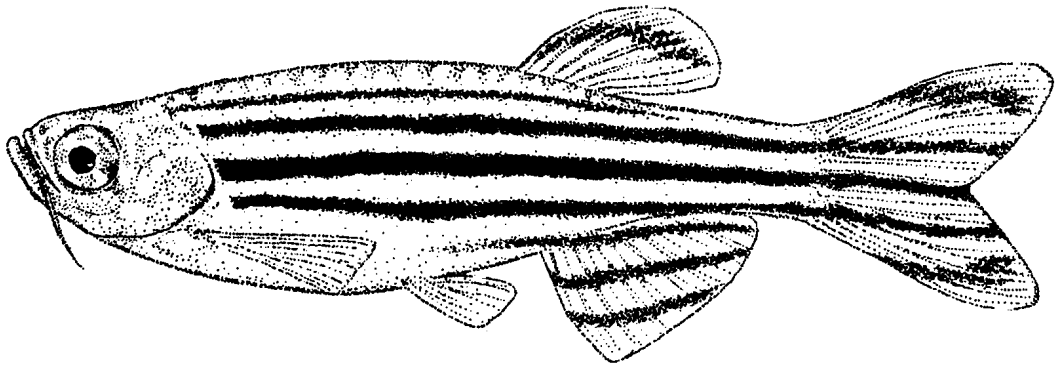
The alpha level classification of *Danio* is also in disarray. Currently around 26 taxa are recognised as valid species (F. Fang, personal communication), although more than twice this number have been proposed on the basis of differences in colour pattern, barbel morphology, lateral line length and shape. Many currently suggested species synonymies (e.g. Talwar & Jhingran, 1992) remain controversial, and the situation is complicated by incomplete species descriptions, loss of type specimens and changes in pigmentation of preserved fish (Fang &

Kottelat, 2000). The species included in the phylogeny of Meyer et al. (1995) provide an example of the current confusion. Of the six taxa used to represent the '*Brachydanio*' group, three, '*D. tweediei'*, *'D. pulcher'* and *'D. albolineatus'* are currently considered to represent just one species, although their exact status remains unclear (Fang & Kottelat, 2000), whilst a fourth, '*D. frankei'* is an aquarium variant of *D. rerio* (a *leo* mutant, see chapter 7).

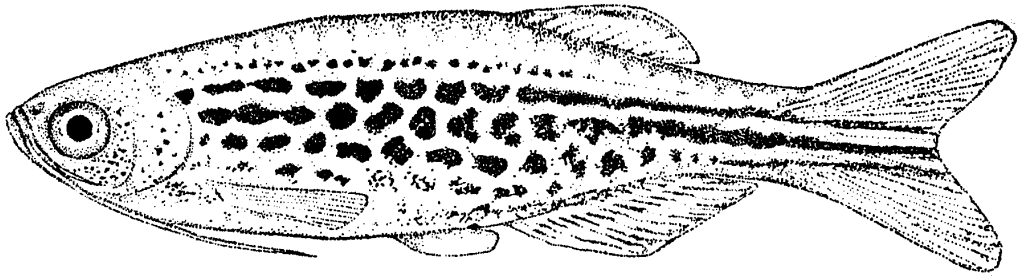
2.2. Distribution of the zebrafish and close relatives

It appears that *Danio rerio* is frequently overlooked in fish surveys, both because it escapes from fishing nets with standard size mesh (personal observation) and because it is of little or no value as a food fish, even non-commercially; Dutta (1993) refers to it as a 'trash fish'. The species also appears to be most common in small water bodies which may be sampled only rarely. The data that are available, however, suggest that the zebrafish is present over much of the Indian subcontinent, occurring from eastern Pakistan (collection of the British Natural History Museum), to the north-eastern India-Myanmar border (R. P. Barman, personal communication), and recorded as far south as Mysore in southern India (collection of the Swedish Museum of Natural History) and as far north as Jammu in the region of Jammu and Kashmir (Dutta, 1993). In addition, feral populations of the zebrafish have been recorded in Columbia, Malaysia and the southern USA and, given the species popularity as an aquarium fish, are likely to have become established elsewhere.

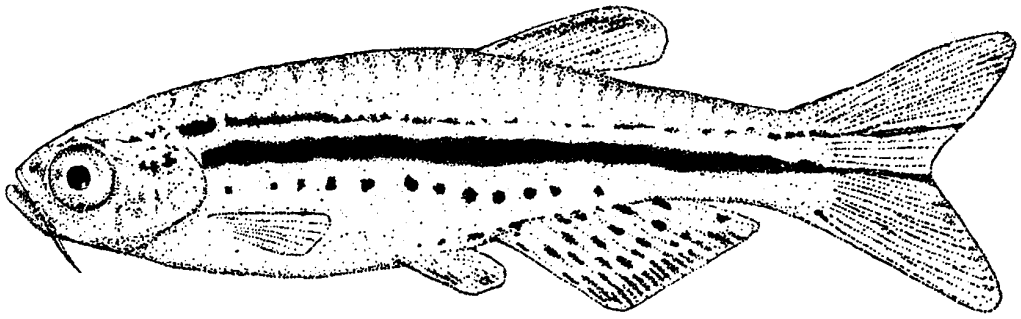
Throughout most of its distribution the zebrafish appears to be the only member of the '*Brachydanio*' subgroup present, although it is frequently sympatric with the '*Danio*' species *D. danglia*, *D. devario* and *D. aequipinnatus*. Three other small *Danio* species have been described from north-eastern India (*Danio (Brachydanio) acuticephala*, Hora, 1921; '*Danio (Brachydanio) horai'*, Barman, 1983; '*Danio deyi*', Sen & Dey, 1985) and it is possible that their range overlaps with that of the zebrafish. However, the only one of these currently recognised as a valid species, *D. acuticephala*, is endemic to Manipur, a region where the zebrafish appears to be absent (Hora, 1921; Menon, 1954; Vishwanath et al., 1998). The zebrafish has previously also been recorded from northern Myanmar (Prashad & Mukerji, 1929) but specimens from this locality have recently been described as a new species, *Danio kyathit* (Fang, 1998). *Danio rerio*, *D. kyathit* and a third species occurring in central and Southern Myanmar, *D. nigrofasciatus*, appear to be very closely related and are suggested by Fang (1998) to represent sister species within the *D. danglia* group. The three species differ in aspects of barbel length and anal fin width but most strikingly in their coloration, as shown in Figure 2.1.



Danio rerio



Danio kyathit



Danio nigrofasciatus

Figure 2.1. Pigmentation pattern of the zebrafish and two closely related species.

The zebrafish exhibits a striking pattern of up to five blue longitudinal stripes on the sides of the body, alternating with gold interstripes and extending into the caudal fin (Figures 1.1, 2.1 & 6.1). The anal fin is similarly striped whilst the dorsal fin has a blue upper edge, bordered with white. In *D. kyathit*, (Fig. 2.1), the stripe pattern typical of *D. rerio* is broken up anteriorly into spots, with only three stripes extending into the caudal fin and weak striping on the anal fin. There appears to be variation in the degree of spotting, with some individuals perhaps approaching more closely the typical *D. rerio* striped pattern (Fang, 2000). *D. nigrofasciatus* retains the central body stripe of *D. rerio*, (Fig. 2.1) however the stripe above this is considerably narrowed and the stripe below replaced with a line of spots. The anal fin is similiarly spotted and the caudal fin bears only two narrow stripes. A fourth member of the *D. danglia* group from Thailand, *D. kerri*, also exhibits a striped pattern, however, the blue melanophore stripes are fewer in number and thicker than those of the zebrafish and do not extend into the caudal fin. This species may also prove to be closely related to the zebrafish, as might possibly be the poorly described *'D. deyi'* from north-eastern India (K. -E. Witte, personal communication). Also present in Myanmar are the three other species that Fang (2000) includes in the *D. danglia* group: *D. albolineatus* (the pearl danio), *D. choprae* (a species with a barred colour pattern but which does not share other characteristics of the 'barred *Danio* group') and *D. feegradei*, as well as several species attributed to the 'barred *Danios*'. The zebrafish will hybridize in captivity with both *D. nigrofasciatus* and *D. albolineatus*, however the offspring of such crosses are generally described as 'infertile' (Kirschbaum, 1977; Meyer et al., 1995).

The apparent diversity of small *Danio* species in northern and central Myanmar, compared to the ubiquity of *Danio rerio* throughout much of India, Bangladesh and Nepal suggests that the Myanmar region may be a centre of speciation for the *rerio* group. Investigation of the genetic basis and behavioural significance of colour pattern variation within and between *D. rerio* populations in different geographical regions should shed light on the evolution of the colour pattern differences within this group and their role, if any, in speciation. Such work will be aided by the current efforts to identify the genetic and developmental basis of stripe pattern formation in the zebrafish using developmental mutants (see Chapter 7). However, detailed information about the distribution of the *rerio* sister species, in particular within their potential contact zones in northern Myanmar is also required.

2.3. Collection of wild zebrafish

Zebrafish were collected in Nepal in February 1996 and in Bangladesh in March 1997. Collection of fish from India was precluded by the limited time available to arrange such a visit.

All fish were collected by local fishermen using various methods, including fine-mesh seine nets (Figure 2.3), scoop nets and pond drainage. Fish collected in Nepal were transported by air and road to Godavari Fish Farm, Kathmandu where they were housed in a stream-fed tank for several weeks prior to transfer to the UK. Fish collected in Bangladesh were transported by air or road to the Bangladesh Aquaculture and Fisheries Resource Unit guesthouse in Dhaka where they were kept in large buckets for up to three weeks prior to export. Aeration was supplied to these buckets and water changed frequently. Fish were imported into the U.K. under a MAFF Tropical Fish Import Licence (DOF 8C) accompanied by the relevant health certificates. All fish were packed for air transportation in double skin polythene bags containing pure oxygen over 1.5 litres of water. Maximum fish density was 20 fish per litre. Bags were packed in heavy-duty cardboard boxes (6 bags/box). Fish collected in Nepal were carried as hand luggage in the aeroplane cabin whilst fish from Bangladesh were transported in a cargo hold heated to 15-20°C.

2.3.1. Collection Sites of Experimental Populations

Collection sites for the experimental populations are shown in red on Figure 2.2. Between 50-100 individuals were collected from each site, however in the collections from Bangladesh mortality prior to arrival in the UK reduced this sample size for most sites.

Nepal: Zebrafish were collected from shallow ditches and an adjacent pond (Figure 2.3) on the Rampur campus of the Royal Nepal Agricultural College, Chitwan, Nepal. These small water bodies had clear, slow-flowing or still water with a silt substrate and some vegetation. Zebrafish occurred at high densities in this habitat and were numerically dominant to the two other species recorded, a barb, *Esomus* sp., and an unidentified catfish. Water temperature at the time of collection was 15°C and it is expected that this might fall further overnight.

Tangail: Zebrafish were collected from a medium-sized pond, approximately 10km north of Tangail adjacent to the Tangail-Madhupur highway in Bangladesh. This pond was steep sided with clear water, a silt substrate and significant amounts of vegetation, in particular water hyacinth covering the surface. Depth was estimated at 1.5m. Zebrafish could be observed swimming in small groups in mid water. Similar ponds in this area frequently dry up during the dry season (various, personal communication), conversely the Tangail area is extensively flooded during the monsoon season.

Figure 2.2. Collection sites of zebrafish in Nepal and Bangladesh.

Figure 2.3. Collection of zebrafish from a pond in Nepal using a seine net. This photograph shows typical zebrafish habitat.

Santal: Zebrafish were taken from small shallow pools in a dry river bed and from an adjacent spring-fed pond in a village of the Santal tribal group near to the India-Bangladesh border. These habitats are connected during the rainy season. Some aquatic vegetation was present, and zebrafish co-occurred with one or two other small fish species (tentatively identified as *Esomus* spp. and *Puntius* spp.).

Canal: Zebrafish were taken from an artificial concrete channel at the Northwest Fisheries in Saidpur, Bangladesh. Vegetation was absent and water in the channel was still and extremely turbid, appearing white. Zebrafish occurred at high densities at this site, together with a species of *Esomus* and freshwater prawns. All fish collected from this site were uniformly pale with no evident stripe coloration, and mortality in the net was unusually high. Normal coloration developed over a period of several days once zebrafish had been moved to clear water. Length of isolation of this population was unknown.

2.3.2. Other Records

Zebrafish were additionally found to be present at the following sites in Bangladesh, indicated on Figure 2.2.

Saidpur: (on Fig. 2.2 as 'Canal'): small numbers of zebrafish were caught from a shallow river, together with other small fish species.

Sunamganj: several zebrafish were found in scoop-net samples of a village pond just south of Sumanganj. A diversity of larger fish species were also present. No zebrafish, however, were evident in an exhaustive village fish catch from a second, more extensive pond.

Srimigal: zebrafish were found in a shallow, mud-bottomed pond just south of Srimigal on the road to Dhaka.

Chittagong: zebrafish were collected from village fish culture ponds just south of the Karnafuli bridge on the Chittagong-Cox's Bazar highway. These ponds had silty water with little natural vegetation but with brushwood added as cover for fish fry.

Bandarban: a group of four zebrafish was observed in hiding under an overhang in a small river off the Chittagong-Bandarban highway. The river was fast-flowing and extremely

shallow with a sandy bed, and apparently originated higher up in the Bandabaran Hill Tracts on the Bangladesh-Myanmar-India border.

2.3.3. General Observations

Zebrafish in this study were recorded from a variety of habitats, and are also expected to be present in larger rivers and lakes that could not be sampled with the equipment available. Most typically however the species was found at high densities in small bodies of water, commonly co-occurring with a single other fish species of the genus *Esomus*. Both of these fish species appear to act as r-selected strategists, reproducing rapidly in habitat where there are no fish predators present and which may be unsuitable for other species. In contrast, larger water bodies with a high fish species diversity appeared to support only small zebrafish numbers.

In Bangladesh, many of the pools found to contain zebrafish in March were expected to be completely dry before the start of the monsoon season in June. This suggests that zebrafish in such habitat go through an annual cycle of extinction and recolonization, with pools repopulated during the extensive monsoon floods by zebrafish derived from more permanent bodies of water. Zebrafish require a long day length to come into reproductive condition, indicating that, as with many other species in this region (Harikumar et al., 1994), spawning may coincide with the onset of the monsoon rains. The monsoon floods in Bangladesh are extensive, and may effectively lead to a panmictic population throughout much of the country, a situation which may also occur in other parts of the species' range. Such a cycle is expected to severely limit the potential for long-term genetic divergence between different populations, and may partly explain the apparent phenotypic uniformity of *Danio rerio* throughout its recorded distribution. In contrast, the diversity of the *Danio* in Myanmar may to a large extent be due to the greater potential for geographical isolation of populations in different river valleys.

Not all parts of the zebrafish's range however, are likely to be so strongly affected by flooding. The fish collected from Nepal for example, may represent a more isolated population. Similarly, the Chittagong region of Bangladesh represents a separate drainage basin from the rest of the country. Even in the absence of physical barriers between populations, several other mechanisms could act to limit gene flow. For example, if zebrafish exhibit philopatry, remaining and spawning in their natal area rather than dispersing during the floods, then local differences in gene pool due to adaptation or stochastic processes will be retained despite the potential for mixing. Site philopatry as a result of habitat preference has been suggested to promote speciation in cichlids (Markert et al., 1999), and several other fish species have been

shown to exhibit preferences for particular sites within their habitat range (e.g. Clough and Ladle, 1997). Anecdotal evidence suggests that zebrafish are common in flooded paddyfields following the rainy season in Bangladesh (various, personal communication), suggesting at least a certain degree of dispersal from their dry season refuges, but the extent of this remains unknown. Additionally, certain shoaling preferences, such as a tendency to associate with siblings or familiar fish, might in some circumstances affect gene flow.

Chapter 3
General Methods

3.1. Zebrafish Husbandry

Zebrafish were housed in 12-15 litre tanks, which were arranged on shelves in a self-contained stock room within the School of Biology aquarium unit. The room was heated to a temperature of 23–25 °C using an 'Autoheat' greenhouse fan heater (Findlay Irvine) in combination with a built-in room temperature control. Lighting was provided by fluorescent ceiling lights and was set to a 14h light: 10h dark cycle. Compressed air was supplied to the room from a central source in the aquarium.

Fish in the tanks were provided with a gravel substrate and imitation plastic plants made out of green mesh. Filtration was supplied by one Algard 'Biofoam 45' air-driven biological filter unit per tank. Maximum density of fish was 35 adults per tank. Tank water was topped up where necessary, with a 50% water change done every two to three months, and a complete cleaning of tanks, gravel and filters performed every 5-6 months. All tap water was aged in buckets for a minimum of two days before introduction to tanks, and API 'Stress Coat' aquarium water conditioner was added to aid chlorine removal. Fish were fed once a day with Aquarian 'Tropical' flake food, occasionally supplemented with live brine shrimp. Fish were monitored daily for signs of distress, with extra water changes being performed when required.

In the later stages of the project a large stock of Nepalese zebrafish was housed in a 180 litre fibreglass tank fitted with an Eheim 2215 external filter unit. Gravel, imitation plants and food were provided as detailed above.

3.2. Collection of eggs and rearing of offspring

Zebrafish have external fertilization, scattering non-adhesive eggs with no prior substrate preparation. The species requires a long day length to come into spawning condition. Female zebrafish ovulate overnight and spawning generally occurs immediately after 'daybreak'. Females can produce several hundred eggs per morning, and domestic strains of zebrafish may spawn daily in optimum conditions.

A clean 12 litre tank was prepared for egg collection by lining the bottom with 1mm plastic mesh. Eggs sink down through this mesh and are thereby protected from consumption by the individuals in the spawning group. The tank was filled with clean water to an approximate depth of 10cm. Plastic plants, a thin layer of gravel substrate and 200-500 ml of water from an established stock tank were provided in order to encourage egg production. Although domestic zebrafish strains will spawn readily over mesh alone, the addition of gravel appears to be an important factor in encouraging wild fish to produce eggs (K. -E. Witte & personal observation). Spawning pairs or groups were introduced to this tank 4-5 hours before the end of the light cycle and left undisturbed until the following morning. Two or three hours after 'daybreak' the fish were taken out of the tank. Plants, gravel and mesh were shaken in the tank water in order to dislodge attached eggs and then removed. Any eggs present in the bottom of the tank were collected using a plastic pipette (2.5ml) with the tip cut off to increase the intake aperture. Eggs were subsequently pipetted between several containers of clean water in order to remove any associated debris, and those showing any sign of fungal infection were discarded. Following egg collection, tanks, plastic mesh and plants were carefully rinsed with hot water to prevent any carry-over of viable eggs to the next spawning session, while gravel was autoclaved before being re-used. Occasionally, if no eggs were evident on the first morning, fish were left in the spawning tanks for several days until eggs were laid or the water in the tank became unacceptably dirty.

Initial attempts to rear zebrafish fry following laboratory protocols detailed in 'The Zebrafish Book' (Westerfield, 1994) proved very time consuming and resulted in extremely high larval mortality (90-100%). This mortality was most obviously the due to contamination of water in small rearing containers, which was not solved by more frequent water changes, although insufficient food may also have been a factor. Both problems probably resulted from difficulties with preparing suitable infusoria cultures for larval feeding, although trials with 'Liquifry' (Interpet) as a food source gave similar results. A novel larval rearing protocol was therefore developed which reduced mortality to an acceptable level (20–50%) and additionally required much reduced daily input levels. Such a protocol is likely to be of use to others working on small-scale zebrafish projects without access to specialized filtration systems or technical assistance. It must be noted, however, that several attempts to rear laboratory mutant lines (supplied by the Max-Planck-Institut für Entwicklungsbiologie, Tübingen, Germany) from the late egg stage using this protocol ended in failure, and it may therefore only be suitable for wild-derived or less specialized stocks.

Under this protocol, zebrafish fry were reared in the same type of 12 litre tanks that were used for housing adult fish. Several days before a spawning was planned, a new stock tank was

set up as normal with clean water, a gravel substrate and an air-driven filter set at a very low level. Approximately one litre of water from an established tank was added in order to encourage the growth of infusoria. Three to seven days later, 50-100 washed eggs were introduced to the tank. Within 4-5 days of fertilization, zebrafish eggs hatch into non-motile larvae which cling to the sides of the tank and continue to receive nourishment through the yolk. Once these larvae reached the free-swimming stages, at around 1 week after spawning, they were fed small amounts of a commercially available invertebrate food which was mixed with water and added to the tank twice daily (2-5 drops per feeding). Approximately one week later food was changed to Tetra Min Baby Fish Food 'E', on which the larvae were fed until they became large enough to take crumbled flake food. Level of filtration was increased as the fish grew.

3.3. North Carolina II Breeding Scheme

In order to investigate the genetic basis of morphological variation within a population of zebrafish, a North Carolina II breeding scheme was implemented (Comstock & Robinson, 1948). In this breeding design, a set of *n* males is mated to a set of *m* females in every possible pairwise combination, such that there are *nm* families, each having (*n*-1) families of paternal half sibs and (*m*-1) families of maternal half sibs. Genetic variation underlying the phenotypic variation within the population can then be estimated by comparing the different classes of half and full sibs. An advantage of the North Carolina II family design is that it enables the phenotypic variation observed in the offspring to be partitioned into that due to additive genetic variation, that due to genetic dominance effects, and that due to maternal effects (Lynch & Walsh, 1998). In this respect it is superior to the more commonly used methods such as nested full sib - half sib breeding designs which only allow estimations of some of these effects. The requirement for multiple matings means that the North Carolina II design can only rarely be applied to animal species, however, the zebrafish, with external fertilization, frequent spawning and high volume egg production is an ideal subject. In consideration of time and space constraints, five male and five female Nepalese zebrafish were chosen as the parentals, so that the complete set of reciprocal crosses would give 25 families. Each full-sib family was divided into two groups which were reared in isolation from each other in order to separate possible tank effects from family effects.

The five male and five female parents were selected to represent the range of phenotypes observed in the Nepal fish. Eggs were obtained from each pair using the spawning protocol detailed above. Depending on the number of eggs produced in a spawning, eggs were either

divided into two tanks to give the two family replicates or offspring reared together in single tank and the cross repeated at a later date to give the second replicate. Parents were kept in single-sex groups when not involved in spawning, and the diet of these individuals was regularly supplemented with live brine shrimp in an attempt to encourage egg production.

Families were obtained from all male-female combinations except four. 'Male 5' died while fish stocks and equipment were being moved to new building accommodation and therefore only mated with two females. A sixth male was introduced to replace this individual but was only involved in two spawnings, which were not retained as families. Most pairings of females with 'Male 3' resulted in small numbers of eggs being produced, with no offspring resulting from a 'Male3-Female 1' cross, additionally, the few offspring produced from the second 'Male 3-Female 5' cross died meaning that this family only had a single replicate tank. Wild-collected zebrafish in general do not appear to spawn as readily as domestic strains, a problem that has been noted in several zebrafish labs (S. Johnson, personal communication). Together with larval rearing problems and interruptions to the breeding program, this meant that establishment of all possible families took approximately 12 months. Such temporal spacing of families could potentially lead to effects of parental age being confounded with other sources of variation, however as males and females were essentially paired at random this is unlikely to have a systematic effect on the results (Mather & Jinks, 1982).

Fish from all families were reared as previously described. Fish density was standardized to a maximum of 35 fish per tank when each family was approximately 6 months old. Tanks were arranged in a haphazard method on the shelves in the stock room, with pairs of tanks containing the same family spaced away from each other.

3.4. Collection of morphological data

All morphological data in this study were collected from live fish, photographed using the photographic set-up illustrated in Figure 3.1. Fish were anaesthetized for photography by reduction of their body temperature. An individual fish was placed in water in a small container and iced water slowly added to decrease temperature. Since zebrafish are poikilotherms reduction of environmental temperature in this way causes the fish to gradually become immobile. The anaesthetized fish was immediately placed on its right-hand side into the petri dish with sufficient cold water to cover it. The petri dish was positioned under the camera such that the ventral side of the fish was towards the mirror and a photograph taken.

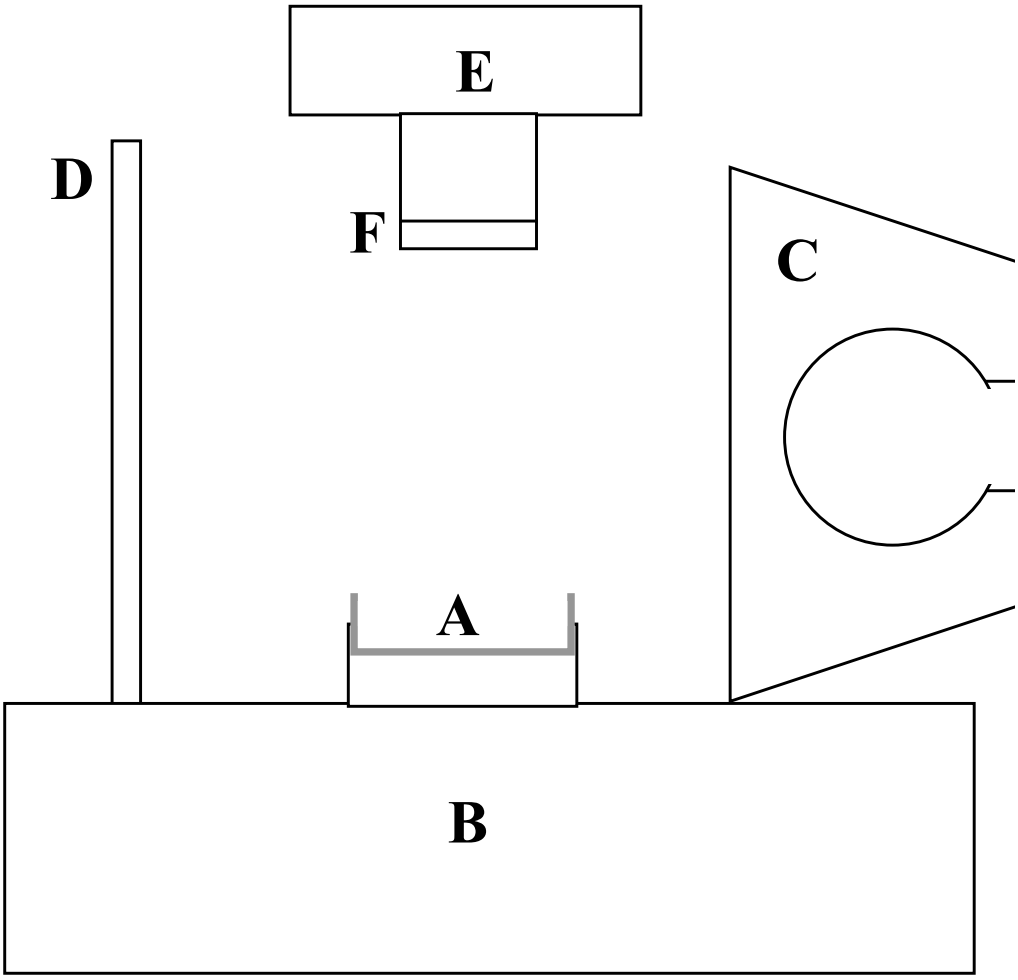


Figure 3.1. Apparatus for photographing zebrafish for morphometric analysis. The anaesthetized fish was placed in water in a glass petri dish (**A**). Illumination was provided from below by a standard light box (**B**), and from the side and top by a studio lamp fitted with a 100watt daylight bulb (**C**). A mirror (**D**) was placed opposite to increase light intensity. Photographs were taken using an Olympus OM 30 or OM 1N SLR camera (**E**) fitted with a 50mm macro lens and a +4 close-up filter (**F**) and with Kodak Royal Gold 100 ASA print film. Aperture was held constant at f22 and shutter speed at ¼ second. The camera was supported on a stand at a height of 25mm above the petri dish, with focus distance of the camera lens fixed for all photographs. Minor variations to this method were used when photographing wild-collected populations and fish used in behavioural trials.

Following photography the fish was removed into a recovery chamber and sufficient warm water added for visible gill movement to recommence and the fish to be able to right itself. The fish was left to further warm up gradually and subsequently returned to its home tank.

Westerfield (1994) recommends reduction of body temperature by freezing as a humane euthanasia method for zebrafish, with the justification that they 'show no signs of pain or distress'. This is not completely true; zebrafish exhibit an avoidance reaction to cold water and probably suffer short-term distress as a result of the treatment described, as evidenced by extension of the dorsal and anal fins which is commonly seen in fright reactions. In addition, there are possible longer-term consequences of temperature shock. The most commonly recommended chemical anaesthetic for fish is MS222 (Tricaine). However, preliminary trials showed that zebrafish anaesthetised in this way clamped their fins against their body, precluding the analysis of fin patterns which may have important behavioural roles. Under these circumstances it was considered justified to use the cold water technique. Care was taken to minimize the exposure time of fish to the treatment and the majority of individuals recovered with no apparent ill effects. Mortality rate was less than 1% and of those individuals that did die most appeared to be already in ill-health, frequently emaciated compared to their tank mates.

Negatives were developed and images scanned into a computer using a Minolta Dimage Scan Dual negative scanner in combination with Adobe Photoshop (version 5.0 LE) software. Each image was scanned at the maximum resolution of 2400 dpi with exposure adjusted to + 10 and contrast to + 20. Brightness and contrast were further adjusted in Photoshop using the 'Auto Levels' option, a procedure which was found to improve image clarity. A reference line was then drawn from the anterior insertion point of the dorsal fin to the anterior insertion of the anal fin (Figure 3.2). The image was saved with a random numerical file name so that all morphological analyses were performed without knowledge of a fish's family identity. File numbers and family identities were stored on a Excel spreadsheet. Images were scanned in blocks of approximately 300 and then transferred to compact disc using a CD rewriter. Image files were analysed in numerical order.

All morphometric data were collected using the image analysis program UTHSCSA Image Tool 2.0 (developed at the University of Texas Health Science Center, San Antonio, Texas and available free at http://pacer.uthscsa.edu/dig/itdesc.html). The co-ordinates of landmark points were recorded using the 'points' tool in Image Tool and data were then transferred to an Excel spreadsheet where distances (in pixel units) between landmarks were calculated.

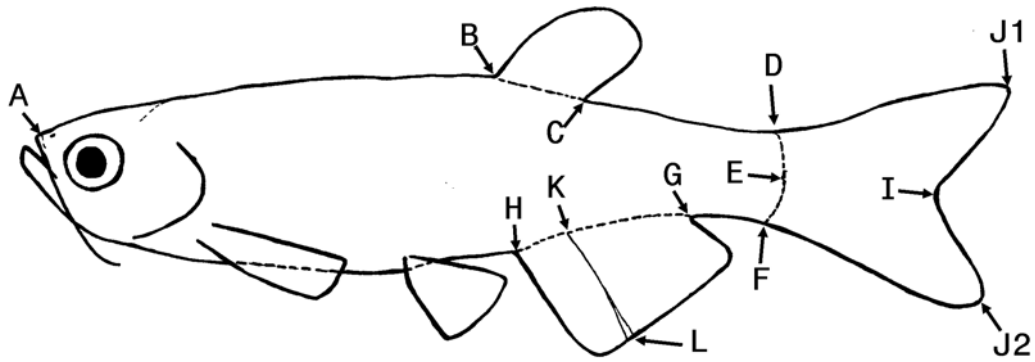


Figure 3.2. Landmark points for fish body measurements.

3.4.1. Body measurements

Landmark points for body measurements are shown in Figure 3.2, and measurements were as follows:

I. **Standard length:** distance from tip of nose **(A)** to centre of caudal peduncle **(E)**.

II. **Width:** distance from anterior insertion of dorsal fin **(B)** to anterior insertion of anal fin **(H)**.

III. Nose **(A)** to anterior dorsal fin insertion **(B)**.

IV. Nose **(A)** to anterior anal fin insertion **(H)**.

V. **Width of dorsal fin base:** distance from **(B)** to **(C)**.

VI. **Width of anal fin base:** distance from **(G)** to **(H)**.

VII. Posterior dorsal fin insertion **(C)** to start of caudal fin **(D)**.

VIII. Posterior anal fin insertion **(G)** to start of caudal fin **(F)**.

IX. **Width of caudal peduncal:** distance from **(D)** to **(F)**.

X. **'Tail 1':** distance from centre of caudal peduncal **(E)** to centre of caudal fin fork **(I)**.

XI. **'Tail 2':** mean distance from centre of caudal peduncle **(E)** to outermost points of caudal fin = ½ ((**E** to **J1**) + (**E** to **J2**)).

XII. **Length of anal fin:** distance from start (**K**) to end (**L**) of ray 5 of the anal fin. Rays are counted as shown in Figures 3.3b & 3.3c.

3.4.2. Stripe measurements

Melanophore stripes on the body are termed following Fang (1998) as shown on Figure 3.3a. The P (primary) stripe runs through the middle of the caudal peduncle, with stripes P+1 and P-1 respectively above and below it. Developmentally, P and P+1 are the first stripes to be laid down as the adult coloration develops (McClure, 1999). The xanthophore stripe between these two melanophore stripes is termed, for the purpose of this study, 'IS1' (interstripe 1). In this study, the P-1 stripe in zebrafish was frequently truncated or broken and was therefore not included in any analyses.

Anal fin stripes are termed according to their position along ray 5. The first stripe immediately adjacent to the body, whether blue or gold, is not included in any analyses since the width of this stripe is limited by the anal fin border. The B1 (blue 1) stripe is then the first blue melanophore stripe after this stripe, and G1 the first gold stripe. The B1 stripe defined in this way corresponds to the 'A stripe' of Fang (1998). In most zebrafish the G1 stripe occurs above the B1 (Figure 2b) but in the cases where the stripe adjacent to the body is gold it occurs below (Figure 2c). Subsequent stripes are named B2, G2 and so forth. For measurement purposes, the definition of a G stripe in this study includes not only the actual xanthophore stripe, but the unpigmented regions either side of it bordering the B stripes. This definition was chosen as the boundary of the xanthophore stripe was not clear. For both body and fin measurements, melanophore stripe boundary was defined as the outer edge of the outermost melanophores along whichever reference line was being used.

The following measurements were taken for use in morphometric analysis:

Body stripes: Width of P, P+1 and IS1, measured along the reference line extended between the anterior insertions of the dorsal and anal fins (Fig. 3.3a).

Anal fin stripes: Width of B1, G1 and where applicable B2 & G2, measured immediately anterior to ray 5. Width of G stripes was defined as the distance between the boundaries of the adjoining B stripes.

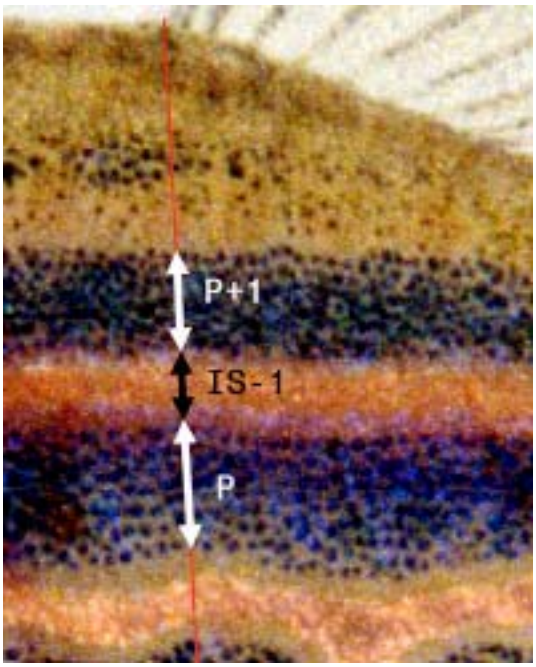


Figure 3.3 a: Body stripe measurement. Width of stripes P, P+1 and IS1 are measured along the reference line, shown in red.

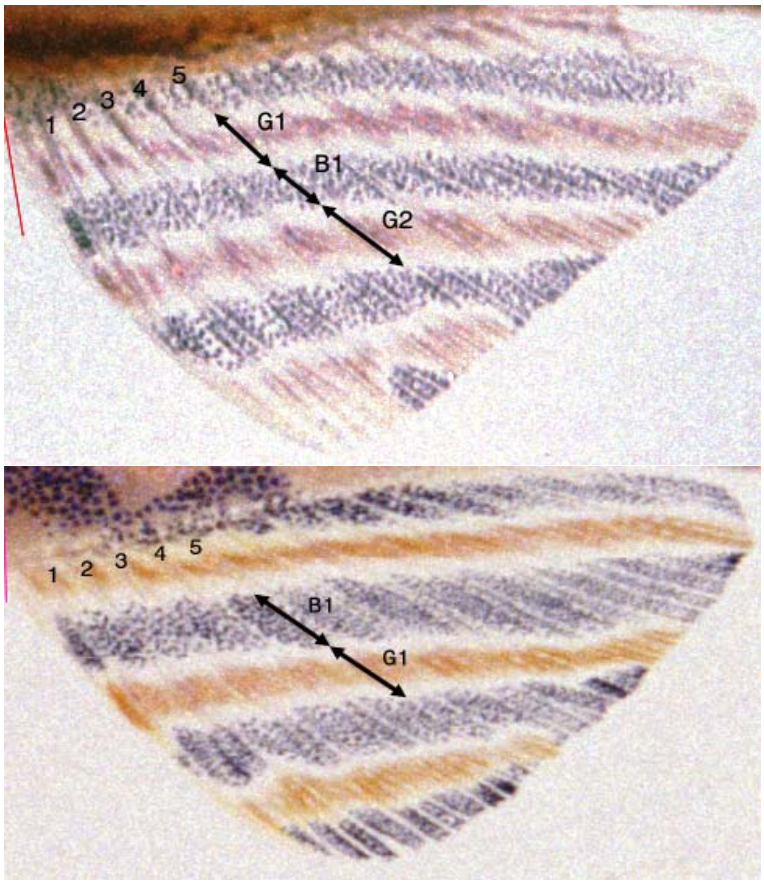


Figure 3.3 b & c: Measurements of anal fin stripes along ray 5. Fin rays are numbered as shown. In **2.3b**, G1 as defined in the study occurs above B1; in **2.3c**, it is below.

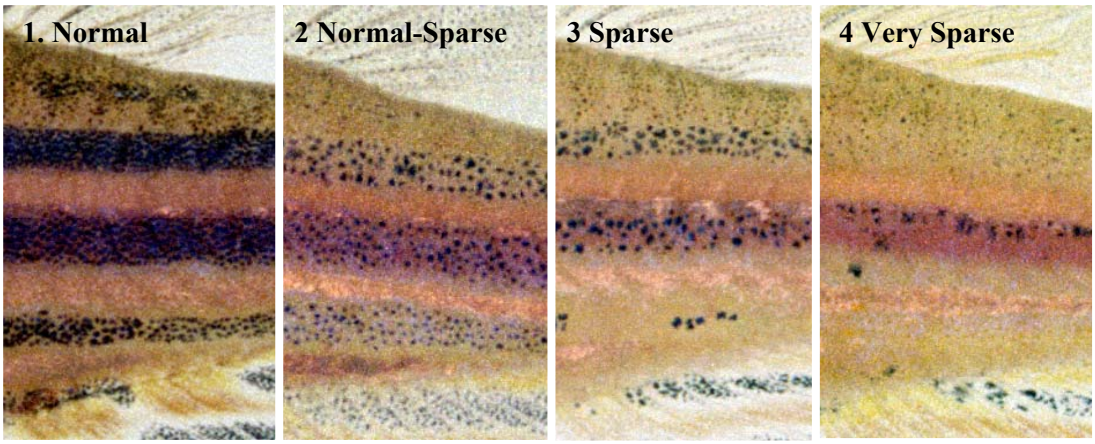


Figure 3.4. Classification of stripe melanophore density

3.4.3. Other morphological features

In addition to morphometric measurements, several morphological features were scored by eye. These are as follows:

I. **Density of stripe melanophores:** from 1 (nearly all missing) to 4 (normal) (Fig. 3.4)

II. **Appearance of melanophore stripes:** 1 = blotchy, 0 = normal.

III. **Colour of each G stripe on anal fin:** 1 = brown, 2-3 = yellow-brown, 4 = yellow, 5 = orange.

IV. **Presence/ absence of cloacal aperture** (anterior to anal fin): 2 = present, 0 = absent, 1 = unclear.

Scores for melanophore density and colour of anal fin are by necessity subjective, and colour values in particular can be affected by variations between film batches and slight differences in lighting and film scanning conditions. Nevertheless, such scoring over a large sample of fish can provide qualitative information about overall patterns, such as difference in pigmentation between the sexes.

Chapter 4
Sexual and Aggressive Behaviour in the Zebrafish

> '...many of the carnivorous Cyprinidae in India are ornamented with 'bright longitudinal lines of various tints'..... On the whole, the most probable view in regard to the fishes, of which both sexes are brilliantly coloured, is that their colours have been acquired by males as a sexual ornament, and were transferred equally, or nearly so, to the other sex.'
>
> Charles Darwin, *The Descent of Man, and Selection in Relation to Sex*, 1871.

4.1. Introduction

4.1.1. Studies of mate choice in fish

Despite the vast diversity of sexual strategies, courtship behaviour and potential sexual signals exhibited by teleost fishes, studies of mating preference have concentrated on relatively few groups (Ptacek, 2000). A number of these groups have become important model systems for addressing questions about the evolution of sexually selected traits and their potential role in speciation. Work on Trinidadian guppies (*Poecilia reticulata*) and threespine sticklebacks (*Gasterosteus aculeatus* complex), taxa in which male carotenoid pigmentation is an important sexual signal, has shown, for example, how sexually selected cues may provide information about mate quality, (Lopéz, 1998; Milinski & Bakker, 1990; Kodric-Brown, 1989), how male traits and female preferences may co-vary geographically (Houde, 1988; Houde & Endler, 1990) and be genetically correlated (Bakker, 1993; Houde, 1994), how these traits and preferences may be shaped by the biotic and abiotic environment (Endler, 1983; Luyten & Liley, 1991; Endler & Houde, 1995; McDonald et al., 1995), and how assortative mating might maintain genetic divergence between co-occurring ecotypes (Ridgeway & McPhail, 1984; Rundle et al., 2000). Investigations into female preference for tail-sword length in the genus *Xiphoporus* (swordtails and platyfish) have demonstrated a potential role of pre-existing female bias in promoting the emergence of particular male traits (Basolo, 1990, 1995; Rosenthal & Evans, 1998; but see Meyer et al., 1994), whilst studies on male mating success in other members of the family Poeciliidae have provided functional explanations for sexual size

dimorphism in this group (Bisazza & Marin, 1995; Pilastro et al., 1997). Divergence in male coloration and associated female mate choice appears to be an important factor in generating and maintaining the explosive diversity of haplochromine cichlids in the East African Great Lakes (Seehausen et al., 1997; Seehausen & van Alphen, 1998), and has also been suggested to promote speciation in several coral reef taxa (Domeier, 1994; Thresher & Moyer, 1993; but see McMillan et al., 1999).

4.1.2. Sexual selection in the zebrafish?

A characteristic of the *Danio* genus is the diversity of colour patterns seen in these fishes (Chapter 2). This raises the possibility that variation in colour pattern may play a role in creating and maintaining reproductive barriers within the genus. Several authors, most notably Darwin (1871), have suggested that these colour patterns may be sexually selected. Divergence between geographically isolated or distant populations for sexually-selected traits and associated preferences, resulting in ethological isolation, is widely accepted as a potential mechanism of species formation (Fisher, 1958; Panhuis et al., 2001). Divergence in mate choice systems as a result of selection against hybrids is also suggested to promote reproductive isolation between newly sympatric populations which have genetically diverged in allopatry ('reinforcement': Dobzhansky, 1970; see Butlin, 1989). More controversially still, divergent sexual selection has also been suggested as a mechanism to allow speciation in sympatry (e.g. Turner & Burrows, 1995; Seehausen & van Alphen, 1998; Higashi et al., 1999). In all cases, the potential for divergence is dependent upon the amount of genetic variation in sexual traits and preferences present in the ancestral populations and the degree of genetic covariance between them. As noted in Chapter 1, a full understanding of the process of speciation by ethological isolation requires that the genes themselves be identified. The vast amount of work already done on the genetics of the zebrafish raises the possibility that fishes of the genus *Danio* may be suitable models for such a task.

Despite years of research into zebrafish development, and the fish's extensive use as a bioassay, relatively little attention has been paid to the mate choice behaviour of this species. Anecdotal evidence ranges from protestations of almost complete monogamy (Riehl & Baensch, 1986) to suggestions that a female is 'forced' to spawn by a group of males. There are several reasons for the absence of such studies. Investigations into the role of sexual selection in driving morphological evolution in fish have concentrated on species exhibiting striking sexually dimorphic male characteristics, precisely because these can immediately be identified as good candidates for sexually selected traits. Such species, of course, have the additional advantage

that the sexes can be easily distinguished. The occurrence of striking monomorphic coloration, such as the stripes of the zebrafish, is more problematic. One view of such monomorphism is that traits sexually selected in the male as a result of male-male competition or female choice are expressed in the female as a result of the genetic correlation between the sexes (Darwin, 1871; Lande, 1980). In most cases, such traits are expected to be selectively disadvantageous causing their expression to become sex-limited (Fisher, 1958). However, in species such as shoaling fish where the 'oddity effect' (the increased predation risk for phenotypically distinct individuals in a group) selects for phenotypic uniformity (Landeau & Terborgh, 1986), retention in females of traits sexually selected in males may be selectively neutral or even advantageous. Selection for monomorphism as a mechanism for individuals to avoid sexual competition or harrassment has also been proposed (Langmore & Bennett, 1999). More recently, attention has focused on a role of monomorphic ornaments in mate choice and intrasexual competition in both males and females (e.g. Jones & Hunter, 1999), and in birds at least there frequently appears to have been directional selection for monomorphism of conspicuous traits (Amundsen, 2000). The weak sexual dimorphism of *Danio* species therefore in no way precludes a role of sexual selection in generating pattern divergence within the genus.

Studies of sexual selection in fish have additionally tended to avoid those species with external fertilization and the potential for group spawning, such as the zebrafish, due to doubts as to whether female choice is an important determinant of fertilization success in such a system (Berglund, 1996). However there is a growing body of evidence that in many so-called 'promiscuous' fish species mating is not random (e.g. Iguchi & Maekawa, 1993: ayu fish *Plecoglossus altiveli;* Hutchings et al., 1999: Atlantic cod *Gadus morhua*). Additionally it is now well known that in many fish species held up as models of the sexual selection principle female choice is frequently subverted by the activities of sneaky maters (e.g. Magurran & Seghers, 1994).

4.1.3. Sexual behaviour in the zebrafish

As previously described, *Danio rerio* is an oviparous fish with external fertilization and no parental care, scattering non-adhesive eggs with no prior substrate preparation. Several studies have established a role of olfactory cues in mediating reproductive behaviour in the species. Males appear to be attracted to ovulated females by a mixture of steroid glucuronides produced in the ovaries, which may also help stimulate male courtship behaviour (Van den Hurk & Lambert, 1983). Similarly, in females ovulation is promoted by exposure to steroid glucuronides produced in the testes of males (Van den Hurk & Resink, 1992). Bloom &

Perlmutter (1977) and Golubev (1984) have suggested that pheremonal cues may also act to facilitate aggregation of males and females, perhaps for spawning purposes, and additionally that these cues may be species specific (Bloom & Perlmutter, 1978).

Although such olfactory stimuli may be important as physiological cues to bring an individual into breeding condition, zebrafish additionally have a sophisticated visual system (Orger et al., 2000) with large eyes and tetrachromatic colour vision that extends into the UV (Robinson et al., 1993). It is therefore likely that, as in many other fish species, visual and behavioural cues are important determinants of mate choice (Van den Hurk & Lambert, 1983).

A male zebrafish encountering a female shortly after 'lights on' in the laboratory will first begin to follow her in a jerky swimming motion with the dorsal fin held erect and attempt to nudge her in the region of the cloacal aperture (personal observation). Frequently this develops into an immediate spawning attempt in which the male rapidly swims up to the female and curves his body around her, positioning his genital pore next to hers and jerking the posterior part of her body upwards in an attempt to elicit egg scattering. If this first attempt is unsuccessful, the male will commonly begin courtship behaviour, positioning himself just above the substrate, facing the female and with his body slightly inclined downwards ('hang'). From this position, he frequently executes two actions: a rapid, tight circle on the spot ('circle'), which is sometimes extended to circle the female, and a rapid swim towards the female and back to his previous position on the substrate ('dash'). Throughout courtship he adjusts his position to remain facing the female and, if she moves too far away, will swim to a new spot in front of her and continue this behaviour. Periodically, he will make another spawning attempt. Females exhibit no obvious acceptance behaviour other than allowing a male to spawn with them, but will respond to unwelcome advances with aggressive behaviour and frequently chase the male away. A male who has been rebuffed in this manner will continue to approach a female in a 'submissive posture', hanging in front of her with fins down. Strategies adopted by males and females in a courtship situation appear to depend on their relative dominance status: a male approaching a larger or apparently more dominant female will commonly use courtship behaviour from the outset, whereas one encountering a smaller female will often continue to chase her in an attempt to elicit spawning without exhibiting any courtship. A spawning is characterised by males and females curling their bodies together in the position described earlier and simultaneously ejecting eggs and sperm. Females generally do not scatter all their eggs in a single spawning, instead coupling repeatedly in a session, sometimes with different males. Other males in the tank may attempt 'sneaky fertilization' of ejected eggs by joining in with a spawning pair. Individuals engaged in sexual behaviour will frequently break off this activity to drive other fish out of the vicinity.

4.1.4. Use of association times in studies of fish mate choice

A common method of assessing mate choice in fish is to give an individual a choice between two potential mates differing in the character of interest. Frequently these alternatives are presented at either end of a test tank and mate preference gauged by looking at the relative time the test fish spends associating with each one. A significant advantage of this approach is that it reduces the complication of male-male dominance interactions affecting the behaviour of the female. As many species of fish shoal, however, it is important to establish that the test fish in question is indeed exhibiting a sexual preference and not merely a shoaling preference. Only a few authors have attempted to test directly whether an association preference corresponds with a spawning preference (Kodric-Brown, 1993: guppies; Pyron, 1995: orangethroat darters *Etheostoma spectabile*; Howard et al., 1998: Japanese medaka *Oryzias latipes*). In a number of fish species, performance of cues associated with sexual behaviour may be sufficient to indicate whether a sexual preference is being expressed, for example 'glides' by female guppies (Lopéz, 1998), arching displays by mosquitofish (*Gambusia affinis* Hughes, 1985), follow behaviour by cichlids (Seehausen & van Alphen, 1998) and head up displays by sticklebacks (Milinski & Bakker, 1990). Other studies have used more indirect methods. Ptacek & Travis (1997) compared association times of sexually receptive and non-receptive female sailfin mollies (*Poecilia latipinna*) with males of different sizes and concluded that, since only receptive females showed a significant preference, this represented a mate choice. A more recent study (Gabor, 1999), however, has cast doubt on whether such observed association preferences do indeed represent a sexual preference in this species, as fish prefer to associate with larger individuals regardless of sex. It has also been suggested that presenting stimulus males behind glass may result in females appearing to prefer traits that would have less importance if they were able to view the male more freely (Nilsson & Nilsson, 2000: sticklebacks).

4.1.5. Fish mate choice and dominance hierarchies

Although female association preference tests can gauge a female's preference for certain traits, in a natural situation matings are likely to be influenced by a large number of factors, including both intra- and interspecific dominance interactions. In guppies, for example, antagonistic interactions influence male mating success (e.g. Farr, 1980), meaning that a male preferred by a female in a two-way choice test may fail to achieve a mating with that same female in an open-tank situation if the alternative male is dominant (Kodric-Brown, 1993). Studies of fish species with similar reproductive strategies to that of the zebrafish, that is, external fertilization, frequent spawning and no parental care, have in many cases demonstrated

a role of dominance status in male mating success (e.g. Hutchings et al., 1999: Atlantic cod; Howard et al., 1998: medaka) with less dominant individuals nevertheless able to gain fertilization opportunities via 'sneaky spawning'. Intersexual aggression also appears to have a role in determining male mating success in some of these species (Järvi, 1990: Atlantic salmon *Salmo salar*; Petersson et al., 1999: brown trout *Salmo trutta*).

In the extreme situation where fertilization success is determined entirely by male dominance rank with females having no mate preference, then sexual selection for male coloration acts via intrasexual aggression. In this case although divergence between isolated populations in sexually selected traits may still occur, this divergence is unlikely to give rise to ethological isolation between them. However, if there is any opportunity for less dominant males to gain matings, female inclusive fitness will be increased by mating preferences for heritable morphological characters that reliably signal dominance in males. Signals used in male-male competition are therefore expected to become co-opted as signals for female choice. This has been suggested by Fisher (1958) as one of the starting points for runaway co-evolution of traits and preferences involved in mate choice.

4.1.6. Aggressive behaviour in the zebrafish

Both male and female zebrafish engage in visually striking antagonistic displays by which they establish dominance status (personal observation). An individual will typically initiate an antagonistic interaction by extending its dorsal and anal fins and swimming with a stiff body parallel to the potential opponent. This fish most commonly responds by taking up the same posture and the fish then engage in a pair-wise contest, circling head to head and frequently biting at each other in the gill region. Direction of circling is repeatedly reversed and the fighting fish rise in the water column, periodically breaking off the contest and resuming it again lower down. Apparent attempts of one individual to force the other down onto the substrate are also seen. From the onset of antagonistic interactions, both fish exhibit an intense darkening of melanophore stripes on the body and fins. A fight most commonly ends with one of the opponents breaking off, immediately fading its colour and lowering its fins, and being chased away by the victor.

Such antagonistic interactions were frequently observed among the zebrafish in this study, both in the stock tanks and in mate choice trials, where male-male, female-female and male-female fights all commonly occurred. Although antagonistic interactions in zebrafish may

be increased under captive conditions (Magurran & Bendelow, 1990), it appears that dominance interactions may be important in the social system of this species.

4.2. Tests of female association preference and mate choice

As little is known about the mate choice behaviour of zebrafish I chose to investigate female association preferences for a subset of males using two-way choice tests. Males were chosen to reflect the range of morphological variation observed in the wild fish, particularly with regards to tail pattern which was the most obviously varying trait. By pairing each male against each other male in a 'round-robin' experiment I could assign each individual male a rank from 'most preferred' to 'least preferred'. I was then able to investigate firstly whether female association time was a good measure of mating success, using spawning latency tests with the same males, and secondly whether a male's ranking correlated with specific morphological traits whose role could then be further investigated. In these experiments I used wild-collected fish as these were known to be sexually mature at the time of the trials. Eight males were used in the round-robin experiment.

4.2.1. Methods

Tests of Female Association Preference

The test apparatus consisted of a four-chambered glass test tank (Figure 4.1), comprising a central test chamber (30cm x 15cm x 15cm high) with two stimulus chambers (15cm x 15cm x 15cm) at either end and a companion chamber (15cm x 15cm x 15cm) positioned directly behind the test chamber. Chambers were separated by glass partitions fixed in place with silicon sealant, such that there was no water exchange between them. Two opaque green plastic partitions were positioned at either end of the test chamber directly in front of the glass partitions. These could be raised or lowered using a remote pulley system allowing the test fish's view of the stimulus fish to be controlled. Within the test chamber I defined two preference zones extending 5cm from each of the end partitions and marked using permanent

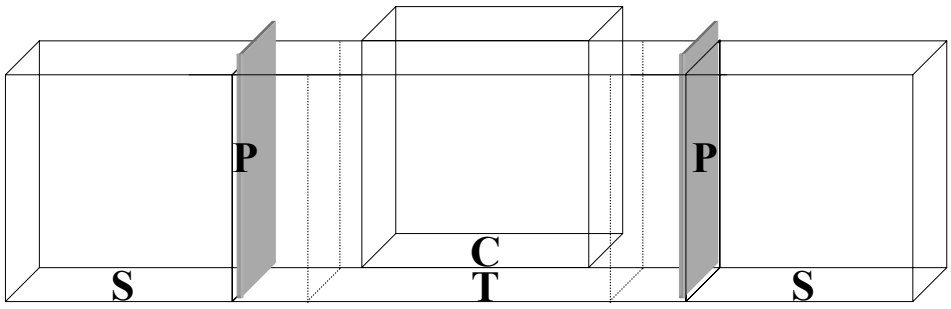


Figure 4.1. Test tank set-up for mate choice trials showing central test chamber (T), stimulus chambers (S), companion chamber (C) and moveable partitions (P). Borders of preference zones are indicated by dotted lines.

pen on the walls of the tank. All chambers were filled with water at 24°C to a depth of 12cm over a layer of gravel. The sides and back of the test apparatus were covered with grey plastic and the apparatus was illuminated by means of a 58 watt fluorescent strip light fixed on the wall 25cm above the tank and extending across all chambers. As preliminary trials indicated that fish were disturbed by bright illumination, light levels were reduced by fixing dark green paper inside the cover of the strip light. Additional weak illumination was provided by the room lights which were set to a 14h:10h light-dark cycle. Experiments were performed in five of these test tanks which were arranged adjacent to each other in the stockroom.

Experimental fish were adult zebrafish that had been collected from the wild a year previously ('Santal' population). All fish had originally been housed together but males and females had been separated into different tanks three months before the start of the experiments. Each trial used two stimulus males and one test female together with one companion female. When first moved from the stock tanks into the stimulus chambers, where they did not have visual access to other fish, stimulus males generally remained hiding on the gravel bottom for several days. In order to accustom them to the test tank environment, therefore, all stimulus males were introduced into the stimulus chambers three weeks before the start of experiments and remained housed in them for the duration of the trials. A stimulus male was moved into a new chamber for each trial. Test females, who had visual access to a companion female at all times, rarely exhibited such stress reactions.

All fish were introduced into the relevant chambers of the test apparatus 4-6 hours before 'lights out' on the day before the trial. Since female zebrafish ovulate more readily in the presence of male pheromones (Van den Hurk & Resink, 1992), 50ml of water from a stock tank

containing male zebrafish from the same population was also added to the test chamber. Opaque partitions were lowered to prevent visual contact between test and stimulus fish and the overhead strip light was set using a timer to switch off half an hour before the room lights. Immediately following 'lights on' the next morning all experimental fish were fed in their test chambers from behind a plywood hide. The strip light was then turned on and after a five minute period trials were started. Opaque partitions were carefully raised in one of the test tanks and female behaviour was recorded for a 15 minute period using a video camera positioned behind the hide. Four trials were performed per day, and all were completed within an hour and a half of 'lights on' in the room. Videotaped trials were analysed using a Turbo Basic program running on an Amstrad PC1512, which enabled an observer to record each time the test female started and stopped associating with a stimulus male. A female was defined as associating with a male when she was within a preference zone and orientated towards him.

Each of the 28 possible pairings of stimulus males was used three times, giving a total of 84 trials. Each of these three replicates was performed in a different test tank with the position of males being swapped between trials and a gap of at least 48 hours between them. The limited number of females (n=24) available meant that test females were re-used. An individual female was never used more than once with a given pair of stimulus males and never more than twice with a given individual male, with a minimum of 15 days between the two tests with the same male. The maximum number of trials any test female was used in was four.

Spawning latency tests

Test apparatus comprised a glass tank 30cm x 15cm x 15cm high, filled with water at 24°C to a depth of 12cm over a gravel substrate. Sides and back of the tank were covered in grey plastic. A test male and two females were placed in the tank 4-7 hours prior to 'lights out' on the day before the trial. Females were selected that appeared in good condition to spawn, indicated by plumpness of the belly (Westerfield, 1994). Fish were left in the tank overnight and flake food added just before 'lights on' the following morning. Behavioural observations began immediately following 'lights on'. I recorded the time from 'lights on' until spawning first occurred. Elements of courtship and aggressive behaviour occurring in the trials were also noted. If spawning had not occurred within an hour then the test was ended, unless fish were still exhibiting sexual behaviour.

Each of the eight males was tested four times, with a different pair of females each time. As the number of suitable test females was limited, some females were re-used with other

males. Since spawning events were not easy to identify on video, all behavioural observations were done in real time with the observer sitting in front of the tank. Room lights were turned off to minimize observer conspicuousness to the fish.

Male morphology

Following all experiments, test males were anaesthetised using cold water and photographed using the standard photographic set-up described in Chapter 3. All males recovered rapidly from this treatment and did not appear to suffer any long-term ill-effects. The following morphometric measurements were taken, as described in Chapter 3: standard length, width of body stripes P, P+1 and IS1, length of anal fin at ray 5 and width of G1 and B1 stripes on the anal fin. Each measurement was taken twice from the same photograph on different days and all calculations done using the mean of the two replicate measurements. The following measures were used in the analysis:

I. **Standard length**

II. **Relative anal fin length:** anal fin length at ray 5 divided by standard length.

III. **Body stripe width:** mean width of stripes P and P+1.

IV. **Body stripe-interstripe ratio:** relative width of melanophore body stripe to interstripe. Calculated as width of P divided by width of IS1.

V. **Ratio of body stripe P to stripe P+1.** This measurement was chosen in view of the difference in width of the P+1 stripe between *D. rerio* and its close relative *D. nigrofasciatus*.

VI. **Width of B1 and width of G1**

VII. **B1:G1 ratio:** width of anal fin stripe B1 divided by width of G1.

In addition, **bilateral symmetry of tail pattern** on either side of the tail mid-stripe was assessed by presenting photographs of the males to 10 independent observers who were asked to rank tail pattern as either ‘symmetrical’ or ‘asymmetrical’. Tails were then classified as symmetrical or asymmetrical according to the majority opinion. For all males, this opinion was shared by at least 80% of observers.

.

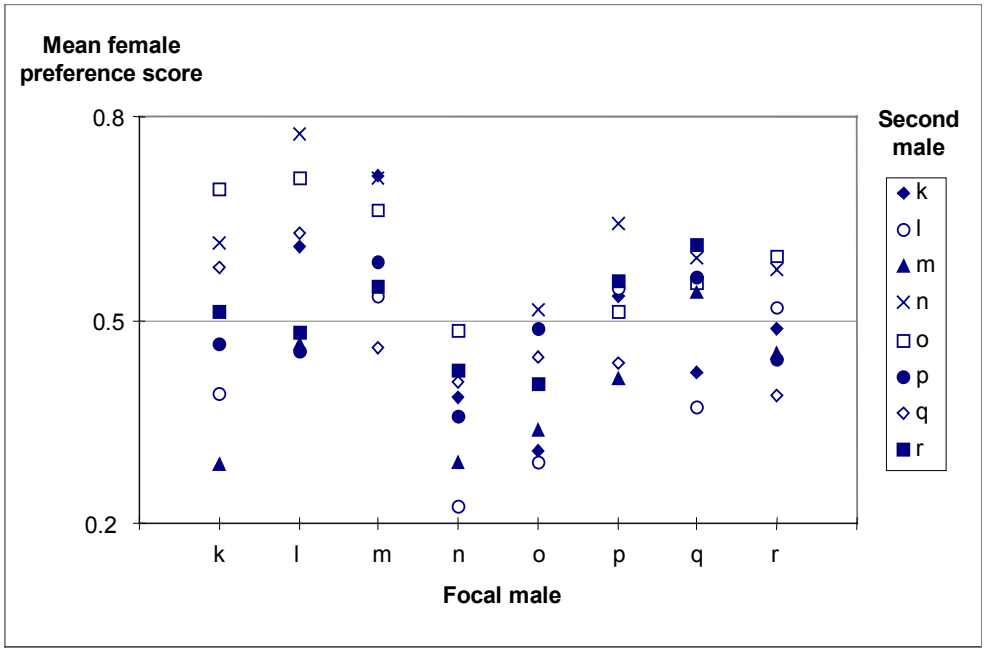


Figure 4.2. Mean female preference score over the three replicate trials for a focal male paired with each of the other seven males. A score of 0.5 indicates no overall preference for either male.

4.2.2. Results

Tests of Female Association Preference

During the female association trials, stimulus males typically spent much of their time swimming to and fro against the partition separating them from the test female. An approaching female elicited more active efforts to reach the test chamber and mirroring of her swimming against the partition. Males occasionally exhibited 'dashes' to and from the female and 'hangs' behind the partition although other elements of male courtship such as the circling displays were not observed in the test tanks. Females in the trials spent on average 29% of the trial associating with the stimulus males (range = 4.5% to 69%). The remaining time was most commonly spent swimming up and down the sides of the tank, foraging, or associating with the companion female (approximately 25% on average of total trial time).

For each trial I calculated a 'preference score' for each male, defined as the time spent associating with that male divided by the total time spent associating with both males. Figure 4.2 shows the mean preference score for each male over the three trials paired with each other male. For example, the mean preference score for male k over the three trials paired with male o was 0.7. It is immediately obvious that there is variation in success between individual males

with, for example, females nearly always spending less time on average with male n and more time with male k irrespective of the identity of the alternative male. The mean preference score for each male over the 21 trials in which he was involved was as follows:

Male	**m**	**l**	**q**	**p**	**k**	**r**	**o**	**n**
Mean	0.6	0.59	0.52	0.52	0.51	0.50	0.40	0.37

From these scores it is clear that two males, m and l, can be said to be 'more attractive' and two, o and n, to be 'less attractive'. However, the remaining four males have very similar mean preference scores and a ranking cannot reliably be resolved.

An alternative method for ranking the males is to take each pair-wise trial in turn and decide a 'winner' and 'loser', the winner being the male most preferred by the female. In these experiments a male was declared to have 'won' in a trial where the female spent more than 60% of her total shoaling time with him (i.e. preference score of over 0.6). Trials where the female did not spend more than 60% of her time with either male were declared a draw. Males were assigned a score of 2 for a win, 1 for a draw and 0 for a loss in each trial. The resulting score matrix is shown in Table 4.1; for example, as male k 'won' in two of three trials with male q a score of 4 is shown in row k and column q. Kendall (1955) describes a method for ranking competitors using this type of data which allows both an individual's total score and his performance against other 'high' or 'low' ranking individuals to be taken into account. This is done by repeatedly squaring the score matrix until a stable ranking is reached. For these data, the following stable ranking was reached at the third iteration:

Male	**m**	**l**	**p**	**r**	**q**	**k**	**o**	**n**
Rank	1	2	3	4	5	6	7	8

Spawning Latency Tests

Table 4.2 shows the number of seconds from 'lights on' to first spawning for the eight males in each of the 4 replicates: 'n' indicates that a spawning did not occur in the trial. Only four spawnings were observed in the 36 spawn latency trials performed, all in the first replicate. It is unclear why test fish were reluctant to spawn in subsequent trials. Although some elements of male courtship, in particular 'hang' were observed, the predominant behaviours that occurred in these trials were female-male and female-female aggressive interactions.

	k	l	m	n	o	p	q	r	Total
k	-	1	1	5	6	3	4	3	**23**
l	5	-	3	6	5	3	4	2	**28**
m	5	3	-	6	5	4	3	4	**30**
n	1	0	0	-	3	3	2	3	**12**
o	0	1	1	3	-	2	4	1	**12**
p	3	3	2	3	4	-	3	4	**22**
q	2	2	3	4	2	3	-	4	**20**
r	3	4	2	3	5	2	2	-	**21**

Table 4.1. Score matrix for the eight males used in the round-robin experiment. Focal male is shown in the left hand column.

	k	l	m	n	o	p	q	r
1	200	n	807	n	n	580	465	n
2	n	n	n	n	n	n	n	n
3	n	n	n	n	n	n	n	n
4	n	n	n	n	n	n	n	n

Table 4.2. Results of the spawning latency tests

Variable	Correlation coefficient	p
Standard length	-0.286	0.493
Anal fin–body length ratio	-0.190	0.651
P1 stripe width	0.119	0.779
P1 - P+1 ratio	0.381	0.352
Stripe-interstripe ratio	-0.190	0.651
B1 stripe width	-0.667	0.071
G1 stripe width	-0.286	0.493
Anal fin B1:G1 ratio	-0.476	0.233

Table 4.3. Correlation between male rank and morphological features

The paucity of successful spawnings means that male rank in the association tests cannot reliably be related to his spawning success. Although it may be of interest to note that the two males with the lowest ranking, o and n, also failed to achieve a spawning in the first round of trials, this was also the case with l, the second highest ranked male.

Morphology

Morphological measurements were tested against Kendall rank of the males using Spearman rank correlation. Results are shown in table 4.3: no significant correlations were found. Ranks of males with symmetrical and asymmetrical tail patterns were compared using a Mann-Whitney U test: symmetrical males had higher ranks ($U = 0$; $p = 0.025$), however this result is not significant if alpha levels are adjusted to take into account multiple testing (Bonferroni corrected $\alpha = 0.006$).

4.2.3. Discussion

The outcome of the round-robin trials hints at a role of male tail symmetry in influencing female association preference. Nevertheless, the results should be interpreted with caution. The experiment suffers from a low sample size of males and an influence of previous female experience with given stimulus individuals cannot be discounted. In addition, female association preference cannot be directly related to female mate preference due to a lack of spawnings in the spawning latency experiments. Several other methods were used in an attempt to relate association preference to spawning success in the course of this study (unpublished data), however none of these yielded better results.

The symmetry of morphological traits has received much attention recently as a potential 'good genes' indicator in mate choice (reviewed in e.g. Watson & Thornhill, 1994). While a preference for more symmetrical traits has been reported in many species (e.g. guppies, Sheridan & Pomiankowski, 1997; Brooks & Caithness, 1995; bower-holding cichlids *Cyathopharynx furcifer*, Karino, 1997), only a few studies have demonstrated that the symmetry itself, and not some correlated property of the individual bearing the trait, is the signal being used (Swaddle, 1999). In zebrafish, for example, bold individuals could develop more symmetrical tail patterns as a result of higher foraging success during tail pattern ontogeny, and also attract females by being more active in the test tanks. By manipulating the numbers of pigmented bars on the flanks of swordtails (*Xipophorus helleri*), Morris (1998) showed that

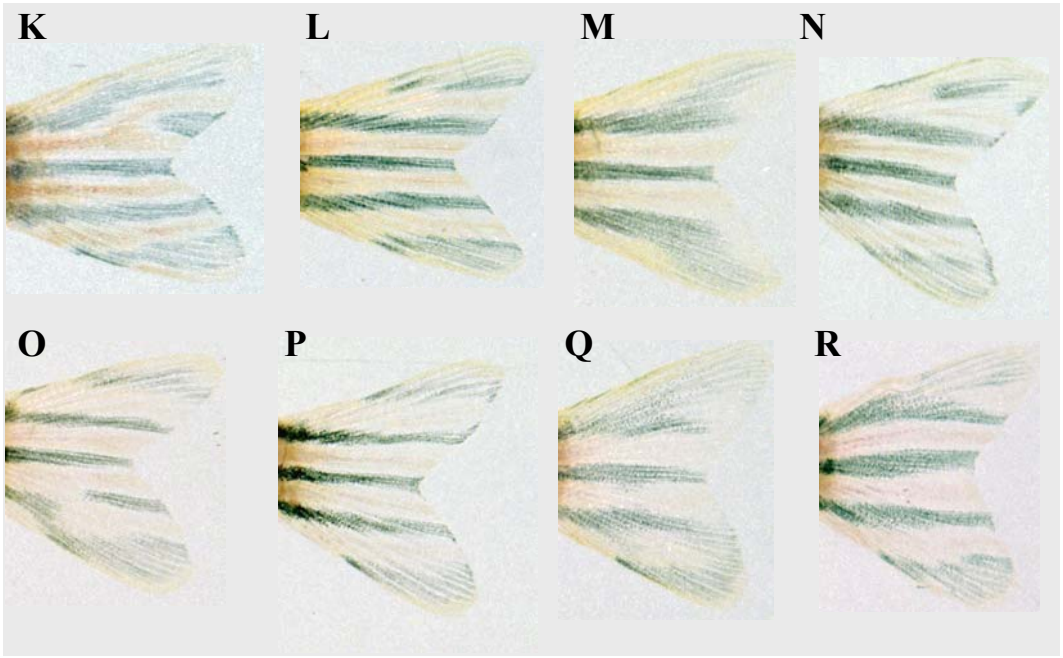


Figure 4.3. Tail patterns of the eight males used in the round-robin experiment. Males L, M & P were classified as having symmetrical tail patterns, the remaining individuals as having asymmetrical patterns.

females prefer to associate with males with the same number of bars on each side, whilst Schluter et al. (1998) found a similar preference in sailfin mollies presented with model males. Uetz & Smith (1999), using video stimuli, have shown a sexual preference for symmetrical foreleg ornaments in the wolf spider *Schizocosa ocreata,* and a potential signalling role of plumage pattern asymmetry has also been demonstrated in the zebra finch (e.g. Swaddle & Cuthill, 1994) and both male and female bluethroats *Luscinia svecica svecica* (Hansen et al., 1999; Fiske & Amundsen, 1997).

The tail pattern of the zebrafish is extremely variable, at least in the wild-derived fish used in our study (Figure 4.3) and asymmetry may therefore be a reliable indicator of developmental homeostasis as a result of 'good genes'. In addition, asymmetry of zebrafish tail pattern is likely to be perceptually easier to assess by a fish than the left-right bar asymmetry in swordtails and mollies (Swaddle, 1999). Assessment of tail symmetry, therefore, may well be important in mate choice in this species. This possibility requires further investigation in the form of more extensive mate choice trials and experiments presenting zebrafish with manipulated stimuli.

Although no other significant correlations of rank and morphology were found in this study, this could again be due to the limited sample size, and the potential involvement of several elements of zebrafish morphology in mate choice deserves more investigation. In particular, a role of carotenid pigmentation was not investigated in this study, due to the potential difficulties in accurately assessing colour (Endler, 1990). However, male zebrafish exhibit more orange coloration on their anal fins than females (Chapter 7), suggesting that, as in many other fish species, such coloration may play a role in mate choice. In the zebrafish there is also a potential role of signals in the UV range, as has recently been described for several species of bird (e.g. blue tits *Parus caeruleus,* Andersson et al., 1998).

4.3. Repeatability of female preferences

Female zebrafish have been observed to produce eggs daily in captivity (Westerfield, 1994) and may spawn with equal frequency over the reproductive season in the field. It is therefore of interest to know whether fish presented with the same individuals over successive spawning opportunities will retain a preference for one mate, or whether the preference will change as a result of previous experience. The inclusive fitness benefits to males of having multiple mates are well known, and male guppies have been shown to preferentially associate with unfamiliar females, presumably for this reason (Kelley et al., 1999). However, multiple mating is also common in females of many species, and in many cases this results in multiple paternity of offspring (e.g. Lake Malawi cichlids, Kellogg et al., 1998). The adaptive reasons for this phenomenon remain under debate (Jennions & Petrie, 1999).

4.3.1. Methods

Tests of female association preference were carried out in an identical manner to those described in section 4.2.1, with stimulus males presented at either end of the test chamber. The stimulus fish were ten wild-collected Santal males, different from those used in the previously described experiment. Ten wild-collected Santal females were used as the test and companion fish.

Each of the ten test females was presented with the same two test males and the same companion female for four consecutive trials. Each of these four replicate trials was performed

in a different test tank with the position of males swapped between replicates. Five females were tested once each day, so that there was a gap of 48 hours between replicate trials. All ten stimulus males were used on both days, so that each male was used as a stimulus fish for two different test females, but paired with a different male each time. A test female used on one day was used as a companion female on the following day, in a trial that did not involve her own companion female or either of her stimulus males.

4.3.2. Statistical analysis

Preference score for each male in each trial was calculated as previously described and arcsine transformed for statistical analyses. From the first set of replicates, a 'preferred male', was identified for each female, defined as the stimulus fish with which she spent the most time associating. The preference scores for these males were compared over the four replicates using a repeated-measures ANOVA to test whether replicate number had a systematic effect on female behaviour. Total time spent associating with either male over the four replicates was also investigated using a repeated measures ANOVA.

All data were checked for deviations from normality using the D'Agostino-Pearson Z^2 test (Zar, 1990) prior to statistical analysis, and data used in the repeated measures ANOVA were additionally checked for sphericity using Maunchly's W test. Statistical analyses were carried out using Microsoft Excel 97 and SPSS for Windows (Release 9.0.0).

4.3.3. Results

Total time spent associating with one or the other stimulus males is shown in figure 4.4. The time that females spent associating with males decreased over the four replicates: Repeated measures ANOVA: $F_{3, 27} = 3.451$, $p = 0.030$.

Of the ten test females, five preferred a male to the right of the test tank and five preferred a male to the left in the first replicate. Preference scores for the 'preferred male' over the four replicates are shown in figure 4.5. The repeated-measures ANOVA shows a significant effect of replicate on preference score: $F_{3, 27} = 3.979$, $p = 0.018$. It is clear that, on average, the amount of time a female spends with the 'preferred male' in replicate 1 declines in replicate 2 and continues to be lower in replicates 3 and 4.

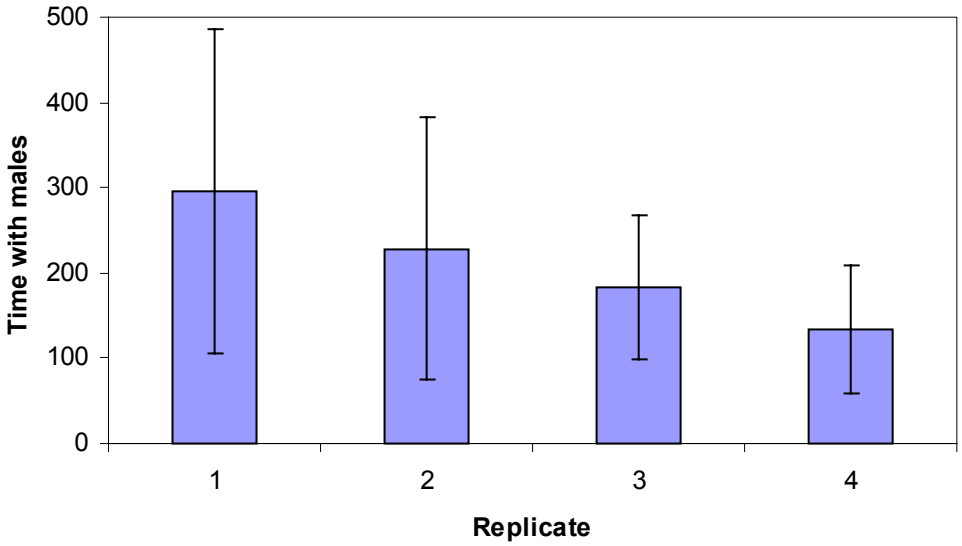


Figure 4.4. Time in seconds (mean ± standard deviation) spent by the test female associating with either male in the four replicate trials.

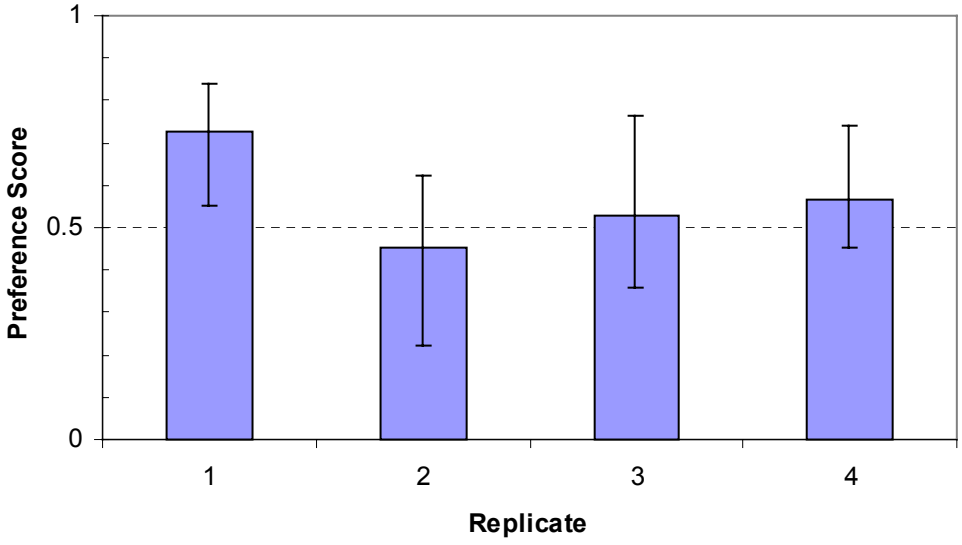


Figure 4.5. Preference score (mean ± standard deviation) for the male preferred in the first replicate over the four replicate trials.

4.3.4. Discussion

These experiments clearly show a systematic effect of replicate on the time that a female zebrafish spends associating with a male in the test situation: all females spent a smaller proportion of total association time with the 'preferred' male in replicate 2 than in replicate 1. Six out of the ten test females reversed their association preference, defined as the male with which they spent the greatest amount of shoaling time, between replicate 1 and replicate 2. This effect does not appear to be due to an overall side bias on the part of the test females as equal numbers of preferred males were on the left and right hand side: it could perhaps be explained by different females having different side biases but these do not appear to be retained in replicates 3 and 4 and are also likely to change when the test fish are moved between tanks. A more likely explanation is that a female's experience in one trial is influencing her behaviour in subsequent trials.

If female association preference within a trial does in fact correspond with her mate choice preference, then one possible explanation for these results is that females are selecting for paternal diversity of their offspring. Further examination of this question requires day-to-day observations of groups of zebrafish under more natural conditions. There are, however, several alternative explanations which involve the artificial trial situation rather than adaptive female choice. For example, the female may be remembering the position of the previously preferred male in the test apparatus. Alternatively, females may have learnt from one trial that they cannot reach one of the males and instead attempt to reach the other, or indiscriminately associate with males in the first trial as a reaction to the novel environment and only exhibit a mate preference in the second trial when they have become accustomed to the situation. The decline in total association time over the replicates supports both these possibilities. If the trial situation is indeed generating the observed results, then this has implications for the treatment of repeatability of female preference for specific male traits (e.g. coloration in guppies, Godin & Dugatkin, 1995), which is calculated using repeated trials with the same individual. Repeatability is often used as an estimate of the upper limit of heritability of female preferences (Boake, 1989). If the design of the trial generates a reversal of female preference where this would not occur in a natural situation, however, then the upper limit to heritability calculated from repeatability will in fact be an underestimate.

4.4. Dominance interactions between zebrafish.

As noted in section 4.1.6., fertilization patterns in fish are frequently influenced by male-male dominance interactions as well as female choice. In many species, the traits that are sexually selected by females also appear to be important in male-male competition (e.g. vertical bars on *Xiphoporus multilineatus,* Morris et al., 1995). However, this is by no means universal: in Atlantic salmon, for example, dominance between males appears to depend on relative size of the kype (an extension of the lower jaw), while females appear to select between males on the basis of adipose fin size (Järvi, 1990). Here I use the set of males previously used in the mate choice experiments to investigate whether males preferred by females are also more dominant, and whether this dominance can be related to morphological features of the individuals concerned.

4.4.1. Methods

The experimental apparatus was a tank 30cm x 15cm x 15cm high, filled with water at 24°C to a depth of 12cm over a gravel substrate. Sides and back of the test tank were covered in grey plastic and the tank was illuminated by means of a strip light as described in the previous experiments. Within the test tank were two transparent plastic cylinders, fixed together by a stick passed through their top ends so that they were 15cm apart, measured from their centres. These cylinders could be raised and lowered simultaneously by means of a remote pulley system.

Tests were undertaken as follows. A fish was introduced to each of the two cylinders within the test tank. Fish were allowed a five-minute period to acclimatise, during which time they could see their potential opponent. Cylinders were then raised and the males allowed to interact with each other in the aquarium.

Male behaviour was recorded using a Turbo Basic program running on an Amstrad PC1512. For each fight I recorded time to start of first fighting bout, total duration of all fight bouts, approximate number of bites and identity of winner. Number of bites performed by each individual and an accurate bite count could not be made due to the speed and frequency of biting in many fights. The two individual fish in each trial were distinguished from each other by natural variation in body and fin pattern.

As test fish I used the eight wild-collected Santal males that had previously been used in the female association preference tests (section 4.2). When not being used in tests, males were housed individually in 3 litre containers (stimulus chambers of the mate choice tanks) without visual access to other fish. Each male was pitched against each other male in a round-robin competition. A period of 48 hours was left between each test with the same male. Because the 'loser effect' has been shown to influence the performance of a contestant in subsequent trials (Beaugrand & Goulet, 2000), the majority of trials were arranged so that the two contestants had both either been winners or losers in their previous trial. Because not all of the male combinations could be performed if this rule was strictly adhered to, it was occasionally necessary to pit two males who had won and lost two days previously. Test fish were separated immediately one male won the contest to prevent continued harassment of the subordinate male. Although fighting between fish could occasionally be intense, with high frequencies of biting behaviour for an extended period of time, no obvious physical injury to any of the competing males was observed.

The round-robin trials were performed twice to assess the constancy of dominance status, with not less than 14 days between trials with the same two test fish. Dominance rank for males in each of the two rounds was calculated using the method of Kendall (1955) as described in section 4.4.2. Dominance rank was then compared to rank in mate choice tests and male morphology using Spearman Rank Correlation and the Mann-Whitney U test, again as previously described.

4.4.2. Results

Out of 56 trials, 38 resulted in a full antagonistic contest out of which one male emerged as the clear winner. In eight of the other trials, fights did not end in a clear victory, although one of the fish subsequently appeared dominant in six of these trials, chasing the other around the tank. In a further 7 trials one of the males was obviously submissive, and aggression by the dominant male, but no fighting behaviour, was observed. In the remaining three trials, male o attempted to court the second male and the trial was aborted. In trials where one male was obviously dominant to another this male was declared the 'winner'. In trials where there was no obvious dominance or where one male exhibited courtship behaviour then a draw was declared. For ranking purposes, a fish was assigned 0, 1, or 2 for a loss, draw or win respectively.

When fights occurred, they all started within 35 seconds of the fish being released from the cylinders, and commonly much sooner (mean time to fight = 7.3s). Total fight duration ranged from six seconds to just over 30 minutes (mean duration = 428s). Bites were observed in all but four of the fights, unsurprisingly, number of bites correlated with fight duration (Spearman's rho = 0.944; $p < 0.0001$).

Ranking for the first and second sets of trials differed, primarily because male n, from losing all fights in round 1, came back to win them in round 2. This male rarely became involved in fights in round 1, always being recorded as submissive, and his success in round 2 may reflect costs borne by the other individuals from engaging in repeated conflicts in round 1. Ranks for the two rounds, and overall rank calculated from all fight scores were as follows:

Rank	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**
Round 1	l	r	q	m	o	k	p	n
Round 2	n	l	q	m	k	r	p	o
Overall	**l**	**r**	**q**	**m**	**n**	**k**	**o**	**p**

All statistical results shown are calculated using the overall ranking from the two sets of round-robin trials, however, using the ranking from either the first or the second round does not affect the results. Spearman rank correlation coefficients and associated levels of significance are shown in Table 4.4. No significant correlation was found between male rank calculated from the female association preference trials and rank calculated from the male-male dominance trials. Neither did male dominance rank correspond with any of the morphological measurements investigated, including tail symmetry (Mann-Whitney U test: $U = 7$, $p = 0.88$).

4.4.3. Discussion

Antagonistic interactions between males in these trials began quickly and were often extended and violent. It is likely that aggression amongst males was increased as a result of their long-term isolation in their own chambers (Halperin & Danham, 1993). Similar trials using unfamiliar males and females kept in communal tanks (unpublished data) resulted less frequently in escalated fights, although antagonistic interactions were observed in nearly all cases. Nevertheless, a male's success in pairwise antagonistic interactions is expected to reflect

Variable	Correlation coefficient	p
Mate choice rank	0.381	0.352
Standard length	-0.143	0.736
Anal fin–body length ratio	-0.214	0.610
P1 stripe width	-0.500	0.207
P1 - P+1 ratio	0.214	0.610
Stripe-interstripe ratio	-0.214	0.610
B1 stripe width	-0.619	0.102
G1 stripe width	-0.214	0.610
Anal fin B1:G1 ratio	-0.571	0.139

Table 4.4. Correlations between overall male rank in the dominance trials and other variables measured.

his dominance rank in a communal situation. With the exception of changes in rank of males n and r, the dominance rank of the eight males was well matched over the two replicates.

Male rank in this experiment did not correlate with any of the morphological features measured, although again this study suffers from a small sample size. There is good reason to believe that stripe pattern on the body, and on dorsal, caudal and anal fins may be an important signalling trait used by both sexes in antagonistic interactions. An individual taking part in a fight expands its fins and darkens its melanophores, therefore emphasizing its striping pattern to a dramatic degree. If it loses the fight, this individual will fade its stripes to an intensity well below that seen normally.

One possible function for the greatly enhanced striping in zebrafish antagonistic interactions is as an amplifier, allowing opponents to gauge each other's size more accurately. Dark striping appears to have such a role in the pipefish (*Syngnathus typhle*, Berglund, 2000), a species in which males prefer to mate with larger females. In the case of the zebrafish the striping appears the emphasize the combined width of the body and expanded dorsal and anal fins. Since zebrafish lay down more body and anal fin stripes as they grow, large size differences are particularly obvious. However, perception of size differences in fish with equal stripe numbers may be influenced by factors such as relative stripe-interstripe width and stripe regularity. Additionally, there may be selection for increased size of the dorsal and anal fins as

this may result in the overall size of a fish being perceived as larger in an antagonistic interaction.

Both male and female zebrafish are expected to benefit from establishing a dominant position within their local population. Behavioural dominance in high-density zebrafish populations may determine survivorship by ensuring access to limited food resources. The observation of frequent inter-sexual fighting and female aggression towards courting males suggests that both inter and intrasexual dominance relationships may also influence fertilization patterns. A dominant female, for example, may be able to avoid sexual harassment from less dominant males and so exercise more active mate choice. This suggests the possibility that the evolution of the colour pattern in both males and females may be influenced by assortative fertilization and survival patterns as a result of a role of the stripe pattern in establishing dominance.

4.6. Why does the zebrafish have its stripes?

Contrasting stripes are a colour pattern frequently observed in nature, but the mechanisms driving the evolution of this pattern remain poorly understood. The well known stripe pattern of the zebrafish's equine namesake (*Equus* spp.), for example, has been variously ascribed a role in camouflage from predators or parasites, conspecific signalling and thermoregulation (Ortolani, 1999). This chapter has concentrated on a potential role of zebrafish coloration as a signal in mate choice and the establishment of dominance hierachies. Nevertheless, other functions of the zebrafish's striking pattern must be considered.

Several authors have proposed that vertical or horizontal stripes may act to camouflage an organism against certain backgrounds (e.g. Ortolani, 1999, carnivorous mammals), with Khoda & Watanabe (1988) going so far as to suggest that species of fish preferentially choose a background that matches the direction of their striping. This explanation is unlikely to hold for zebrafish which spend much of their time in open water, and fade the intensity of their stripes when hiding in cover (personal observation). Many shoaling fish species exhibit similar coloration, however, and it is possible that longitudinal striping, particularly in combination with iridescence as seen in the zebrafish (Chapter 7), increases the 'confusion effect' of a shoal (Landeau & Terborgh, 1986) and thereby reduces predation risk for the shoal members. Additionally, combinations of dark stripes and iridescent elements may communicate accurate information about a fish's position and movement to other members of the shoal (Denton &

Rowe, 1998). Interestingly, a number of predators of shoaling marine fish have longitudinal striping which appear to confuse their prey (Pitcher & Parrish, 1993). These potential roles of striping for shoaling fish have received surprisingly little attention in the literature. Seehausen et al. (1999) found a phylogenetic association of longitudinal melanophore striping in East African cichlids with both shoaling and predation on evasive prey, while horizontal striping was associated with complex habitats, where it may act to improve crypsis. Similarly, Hailman (1982) notes that certain species of coral reef fish may adjust their colour pattern according to their current behaviour, displaying longitudinal stripes when swimming and vertical bars when at rest. Such observations suggest that the various striped, barred and spotted colour patterns in the *Danio* genus may at least partly be adaptive to different habitat types. It seems likely that both behavioural and ecological factors have shaped the evolution of pigment patterns within *Danio*.

Chapter 5
Zebrafish Shoaling Preferences

5.1. Introduction

As discussed in Chapter 2, the occurrence of monsoon flooding throughout much of the zebrafish's range may permit mixing and gene exchange between populations that have been isolated during the dry season. A number of mechanisms including philopatry and assortative mating may, however, act to restrict this gene flow. In certain circumstances, shoaling preferences, for example for population-specific phenotypes, kin or familiar fish, could have a similar effect, depending on whether zebrafish tend to spawn within a shoaling group, or, as in the case of guppies, preferentially chose unfamiliar mates (Kelley et al., 2000).

Irrespective of a potential role of zebrafish shoaling preferences in regulating gene flow between populations, the question of the influence of familiarity, kin and population on fish shoaling preferences is important in its own right. Fish shoals have become a model system for investigating the functions and mechanisms of group living, and much work has been done on the role of association preferences in determining fish shoal composition and dynamics. However, these studies have so far been limited to very few species, and more are needed in order to assess the generality of the results so far obtained. In addition, investigations of shoaling behaviour in the zebrafish raise the possibility that genetic basis of different shoaling tendencies between different populations could be investigated.

5.1.1. Shoaling preferences for population

Fish have been shown to take a wide range of factors into account when choosing shoal associates, including species identity and intraspecific phenotypic characteristics (see Krause et al., 2000 for a review). Divergence between isolated populations in morphological or behavioural traits, irrespective of whether this reflects underlying genetic divergence, could lead to assortative shoaling if these populations again have the opportunity to mix. Many fish species exhibit a high degree of phenotypic plasticity (e.g. Pettersson & Bronmark, 1999: crucian carp *Carassius carrasius*; Robinson & Wilson, 1996: pumpkinseed sunfish *Lepomis gibbosus*; Robinson & Wilson, 1995: Trinidadian guppies), and morphological divergence between

populations experiencing different ecological conditions, even if these are only isolated for a short time, is therefore expected to be common. Intraspecific divergence in shoaling tendency and other elements of anti-predator behaviour between populations is also well documented (e.g. Brown & Warburton, 1997: rainbowfish, *Melanotaenia eachamensi*; Gelowitz et al., 1993: brook stickleback, *Culaea inconstans*; Mathis et al., 1993: fathead minnows, *Pimephales promelas*; Magurran & Seghers, 1991: guppies).

Investigations of the role of population in mediating shoaling preferences have focussed on salmonids, where preference for population-specific odours is suggested to have some role in mediating spawning migrations to natal streams (Nordeng 1971). Work by Quinn & Tolson (1986) and Courtenay et al. (1997) has shown that coho salmon (*Oncorhynchus kisutch*) may indeed recognise and preferentially respond to population specific odours, however, this response is not strong and can be altered by factors such as odour familiarity and relative odour concentration (Courtenay et al., 1997). Other studies have failed to control for the likely confounding effects of sibship (e.g. Folke et al., 1992) and familiarity (Groot et al., 1986) on odour preferences.

5.1.2. Shoaling preferences for familiars

Individual fish are, on average, expected to gain fitness advantages by remaining with a familiar group rather than joining an unfamiliar one, since they are able to make behavioural decisions to maximize their fitness based on their information about other group members. For example, the frequency and duration of antagonistic interactions is reduced in groups of familiar fish, probably as a result of individuals' knowledge of their companions' comparative dominance status (Höjesjö et al., 1998; O'Connor et al., 2000). Dugatkin & Wilson (1992) and Metcalfe & Thomson (1995) demonstrated that fish can remember the past foraging ability of shoalmates and may choose to associate with them based on this information. Furthermore, workers including Milinski et al. (1990) and Dugatkin & Alfieri (1991) have shown that fish may choose partners for risky predator inspection behaviour based upon past co-operation. It is also possible that familiarity within a shoal may enhance group anti-predator behaviour (Chivers et al., 1995).

Several authors have reported shoaling preferences for familiar fish in laboratory studies, and the idea that 'fish prefer familiars' is now a widely quoted tenet (e.g. Griffiths & Magurran, 1999). A closer examination of the literature, however, suggests that the role of familiarity in shoal choice is less well demonstrated than is often claimed. A number of studies have used fish

collected from the wild and designated as 'familiar' those individuals that are associating together when caught (Brown & Smith, 1994: fathead minnows; Brown & Colgan, 1986: bluegill sunfish *Lepomis macrochirus*; Griffiths, 1997: European minnows *Phoxinus phoxinus*; Griffiths & Magurran, 1998: Trinidadian guppies). An observed preference for these 'familiars' does not rule out the possibility that some factor other than familiarity may be mediating shoal choice both in the wild and the test environment. Indeed, a wide variety of other factors have been shown to influence shoal choice decisions (Krause et al., 2000), many of which are not explicitly controlled in these studies. In addition the duration of association of these 'familiar' fish prior to their capture is not known. Brown and Smith's (1994) observation that minnows preferred original shoalmates after three months separation is interpreted as evidence for long-term memory for familiars but would also be expected if another discrimination factor were being used. The results of such studies, however, do support the idea that fish shoals are long-term, stable associations and as such familiarity may be an important element of intra-shoal interactions even if it is not a direct cause of shoaling preferences. Fish do not always prefer the shoals in which they were collected: Griffiths and Magurran (1998) found a preference for shoalmates in female, but not male, Trinidadian guppies and Brown and Colgan (1986) found shoalmate preference in bluegill sunfish but not pumpkinseed sunfish, two closely related species with differing shoaling habits. Evidence from field studies for stable fish shoal composition remains scanty (Krause et al., 2000).

A more robust test involves generating familiar groups at random in a laboratory environment. Preference for familiars using this method has been demonstrated in bluegill sunfish (Dugatkin & Wilson, 1992), female Trinidadian guppies (Magurran et al., 1994; Griffiths & Magurran, 1997a; Lachlan et al., 1998), mixed-sex guppy fry (Warburton & Lees, 1996; Griffiths & Magurran, 1999), and threespine sticklebacks (Barber & Ruxton, 2000). In female guppies this preference gradually increases over a period of 12 days (Griffiths & Magurran, 1997a), suggesting that a certain degree of temporal shoal cohesion is required before preferences for familiars can become a factor mediating shoal choice.

The role of familiarity in mediating shoal choice is constrained by the ability of individuals to recognise familiars. Van Havre and Fitzgerald (1988) collected female sticklebacks from two large, temporarily separated, tidal pools and found that individuals preferred to associate with fish from their own pool. They attributed this result to a preference for familiar fish, an interpretation which requires sticklebacks to be able to identify several hundred fish as familiar. However, a study of wild guppies, temporarily separated in small riverbed pools for a similar length of time (Griffiths & Magurran, 1997b), suggested that the number of familiars a female guppy can recognise is much more limited, around 40 or less. It is

possible that these two species are using different criteria to recognise familiars in these experiments, with sticklebacks perhaps using a general cue such as a group odour. Cues involved in discriminating familiars have indeed been found to vary between species. Guppies are able to recognise familiars using either visual or olfactory cues (Griffiths & Magurran, 1999). Sticklebacks, however, cannot discriminate poolmates using visual cues alone (Van Havre & Fitzgerald, 1988) and in fathead minnows shoalmates seem to be recognised solely by smell (Brown & Smith, 1994).

5.1.3. Shoaling preferences for kin

In addition to the potential fitness benefits of shoaling with familiars, shoaling with kin may also be selectively advantageous, primarily as a result of inclusive fitness benefits (Hamilton, 1964). As shoal size increases, per-capita predation risk for shoal members is expected to decrease as a result of group vigilance and the 'confusion effect' (Landeau & Terborgh, 1986), and a shoal may also be able to find and access food resources more readily (Ryer & Olla, 1992; Chapman & Kramer, 1996). By joining and thus increasing the size of a shoal of relatives, therefore, a fish may increases both its direct and inclusive fitness. Additionally, if genetic homogeneity within a shoal translates into phenotypic homogeneity this may further enhance the confusion effect. Such phenotypic homogeneity is also expected to increase competition for food within a shoal although this may be reduced by individuals exercising 'competitive restraint' when competing with kin (Waldman, 1988). Certain studies, with fish and anurans, have shown enhanced growth rates of individuals in kin groups compared to those in non-kin groups (e.g. Brown & Brown, 1996b), however, this result is by no means universal (Blaustein & Waldman, 1992).

Investigation of kin discrimination in fish has mainly focused on the use of olfactory cues by young salmonids, specifically coho salmon, Arctic charr (*Salvelinus alpinus*), Atlantic salmon (*Salmo salar*) and rainbow trout (*Oncorhynchus mykiss*). Preference for the scent of sibs rather than non-sibs has been demonstrated in all four species (Olsén, 1989; Quinn & Busack, 1985; Brown & Brown, 1992). As test fish in these studies were reared in kin groups, these results may be due to preference for familiar kin-specific odours rather than an innate kin recognition mechanism. Quinn and Hara (1986) showed that coho salmon reared with sibs and non-sibs showed no discrimination between them. Furthermore, Winberg and Olsén (1992) showed that young Arctic charr reared in isolation did not show a preference for siblings over non-siblings. A recent study by Olsén et al. (1998), however, demonstrated that association preferences of Arctic charr are at least partly based on MHC genotype, suggesting that

assessment of genetic similarity may be playing a part in association decisions. Juveniles of all these species are territorial, and it appears that recognition of kinship at this stage may have a role in mediating territorial aggression. Nevertheless, it is possible that such cues also play a role in the shoaling preferences of salmonids of different species and life stages, for example during migration to the natal spawning grounds (Brown & Brown, 1996a).

Kin preferences have also been investigated in sticklebacks. Van Havre and Fitzgerald (1988) found that stickleback fry preferred to associate with sibs rather than non-sibs when given visual and olfactory cues. This preference was shown by individuals reared in isolation and those reared with non-sibs only, suggesting that sib recognition is innate. Fitzgerald and Morrissette (1992) also reported a preference for sibs, but in this study sibship appears to have been confounded with familiarity. More recently, Steck et al. (1999) found no preference for sibs in stickleback fry presented with olfactory cues alone. Arnold (2000) reports association preferences for kin in the rainbowfish, which vary depending on the sex of the test and stimulus fish. Griffiths & Magurran (1999), however, found no shoaling preference for kin in juvenile Trinidadian guppies.

Here I investigate the influence of familiarity, kinship and population on shoaling preferences in the zebrafish. I use the 'two way choice' method, which has been used in many studies of this type.

5.2. General Methods

The experimental apparatus (Figure 5.1) consisted of a glass test tank (60cm x 15cm x 15cm high) divided into three sections by partitions placed 15cm from each end. Within the central section I defined two 'preference zones' extending 5cm from each partition and marked using permanent pen on the walls of the tank. The test tank was filled with water to a depth of 12cm over a layer of gravel and the sides and back of the tank were covered with grey sticky-back plastic. A rigid transparent cylinder (8cm in diameter x 15cm high) with a rectangular opening 2cm x 12cm high was placed in the middle of the central section with the opening facing the front of the tank. Within this was a second cylinder which could be raised and lowered by means of a remote pulley system. The apparatus was illuminated by means of a 58 watt fluorescent strip light fixed on the wall 25cm above the tank and extending across all

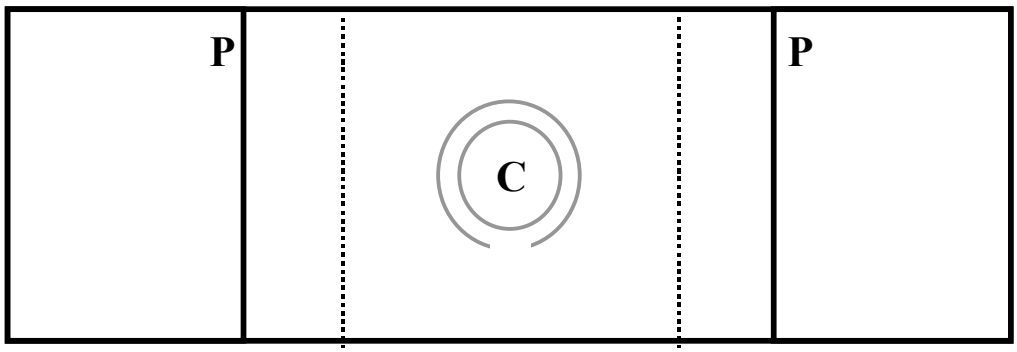


Figure 5.1. Test tank for shoaling preference experiments, showing position of the partitions separating stimulus and test fish (**P**) and placement of the transparent cylinders (**C**) into which the test fish is introduced. Boundaries of preference zones are indicated by the dotted lines.

three sections; light levels were reduced by fixing dark green paper inside the cover of the strip light. Four of these test tanks were arranged adjacent to each other.

All fish were fed in their holding tanks immediately before the start of a day's trials. Due to problems with separating sex I used mixed-sex groups in these experiments. Although morphological differences between the sexes are described in section 6.2.1, these are difficult to observe in non-anaesthetized fish and separation of each tank into male and female groups for the duration of the trials was precluded by space considerations. Differences in shoaling preferences of males and females has been found in guppies and sticklebacks (Griffiths & Magurran, 1998; Van Havre & Fitzgerald, 1988), however, similar studies using other cyprinid species (e.g. Brown & Smith, 1994) have not made any attempt to control for sex. No trials were performed within three hours of 'lights on' in the stockroom in order to avoid any influence of sexual behaviour on the results, and no courtship behaviour was observed in any of the trials. Stimulus and test fish within a trial were carefully size-matched by eye, a method that was considered to be equally or more accurate than measuring the fish with the additional advantage of minimizing stress to the experimental subjects. In cases where there was doubt about the accuracy of size matching, a second opinion was obtained from an independent observer.

Experiments were undertaken as follows. Stimulus shoals of 5 fish were introduced into each of the two outermost compartments and allowed to settle for at least an hour. A size-matched test fish was then introduced into the inner of the two cylinders. Following a 5 minute

acclimation period the inner cylinder was carefully raised and the test fish allowed to leave the outer cylinder by means of the hole. This procedure ensured that the test fish was not facing towards either shoal at the time of its release. Data recording began immediately the test fish left the cylinder and continued for a 10 minute period. All data were logged using a Turbo Basic program running on an Amstrad PC1512, which enabled an observer to record each time a fish's head crossed a line delineating a preference zone. Data was recorded in real time by the observer sitting in front of the tank; main lights in the room were turned off to minimize observer conspicuousness to the fish and fish did not seem disturbed by my presence. For each trial I recorded the identity of the first shoal visited by the fish after it left the cylinder and the time spent in each preference zone during the 10-minute period.

Individuals were never used as test fish more than once in a set of experiments, and were placed in a 'used test fish' tank following the trial. Stimulus fish were selected at random, given size-matching constraints, from the holding tanks and returned after use. Stimulus fish could be re-used as test fish, and test fish were sometime re-used as stimulus fish by selecting a stimulus group from the 'used test fish' tank. Occasionally in a trial test or stimulus fish showed fright reactions such as freezing or predator evasion manoeuvres. When this was observed the trial was aborted and re-run using different individuals. Additionally, although most test fish left the outer cylinder within 30 seconds of the inner cylinder being raised, a few took much longer to encounter the exit hole. A test was aborted when a fish had failed to leave the outer cylinder after 10 minutes.

For each trial, '*total shoaling time*' was defined as the total time spent by the test fish in either preference zone. The '*preference score*' for a given shoal was then calculated as time spent in that shoal's preference zone divided by total shoaling time. All proportional data were arcsine square-root transformed, and all data checked for deviations from normality using the D'Agostino-Pearson K^2 test (Zar, 1996), prior to statistical analysis. Statistical tests were performed using Microsoft Excel 97 and SPSS for Windows 9.0.0. All tests are two-tailed.

In a number of trials, test fish visited only one shoal. Although this can be interpreted as a shoaling decision made while viewing both test shoals from the cylinder, Griffiths & Magurran (1999) suggested that test fish in this situation may not be aware of the alternative shoal and therefore excluded these results from their analysis. In population preference experiments, trials where test fish failed to visit each shoal at least once were discarded and the trial repeated with a new test fish. On further consideration, this procedure was considered to be unjustified and therefore in all other sets of experiments, results from trials where only one shoal was visited

were retained. Statistical analyses were then performed both with these trials included and these trials excluded to investigate whether this had any influence on the outcome.

5.3. Preference for population

5.3.1. Methods

Association preference tests

The experimental subjects were first generation offspring of fish collected from Nepal and Tangail. For each population I used stimulus fish from four tanks, each containing 20-25 individuals. Fish in these tanks were the offspring of group spawnings and had previously been mixed several times, therefore they were considered to be equally related to each other. Tanks of Tangail fish had been separated visually and olfactorily from each other for a minimum of eight months, however Nepal fish had been separated for only four months.

Tangail test fish were taken from the same four tanks that provided the stimulus fish, with fish from different tanks being considered unfamiliar. Since the four Nepal groups had been separated for a shorter period of time, however, Nepal test fish were taken from tanks housing the families in the North Carolina II breeding scheme that had never previously encountered the stimulus fish. A single test fish was taken from each of 30 tanks and returned after use. Tangail test fish had previously had visual access to fish from Nepal although never those individuals used in the experiments. No Nepal test fish had had previous experience of any Tangail fish.

For each experiment two size-matched stimulus shoals, one from each population, were placed at either end of the test tank. A trial was performed with a test fish from one of the populations that was unfamiliar with either stimulus shoal. Following a break of at least 45 minutes, the trial was repeated with a test fish from the alternative population. Test tank partitions were made of glass fixed in place with silicon sealant, such that all test fish in the trials had access to visual cues only. Tangail and Nepal test fish were used first in the trials an equal number of times. Each of the four tanks of Nepal and Tangail stimulus fish were used in as many different combinations as size-matching allowed, with side of population alternated between each pair of trials.

1	**2**	**3**		**3**	**2**	**1**
4	**5**	**6**	**Test Chamber**	**6**	**5**	**4**
7	**8**	**9**		**9**	**8**	**7**

Figure 5.2. Numbering of grid squares on stimulus chambers of test tank.

Trials where the test fish was from Nepal and those where the test fish was from Tangail were analysed separately. Arcsine transformed preference scores of the test fish for its own population were compared to the null expectation of 50% of time spent with each stimulus shoal using a one-sample t-test. Identity of first shoal visited was investigated by comparing the results to the null expectation using binomial tests.

Population differences in behaviour of stimulus shoals

Preliminary observations suggested that Nepal and Tangail fish might exhibit different behaviour in the stimulus chambers that might influence shoaling preferences. This was investigated further by looking at the positions of stimulus fish from different populations within the test tanks. Size-matched shoals of five fish each from Nepal and Tangail were introduced into the stimulus chambers of a test tank, as in a normal experiment, such that they had visual access to each other across the test chamber. No test fish was present. The front of the stimulus chamber as viewed by the observer was marked with a 3 x 3 grid of squares so that the position of each stimulus fish could be described. Squares were numbered as shown in figure 5.2. Following a settling period of 1 hour, the behaviour of shoals in each stimulus chamber was recorded for a 15 minute period using a Panasonic S-88 S-VHS video camera. No observer was present in the room while videos were being recorded. Since the process of setting up the video camera potentially disturbed the fish, behaviour was scored for only the final 10 minutes of each 15 minute video. Each video was played back using a Panasonic NV-HS 900B video recorder attached to a Hantarex monitor and the number of fish in each of the nine grid squares was noted at 20 second intervals. A fish was considered to be within a grid square when the major part of its head was present in that square.

Fifteen shoals were recorded from each population, five each from three of the four tanks containing the stimulus fish. Each population was placed on the left and right hand side of the test tank an approximately equal number of times, and all four test tanks were used. For each shoal, mean number of fish in each grid square was calculated by dividing the cumulative number of fish recorded in that square by 30, the total number of observations. Following visual examination of the data, observations from multiple grid squares were combined so that vertical and horizontal distributions of the fish could be examined seperately. For investigation of vertical distribution the tank was divided into 'top' (grid squares 1,2 and 3), 'middle' (squares 4, 5 and 6) and 'bottom' (squares 8, 9 and 10). For horizontal distribution it was divided into 'near' (squares 3, 6 and 9), 'mid' (squares 2, 5 and 8) and 'far' (squares 1, 4 and 7). Data were analysed using ANOVA, with 'tank' nested within 'population'. Tank was included as a factor since potential differences in behaviour between tanks, for example because fish are accustomed to different light intensities, could create the impression of a population-level difference if tank results were pooled.

5.3.2. Results

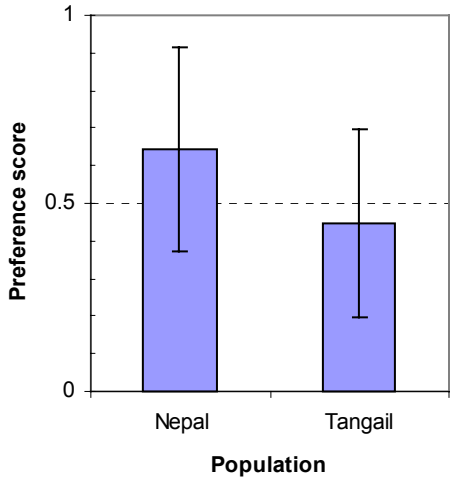
Association preference tests

Nepal and Tangail fish did not differ in the total time they spent associating with either stimulus shoal (Total shoaling time (mean ± SD): Nepal: 452.0 ± 89.8s; Tangail: 465.1 ± 80.1s; $t_{58} = 0.598$, $p = 0.550$). Test fish from Nepal exhibited a significant association preference for fish from their own population ($t_{29} = 2.975$, $p = 0.006$). Fish from Tangail did not exhibit any significant association preference, however, there was a trend for them to spend more time associating with the stimulus fish from Nepal ($t_{29} = -1.202$, $p = 0.239$). Comparing preference scores of Nepal and Tangail test fish for their own population showed a significant difference ($t_{58} = 3.035$, $p = 0.004$), and Tangail test fish did not prefer Nepal shoals significantly less than did Nepal test fish ($t_{58} = 1.461$, $p = 0.149$; Figure 5.3).

Binomial tests showed no significant influence of population on identity of first shoal visited (Nepal: $p = 0.86$, N.S.; Tangail: $p = 0.1$, N.S.), although Tangail test fish visited Tangail first twice as often as they visited Nepal.

Figure 5.3.

Preference score (mean ± standard deviation) of test fish for stimulus fish from their own population. A score of 0.5 indicates no preference for either shoal.



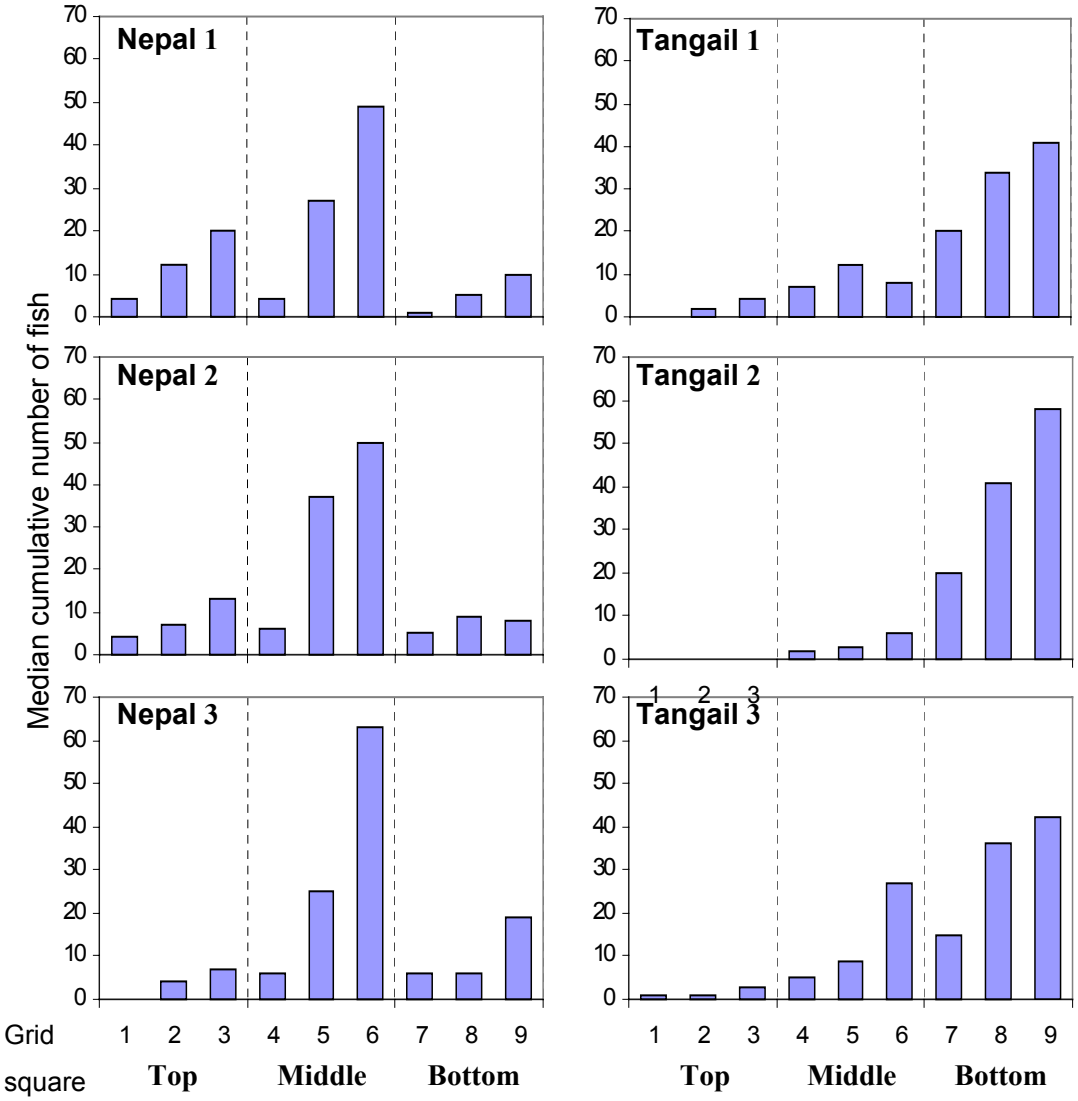


Figure 5.4. Position of fish in the stimulus chamber over the 10 minute observation period for two test populations. Squares 3, 6 and 9 are those nearest the alternative stimulus shoal.

Population differences in behaviour of stimulus shoals

Figure 5.4 shows the median cumulative number of fish in each grid square for each tank from each population. It is clear that there is a consistent difference between the two populations in the behaviour of stimulus fish within the test tank. There is a significant influence of population on numbers of fish in the top, middle and bottom sections of the water column. While Nepal fish tend to spend their time in mid-water, fish from Tangail remain near the bottom of the tank (ANOVA: Top: population: $F_{1, 24} = 11.54$, $p = 0.002$; tank $F_{4, 24} = 2.78$, $p = 0.05$. Middle: population: $F_{1, 24} = 39.38$, $p < 0.001$; tank: $F_{4, 24} = 0.99$, $p = 0.43$. Bottom: population: $F_{1, 24} = 52.4$, $p < 0.001$, tank $F_{4, 24} = 1.22$, $p = 0.33$). Examination of the graphs suggests that both populations also spend more time on the side of the stimulus chamber facing the alternative shoal. There is, however, some population influence on number of fish in the side of the chamber furthest from the alternative shoal (ANOVA: Near: population: $F_{1, 24} = 4.34$,$p = 0.11$; tank: $F_{4, 24} = 0.503$, $p = 0.62$. Mid: population: $F_{1, 24} = 0.04$, $p = 0.86$; tank: $F_{4, 24} = 0.56$, $p = 0.70$. Far: population: $F_{1, 24} = 23.14$, $p = 0.009$; tank $F_{4, 24} = 0.27$, $p = 0.90$), suggesting that Tangail stimulus fish may spend more time further from the partition than Nepal stimulus fish.

5.4. Preference for familiar fish

5.4.1. Methods

The experimental subjects were first generation offspring of fish collected from Nepal and Tangail. For each population I again used experimental fish from four tanks, each containing a group of 20-25 individuals which were considered to be equally related to each other. Each group had been separated visually and olfactorily from the other groups for a minimum of six months, with the exception of short-term visual encounters with the other groups in population choice trials two months previously. Fish from the same tank were designated as ‘familiar’ and those from different tanks ‘unfamiliar’. Test and stimulus fish were used from each of the four tanks in approximately equal numbers.

Additionally for the Nepal stock I investigated preference for familiar and unfamiliar siblings. I used eight full-sib families that had been divided into two groups (‘a’ and ‘b’) at the egg stage. Each tank contained 30-35 fish. Siblings from the same tank were designated as ‘familiar’ and those from different tanks ‘unfamiliar’.

For each experiment two stimulus shoals unfamiliar to each other were placed at either end of the test tank. A trial was then performed with a test fish familiar with one of the shoals. Following a break of at least 45 minutes, the trial was repeated with a test fish familiar with the alternative shoal.

This study investigated shoaling preferences under two different types of cue availability:

Visual cues only: Test tank partitions were made of glass fixed in place with silicon sealant. This prevented any exchange of olfactory cues between test and stimulus fish. Test tanks were emptied and rinsed at the end of every day.

Visual and olfactory cues: Test tank partitions were made of transparent Perspex into which were drilled forty 1.5mm diameter holes. Partitions were held in place by slots on the walls of the tank. After the removal of each pair of stimulus shoals, test tanks and partitions were rinsed several times with hot water, wiped with a mixture of ethanol and ethanoic acid, rinsed again and allowed to dry overnight in order to remove any remaining olfactory cues before being used for the next experiment.

5.4.2. Statistical analysis

For each pair of trials using the same two stimulus shoals I designated one of the shoals as the '*reference shoal*'. For this reference shoal there are two preference scores, one where the test fish is familiar with the shoal and one where it is unfamiliar. I compared these two preference scores over all trials using a paired t-test to investigate whether test fish familiar with the reference shoal spent more shoaling time with it than test fish unfamiliar with the reference shoal. Using this approach enabled me to investigate the influence of familiarity on association preference whilst controlling for potential overall differences in the attractiveness of alternative stimulus shoals. Since the preference scores for the two alternative shoals by definition add up to unity, the identity of the shoal selected as the 'reference shoal' does not alter the results.

Following any non-significant t-test result, I performed a power test (Zar, 1996) to ask what difference would be required between time spent with a shoal when it was familiar and when it was unfamiliar in order for the paired t-test to have a 95% probability of detecting it ($\beta = 0.05$). This is termed the 'minimum detectable difference' (δ).

Identity of first shoal visited was investigated by treating each trial as a separate replicate and comparing the results to the null expectation using binomial tests.

5.4.3. Results

Visual cues only

There was no significant deviation from the null expectation of equal time spent with a reference shoal when the test fish was familiar and when it was unfamiliar (Figure 5.5: Nepal: $t_{19} = -1.179$; $p = 0.253$; Tangail: $t_{17} = 0.559$, $p = 0.292$; Nepal Sibs: $t_{14} = 0.122$, $p = 0.905$). Removing trials where the test fish visited only one shoal did not substantially alter these results: (Nepal: $t_{18} = 0.983$; $p = 0.338$; Tangail: $t_{15} = 0.474$, $p = 0.642$; Nepal Sibs: $t_{10} = -0.437$, $p = 0.671$). As there is no a-priori reason to suspect that zebrafish from different populations should differ in their response to familiar fish, I pooled all results to examine whether zebrafish in general exhibit a shoaling preference. Again, there was no influence of familiarity on time spent with the reference shoal. (All data: $t_{52} = 1.039$; $p = 0.303$; 'One shoal only' removed: $t_{45} = 0.551$; $p = 0.585$; Figure 5.5). The minimum detectable difference between mean preference score for the reference shoal with familiar and unfamiliar test fish was 0.265. This was not substantially changed where the trials where only one shoal was visited were removed ($\delta = 0.276$).

There was no significant deviation from the null expectation of first shoal visited being independent of familiarity (Binomial tests: Nepal: $p = 0.635$, N.S.; Tangail: $p = 0.868$, N.S.; Nepal sibs: $p = 0.584$, N.S.; Pooled data: $p = 0.627$, N.S.).

Visual and olfactory cues

Again, no significant influence of familiarity on association preference was found for any of the groups tested (Nepal: $t_{19} = -1.289$, $p = 0.213$; Tangail: $t_{19} = 0.274$; $p = 0.787$; Nepal Sibs: $t_{14} = 1.26$, $p = 0.227$). Removing the pairs of trials where only a single shoal was visited did not change these results: (Nepal: $t_{15} = -0.405$; $p = 0.691$; Tangail: $t_{17} = 0.487$, $p = 0.632$; Nepal Sibs: $t_{9} = 0.412$, $p = 0.690$). Neither did pooling the data to investigate the response of zebrafish in general to familiar and unfamiliar fish: All data: $t_{54} = 0.225$; $p = 0.823$; One shoal only removed: $t_{44} = -0.147$; $P = 0.883$.

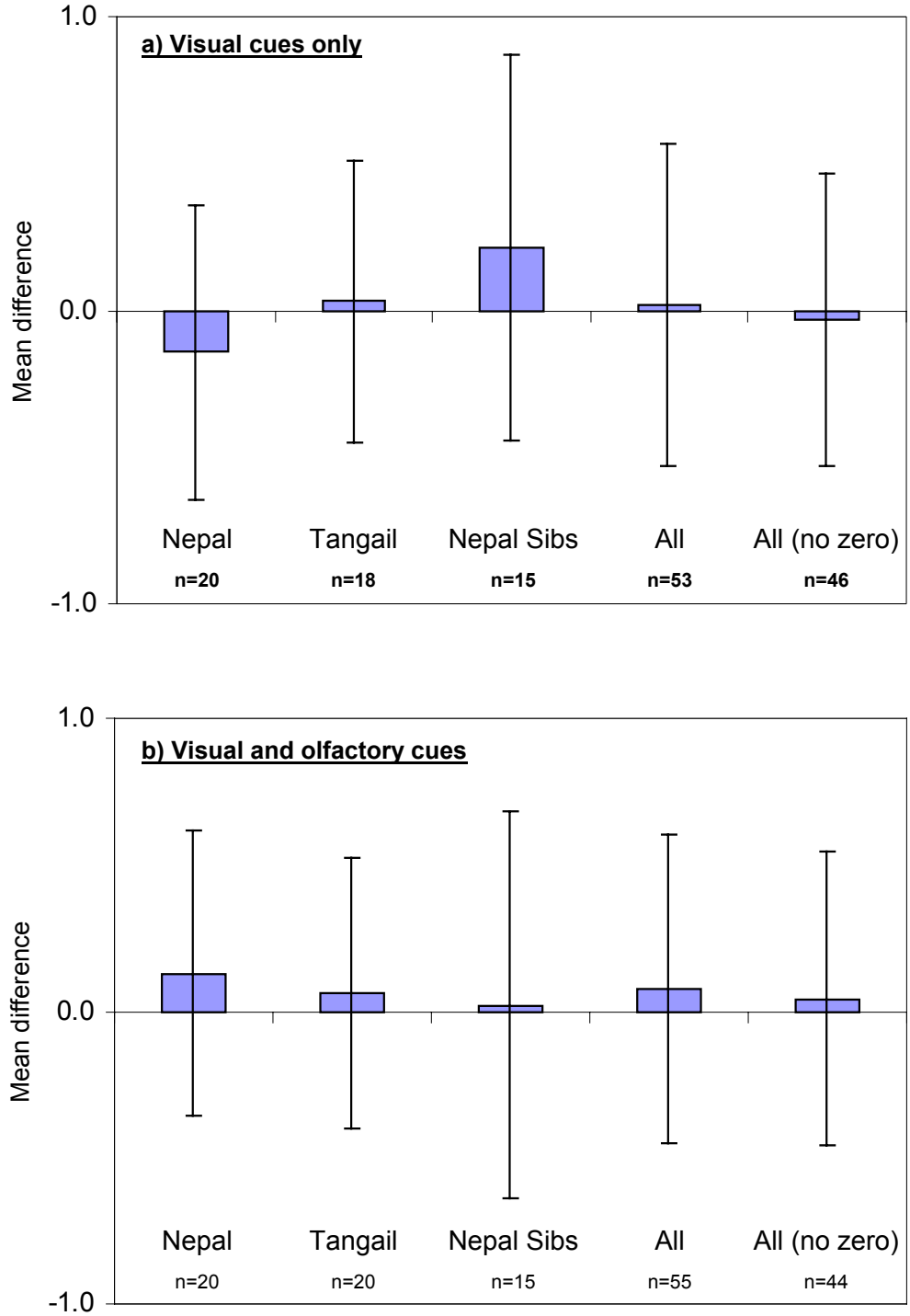


Figure 5.5. Difference (mean ± standard deviation) between familiar and unfamiliar fish in preference score for the reference shoal. A positive value indicates that a familiar fish spends more time with the reference shoal than an unfamiliar fish. 'All (no zero)' shows the results of the pooled trials with experiments where the test fish visited one shoal only removed.

Minimum detectable difference for the pooled data was, for all data, $\delta = 0.272$, and for the data with pairs of trials where only one shoal was visited removed, $\delta = 0.269$**.**

There was no influence of familiarity on the identity of the first shoal visited (Binomial tests: Nepal: $p = 1.00$, N.S.; Tangail: $p = 0.268$, N.S.; Nepal sibs: $p = 0.855$, N.S.; Pooled data: $p = 0.634$, N.S.).

5.5. Preference for siblings

5.5.1. Methods

The experimental subjects were zebrafish from 5 full-sib families generated by pair-wise spawnings of zebrafish collected in Nepal. Each family comprised two groups ('a' and 'b') of 30-35 fish each that had been separated as eggs and reared in isolation from each other. An individual fish therefore had both familiar and unfamiliar siblings. Fish from different families may differ in their degree of preference for siblings, and in their 'attractiveness' to the test fish as a result of genetic factors. I addressed this question by keeping one of the families, 'f4m2', constant over all trials and pairing it with each of the other four families.

For each experiment size-matched stimulus shoals were selected from two families and introduced to the test tank. A trial was performed using a non-familiar sibling of one of the stimulus shoals as the test fish. Following a gap of at least 45 minutes, the trial was repeated using an unfamiliar sibling of the other stimulus shoal. All experiments were performed with the fish having access to visual cues only.

A total of 128 trials were performed, comprising 16 pairs of trials with each of the four alternative families. Therefore there were in total 64 trials with a fish from f4m2 as the test fish and 16 trials each with test fish from the 4 other families. Each possible combination of 'a' and 'b' groups was used an equal number of times. Results from the 64 trials with f4m2 and 64 trials with the other test fish were analysed separately.

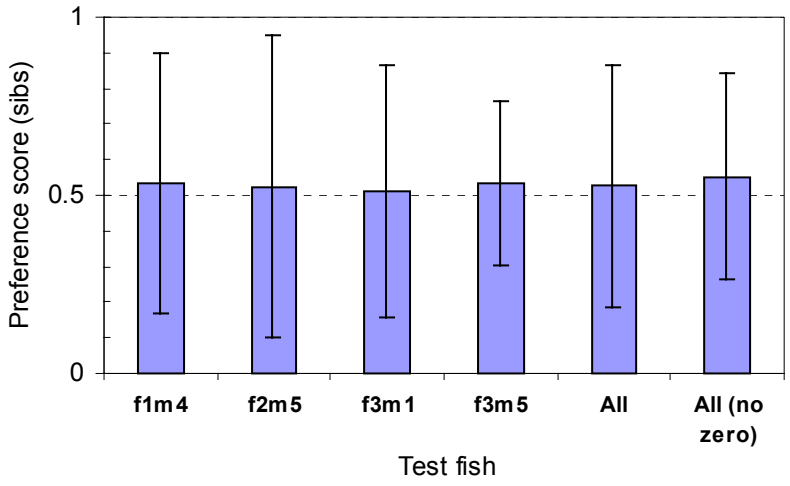


Figure 5.6 (a). Preference score (mean ± standard deviation) of fish from four different families for the sibling shoal in the two-way choice experiments. 'All (no zero)' shows the pooled results from the four families with trials where the test fish visited only one shoal removed.

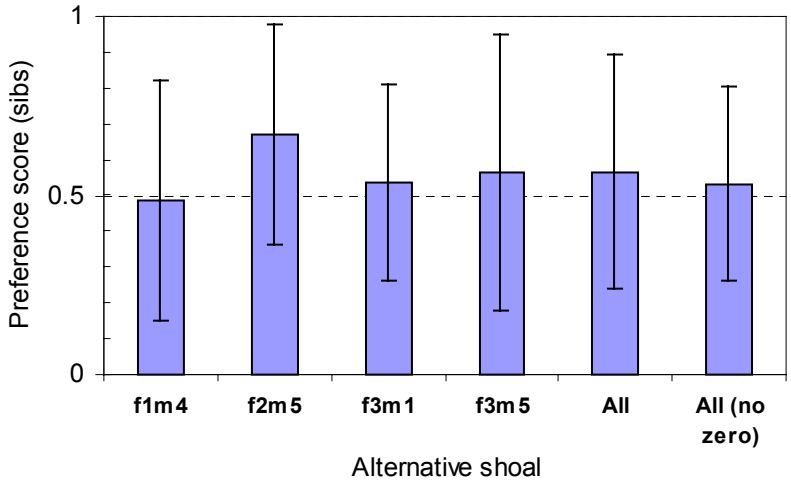


Figure 5.6 (b). Preference score (mean ± standard deviation) of fish from family f4m2 for the sibling shoal, given four different families as the alternative stimulus. 'All (no zero)' shows the pooled results of experiments with the four alternative families with trials where the test fish visited only one shoal removed.

5.5.2. Results

i) Do test fish from four different families differ in their response to siblings when the alternative family ('f2m4') is held constant?

A Levene's test revealed significant between-family heterogeneity in variance of time spent with sibs (Levene statistic = 5.599, p = 0.002), which was investigated further by plotting histograms. This suggested that there were differences between families in their reaction to the test apparatus. In particular, the distribution of preference scores for both f3m5a and f3m5b was approximately normal whereas both f2m5a and f2m5b spent the majority of their time either with one shoal or another. The similarity of the two family groups in each case suggests that this may be a genetic, rather than an environmental effect, perhaps indicating an elevated threat sensitivity in family f2m5 compared to f3m5. Differences of this kind between families deserve further investigation.

Given the non-normality of parts of the data set I tested for differences between the families in the amount of time spent with the related shoal using a Kruskal-Wallis test. This showed that families did not significantly vary in the proportion of time spent with the sibling shoal ($H_2 = 0.056$, p = 0.997) (Figure 5.6a).

ii) Does the family identity of fish in the alternative shoal influence preference for siblings in the f4m2 test fish?

Test fish from the reference family, f4m2, did not vary in their response to siblings according to the identity of the alternative shoal (Kruskal Wallis test: $H_2 = 2.833$, p = 0.418, Fig. 5.6b).

Given these results I pooled the data from all of these tests to ask the question: is there an overall preference for siblings in these experiments? The data were treated as previously described in section 5.4.2, with a paired-sample t-test performed to investigate whether there was a difference in shoaling response of a test fish depending whether the reference shoal consisted of siblings or non-siblings. Fish did no show any significant shoaling preferences: difference in preference score (mean ± SD) = 0.091 ± 0.449; $t_{63} = 1.518$, p = 0.134. Removing trials where the test fish visited only one shoal did not change these results: difference (mean ± SD) = 0.110 ± 0.419; $t_{44} = 1.511$, p = 0.138. A power analysis showed the minimum detectable difference with $\beta = 0.05$ to be 0.283. With trials where only a single shoal was visited removed, this remained very similar at $\delta = 0.292$.

5.6. Discussion

In this study I found a significant association preference of Nepal fish for unfamiliar fish from their own population when provided with visual cues. Tangail fish did not exhibit a significant shoaling preference but also tended to spend more time with Nepalese fish. These results may be largely explained by differences in behaviour of stimulus fish from the two populations. Nepalese fish tended to spend more time in the mid-water column and near the partition next to the test chamber, and might therefore be more conspicuous and attractive to the test fish. The consistency of this pattern irrespective of which stock tank the stimulus fish were taken from suggests that it reflects a genetic difference between the two populations in their reaction to the test tank situation. Such differences may perhaps have been shaped by different predation regimes at the collection sites. It may be notable that, while the parents of the Nepal fish were derived from an extremely shallow habitat, those from Tangail were collected from a much deeper pond, where movement towards the bottom is more likely to be an effective defense against predators such as wading or diving birds.

Although Nepal stimulus fish are expected to be more attractive to the test fish than those from Tangail, the preference of Tangail test fish for Nepal was less strong than that of Nepal fish. In addition, Tangail fish showed a non-significant tendency to approach their own population first in the trials. These results suggest that, despite the attractiveness of the Nepal shoal, some degree of population assortment may be occurring, with Tangail fish being more attractive to other Tangail individuals than they are to those from Nepal. This effect may have been more significant if Tangail fish had not been reared in visual contact with fish from Nepal.

No evidence of an association preference by the zebrafish for either familiar fish or kin was found in this study. In the experiments investigating the influence of familiarity, this result was unchanged whether the test fish had access to visual cues alone or both visual and olfactory cues. In experiments investigating the influence of kinship, only visual cues were presented to the test fish. Lack of a significant preference using a statistical test does not, however, mean that an association preference for either familiars or siblings does not exist in zebrafish. In order to address this question, the power of the test also has to be investigated, for example by calculating the minimum difference detectable with a sufficient level of probability given the data and statistical test used. This minimum difference can then be compared with the results of other studies investigating the same problem.

For the trials investigating association preference of zebrafish for familiars, providing visual cues alone and visual and olfactory cues together, minimum detectable differences calculated using arcsine-transformed data were 0.265 and 0.272 respectively. In both cases this means that, despite the relatively large sample size, the paired t-test will only detect with 95% probability an effect analogous to 64% or more of total shoaling time spent with the familiar shoal. Griffiths & Magurran (1999) estimate that guppies in familiarity experiments spend between 59-65% of total association time with the familiar fish. In this study, therefore, I could only detect with confidence a preference for familiars near the upper end of that seen in guppies. The low power of the t-test is due to the large variances seen in the data set. Zebrafish frequently spent nearly all of a trial with one or the other shoal, independent of its familiarity or kinship, visiting the other shoal only briefly. This effect may indicate some reluctance to cross the open area of the tank to associate with the alternative shoal. Shoaling preferences for familiars in zebrafish need to be further investigated, for example using the 'nearest neighbour' approach described in Barber & Ruxton (2000), before any influence of familiarity on shoaling preference can be ruled out. Nevertheless, it is clear that, if a shoaling preference for familiars does occur in zebrafish, it is not particularly strong and may be unlikely to lead to strong shoal fidelity in the wild.

Minimum detectable difference in the sib preference experiments was 0.283, which again translates to approximately 64% of time spent with siblings. Van Havre & Fitzgerald (1988), investigating sibling stickleback fry under various treatments, found that test fish spent on average 80% of their total shoaling time with siblings. Arnold (2000) did a similar experiment with female rainbowfish and found that the median proportion of time spent with full sibs rather than non-sibs was 75%. If zebrafish have a preference for siblings, it is not as strong as that found in these experiments. It must be pointed out, however, that all studies investigating shoaling preference for siblings have provided fish with olfactory cues rather than, or in addition to, visual ones. Although it is quite possible that sib recognition can occur by visual cues alone, the absence of trials providing olfactory cues to the test fish is a failing of this study and should be addressed.

In conclusion, this study provided evidence of population assortative shoaling behaviour in the zebrafish, probably as a result of behavioural differences between the two populations used that may reflect genetic-level adaptations to different predation regimes. However neither familiarity or sibship, factors which have been demonstrated as important in other species, have a strong influence on zebrafish shoaling preferences. This question nevertheless deserves further investigation using different experimental techniques before a role for these factors in structuring zebrafish shoals can be completely ruled out.

Chapter 6
Zebrafish morphology: sex and population differences

Divergence between populations in the traits and preferences involved in mate choice is a necessary step towards the establishment of ethological isolation. Such divergence can in theory result from a number of processes, including sexual selection acting in different directions in different populations, adaptation of the sexual signal-response system for improved communication under differing environmental conditions and genetic drift in populations isolated in allopatry (Otte & Endler, 1989).

Inter-population variation in signals involved in mate choice has frequently been documented (e.g. Houde & Endler, 1990: Trinidadian guppies; Claridge & Morgan, 1994: planthoppers *Nilparvata bakeri;* Ryan et al., 1996: tungara frogs *Physalaemus pustulosus*; Uy & Borgia, 2000: bowerbirds *Amblyornis inornatus*) and in many cases this may generate assortative mating (e.g. Maksymovitch & Verrell, 1993: salamanders *Desmognathus santeetlah*, but see Magurran, 1998: guppies). Study of the factors driving such divergence, and of the genetic basis of the traits and preferences involved, has the potential to provide major insights into how speciation via ethological isolation might proceed. This approach has several advantages over the alternative of comparing closely related species. Although inter-species comparisons have shed much light on the speciation process (e.g. Coyne & Orr, 1989, 1997), it is difficult in these cases to distinguish traits that may have contributed to the establishment of sexual isolation from those which have diverged subsequently. Additionally, genetic analysis of trait differences between species is frequently complicated by problems such as the sterility of hybrid offspring (e.g. Coyne & Orr, 1989, but see Stratton & Uetz, 1986).

Examination of divergence between populations in the signal-response systems involved in mate choice requires that the signals involved first be identified. When attempting to identify morphological traits that may be important in mate choice in a species, traits exhibiting some degree of sexual dimorphism provide an obvious starting point. Documentation of sexual dimorphism in a species, however weak, is therefore a useful step. Identification of sexual dimorphism in a species is also important from a practical point of view, since accurate discrimination of males and females is vital when investigating mate preferences in a laboratory setting.

This chapter investigates variation in morphology and colour pattern of wild-collected zebrafish deriving from four different collection sites, and their laboratory-reared offspring. I

examine differences between males and females in order to identify sexually dimorphic traits that are potentially important for mate choice. I also investigate geographical divergence in these and other traits.

6.1. Methods

Wild fish were collected from Nepal and Bangladesh as described in Chapter 2. Laboratory-reared fish deriving from the Bangladesh populations were obtained from group spawnings involving the following minimum numbers of wild-collected fish: Canal: 4 females, 11 males; Santal: 24 females, 20 males; Tangail: 9 females, 4 males. Fry obtained from these group spawnings were mixed several times in the first few months of life and subsequently reared in four separate tanks per population, with density standardised to 25 or fewer fish per tank. A sample of wild fish from Nepal was used to generate the families in the North Carolina II breeding scheme (Chapters 3 & 7). Due to the great disparity in sample size, data from these laboratory reared Nepalese families are not included in statistical tests in this chapter, although results of morphometric analyses of these fish are included in figures for qualitative comparison with the other lab-reared populations. Relevant statistical results from the North Carolina II fish are presented in Chapter 7.

All fish were photographed and all morphological measurements taken as described in Chapter 3. Age of wild-collected fish at time of photography was at least 12 months. Laboratory reared fish were photographed at an approximate age of 12-18 months. All data were checked for normality and homogeneity of variance where applicable, and transformations applied as described in the relevant section. All statistical analyses were performed within SPSS for Windows (Release 9.0.0). In all ANOVAs, 'sex' was defined as a fixed factor and 'population' and 'tank' as random factors.

6.1.1. Sexual dimorphism in the zebrafish

As previously noted, the zebrafish is not strongly sexually dimorphic. Males are generally described as 'more yellow' and females 'plumper' and 'duller' (Westerfield, 1994), however in practice these distinctions are difficult to apply, especially when comparing non-breeding fish. A single note of sexual dimorphism in tail shape in one Indian population (Ansari & Kumar, 1982) appears to be spurious. I used qualitative scores of morphology in wild-collected and lab-reared fish from all populations in an attempt to identify combinations of

morphological features that could easily be used to discriminate male and female fish. The reliability of these features for sexing purposes was confirmed by examining their expression in those fish whose sex was already known as a result of behavioural observations.

6.1.2 Repeatability of measurements

Thirty four individual fish (10 wild-collected and 24 from one of the families in the North Carolina II breeding design) were photographed twice each, with a gap of several months between duplicate photographs. These photographs were assigned random file numbers and included with all others in the morphometric analysis. Measurements from the duplicate fish were then compared to investigate repeatability of morphometric measurements between photographs and over time. Repeatabilities were calculated following Lessells & Boag (1987).

6.1.3. Body shape and fin measurements

Fish body measurements were taken as described in Chapter 3. For the purpose of this study, 'body shape' measurements, defined in section 3.4.1 as measurements I - IX and 'fin' measurements, defined in section 3.4.1 as measurements X - XII, were analysed separately. This approach was taken because a number of laboratory-reared fish exhibited skeletal deformities, a problem that has been noted by other workers (Ferreri et al., 2000), and body shape analysis was therefore performed on wild-collected fish only.

All body shape measurements were significantly correlated with standard length (Pearson correlations using untransformed data: $n = 92$; width: $r = 0.94$; nose-dorsal fin: $r = 0.97$; dorsal fin base $r = 0.77$; dorsal fin-tail: $r = 0.86$; caudal peduncal: $r = 0.91$; tail-anal fin: $r = 0.684$, anal fin-nose: $r = 0.972$; all $p < 0.001$). The 'curve-fit' function within SPSS confirmed that this relationship could best be explained by a linear regression in all cases. In order to control for varying fish sizes, therefore, all body shape measurements were standardized by dividing them by fish standard length.

The seven standardized body shape measurements listed above were included in a Principal Component Analysis with varimax rotation to investigate whether there were any differences between sexes or between populations in overall body shape. Investigation of difference between the sexes is a necessary first step to ensure that these do not mask population differences in the analysis. Apparent separation of groups along principle component axes was

further investigated by performing a stepwise discriminant function analysis, to find out whether elements of body shape could reliably be used to assign fish to specific groups.

Anal and caudal fin measurements were analysed for both wild-collected and laboratory-reared fish. A small number of individuals exhibited minor fin abnormalities as a result of fungal infection, apparent fight injuries or developmental effects and were therefore excluded from the analysis. Zebrafish fins are known to exhibit isometric growth (Iovine & Johnson, 2000) and, as expected, fin size was linearly related to standard length (Wild fish: anal fin length: $r_{90} = 0.673$; tail 1: $r_{90} = 0.817$; tail 2: $r_{90} = 0.673$; Lab-reared fish: anal fin; $r_{118} = 0.62$; tail 1: $r_{140} = 0.73$; tail 2: $r_{144} = 0.77$. All $p < 0.001$). All analyses were therefore performed on fin measurements expressed as a proportion of standard length. I also calculated a third measurement of caudal fin shape, 'tail 3' , defined as the ratio of tail 1 to tail 2 and hence a measure of caudal fin fork depth. I used this ratio to test for possible sex differences in tail fork depth as reported by Ansari & Kumar (1982). Effects of population and sex on fin measurements were investigated using ANOVA.

6.1.4. Body stripe measurements

In a proportion of both wild-collected and laboratory-reared individuals body melanocytes were sparse, and in some they were lost altogether. Other individuals exhibited 'blotchy' stripe appearance (Chapter 3). Such reduced melanophore density does not appear to be developmental in origin, as several individuals that were photographed twice showed reductions in melanocyte numbers between the two photographs. In some cases this phenomenon may be the result of tissue damage from the cold water treatment, however it was also noted in fish that had not previously been photographed. Loss of melanocytes in this way means that measurement of the width of the body stripes is unreliable, therefore fish recorded as having 'sparse' or 'very sparse' melanocytes were excluded from body stripe analysis. I also excluded fish that had breaks, bends or other irregularities in their melanocyte stripes where measurement was to be taken. Fish excluded from stripe analysis included all individuals in one of the four rearing tanks of the Santal population.

Body stripes were measured along a reference line drawn between the dorsal and anal fin insertion, as described in Chapter 3. Although this provides a measurement at a standardized point on a fish's body, inter-individual differences in body shape can result in this line passing at a different angle across the stripes and so influence stripe width measurements. I controlled for this problem by performing all analyses on the ratio of stripe P to interstripe 1. This provides

a measure of the relative widths of melanophore and xanthophore stripes on the body, a character which is known to be affected in the developmental mutants *ase* and *obe* (Chapter 7) and is additionally altered in *Danio kerri*, an apparent close relative of the zebrafish. Widths of xanthophore and melanophore stripes are developmentally interdependent (Chapter 7). Stripe-interstripe ratio showed no consistent relationship with fish standard length (Pearson correlation: wild Canal: $r_{13} = 0.08$, $p = 0.80$; wild Nepal: $r_{26} = 0.13$, $p = 0.54$; wild Santal: $r_{36} = 0.26$, $p = 0.13$; wild Tangail: $r_{13} = 0.54$, $p = 0.06$; lab-reared Canal: $r_{44} = -0.33$, $p = 0.03$; lab-reared Santal: $r_{34} = 0.20$, $p = 0.26$; lab-reared Tangail: $r_{43} = 0.20$, $p = 0.20$). Data were $\log_{10}$ transformed prior to analysis to correct for a left-skewed distribution in some populations.

Additionally, I investigated differences between populations in the ratio of width of stripe P to stripe P+1, which is greatly increased in another of the zebrafish's close relatives, *Danio nigrofasciatus*. Again this was uncorrelated with body size for most populations (Pearson correlations: wild Canal: $r_{13} = -0.444$, $p = 0.13$; wild Nepal: $r_{26} = -0.24$, $p = 0.23$; wild Santal: $r_{36} = -0.33$, $p = 0.05$; wild Tangail: $r_{13} = -0.265$, $p = 0.382$; lab-reared Canal: $r_{44} = 0.16$, $p = 0.29$; lab-reared Santal: $r_{34} = -0.03$, $p = 0.86$; lab-reared Tangail: $r_{43} = 0.20$, $p = 0.23$). Analysis of the P/P+1 ratio was performed using untransformed data.

6.1.5. Anal fin stripe measurements

In several fish, anal fin striping was irregular or broken, with stripes frequently merging into each other. Those fish in which anal fin stripe at ray 5 was not a reliable representation of overall stripe width were excluded from the analysis. Several fish also had blurry fins on the photograph due to fish movement and were also excluded.

Differences in anal fin striping, as with body stripes, were investigated by calculating the ratio of the width of the blue 1 (B1) and gold 1 (G1) stripes. This ratio was again independent of fish standard length (Pearson correlations: wild Canal: $r_{12} = -0.023$, $p = 0.94$; wild Nepal: $r_{24} = 0.098$, $p = 0.65$; wild Santal: $r_{40} = 0.382$, $p = 0.02$; wild Tangail: $r_{13} = -0.083$, $p = 0.787$; lab-reared Canal: $r_{41} = 0.11$, $p = 0.51$; lab-reared Santal: $r_{36} = -0.3$, $p = 0.23$; lab-reared Tangail: $r_{46} = -0.03$, $p = 0.84$). Since some populations exhibited a left-skewed distribution, data were square-root transformed prior to analysis. ANOVA was used to investigate the influence of sex, population and rearing tank on the blue-gold ratio.

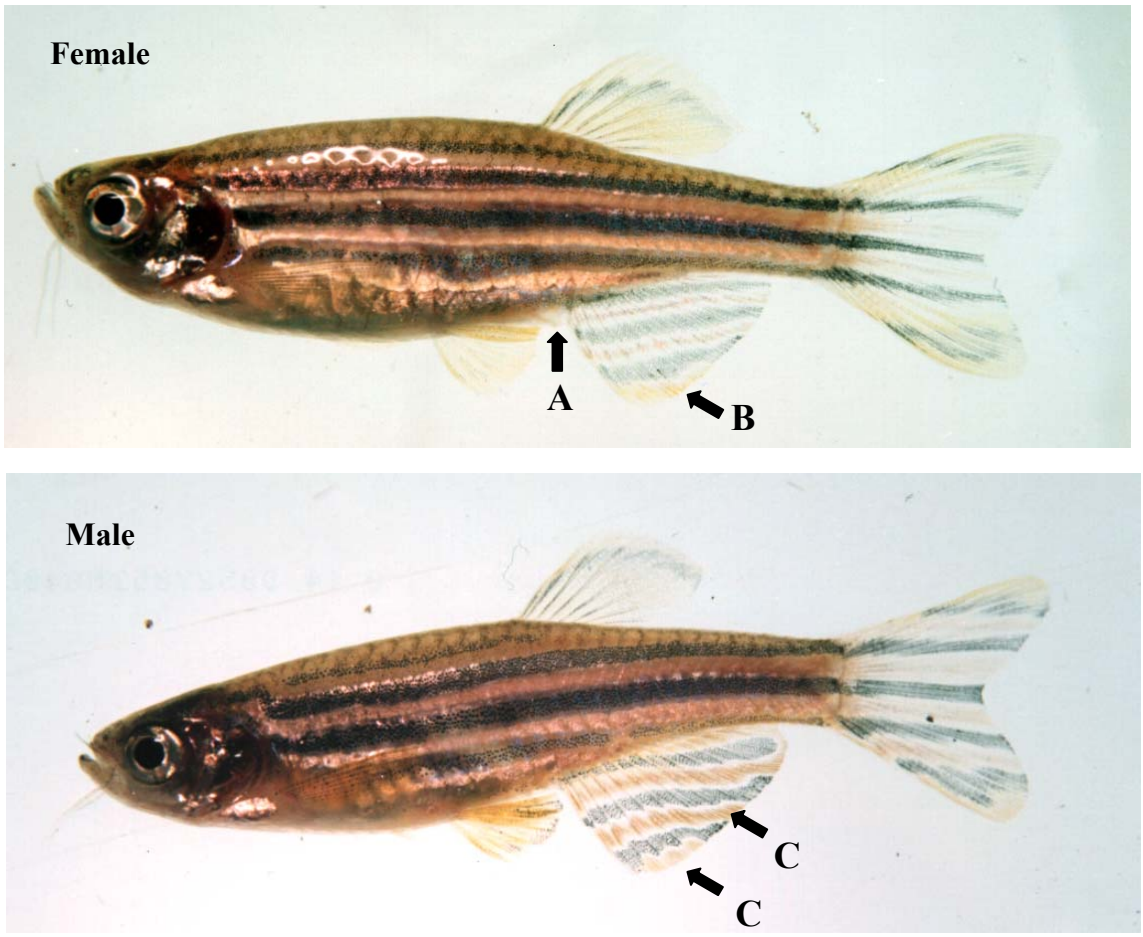


Figure 6.1. Morphological features used to separate male and female zebrafish

Measurement	n	r	Measurement	n	r
body width	34	**0.90**	tail1	34	**0.84**
nose - dorsal fin	34	**0.92**	tail2	34	**0.83**
dorsal fin base	34	**0.58**	anal fin length	34	**0.81**
dorsal fin - tail	34	**0.72**	P / P+1 ratio	27	**0.68**
caudal peduncle	34	**0.88**	stripe-interstripe ratio ($\log_{10}$ transformed)	27	**0.87**
tail - anal fin	34	**0.72**			
anal fin base	34	**0.62**	Blue-gold ratio (square-root transformed)	33	**0.81**
anal fin - nose	34	**0.86**			

Table 6.1. Repeatability of measurements used in morphometric analysis

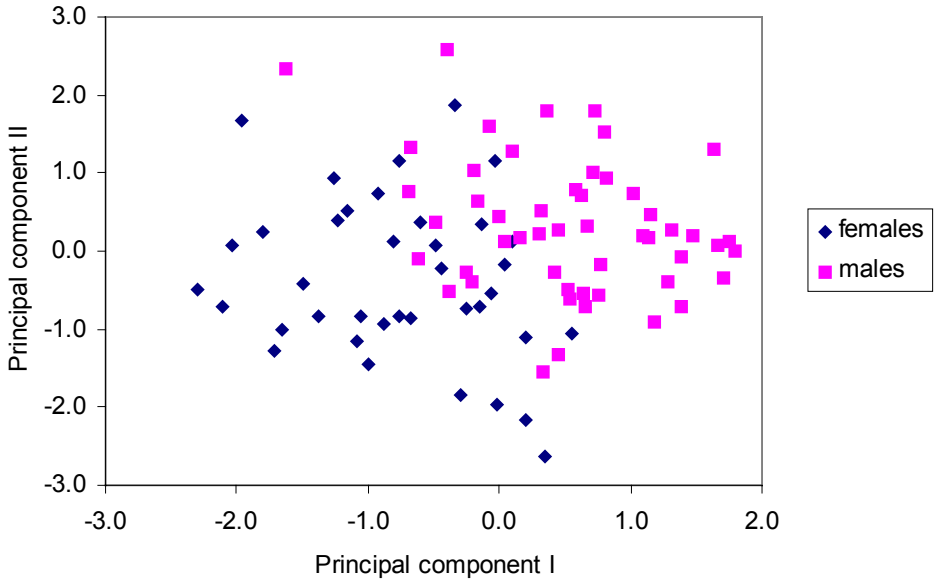


Figure 6.2. Plot of first two principal components derived from analysis of body shape measurements.

6.2. Results

6.2.1. Sexual dimorphism in the zebrafish

Examination of photographs of wild-caught and lab-reared fish in this study revealed a combination of morphological features that appeared to reliably discriminate males and females. These are shown in Fig 6.1 and are as follows:

A Presence of conspicuous cloacal aperture in female

B Pure yellow coloration on extreme of female anal fin

C Orange coloration throughout male anal fin.

6.2.2. Repeatability

Of 34 fish used, seven individuals showed clear melanocyte loss between the two photographs and were excluded from the body stripe analysis. A single individual showed interruptions to the stripe pattern on the anal fin. Repeatabilities are shown in Table 6.1.

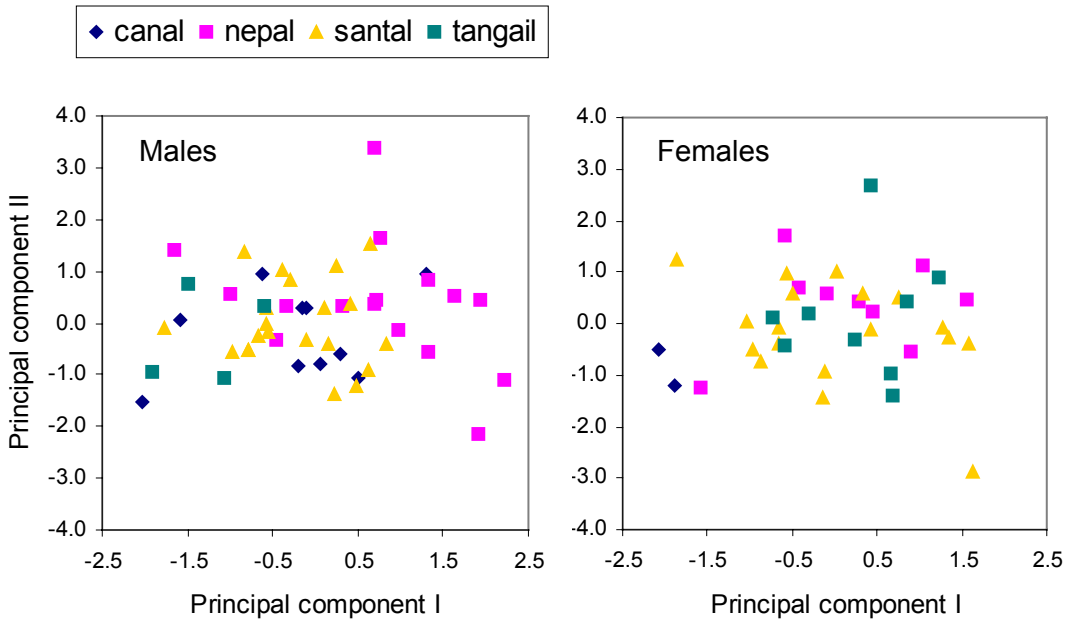


Figure 6.3. Plot of first two principal components derived from analysis of body measurements for males and females separately.

6.2.3. Body shape and fin length

Figure 6.2 shows the plot of the first two principal components calculated from the eight body shape measurements used for all wild-collected fish (n = 90). Sex of these fish was assigned using the combination of morphological features described in section 6.2.1. Weightings of the rotated component matrix are as follows:

	body width	nose - dorsal fin	dorsal fin base	dorsal fin - tail	caudal peduncal	tail - anal fin	anal fin base	anal fin-nose
PC1	**0.097**	**0.868**	**-0.143**	**-0.693**	**-0.010**	**-0.541**	**-0.329**	**0.844**
PC2	**0.853**	**-0.127**	**0.211**	**-0.036**	**0.846**	**-0.232**	**0.076**	**0.223**

It is clear that there is some separation of males and females, principally along PC I. A stepwise discriminant function analysis using 'sex' as the classifying group showed that a function involving just three elements of shape, distance from nose to anterior insertion of anal fin, width of anal fin base and width of caudal peduncle, could classify 94.5% of individuals to the correct sex.

Given the body shape difference between the sexes, further principal components analyses to investigate shape differences between populations were performed on males and females separately. Populations did not separate along any of the four principal component axes tested. Plots of PC I against PC II are provided as an example (Figure 6.3).

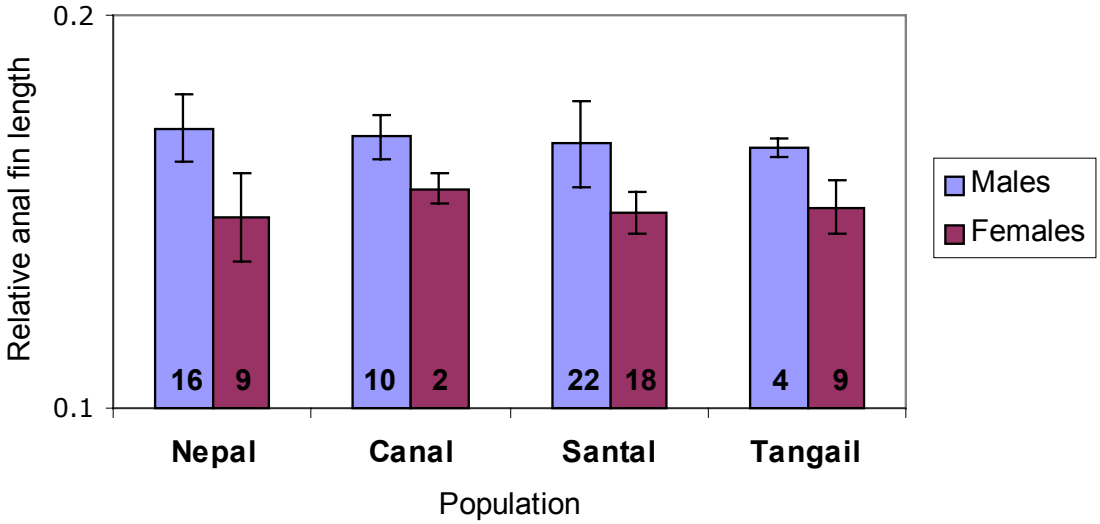


Figure 6.4.a. Relative anal fin length (mean ± standard deviation) for wild male and female zebrafish collected from different localities. Number of fish is indicated on each bar.

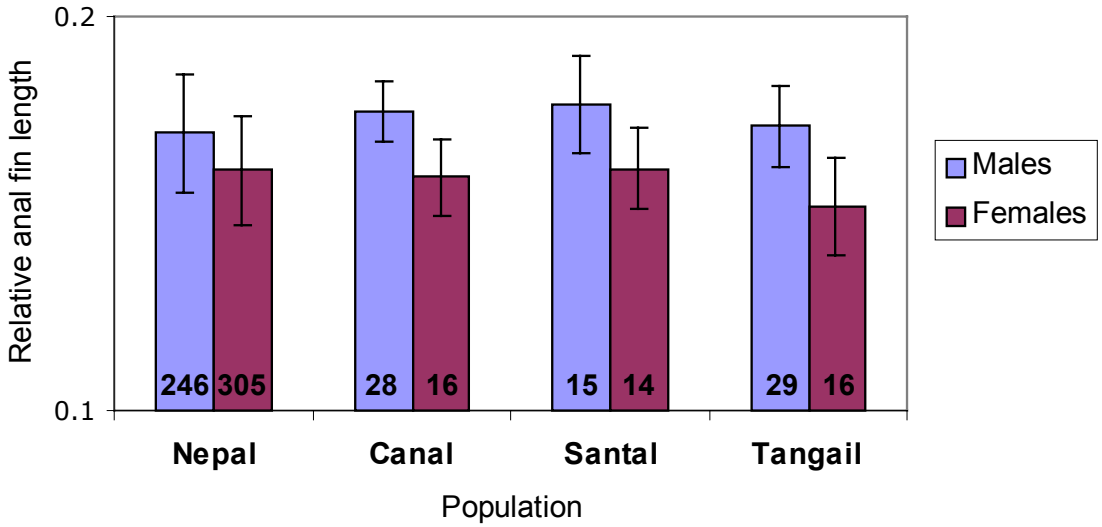


Figure 6.4.b. Relative anal fin length (mean ± standard deviation) for male and female laboratory-reared zebrafish deriving from different localities. Number of fish is indicated on each bar.

For both wild and laboratory-reared fish, relative anal fin length was significantly affected by sex (Figure 6.4). In all cases, males had longer anal fins relative to their standard length than did females (ANOVA: Wild fish: sex: $F_{1, 5.98} = 60.47$, $p < 0.001$; population: $F_{3, 3} = 0.59$, $p = 0.66$; sex * population: $F_{3, 82} = 0.83$, $p = 0.48$; Lab-reared fish: sex: $F_{1, 2.11} = 122.8$, $p = 0.007$; population: $F_{2, 1.95} = 8.35$, $p = 0.11$; sex * population: $F_{2, 26.06} = 0.5$, $p = 0.61$; sex*population*tank: $F_{18, 94} = 1.09$, $p = 0.37$).

Neither of the two caudal fin measurements used was altered by the sex or population of laboratory-reared fish, although there was an effect of rearing tank on Tail 2. (Tail 1: ANOVA: sex: $F_{1, 2.01} = 0.03$, $p = 0.89$; population: $F_{2, 1.98} = 3.45$, $p = 0.23$; sex * population: $F_{2, 24.9} = 1.29$, $p = 0.29$; sex * population * tank: $F_{19, 115} = 1.23$, $p = 0.24$; Tail 2: ANOVA: sex: $F_{1, 2.0} = 0.08$, $p = 0.80$; population: $F_{1, 2.0} = 1.28$, $p = 0.44$; sex * population: $F_{2, 21.6} = 1.04$, $p = 0.37$; sex * population * tank: $F_{19, 119} = 2.065$, $p = 0.001$). Tail 3, the estimate of caudal fin fork depth, was independent of sex or population in wild fish but in laboratory-reared fish was somewhat influenced by population and much more strongly, by rearing tank (Tail 3: Wild fish: sex: $F_{1, 9.2} = 3.43$, $p = 0.09$; population: $F_{3, 3} = 4.50$, $p = 0.12$; sex * population: $F_{3, 81} = 0.45$, $p = 0.72$; Lab-reared fish: sex: $F_{1, 2.07} = 0.63$, $p = 0.51$; population: $F_{2, 1.9} = 28.45$, $p = 0.04$; sex * population: $F_{2, 22.2} = 0.19$, $p = 0.83$; sex * population * tank: $F_{19, 115} = 2.23$, $p = 0.005$).

6.2.3. Body stripes

Wild collected fish: neither sex nor population overall had a significant influence on stripe-interstripe ratio ($\log_{10}$ transformed), however there was a significant population-sex interaction (Figure 6.5.a; ANOVA: sex: $F_{1, 3.42} = 4.74$, $p = 0.11$; population $F_{3, 3} = 5.34$, $p = 0.10$; sex by population $F_{3, 80} = 4.50$, $p = 0.006$). Analysis of each sex separately showed a significant effect of population in both males and females (ANOVA: Males: population: $F_{3, 50} = 30.75$, $p < 0.001$; Females: population: $F_{3, 30} = 8.25$, $p < 0.001$).

Values for stripe-interstripe ratio for wild-collected fish are shown in figure 6.5.a. It is apparent that stripe-interstripe ratio is higher in females than males in all Bangladesh populations, although results for Canal females and Tangail males should be treated with some caution as they are only based on two and four individuals respectively. There is no influence of sex on stripe-interstripe ratio in the Nepalese fish. Wild-collected males from Nepal, and to a lesser extent those from Tangail, appear to have wider melanophore stripes relative to interstripes than those from either Canal or Santal.

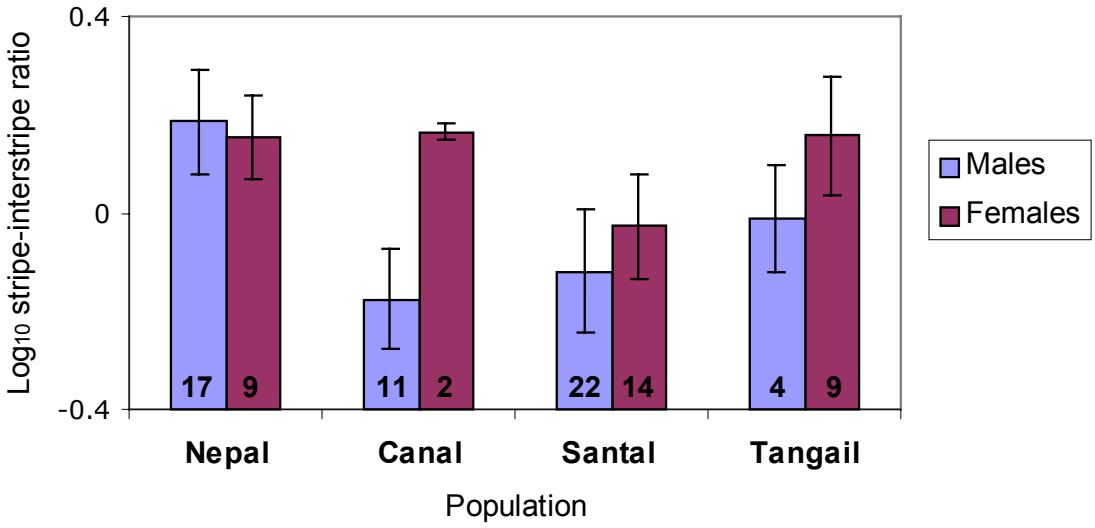


Figure 6.5.a. Body stripe-interstripe ratio (mean ± standard deviation) for wild male and female zebrafish collected from different localities. Number of fish is indicated on each bar.

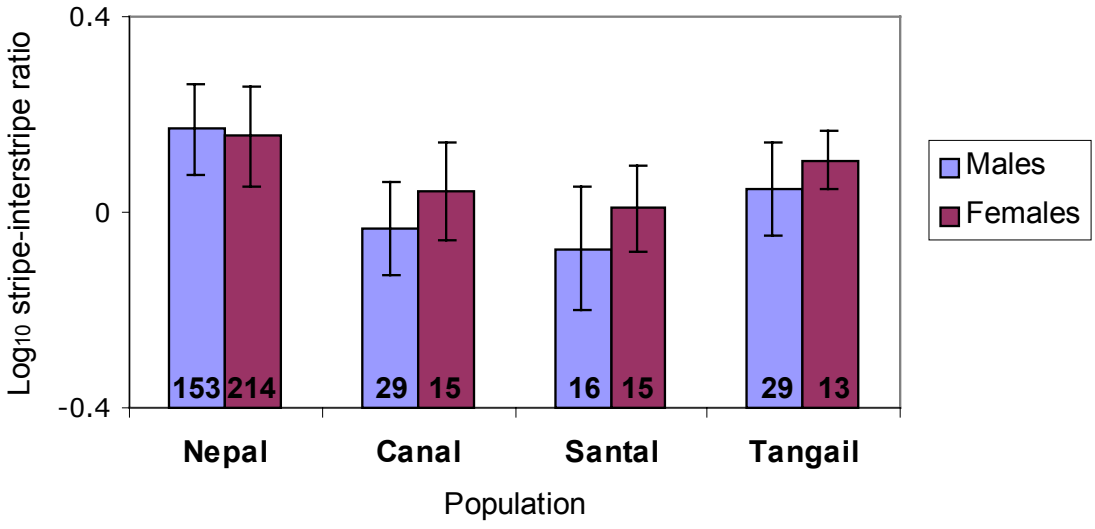


Figure 6.5.b. Body stripe-interstripe ratio (mean ± standard deviation) for male and female laboratory-reared zebrafish deriving from different localities. Number of fish is indicated on each bar.

P/P+1 ratio was not significantly influenced by sex or population in wild fish (ANOVA: sex: $F_{1, 4.35} = 1.40$, $p = 0.30$; population: $F_{3, 3} = 0.76$, $p = 0.59$; sex * population: $F_{3, 80} = 1.49$, $p = 0.23$). However, it is possible that any effect of population or sex on this trait is masked by its relatively low repeatability in this study.

Lab-reared fish: both sex and population had a significant effect on stripe-interstripe ratio of lab-reared fish derived from Bangladesh, with no significant effect of sex-population interaction or rearing tank (ANOVA: sex: $F_{1, 2.68} = 103.18$, $p = 0.003$; population: $F_{2, 1.68} = 109.90$, $p = 0.016$; sex * population: $F_{2, 23.87} = 0.09$, $p = 0.91$; tank * sex * population: $F_{15, 91} = 1.03$, $p = 0.44$). Figure 6.5.b shows mean stripe-interstripe ratio for the three populations from Bangladesh, together with the values calculated from the fish in the North

Carolina II breeding design which are derived from Nepal. It is apparent that, as in the wild-collected fish, male zebrafish from the Bangladesh populations have lower stripe-interstripe ratios than do females. Of these three populations, fish from Tangail again have wider melanophore stripes compared to interstripes than those from Canal or Santal. Similarly, Nepal fish again appear to have a larger stripe-interstripe ratio than all those from Bangladesh, with again no difference between the sexes in this population.

P/P+1 ratio was not affected by sex or population in laboratory-reared fish, although there was a significant sex-population interaction (ANOVA: Sex: $F_{1, 2.03} = 0.56$, $p = 0.53$; population $F_{1, 1.99} = 0.55$, $p = 0.65$; sex * population: $F_{2, 24.29} = 4.07$, $p = 0.03$; sex * population * tank: $F_{15, 88} = 1.11$, $p = 0.36$). Analysing the sexes separately revealed no overall significant population effect (ANOVA: Females: population: $F_{2, 9.03} = 3.68$, $p = 0.07$; population * tank: $F_{7, 30} = 1.13$, $p = 0.37$; Males: population: $F_{2, 10.16} = 1.33$, $p = 0.31$; population * tank: $F_{8, 58} = 1.11$, $p = 0.37$).

6.2.4. Anal fin stripes

Wild fish: There was a significant effect of population, but not sex or sex-population interaction, on anal fin blue: gold ratio (ANOVA sex: $F_{1, 10.96} = 3.33$, $p = 0.095$; population: $F_{3, 3} = 67.59$, $p = 0.003$; sex * population $F_{3, 78} = 0.29$, $p = 0.84$; Figure 6.6 a). Overall, Nepalese fish appeared to have wider B1 stripes relative to G1 stripes than fish from Bangladesh.

Laboratory-reared fish: Anal fin blue-gold ratio in lab-reared Bangladesh fish was not affected by sex or sex-population interaction, although there was a non-significant influence of

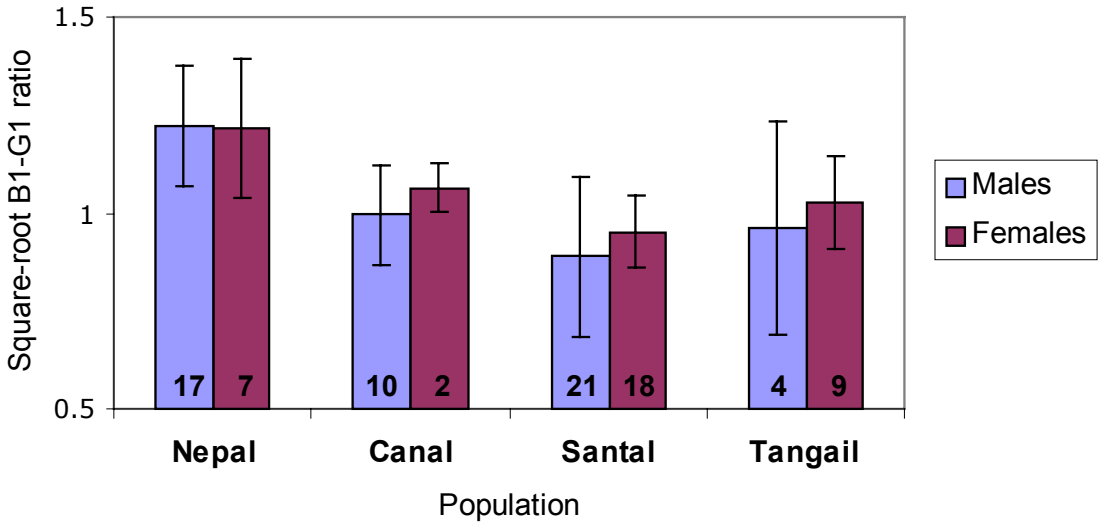


Figure 6.6.a. Anal fin blue-gold ratio (mean ± standard deviation) for wild male and female zebrafish collected from different localities. Number of fish is indicated on each bar.

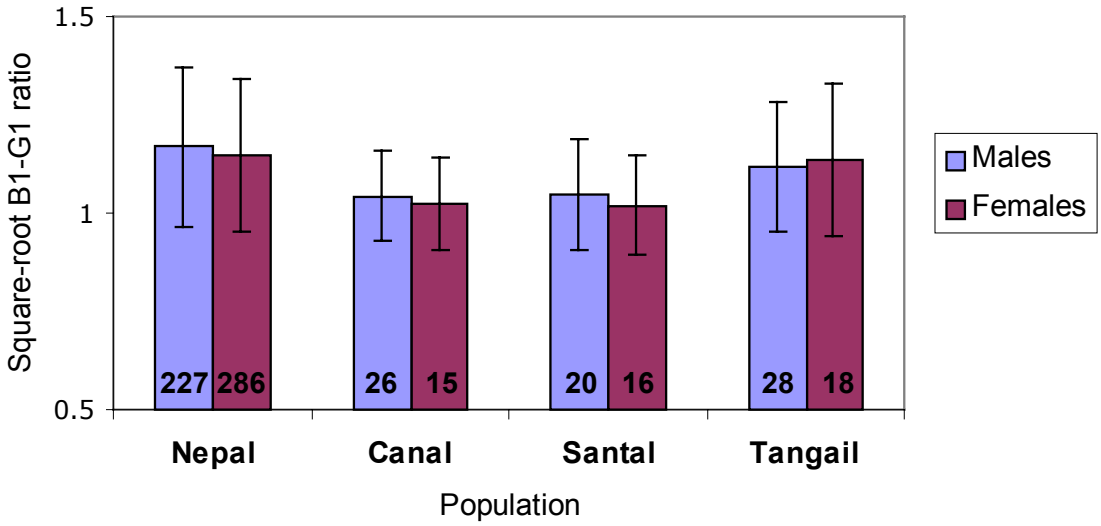


Figure 6.6.b. Anal fin blue-gold ratio (mean ± standard deviation) for male and female laboratory-reared zebrafish deriving from different localities. Number of fish is indicated on each bar.

population. Most of the variation seen in the data set was due to variation between rearing tanks, which may either be the result of environmental differences between tanks or a stochastic effect of the relatively small numbers of males and females available per tank. (ANOVA: sex: $F_{1, 2.03} = 2.39$, $p = 0.26$; population: $F_{2, 1.72} = 25{,}34$, $p = 0.053$; sex * population: $F_{2, 19.60} = 0.052$, $p = 0.95$; sex * population * tank: $F_{17, 97} = 2.78$, $p = 0.001$). Figure 6.6b shows the results from the Bangladesh populations with the results from the North Carolina II fish included. There are no major differences apparent between the populations, although again fish from Nepal may have higher blue-gold ratios than fish from Bangladesh.

For both wild-collected and lab-reared fish, anal fin blue-gold ratio was weakly, but significantly, correlated with body stripe-interstripe ratio (Pearson correlation: Wild fish: $r_{81} = 0.52$, $p < 0.001$; lab-reared fish: $r_{99} = 0.21$, $p = 0.04$).

6.3. Discussion

This study has found evidence for morphological differences in the zebrafish, both between sexes and between geographically separated populations. Patterns of variation documented in wild-collected fish were also observed in their laboratory-reared offspring, suggesting a genetic basis to much of this variability.

Male and female zebrafish differ in body shape but most strikingly in size and coloration of the anal fin, with males from all populations exhibiting relatively larger anal fins bearing more intense orange coloration. This strongly suggests that anal fin morphology is under sexual selection. As noted in Chapter 4, carotenoid-based orange pigmentation has been shown to be a sexually selected trait in a number of other fish species (e.g. guppies, Kodric-Brown, 1989) and may act as a 'good genes' indicator, for example advertising a male's resistance to parasites as proposed by Hamilton & Zuk (1982). Male zebrafish are frequently described as 'more yellow' than females, and the orange coloration on the fin may reflect an overall increase in carotenoids throughout the body of the male that could not be observed in the photographs. Increased orange coloration may be under selection both via direct female mate choice and via a role in male-male dominance interactions. Anal fin size, on the other hand, does not appear to be an important consideration in mate choice as it is not explicitly displayed in courtship. However, anal fin size and stripe pattern are dramatically emphasized during antagonistic interactions (Chapter 4) and evolution of sexual dimorphism in anal fin size may be driven by male-male competition determining access to spawning opportunities. An alternative explanation for sexually dimorphic anal fin size in fish with external fertilization is proposed by Koseki et al.

(2000), who suggest that the size of the anal fin in male medaka has a role in ensuring successful fertilization of eggs, by channelling eggs and sperm together. The relative importance of these contrasting hypotheses for driving sexual dimorphism in anal fin size could be investigated by examining dorsal fin size in the two sexes, an analysis that was not performed in this study due to difficulties with accurately measuring this fin from photographs. Since both anal and dorsal fins are expanded in antagonistic interactions (Chapter 4), selection for increased apparent body size in males as a result of intrasexual competition for mates is expected to increase the size of both of these fins relative to those in the female. However, if increased anal fin size in the male is driven by fertilization considerations then no sexual dimorphism in dorsal fin size is expected.

As well as the sexual dimorphism discussed above, males and females from the Bangladesh populations also differed in width of body stripes, with females having thicker melanophore stripes relative to interstripes than males. It is possible that some form of sexual selection on males for body stripe width may also be occurring. In the absence of direct selection on body stripes, however, sexual selection for increased orange stripe area on the anal fin could result in increased interstripe width on the body as a result of pleiotropic genetic effects. Although development of melanophore striping on the body and anal fin involves partly differing sets of genes (Chapter 7), developmental mutants which alter body stripe-interstripe ratio also alter blue-gold ratio on the anal fin (Haffter et al., 1996). Stripe-interstripe ratio and blue-gold ratio were found to be only weakly phenotypically correlated in the present study, however, the significant effect of rearing tank on the latter character suggests that it may be open to environmental perturbations that could mask an underlying genetic relationship.

In addition to the observed differences between the sexes, there appears to be heritable variation in body stripe-interstripe ratio between wild fish collected from different sites. In particular, fish deriving from Nepal have wider melanophore stripes on both the body and anal fin in comparison to fish deriving from the three sites in Bangladesh. There also appear be some differences in body stripe width between males derived from the geographically close Canal and Santal populations and those from the more distant Tangail population within Bangladesh. Additionally, while fish deriving from Bangladesh exhibit some sexual dimorphism in body stripe-interstripe ratio, those from Nepal do not. It must be noted that these findings do not unequivocally demonstrate inter-population differences in stripe pattern, as it is unknown whether the relatively small numbers of fish collected from each location are a representative sample of the morphology in the area. However, they do provide strong support for this possibility.

Some caveats to the proposed genetic basis of stripe pattern variation between populations must be considered. Since the laboratory-reared fish are first generation offspring of wild-collected fish, there remains a possibility that the rearing environment of the parents may be influencing the phenotype of the offspring. Although zebrafish have external development and no parental care, non-genetic parental influences on offspring phenotype have been documented in this species. For example, vertebral count of zebrafish can be influenced by the temperature at which their parents are housed (Dentry & Lindsey, 1978) and both maternal and paternal genomic imprinting has also been demonstrated in the zebrafish (McGowan & Martin, 1997).

Chapter 7 investigates in more detail the genetic basis of morphological variation discussed in this chapter.

Chapter 7
Zebrafish morphology: quantitative genetic studies

7.1. Introduction

7.1.1. The zebrafish pigmentation pattern: development and genetic control.

The typical adult zebrafish pigmentation pattern (Figures 1.1, 2.1 & 6.1) comprises four different types of cells, all of which are derived from the embryonic neural crest (Haffter et al., 1996). The dark stripes on the body and the tail are formed by melanocytes, cells containing melanin. Scale-associated melanocytes are also responsible for the uniform dark coloration on the dorsal surface. The golden coloration of the interstripes on the body and on the fins is formed by xanthophores, containing carotenoid pigments. Overlying the body stripes are layers of iridophores, cells containing structural elements which reflect light. A fourth class of white cells is present only at the tips of the dorsal, caudal and anal fins (Parichy et al., 2000).

Identification of pigment pattern mutants has enabled the genetic basis and physiological processes of zebrafish pigment pattern development to be investigated (e.g. Kelsh et al., 1996). During the first few weeks of its life a zebrafish develops a larval pattern of pigment cells, which appears to be common to all members of the genus, as well as to other close relatives (McClure, 1999). The adult pattern is generated as a result of migration and proliferation of these larval cells and differentiation of new pigment cells at later stages of development. For example, establishment of the melanocyte stripes on the flanks and fins of an adult zebrafish involves at least three further populations of melanocytes, each under different genetic control (Johnson et al., 1995). The first population, dependent on the c-*kit* gene, arises at the larval stage. These melanocytes are first dispersed across the flanks but later multiply and coalesce to form the P and P+1 stripes of the adult fish, with melanocyte death in the interstripe region (Parichy et al., 1999). At the same time a second melanocyte population, dependent upon functioning *rose (ros)*, *leopard (leo)* and *fms* genes (Parichy et al., 2000), also arises in the stripe region. These two melanocyte populations contribute approximately 50% each to the total number of melanocytes in the adult stripes. Double *kit:rose* or *kit:leo* mutants lack all body melanocytes as adults but continue to exhibit normal stripe patterning on the caudal and anal fin, indicating that a third *kit-, ros-* and *leo*-independent melanocyte population is involved in fin

pigmentation. However, in *kit* mutants tail pattern develops much more slowly than in wild type (Rawls & Johnson, 2000), suggesting that *kit* is involved in normal fin pattern development but that its absence is compensated for by increased proliferation of *kit*-independent melanocytes. *Kit*, *leo, ros* and *fms* have all been mapped on the zebrafish genome.

Several of the genes involved in melanocyte development are also required for the establishment of other pigment cells, and development of the zebrafish stripe pattern involves complex interactions between these various cell types. For example, *fms*, as well as having a role in melanocyte development, is also required for the establishment of both larval and adult xanthophores. It appears that organisation of melanocytes into adult stripes is dependent upon the positions of these xanthophores. In *fms* mutants, which lack xanthophores, *kit*-dependent melanocytes largely remain dispersed over the flanks and gradually die, perhaps as a result of their low density (Parichy et al., 2000). Zebrafish mutant for *fms* also lack fin striping, suggesting a potential role for this gene in the establishment of fin melanocytes. *Ros* has a role in iridophore formation, and correct development of iridophore stripes on the body of the adult zebrafish appears to depend upon melanocyte positioning. Additionally, both *ros* and *leo* appear to have a role in the organization of melanocytes into coherent stripes. Fish mutant at the *leo* locus have stripe anomalies ranging from wavy melanophore stripe boundaries to complete replacement of melanophore striping with fine spotting. In *ros* mutants the P stripe is replaced by a row of spots and the stripes below are absent.

In addition to these well characterised mutants, several others that affect development of the zebrafish adult pigmentation pattern have been identified (Haffter et al. 1996). *Salz* (*sal*) and *pfeffer* (*pfe*) mutants, for example, exhibit interrupted melanocyte stripes, possibly because these genes are involved in larval xanthophore development. Mutations at the *asterix (ase)* and *obelix (obe)* loci affect stripe-interstripe ratio on the body and anal fin and perhaps also cause reduction in the central caudal fin stripe. Several other mutations identified as causing stripe pattern anomalies (e.g. *wanda, hagoromo*; Kawakami et al., 2000) may not directly affect genes involved in adult pigmentation but instead cause developmental stress which disrupts stripe pattern formation.

7.1.2. Zebrafish pigmentation mutants and species differences

The identification of the genes behind zebrafish stripe pattern formation leads to the possibility that the genetic basis of pattern differences between different *Danio* species may be elucidated. Indeed, *fms* has already been implicated in the absence of striping seen in one

member of the *D. danglia* clade, the pearl danio *D. albolineatus* (Parichy et al., 2000), and other mutants such as *ros* exhibit intriguing pattern similarities with other members of the group (compare the description of *ros* mutants with that of *D. nigrofasciatus*, Chapter 2). However no mutant has yet been identified that expresses all colour pattern elements of any of these species, suggesting that multiple loci may be involved in generating the colour pattern differences within the *D. danglia* clade. Whether these loci are few or many in number, and whether those that significantly contribute to inter-species differences also significantly contribute to intra-specific variation remains unknown. If colour pattern evolution within the genus has been driven by sexual selection, a process which requires co-evolutionary change in traits and preferences, then interspecific differences are likely to have arisen via small changes at a large number of loci (Orr & Coyne, 1992). On the other hand, the very limited number of studies that have been performed on the genetics of inter-specific and inter-racial colour pattern differences have frequently found them to be determined by a small number of genes of large effect (e.g. Sheppard et al., 1985; Jiggins & McMillan, 1997: *Heliconius* butterflies; Spurway, 1953: European newts). The dense microsatellite map of the zebrafish genome (Postlethwait et al., 1998) is expected to greatly help in addressing this question in this species.

7.1.3. Quantitative Genetic Analysis

For a given individual in a population, the value of any trait (e.g. fin length, female mate preference) will deviate in some way from the population mean. This deviation will most likely be the result of a multitude of effects, for example the individual's rearing environment, its age and the genes that it carries. The value of the trait may increase (or decrease) the individual's fitness in some way compared to the population mean. However (ignoring the limited cases of cultural or maternal transmission), only if the variation in the trait has some genetic basis can this increased fitness result in this trait value spreading through the population. In addition, the speed of its spread will depend upon how much of the variation is due to these genetic factors. One challenge for a worker interested in the evolutionary potential of a trait, then, is to disentangle the genetic and environmental influences on its expression.

The majority of phenotypic traits exhibiting continuous variation are likely to be influenced a number of genetic loci, each with several alleles. The alleles contributing to a trait over all loci may act additively, each contributing a specific amount of variance. However, interactions within loci (dominance) and between loci (epistasis) will also occur. Since dominance and epistatic effects of alleles are dependent upon the genetic environment in which these alleles are present, phenotypic features of an individual resulting from this type of genetic

variance are not necessarily inherited by the offspring. It is the contribution of the additive genetic variance alone therefore, that determines a trait's evolutionary response to selection. The amount of phenotypic variance in a trait within a population that is due to underlying additive genetic variance is termed its 'heritability'.

Quantitative genetic theory was developed by Fisher (1915), Sewall Wright (1921) and others in order to investigate the genetics of such continuously variable traits. Based on the assumption that such traits are influenced by a large number of genes of small effect, it provides methods to partition the observed variance in such a trait around the population mean into that due to environmental and genetic effects by comparing the expression of the trait in different classes of relatives. Where data are available on traits in parents and offspring, this is done using least-squares regression. Where the traits that are measured are in different classes of sibling, ANOVA can be used to partition the variance into that due to, for example, paternal, maternal and environmental effects. Depending upon the breeding design used the influence of environmental effects, additive, dominance and epistatic genetic effects, maternal effects and (in certain cases) nuclear-cytoplasmic interactions on intra-population variation in a trait can then all be calculated.

7.1.4. The Restricted Maximum Likelihood method

Unfortunately, traditional ANOVA techniques require well-balanced designs with similar family sizes and complete nesting of factors. In practice, data of this sort are rarely available, even in controlled breeding programmes. The method of 'maximum likelihood analysis' (Lynch & Walsh, 1998) has been developed in the context of animal breeding to overcome this problem. This statistical technique iteratively adjusts the relevant variance components until they provide the best fit to the data provided. This is done by incorporating the components into a 'likelihood function' the logarithm of which is then maximized. The version of this method most commonly used in this context is 'restricted maximum likelihood analysis' (REML), which corrects for bias that can be introduced by small sample sizes (Shaw, 1987).

Whereas in ANOVA the significance of the variance components is tested using the F-test, in REML significance can be investigated by comparing the log likelihood of different models (Shaw, 1987; Lynch & Walsh, 1998). For example, if a model has been run to estimate the components of variance due to contributions from the mother and father, the question as to whether the mothers' contribution is significantly different from zero is addressed by running the model again with the factor 'mother' removed. If the maximum log likelihood of the full

model is termed 'L_{max}' and that of the reduced model 'L_0' then the 'likelihood ratio statistic' is simply -2 (L_0 - L_{max}). This is compared to the value of the χ^2 distribution, with degrees of freedom equal to the number of factors that have been removed in the reduced model (in this case, one).

7.1.5. The North Carolina II breeding design.

This study utilizes a North Carolina II breeding design (Comstock & Robinson, 1948) in order to investigate quantitative genetic variation in morphological traits of the zebrafish that may be important in intraspecific communication. In this breeding design, a set of males and females are mated to each other in all possible pair-wise combinations. In this study, each family was split into two tanks for rearing. The variance observed in traits of interest can then be partitioned into the following (Lynch & Walsh, 1998):

I. Variance due to father ('sire'), $\boldsymbol{\sigma^2_s}$ (= *covariance between paternal half sibs*)

II. Variance due to mother ('dam'), $\boldsymbol{\sigma^2_d}$ (= *covariance between maternal half sibs*)

III. Variance due to family (mother-father interaction), $\boldsymbol{\sigma^2_I}$
(= *covariance between full sibs* - $(\sigma^2_s + \sigma^2_d)$)

IV. Variance due to tank environment (within family), $\boldsymbol{\sigma^2_t}$
(= *covariance between tank mates* - $(\sigma^2_I + \sigma^2_s + \sigma^2_d)$)

V. Variance due to all other sources (individual differences, measurement error, etc), $\boldsymbol{\sigma^2_e}$

If no sex linkage is influencing the results then the following can be estimated from the above variance components:

Variance due to maternal effects (egg provisioning, etc) = $\boldsymbol{\sigma^2_d - \sigma^2_s}$
Variance due to additive effects of underlying genes ('heritability') = $\boldsymbol{4\,\sigma^2_s}$
Variance due to non-additive effects of underlying genes = $\boldsymbol{4\,\sigma^2_I}$

In practice, the non-additive variance is normally attributed to dominance effects (Lynch & Walsh, 1998). Sex linkage of genes underlying a trait can be investigated by examining inheritance patterns in males and females respectively (Lynch & Walsh, 1998).

It must be noted that because of the breeding methods used in this study, the two tank replicates of each sib group may be derived from two different spawnings. There is therefore a possibility that tank effects may be confounded with parental effects resulting, for example, from differences in age or nutritional status of the parents between the two different spawnings.

7.1.6. Sex determination in the zebrafish.

Despite the large body of knowledge that has been accumulated about zebrafish genetics, surprisingly little is known about sex determination in this species. Although certain authors have reported heteromorphic sex chromosomes, with the female as the heterogametic sex, (Sharma et al., 1998), the majority of studies of the zebrafish karyotype do not support this (Amores & Postlethwait, 1999; Pijnacker & Ferwerda, 1995, Rishi, 1976). An interesting observation is that clonal lines derived from homozygous female zebrafish develop into both male and female individuals, suggesting an environmental component to sex determination (Streisinger at al., 1981). Sex linkage is likely to be less frequently encountered in the zebrafish than in those species with a chromosomal sex determination system. Nevertheless there remains the potential for linkage disequilibrium between sex-determining loci and those affecting other traits.

7.2. Methods

Families were bred and reared, and individuals of each family photographed, as described in Chapter 3. All families were photographed over a period of three months in 1999. Morphological measurements were taken using Image Tool. In this study I investigated the genetic basis of four traits that have previously been described in Chapter 6: stripe-interstripe ratio on the body, which is known to vary with population, P/P+1 stripe ratio on the body, ratio of B1 and G1 stripe on the anal fin, and relative anal fin length, which is known to vary with sex. Repeatabilities of all measurements are given in Chapter 6.

7.3. Statistical analysis

All traits investigated in this analysis have been previously described in Chapter 6. Since size of fish in this study was variable, the relationship of each trait to fish length was investigated using the Pearson correlation followed by 'curve estimation' within the regression tools of SPSS. Any effect of fish size was corrected for as described in each section.

Since family sizes were unequal and male-female crosses incomplete (Chapter 3), variance components in this study were calculated using the Restricted Maximum Likelihood Procedure. Prior to variance component estimation, all data to be used in the analysis were plotted and outliers and extremes double checked. Estimation of variance components using REML is improved if data are normally distributed (Shaw, 1987) and normality was therefore tested using the D'Agostino-Pearson Z^2 test (Zar, 1996). Most data sets were right skewed and adjusted using either square-root or $\log_{10}$ transformations. Several data sets showed a significant departure from normality even following transformation, however examination of histograms, and skewness and kurtosis values, suggested that this deviation was sufficiently small that further transformation was not warranted.

All analyses were performed using the REML method available in the Variance Components option of the GLM procedure in SPSS for Windows (Release 9.0.0). This procedure returns both variance component estimates and standard errors of the estimates, with the additional option of displaying maximum log-likelihood values. The following random factors were specified in the model: mother, father, mother-father interaction and tank (nested within mother-father interaction). Five models were run for each analysis: a full model including all factors and one with each factor left out in turn. Significance of variance components was then tested using the Likelihood Ratio Test, comparing maximum likelihood value for the full model with that of relevant reduced model. Models were run both for all fish and for males and females separately, to test for any sex linkage.

7.4. General observations

7.4.1. Numbers, age and size distribution of fish.

Age of families when photographs were taken ranged from 18 to 24 months. Age in itself is not expected to have a systematic influence on fish morphology: fish have indeterminate

growth which is affected by, amongst other things, resource availability and dominance status (e.g. Sloman et al., 2000). Reflecting this, all tanks and families contained a range of sizes. However there were significant differences in standard length both between families and between tanks within family (ANOVA with $\log_{10}$ transformed data: $F_{20, 628} = 2.938$, $p = 0.01$; between tanks $F_{20, 628} = 6.130$, $p < 0.001$). Mean fish length in a tank was negatively correlated with fish number (Spearman's rho = -0.74, $p < 0.001$), suggesting a density effect on growth rates. Although numbers in all tanks were standardized to 35 or fewer individuals approximately 6 months after hatching, variation in family sizes prior to this standardization and random fish death following it meant that different families experienced different densities. Numbers of fish per tank at the time of photography ranged from 4 to 32 (median = 15). Most families included a number of very small or severely malformed individuals which were excluded from the analysis (93 individuals out of 747 fish photographed).

7.4.2. Sex of fish

Out of the 654 individuals in the North Carolina II breeding design that were used in the analysis, 84% could be reliably sexed using the qualitative criteria described in Chapter 6. The remainder were mostly small, possibly sexually immature fish (mean length ± standard deviation: sexed = 23.0 ± 2.7 mm, unsexed = 21.1 ± 2.2 mm; using $\log_{10}$ transformed data: $z = 7.115$, $p < 0.001$). Proportion of fish in a tank that could not be sexed ranged from 0% to 35%. Out of the fish that were assigned sex, in total 55% were female and 45% male, representing a slightly, but significantly, female-biased sex ratio (comparing to a null hypothesis of 50% of both sex: n = 549; χ^2 (Yates' corrected) = 5.311, $p < 0.05$). However, this may merely be an effect of more males than females being assigned to the unsexed group. Overall, there was no significant difference in length between males and females (z-test: $z_{534, 2} = -0.194$, N.S.). As previously noted in Chapter 6, males overall have longer anal fins relative to their size than females (z-test: $z_{534, 2} = 7.54$, $P < 0.001$).

7.5. Results

7.5.1 Body stripes

As described in Chapter 6, individuals exhibiting loss of body melanocytes and other stripe irregularities at the reference line were excluded from the data set. This left 436 fish to be

Tables 7.1a, 7.1b, &7.1c: Variance component estimations and likelihood ratio test results for stripe-interstripe ratio.

a) All fish (n = 437)

Source of variation	Variance Component ± Standard Error	% total variation
Mother	0.00	0.0%
Father	0.000032 ± 0.00258	0.3%
Interaction	0.00	0.0%
Tank	0.00180 ± 0.000612	19.1%
Error	0.00758 ± 0.000538	80.6%
Total	0.009412	

Likelihood ratio tests:

Full model: log likelihood = 421.288

Without father: log likelihood = 421.277;

$\chi^2 = 0.022$; N.S.

Without tank: log likelihood = 409.986;

$\chi^2 = 22.6$; $P < 0.01$

b) Males (n=153)

Mother	0.000643 ± 0.000785	7.2%
Father	0.00	0%
Interaction	0.00	0%
Tank	0.00134 ± 0.00081	15.1%
Error	0.00692 ±0.000903	77.7%
Total	0.00890	

Likelihood ratio tests:

Full model: log likelihood = 150.362

Without mother: log likelihood = 149.963;

$\chi^2 = 0.798$, N.S.

Without tank: log likelihood = 148.821;

$\chi^2 = 3.082$, N.S.

c) Females (n = 214)

Mother	0.00	0%
Father	0.00071 ± 0.0008	6.9%
Interaction	0.00	0%
Tank	0.00135 ± 0.00074	13.0%
Error	0.00830 ± 0.00088	80.1%
Total	0.01036	

Likelihood ratio tests:

Full model: log likelihood = 194.683

Without father: log likelihood = 193.519;

$\chi^2 = 2.3278$, N.S.

Without tank: log likelihood = 192.373;

$\chi^2 = 4.62$, $P < 0.05$.

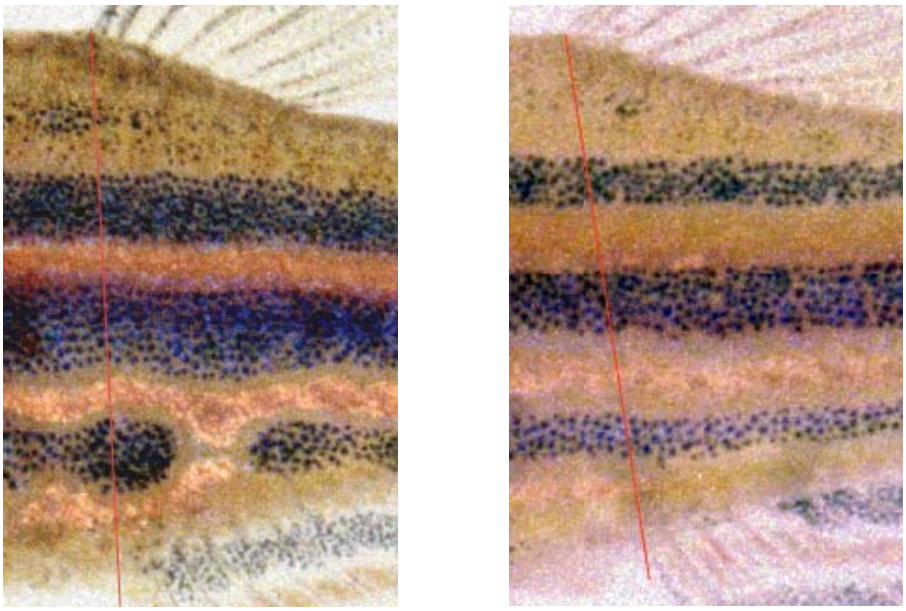


Figure 7.1 a & b. Examples of zebrafish with body stripe-interstripe ratio values at the extremes of the range for this trait.

used in the body stripe analysis. Stripe-interstripe ratio showed a left skewed distribution and was $\log_{10}$ transformed prior to analysis. P/P+1 ratio did not exhibit a significant departure from normality. The distribution of stripe-interstripe ratio was independent of fish standard length (Pearson correlation using transformed data $n = 436$, $r = -0.015$, N.S.). However, for the North Carolina II fish, P/P+1 ratio showed a weak, but statistically significant correlation with length (Pearson correlation using transformed data: $n = 436$, $r = 0.205$, $p < 0.001$). In order to control for any influence of length on P/P+1 ratio, therefore, fish standard length ($\log_{10}$ transformed) was included as a covariant in the variance components model (Packard & Boardman, 1987).

Variance component estimations and results of likelihood ratio tests are shown in Tables 7.1 and 7.2 (a, b & c). It is clear that neither stripe-interstripe ratio or P1/P+1 ratio are significantly influenced by genetic factors. However, there appears to be some environmental effect, as indicated by the significant contribution of rearing tank to the observed phenotypic variance. Since the contribution of the dam to variance is effectively zero there are no obvious maternal effects, although as noted earlier it is possible that a maternal effect may contribute to the between-tank variance. The non-significant result for tank effect on stripe-interstripe ratio when males alone are considered is likely to be due to the smaller sample size; the likelihood ratio test is conservative at such sample sizes (Shaw, 1987). Figure 7.1 demonstrates the phenotypic range of stripe-interstripe ratio and P/P+1 ratio seen in the North Carolina II families.

Tables 7.2a, 7.2b, &7.2c: Variance component estimations and likelihood ratio test results for ratio of stripes P and P+1.

All fish (n = 436)

Source of variation	Variance Component ± Standard Error	% total variation
Mother	0.00	0.0%
Father	0.00	0.0%
Interaction	0.00	0.0%
Tank	0.00217 ± 0.000859	10.3%
Error	0.01605 ± 0.001141	87.2%
Total	0.01740	

Likelihood ratio test:

Full model: log likelihood = 263.862

Without tank: log likelihood =258.862;

$\chi^2 = 10.0$; $P < 0.05$

Males (n=153)

Mother	0.000912 ± 0.00135	5.3%
Father	0.00	0.0%
Interaction	0.00	0.0%
Tank	0.00332 ± 0.00175	19.3%
Error	0.01297 ± 0.00170	75.4%
Total	0.017202	

Likelihood ratio test:

Full model: log likelihood = 100.183

Without mother: log likelihood = 99.774;

$\chi^2 = 0.818$, N.S.

Without tank: log likelihood =97.630;

$\chi^2 = 5.106$, $p < 0.05$.

Females (n = 214)

Mother	0.00	0.0%
Father	0.000132 ± 0.000630	0.8%
Interaction	0.00	0.0%
Tank	0.001299 ± 0.0013	7.7%
Error	0.01553 ± 0.00164	91.6%
Total	0.016961	

Likelihood ratio test:

Full model: log likelihood = 130.212

Without father: log likelihood = 130.184;

$\chi^2 = 0.056$, N.S.

Without tank: log likelihood = 192.373;

$\chi^2 = 4.62$, $P < 0.05$.

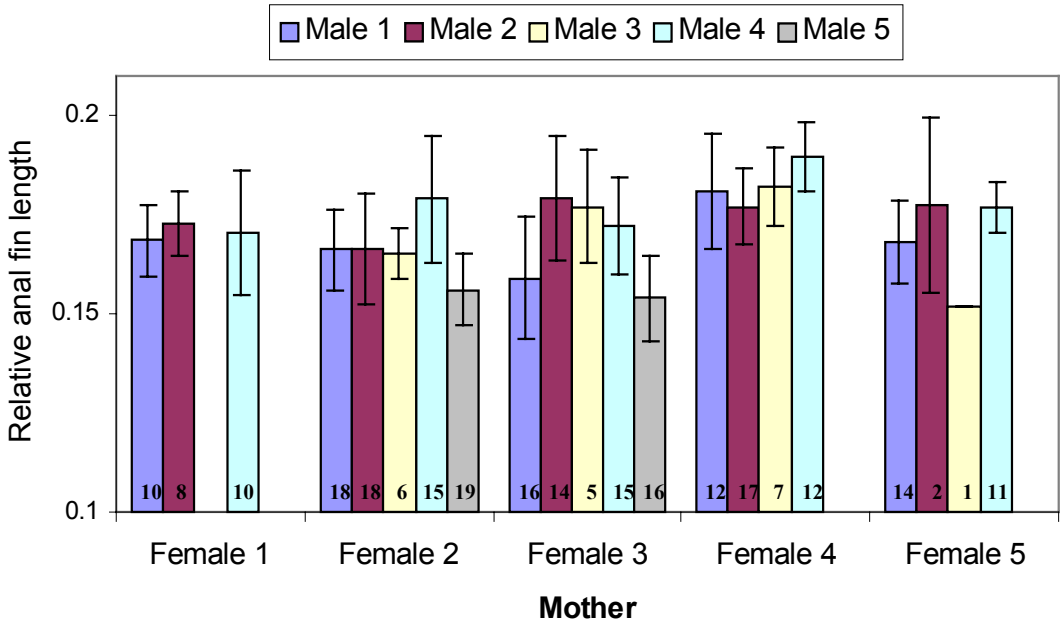


Figure 7.2. Mean ± standard deviation relative anal fin length for males from 21 families in the North Carolina II breeding scheme. Identities of mother and father are as indicated. Numbers of fish are show on the bars.

7.5.2. Anal fin length

Anal fin length standardized for fish size exhibited a normal distribution and analyses were therefore performed using untransformed data. After removal of fish whose anal fin length could not accurately be measured, 664 individuals were available for analysis.

Variance component estimates and results of likelihood ratio tests are shown in Tables 7.3 a, b & c. Maternal, paternal and tank components of observed phenotypic variance are all significant, both when considering the sexes together and when considering them separately. Phenotypic variation for anal fin length observed in males is indicated in Figure 7.2. There appears to be a substantial amount of additive genetic variance underlying this trait. However, estimation of exactly how much is complicated by the fact that, when considering both sexes together and males alone, the paternal component of variance is substantially larger than the maternal component. If taken at face value this implies a substantial non-genetic paternal influence on the trait, which seems unlikely. Examination of the standard errors of the variance component estimates shows that the difference between paternal and maternal variance components is not, in fact, significant. No significant non-additive genetic influences on anal fin length were observed.

Tables 7.3a, 7.3b, &7.3c: Variance component estimations and likelihood ratio test results for anal fin length.

a) All fish (n = 664)

Source of variation	Variance Component ± Standard Error	% total variation
Mother	0.0000132 ± 0.0000125	5.3%
Father	0.0000560 ± 0.0000430	22.4%
Interaction	0.00 ± 0.00	0%
Tank	0.0000233 ± 0.0000085	9.3%
Error	0.0001573 ± 0.0000089	63.0%
Total	0.0002498	

Likelihood ratio tests:

Full model: log likelihood = 1930.658

Without mother: log likelihood = 1928.40, $\chi^2 = 4.512$, $P < 0.05$

Without father: log likelihood = 1923.877, $\chi^2 = 13.562$, $P < 0.01$

Without tank: log likelihood = 1911.814, $\chi^2 = 37.688$, $P < 0.01$

b) Males (n = 246)

Mother	0.000024 ±0.000023	10.3%
Father	0.000046 ±0.000039	19.7%
Interaction	0.00	0%
Tank	0.000036 ± 0.000015	15.4%
Error	0.000127 ± 0.000012	54.5%
Total	0.000233	

Likelihood ratio tests:

Full model: log likelihood = 726.535

Without mother: log likelihood = 724.497; $\chi^2 = 4.076$, $P < 0.05$

Without father: log likelihood = 724.003; $\chi^2 = 5.064$, $P < 0.05$

Without tank: log likelihood = 719.358 $\chi^2 = 14.353$, $P < 0.01$

c) Females (n = 305)

Mother	0.0000084 ± 0.000012	3.8%
Father	0.000061 ± 0.000048	27.7%
Interaction	0.0000089 ± 0.00004	4.0%
Tank	0.000015 ± 0.000011	6.8%
Error	0.00013 ± 0.000011	59.1%
Total	0.00022	

Likelihood ratio tests:

Full model: log likelihood = 911.603

Without mother: log likelihood = 911.193; $\chi^2 = 0.82$, N.S.

Without father: log likelihood = 907.510; $\chi^2 = 8.186$, $P < 0.05$

Without interaction: log likelihood = 911.341; $\chi^2 = 0.524$, N.S.

Without tank: log likelihood =909.217; $\chi^2 = 4.772$, $P < 0.05$.

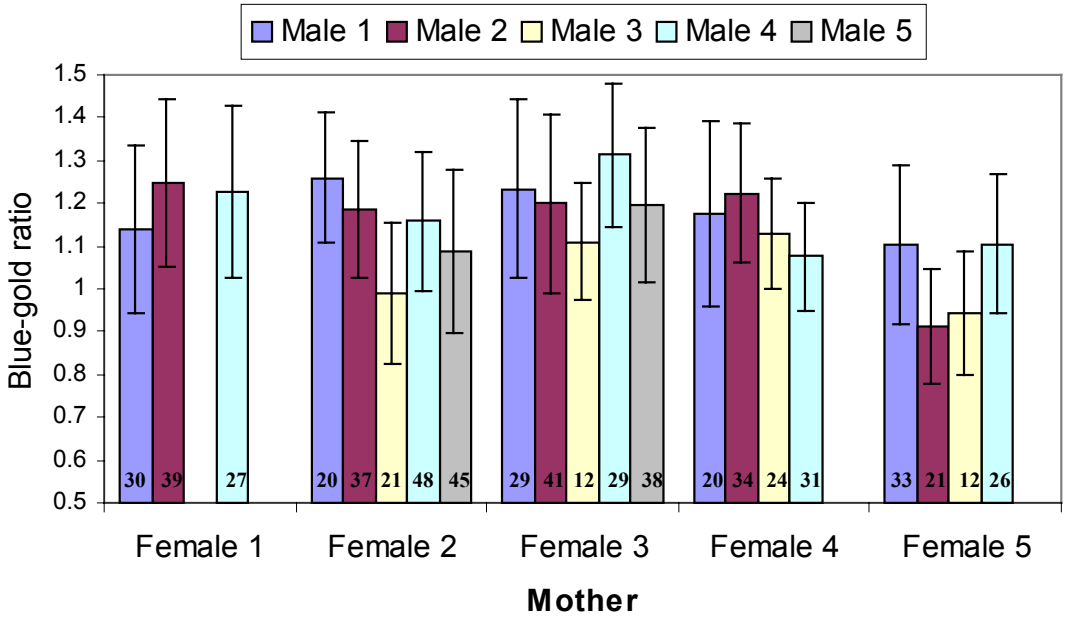


Figure 7.3. Mean ± standard deviation anal fin blue-gold ratio for fish from 21 families in the North Carolina II breeding scheme (both sexes). Identities of mother and father are as indicated. Numbers of fish are show on the bars.

7.5.3 Anal fin stripes

As described in Chapter 6, individuals exhibiting stripe irregularities at anal fin ray 5 or whose anal fin stripes could not accurately be measured were removed from the data set. Exclusion of these individuals left 618 for anal fin stripe analysis. The data exhibited a left skewed distribution and were square-root transformed prior to analysis. Anal fin blue-gold ratio exhibited a weak but significant correlation with length (Pearson correlation using transformed data n = 436, r =-0.105, p = 0.009) and therefore fish standard length ($\log_{10}$ transformed) was again included in the model as a covariate.

Variance component estimations and results of likelihood ratio tests are shown in tables 7.4 a, b & c, and between-family variability on the trait is illustrated in figure 7.3. Where all fish are considered together there is a significant influence of mother-father interaction, suggesting that much of the phenotypic variance seen in the trait (approximately 34%) is due to dominance genetic variance. Although not significant, the additive genetic component contributes approximately 14%. The maternal contribution to variance appears higher than that of the father but examination of the respective standard errors suggests again that this difference is not significant, that is, there is no maternal effect evident.

Tables 7.4a, 7.4b, &7.4c: Variance component estimations and likelihood ratio test results for B1:G1 anal fin stripe ratio.

All fish (n = 618)

Source of variation	Variance Component ± Standard Error	% total variation
Mother	0.00419 ± 0.00397	10.2%
Father	0.00146 ± 0.00209	3.6%
Interaction	0.00393 ± 0.00228	9.6%
Tank	0.00076 ± 0.00090	1.9%
Error	0.03064 ± 0.00180	74.8%
Total	0.04098	

Likelihood ratio tests:

Full model: log likelihood = 176.466

Without mother: log likelihood = 174.638;

$\chi^2 = 3.66$, N.S.

Without father: log likelihood = 176.066;

$\chi^2 = 0.80$, N.S.

Without interaction: log likelihood = 173.08;

$\chi^2 = 6.77$, $P < 0.01$

Without tank: log likelihood = 175.847;

$\chi^2 = 1.24$, N.S.

Males (n = 227)

Mother	0.00156 ± 0.00252	3.8%
Father	0.00109 ± 0.00221	2.6%
Interaction	0.00365 ±0.00255	8.8%
Tank	0.00	0%
Error	0.0351 ±0.00344	84.8%
Total	0.0414	

Likelihood ratio tests:

Full model: log likelihood = 48.703

Without mother: log likelihood = 48.459;

$\chi^2 = 0.488$, N.S.

Without father: log likelihood = 48.630;

$\chi^2 = 0.146$, N.S.

Without interaction: log likelihood = 46.955;

$\chi^2 = 3.496$, N.S.

Females (n = 286)

Mother	0.00361 ±0.00429	9.5%
Father	0.00	0.0%
Interaction	0.00439 ± 0.00328	11.6%
Tank	0.00342 ± 0.00245	9.0%
Error	0.0266 ± 0.00239	70.5%
Total	0.0380	

Likelihood ratio tests:

Full model: log likelihood = 90.907

Without mother: log likelihood = 89.787;

$\chi^2 = 2.24$, N.S.

Without interaction: log likelihood = 89.579;

$\chi^2 = 2.656$, N.S.

Without tank: log likelihood = 88.195;

$\chi^2 = 5.424$, $P < 0.05$

Investigation of the sexes separately shows a similar pattern, with the male-female interaction term being important in all cases, although with these sample sizes the likelihood ratio test is not significant. Rearing environment has a significant influence on variance in blue-gold stripe ratio in females but not in males.

7.7. Discussion

This study used a North Carolina II breeding design in order to investigate heritability of elements of zebrafish morphology that have been found to vary between sexes, between populations or between closely related species and which may have a role as behavioural signals, for example in mate choice or antagonistic interactions. Understanding the heritability of such traits may allow us to make inferences about how traits may have been shaped by selection in the past and respond to selection in the future, and how intraspecific variability in traits may relate to interspecific variation currently observed.

7.7.1. Body stripes

This study demonstrates no significant genetic influence, within the Nepal population, on the variation observed in two elements of zebrafish body stripe pattern: stripe-interstripe ratio ($\log_{10}$ transformed) and stripe P - stripe P+1 ratio. However, there is a significant effect of rearing tank. Such a tank effect may be due to environmental variation between tanks (e.g. in terms of lighting intensity or tank density) or alternatively due to differing age of parents between the two tank replicates for each family.

As apparently heritable differences in stripe-interstripe ratio between different populations have been documented in the zebrafish (Chapter 6), a purely environmental influence on stripe-interstripe ratio variation within the Nepal population appears unexpected. One possible explanation is that heritable variation exists, but that it is masked by environmental effects. It has often been pointed out that heritability documented in the relatively constant environment of a laboratory may not be expressed in the more environmentally heterogeneous conditions of the field (Roff, 1997). Conversely in this study laboratory-reared fish exhibited more skeletal deformity and stripe irregularity than wild-collected fish, and it is possible that environmental effects, perhaps acting in the first few months where rearing difficulties were encountered (Chapter 3) are masking heritable variation that would be

otherwise be expressed in these fish. Variation in heritability of traits as a result of differing environmental conditions has been demonstrated in several other species (e.g. Qvarnström, 1999, collared flycatchers *Ficedula albicollis)*.

Nevertheless, there may be a good reason to expect that the zebrafish stripe pattern within a population may exhibit a low level of additive genetic variance, even if there are genetic differences in pattern between populations. As a result of the elevated predation risk for a phenotypically distinct individual within a group of animals (the 'oddity effect', Landeau & Terborgh, 1986), there is expected to be strong intra-population stabilizing selection on the colour pattern of shoaling fish which co-occur with aquatic predators. Although zebrafish in this study were frequently collected from small water bodies which may lack such predators, many of these appear to be ephemeral habitats re-colonised every year by fish from more permanent bodies of water, where predation pressure may be strong. Such stabilizing selection is expected both to reduce the underlying additive genetic variance and to cause its expression in the phenotype to be limited, for example via selection for linkage disequilibrium between additive loci with opposing effects (Lynch & Walsh, 1998) for modifier loci that canalize the trait (Pomiankowski & Møller, 1995). The latter mechanism is expected, however, to reduce both the genetic and environmental variation of the trait (Roff, 1997).

The finding of a low level of genetic variance for elements of stripe pattern, of course, only applies to the Nepalese population of zebrafish used for this breeding scheme. It was noted in Chapter 6 that, at least in regard to differences between the sexes, this population appears to show less variation in stripe pattern than do the populations from Bangladesh, which may therefore have more underlying genetic variation for this trait.

7.7.2. Anal fin morphology

Relative anal fin length exhibits a substantial amount of additive genetic variance, with an additional significant effect of rearing tank. This pattern was observed both when all fish were examined and where the sexes were analysed separately. Anal fin blue-gold ratio (square-root transformed), on the other hand, exhibits a substantial amount of underlying dominance variance, with a significant effect of tank in females, but not in males.

The hypothesis of a behavioural role for anal fin morphology might tempt one to postulate selective reasons to explain the observed results. The presence of a substantial amount of dominance variance underlying the variation in blue-gold ratio could, for example, indicate

directional selection on this trait, which is expected to erode additive genetic variance faster than dominance variance (Roff, 1997). Similarly, the presence of a significant tank effect in females but not in males might suggest that there is selection for increased developmental stability for this blue-gold in the latter sex, perhaps because it is important in female mate choice (Chapter 4). However, in the absence, thus far, of any direct evidence for such a behavioural role of the anal fin the significance of these results remains unknown. It should be noted that the standard errors of all variance components estimates are high.

Chapter 8
General Discussion

Results of the studies that collectively make up this thesis have been discussed in detail in the relevant chapters. Here I summarise the findings of this research and discuss their implications for the potential use of the zebrafish as a model for investigating the genetics of prezygotic isolation. I also suggest directions for future research in this area.

In Chapter 2, I described the distribution and phylogeny of the zebrafish, documented zebrafish collection in Nepal and Bangladesh and discussed facets of the species' habitat and life history in these regions. The phylogenetic relationships within the *Danio* genus remain unclear, although much useful current work is being performed by Fang (1998, 2000). An understanding of the ecology and distribution of the zebrafish and its sister taxa is vital if we are apply our understanding of zebrafish genetics and behaviour to the processes of speciation within the *Danio* genus. For example, detailed distribution information is required for *D. rerio, D. nigrofasciatus* and *D. kyathit* in order to understand whether these species occur sympatrically without interbreeding, and what aspects of their ecology may have contributed to speciation within the group. Potential regions of interest include the north-western coast of Myanmar and the northern Myanmar-India border, although such work may currently prove logistically demanding. In addition, investigation of zebrafish at the extremes of the species range, for example in eastern Pakistan, southern India, and the hill regions of north-eastern India, might lead to the discovery of populations genetically, morphologically and behaviourally more divergent than those in the current study. Investigations of feral zebrafish populations may also prove interesting from the point of view of documenting evolutionary change in populations whose history and origin is known.

In addition to further work on zebrafish distribution, more comprehensive molecular phylogenies will help clarify the phylogenetic relationships of members of the genus. Preferably, these should use genetic material from wild fish collected from known localities, rather than from domestic stocks whose origin, and frequently species identity, is unknown.

Chapter 3 presented a low-input protocol for rearing large numbers of zebrafish. Although the zebrafish is frequently described as easy to breed and rear, in practice most workers requiring large numbers of fish for genetic analysis utilize specially built systems, often with dedicated technical support. Such a rearing system may facilitate more rapid growth with lower mortality and faster generation times than those found for zebrafish during this work. It

must be noted, however, that the relatively low breeding and growth rate observed in the fish in this study may in part be due to their wild origins. Zebrafish maintained under domestication for several generations are likely to exhibit very different life history and behavioural traits from their wild ancestors as a result of selection pressures unique to the domestic environment. Researchers should therefore be wary of utilizing domestic zebrafish in behavioural experiments if they wish to extrapolate their results to the behaviour of the species in the wild.

Chapter 4 described sexual and antagonistic behaviour in the zebrafish and presents preliminary work to identify morphological traits that may be important with regard to such behaviour. Several problems were encountered in the course of this work which may mean that the zebrafish is less amenable to investigations of mate choice than are many other teleost species currently used in this context. Primary amongst these problems is the weak sexual dimorphism of the zebrafish, which makes identification of males and females difficult. Consistent sexual dimorphism in anal fin colour and size, and in body shape were documented in the morphometric analyses (Chapter 6). However, these have not proved easy to distinguish in non-anaesthetised individuals as a result of the small size and high activity levels of this species. Reliable separation of the sexes under anaesthetic nevertheless remains feasible.

A second drawback encountered during these investigations was the lack of spawnings observed in experiments. The two-way choice test is a widely used method to assess fish mating preferences, however there is a risk of confounding shoaling and sexual preferences unless it can be explicitly demonstrated that a female's behaviour in the choice tank is indicative of her mate preference. So far, this has not been established for the zebrafish. It is unknown what factors are contributing to this reluctance to spawn. Further modifications of the experimental techniques, for example observations of behaviour amongst mixed-sex groups, may provide more productive.

Nevertheless, observations in mate choice and dominance trials, together with results of morphometric analyses detailed in Chapter 6, suggest several phenotypic traits in the zebrafish that may be behaviourally important. These warrant further investigation. Symmetry of tail pattern, appearance of the male anal fin, and a role of melanophore stripes in antagonistic interactions have all been discussed in the relevant chapters. The availability of laboratory strains mutant for pigmentation patterns and developments in video imaging of fish may prove to be useful tools in investigating the behavioural roles of such traits.

Chapter 5 presented investigations on the influence of population, familiarity and kinship on shoaling preferences in the zebrafish. Although not directly concerned with

speciation, shoaling preferences have the potential to influence gene flow between populations. Additionally, shoaling tendency appears to be an easily quantified trait which may be amenable to genetic analysis in the zebrafish. No significant shoaling preferences for either familiar fish or kin were found in this study, results that are discussed in the light of evidence for these preferences in other species. However, there was an influence of population on shoaling preferences probably mediated by different reactions of fish from different populations to the test tank apparatus. This suggests some heritable behavioural variation, potentially representing inter-population differences in anti-predator behaviour or threat sensitivity.

In Chapter 6, I described investigations of inter-population and intersexual differences in morphology within the zebrafish. Differences in relative width of stripes and interstripes on the body, and to some extent on the anal fin, occur between fish collected from different areas of the species' range. These differences continue to be expressed in their laboratory-reared offspring, suggesting that they have a genetic basis. Documentation of such traits in other zebrafish populations should reveal whether these indicate systematic phenotypic differences in zebrafish between different habitats or different geographical areas, or alternatively random between-population variation. An interesting possibility is that intrapopulation colour pattern variation in the zebrafish may be restricted by stabilizing selection acting to increase phenotypic uniformity in shoals. Such selection, for example, may limit the scope for exaggeration of sexually selected traits in males or sex limitation of sexually selected traits in females.

Chapter 7 presented results of a quantitative genetic analysis of several morphological traits found to vary between populations or between the sexes. The lack of underlying additive genetic variation observed in stripe-interstripe ratio suggests that aspects of zebrafish colour pattern may indeed be under stabilizing selection. In terms of understanding the evolutionary forces shaping pigmentation pattern and morphology in the zebrafish, however, it is difficult to interpret such results without more knowledge of the behavioural and ecological significance of the traits under investigation. Nevertheless the compilation of a photographic record of all fish in the North Carolina II breeding design should facilitate genetic analysis of other morphological traits found to be behaviourally important in the future. Identification of underlying genes will be greatly aided by the wealth of molecular markers available in this species.

In conclusion, the mate choice system of the zebrafish may prove less amenable to genetic analysis than had previously been hoped. However the potential of the zebrafish as a tool for investigating the evolutionary process should not yet be dismissed.

References

Amores, A. & Postelthwait, J. H. 1999. Banded chromosomes and the zebrafish karyotype. *Methods in Cell Biology*, **60**, 323-end.

Amundsen, T. 2000. Why are female birds ornamented? *Trends in Ecology and Evolution*, **15**, 129-175.

Andersson, S., Ornberg, J. & Andersson, M. 1998. Ultraviolet sexual dimorphism and assortative mating in blue tits. *Proceedings of the Royal Society of London, Series B,* **265**, 445-450.

Ansari, B. A. & Kumar, K. 1982. A record of the zebra danio (*Brachydanio rerio*) (Cyprinidae) from Uttar Pradesh with notes on sexual dimorphism. *Journal of Advanced Zoology*, **3**, 88-89.

Arnold, K. E. 2000. Kin recognition in rainbowfish (*Melanotaenia eachamensis*): sex, sibs and shoaling. *Behavioural Ecology & Sociobiology*, **48**, 385-391.

Bakker, T. C. M. 1993. Positive genetic correlation between female preference and preferred male ornament in sticklebacks. *Nature,* **363**, 255-257.

Barber, I. & Ruxton, G.D. 2000. The importance of stable schooling: do familiar sticklebacks stick together? *Proceedings of the Royal Society of London, Series B* **267**, 151-155.

Barman, R. P. 1983. A new species of the genus *Danio* Hamilton from India, Pisces: Cyprinidae. *Current Science*, **52**, 177-178.

Basolo, A. L. 1990. Female choice predates the evolution of the sword in swordtail fish. *Science,* **250**, 808-810.

Basolo, A. L. 1995. Phylogenetic evidence for the role of a pre-existing bias in sexual selection. *Proceedings of the Royal Society of London, Series B,* **259**, 307-311.

Beaugrand, J. P. & Goulet, C. 2000. Distinguishing kinds of prior dominance and subordination experiences in males of green swordtail fish (*Xiphophorus helleri*). *Behavioural Processes*, **50**, 131-142.

Berglund, A. 1996. Mating systems and sex allocation. In: J. -G. J. Godin (ed.), *Behavioural Ecology of Teleost Fishes.* pp. 237-265. Oxford University Press, Oxford.

Berglund, A. 2000. Sex role reversal in pipefish: female ornaments as amplifying handicaps. *Annales Zoologici Fennici,* **37**, 1-13.

Bisazza, A. & Marin, G. 1995. Sexual selection and sexual size dimorphism in the eastern mosquitofish *Gambusia holbrooki* (Pisces, Poeciliidae). *Ethology, Ecology and Evolution,* **7**, 169-183.

Blaustein, A. R. & Waldman, B. 1992. Kin recognition in anuran amphibians. *Animal Behaviour*, **44**, 207-221.

Bloom, H. D. & Perlmutter, A. 1977. A sexual aggregating pheromone system in the zebrafish, *Brachydanio rerio* (Hamilton-Buchanan). *Journal of Experimental Zoology,* **199**, 215-226.

Bloom, H. D. & Perlmutter, A. 1978. Possible pheromone mediated reproductive isolation in two species of cyprinid fishes of the genus, *Brachydanio*. *Journal of Fish Biology*, **13**, 47-50.

Boake, C. R. B. 1989. Repeatability: its role in evolutionary studies of mating behavior. *Evolutionary Ecology,* **3**, 173-182.

Brooks, R. & Caithness, N. 1995. Female choice in a feral guppy population - are there multiple cues? *Animal Behaviour*, **50**, 301-307.

Brown, C. & Warburton, K. 1997. Predator recognition and anti-predator responses in the rainbowfish, *Melanotaenia eachamensis*. *Behavioural Ecology and Sociobiology*, **41**, 61-68.

Brown, G. E. & Brown, J. A. 1992. Do rainbow trout and Atlantic salmon discriminate kin? *Canadian Journal of Zoology*, **70**, 1636-1640.

Brown, G. E. & Brown, J. A. 1996a. Kin discrimination in salmonids. *Reviews in Fish Biology and Fisheries*, **6**, 201-219.

Brown, G. E. & Brown, J. A. 1996b. Does kin-biased territorial behaviour increase kin-biased foraging in juvenile salmonids? *Behavioral Ecology*, **7**, 24-29.

Brown, J. A. & Colgan, P. W. 1986. Individual and species recognition in centrarchid fishes: evidence and hypotheses. *Behavioural Ecology and Sociobiology*, **19**, 373-379.

Brown, G. E. & Smith, R. J. F. 1994. Fathead minnows use chemical cues to discriminate natural shoalmates from unfamiliar conspecifics. *Journal of Chemical Ecology*, **20**, 3051-3061.

Butlin, R. 1989. Reinforcement of premating isolation. In: D. Otte & J. A. Endler (eds.), *Speciation and its Consequences*. pp. 158-179. Sinauer Associates Inc., Sunderland, Massachusetts.

Chapman, M. R. & Kramer, D. L. 1996. Guarded resources: the effect of intruder number on the tactics and success of defenders and intruders. *Animal Behaviour*, **52**, 83-94.

Chivers, D. P., Brown, G. E. & Smith, R. J. F. 1995. Familiarity and shoal cohesion in fathead minnows (*Pimephales promelas*): implications for antipredator behaviour. *Canadian Journal of Zoology*, **73**, 955-960.

Claridge, M. F. & Morgan, J. C. 1993. Geographical variation in acoustic signals of the planthopper, *Nilparvata bakeri* (Muir) in Asia - species recognition and sexual selection. *Biological Journal of the Linnean Society*, **48**, 267-281.

Clough, S. & Ladle, M. 1997. Diel migration and site fidelity in a stream-dwelling cyprinid, *Leuciscus leuciscus*. *Journal of Fish Biology*, **50**, 1117-1119.

Comstock, R. E. & Robinson, H. F. 1948. The components of genetic variance in populations of biparental progenies and their use in estimating the average degree of dominance. *Biometrics*, **4**, 254-266.

Courtenay, S. C., Quinn, T. P., Dupuis, H. M., Groot, C. & Larkin, P. A. 1997. Factors affecting the recognition of population-specific odours by juvenile coho salmon. *Journal of Fish Biology*, **50**, 1042-1060.

Coyne, J. A. & Orr, H. A. 1989. Patterns of speciation in *Drosophila*. *Evolution*, **43**, 362-381.

Coyne, J. A. & Orr, H. A. 1997. "Patterns of speciation in *Drosophila* " revisited. *Evolution*, **51**, 295-303.

Darwin, C. 1871. *The Descent of Man and Selection in Relation to Sex.* Murray, London.

Denton, E. J. & Rowe, D. M. 1998. Bands against stripes on the backs of mackerel, *Scomber scombrus* L. *Proceedings of the Royal Society of London, Series* B, **265**, 1051-1058.

Dentry, W. & Lindsey, C. C. 1978. Vertebral variation in zebrafish (*Brachydanio rerio*) related to the prefertilization temperature history of their parents. *Canadian Journal of Zoology*, **56**, 280-283.

Dobzhansky, Th. 1970. *Genetics of the Evolutionary Process.* Columbia University Press, New York.

Domeier, M. L. 1994. Speciation in the serranid fish *Hypoplectrus*. *Bulletin of Marine Science*, **54**, 103-141.

Dugatkin, L. A. & Alfieri, M. 1991. Guppies and the tit-for-tat strategy: preference based on past interaction. *Behavioural Ecology and Sociobiology*, **28**, 243-246.

Dugatkin, L. A. & Wilson, D. S. 1992. The prerequisites for strategic behaviour in bluegill sunfish, *Lepomis macrochirus*. *Animal Behaviour*, **44**, 223-230.

Dutta, S. P. S. 1993. Food and feeding habits of *Danio rerio* (Ham. Buch.) inhabiting Gadigarh Stream, Jammu. *Journal of Freshwater Biology*, **5**, 165-168.

Duyk, G. & Schmitt, K. 2001. Fish x 3. *Nature Genetics*, **27**, 8-9.

Endler, J. A. 1983. Natural and sexual selection on color patterns in poeciliid fishes. *Environmental Biology of Fishes,* **9**, 173-190.

Endler, J. A. 1989. Conceptual and other problems in speciation. In: D. Otte & J. A. Endler (eds.), *Speciation and its Consequences*. Sinauer Associates Inc., Sunderland, Massachusetts.

Endler, J. A. 1990. On the measurement and classification of colour in studies of animal colour patterns. *Biological Journal of the Linnean Society*, **41**, 315-352.

Endler, J. A. & Houde, A. E. 1995. Geographic variation in female preferences for male traits in *Poecilia reticulata*. *Evolution,* **49**, 456-468.

Fang, F. & Kottelat, M. 2000. *Danio roseus*, a new species from the Mekong basin in northeastern Thailand and northwestern Laos (Teleostei: Cyprinidae). *Ichthyological Exploration of Freshwaters*, **11**, 149-154.

Fang, F. 1998. *Danio kyathit*, a new species of cyprinid fish from Myitkyina, northern Myanmar. *Ichthyological Exploration of Freshwaters*, **8**, 273-280.

Fang, F. 2000. Barred *Danio* species from the Irrawaddy River drainage (Teleostei, Cyprinidae). *Ichthyological Research*, **47**, 13-26**.**

Farr, J. A. 1980. Social facilitation of male sexual behavior, intrasexual competition, and sexual selection in the guppy, *Poecilia reticulata* (Pisces: Poeciliidae). *Evolution*, **30**, 707-717.

Ferreri, F., Nicolais, C., Boglione, C. & Bertolini, B. 2000. Skeletal characteristics of wild and reared zebrafish: anomalies and meristic characters. *Journal of Fish Biology*, **56**, 1115-1128.

Fisher, R. A. 1918. The correlation between relatives on the supposition of Mendelian inheritance. *Transactions – Royal Society of Edinburgh*, **52**, 399-433.

Fisher, R. A. 1958. *The Genetical Theory of Sexual Selection.* 2nd Edn. Dover, New York.

Fiske, P. & Amundsen, T. 1997. Female bluethroats prefer males with symmetric colour bands. *Animal Behaviour*, 54, 81-87.

FitzGerald, G. J. & Morrissette, J. 1992. Kin recognition and choice of shoal mates. *Ethology, Ecology and Evolution*, **4**, 273–283

Folke, C., Olsén, H., Winberg, S., Bjerselius, R. & Karlsson, L. 1992. Differences in rheotactic response and attraction to population-specific odours in Baltic salmon (*Salmo salar* L.) parr. *Nordic Journal of Freshwater Research*, **67**, 45-51.

Gabor, C. 1999. Association patterns of sailfin mollies (*Poecilia latipinna*): alternative hypotheses. *Behavioural Ecology & Sociobiology*, **46**, 333-340.

Gelowitz, C. M., Mathis, A. & Smith, R. J. F. 1993. Chemosensory recognition of northern pike (*Esox lucius*) by brook stickleback (*Culaea inconstans*) - population differences and the influence of predator diet. *Behaviour*, **127**, 105-118.

Godin, J. -G. J. & Dugatkin, L. A. 1995. Variability and repeatability of female mating preference in the guppy. *Animal Behaviour*, **49**, 1427-1433.

Golubev, A. V. 1984. Role of chemical stimuli in group and spawning behaviour of *Brachydanio rerio* (Hamilton-Buchanan) (Cyprinidae). *Voprosy Ikhtiologii* **24**, 1020-1027.

Griffiths, S. W. & Magurran, A. E. 1997*a*. Familiarity in schooling fish: how long does it take to acquire? *Animal Behaviour*, **53**, 945-949.

Griffiths, S. W. & Magurran, A. E. 1997*b*. Schooling preferences for familiar fish vary with group size in a wild guppy population. *Proceedings of the Royal Society of London, Series B*, **264**, 547-551.

Griffiths, S. W. & Magurran, A. E. 1998. Sex and schooling behaviour in the Trinidadian guppy. *Animal Behaviour*, **56**, 689-693.

Griffiths, S. W. & Magurran, A. E. 1999. Schooling decisions in guppies (*Poecilia reticulata)* are based on familiarity rather than kin recognition by phenotype matching. *Behavioural Ecology and Sociobiology*, **45**, 437-443.

Griffiths, S. W. 1997. Preferences for familiar fish do not vary with predation risk in the European minnow. *Journal of Fish Biology,* **51**, 489-495.

Groot, C., Quinn, T. P. & Hara, T. J. 1986. Responses of migrating adult sockeye salmon (*Onchorhynchus nerka*) to population-specific odours. *Canadian Journal of Zoology*, **64**, 926-932.

Haffter, P., Odenthal, J., Mullins, M. C., Lin, S., Farrell, M. J., Vogelsang, E., Haas, F., Brand, M., van Eeden, F. J. M., Furutani-Seiki, M., Heisenberg, C. –L., Jiang, Y. -J., Kane, D.

A., Kelsh, R. N., Hopkins, N. & Nüsslein-Volhard, C. 1996. Mutations affecting pigmentation and shape of the adult zebrafish. *Development, Genes and Evolution,* **206**, 260-276.

Hailman, J. P. 1982. Concealment by stripes during movement and bars at rest: Field evidence from color changes in a goatfish and coronetfish. *Copeia*, **2**, 454-455.

Halperin, J. R. P. & Dunham, D. W. 1993. Increased aggression after brief social isolation of adult fish – a connectionist model which organizes this literature. *Behavioural Processes*, **28**, 123-144.

Hamilton, W. D. 1964. The genetical theory of social behavior, I and II. *Journal of Theoretical Biology*, **7**, 1-52.

Hamilton, W. D. & Zuk, M. 1982. Heritable true fitness and bright birds: a role for parasites? *Science*, **218**, 384-387.

Hansen, L. T. T., Amundsen, T. & Forsgren, E. 1999. Symmetry: attractive not only to females. *Proceedings of the Royal Society of London, Series B,* **266**, 1235-1240.

Harikumar, S., Padmanabhan, K. G., John, P. A. & Kortmulder, K. 1994. Dry-season spawning in a cyprinid fish of southern India. *Environmental Biology of Fishes,* **39**, 129-136.

Higashi, M., Takimoto, G. & Yamamura, N. 1999. Sympatric speciation by sexual selection. *Nature*, **402**, 523-526.

Höjesjö, J., Johnsson, J. I., Petersson, E. & Järvi, T. 1998. The importance of being familiar: individual recognition and social behavior in sea trout (*Salmo trutta*). *Behavioral Ecology*, **9**, 445-451.

Hollocher, H., Ting, C. -T., Pollack, F. & Wu, C. -I. 1997. Incipient speciation by sexual isolation in *Drosophila melanogaster*: variation in mating preference and correlation between the sexes. *Evolution*, **51**, 1175-1181.

Hora, S. L. 1921. Fish and fisheries of Manipur with some observations on those of the Naga Hills. *Records of the Indian Museum*, **22**, 165-214.

Houde, A. E. 1988. Genetic difference in female choice between two guppy populations. *Animal Behaviour,* **36**, 510-516.

Houde, A. E. 1994. Effect of artificial selection on male colour patterns on mating preference of female guppies. *Proceedings of the Royal Society of London, Series B,* **256**, 125-130.

Houde, A. E. & Endler, J. A. 1990. Correlated evolution of female mating preferences and male color patterns in the guppy *Poecilia reticulata*. *Science,* **248**, 1405-1408.

Howard, D. J. & Berlocher, S. H. (eds.). 1998. *Endless Forms: Species and Speciation*. Oxford University Press, Oxford.

Howard, D. J., Reece, M., Gregory, P. G., Chu, J. & Cain, M. L. 1998. The evolution of barriers to fertilization between closely related organisms. In: D. J. Howard & S. H. Berlocher (eds.), *Endless Forms: Species and Speciation*. Oxford University Press, Oxford.

Howard, R. D., Martens, R. S., Innis, S. A., Drnevich, J. M. & Hale, J. 1998. Mate choice and mate competition influence male body size in Japanese medaka. *Animal Behaviour*, **55**, 1151-1163.

Hughes, A. L. 1985. Male size, mating success, and mating strategy in the mosquitofish *Gambusia affinis* (Poeciliidae). *Behavioural Ecology and Sociobiology*, **17**, 271-278.

Hutchings, J. A., Bishop, T. D. & McGregor-Shaw, C. R. 1999. Spawning behaviour of Atlantic cod, *Gadus morhua*: evidence of mate competition and mate choice in a broadcast spawner. *Canadian Journal of Fisheries and Aquatic Sciences*, **56**, 97-104.

Iguchi, K. & Maekawa, K. 1993. Female mate preference and male mating success of ayu fish, *Plecoglossus altivelis* (Osmeridae) under a promiscuous mating system. *Ethology,* **95**, 193-201.

Iovine, M. K. & Johnson, S. L. 2000. Genetic analysis of isometric growth control mechanisms in the zebrafish caudal fin. *Genetics*, **155**, 1321-1329.

Järvi, T. 1990. The effects of male dominance, secondary sexual characteristics and female mate choice on the mating success of male Atlantic salmon *Salmo salar*. *Ethology*, **84**, 123-132.

Jennions, M. D. & Petrie, M. 2000. Why do females mate multiply? A review of the genetic benefits. *Biological Reviews*, **75**, 21-64.

Jiggins, C. D. & McMillan, W. O. 1997. The genetic basis of an adaptive radiation: warning colour in two *Heliconius* species. *Proceedings of the Royal Society of London, Series B,* **264**, 1167-1175.

Johnson, S. L., Africa, D., Walker, C. & Weston, J. A. 1995. Genetic control of adult pigment stripe development in the zebrafish. *Developmental Biology*, **167**, 27-33.

Jones, I. L. & Hunter, F. M. 1999. Experimental evidence for mutual inter- and intrasexual selection favouring a crested auklet ornament. *Animal Behaviour*, **57**, 521-528.

Karino, K. 1997. Female mate preference for males having long and symmetric fins in the bower-holding cichlid *Cyathopharynx furcifer*. *Ethology*, **103**, 883-892.

Kawakami, K., Amsterdam, A., Shimoda, N., Becker, T., Mugg, J., Shima, A. & Hopkins, N. 2000. Proviral insertions in the zebrafish *hagoromo* gene, encoding an F-box/WD40-repeat protein, cause stripe pattern anomalies. *Current Biology*, **10**, 463-466.

Kelley, J. L., Graves, J. A. & Magurran, A. E. 1999. Familiarity breeds contempt in guppies. *Nature*, **401**, 661-662.

Kellogg, K. A., Markert, J. A., Stauffer, J. R. & Kocher, T. D. 1995. Microsatellite variation demonstrates multiple paternity in lekking cichlid fishes from Lake Malawi, Africa. *Proceedings of the Royal Society of London, Series B,* **260**, 79-84.

Kelsh, R. N., Brand, M., Jiang, Y. -J., Heisenberg, C. -L., Lin, S., Haffter, P., Odenthal, J., Mullins, M. C., van Eeden, F. J. M., Furutani-Seiki, M., Granato, M., Hammerschmidt, M., Kane, D. A., Warga, R. M., Beuchle, D., Vogelsang, L. & Nüsslein-Volhard, C. 1996. Zebrafish pigmentation mutations and the processes of neural crest development. *Development,***123**, 369-389.

Kendall, M. G. 1955. Further contributions to the theory of paired comparisons. *Biometrics*, **11**, 43-62.

Khoda, Y. & Watanabe, M. 1988. Preference for striped backgrounds by striped fishes. *Zoological Science*, **5**, 501-503.

Kirschbaum, von F. 1977. Zur genetik einiger farbmustermutanten der zebrabarbe *Brachydanio rerio* (Cyprinidae, Teleostei) und zum phänotyp von artbastarden der gattung *Brachydanio*. *Biologisches Zentralblatt,* **96**, 211-222.

Kodric-Brown, A. 1989. Dietary carotenoids and male mating success in the guppy - an environmental component to female choice. *Behavioural Ecology and Sociobiology,* **25**, 393-401.

Kodric-Brown, A. 1993. Female choice of multiple male criteria in guppies: interacting effects of dominance, coloration and courtship. *Behavioural Ecology and Sociobiology*, **32**, 415-420.

Koseki, Y., Takata, K. & Maekawa, K. 2000. The role of the anal fin in fertilization success in the male medaka, *Oryzias latipes*. *Fisheries Science*, **66**, 633-635.

Krause, J., Hartmann, N. & Pritchard, V. L. 1999. The influence of nutritional state on shoal choice in zebrafish (*Danio rerio*). *Animal Behaviour*, **57**, 771-775.

Krause, J., Peukhuri, N., Butlin, R. K. & Pritchard, V. L. 2000. The social organisation of fish shoals: a test of the predictive power of laboratory experiments for the field. *Biological Reviews*, **75**, 477-501.

Lachlan, R.F., Crooks, L. & Laland, K.N. 1998. Who follows whom? Shoaling preferences and social learning of foraging information in guppies. *Animal Behaviour,* **56**, 181-190.

Lande, R. 1980. Sexual dimorphism, sexual selection, and adaptation in polygenic characters. *Evolution*, **34**, 292-305.

Landeau, L. & Terborgh, J. 1986. Oddity and the 'confusion effect' in predation. *Animal Behaviour*, **34**, 1372-1380.

Langmore, N. E. & Bennett, A. T. D. 1999. Strategic concealment of sexual identity in an estrildid finch. *Proceedings of the Royal Society of London, Series B,* **266**, 543-550.

Lessells, C. M. & Boag, P. T. 1987. Unrepeatable repeatabilities: a common mistake. *The Auk*, **104**, 116-121.

Lopéz, S. 1998. Acquired resistance affects male sexual display and female choice in guppies. *Proceedings of the Royal Society of London, Series B,* **265**, 717-723.

Luyten, P. H. & Liley, N. R. 1991. Sexual selection and competitive mating success of males guppies (*Poecilia reticulata*) from four Trinidad populations. *Behavioural Ecology and Sociobiology,* **28**, 329-336.

Lynch, M. & Walsh, B. 1998. *Genetics and Analysis of Quantitative Traits*. Sinauer Associates Inc., Sunderland, Massachusetts.

Magurran, A. E. 1998. Population differentiation without speciation. *Philosophical Transactions of the Royal Society of London, Series B*, **353**, 275-286.

Magurran, A. E. & Bendelow, J. A. 1990. Conflict and co-operation in White Cloud Mountain minnow schools. *Journal of Fish Biology*, **37**, 77-83.

Magurran, A. E. & Seghers, B. H. 1991. Variation in schooling and aggression amongst guppy (*Poecilia reticulata*) populations in Trinidad. *Behaviour*, **118**, 214-234.

Magurran, A. E. & Seghers, B. H. 1994. Sexual conflict as a consequence of ecology: evidence from guppy, *Poecilia reticulata*, populations in Trinidad. *Proceedings of the Royal Society of London, Series B*, **255**, 31-36.

Magurran, A. E., Seghers, B. H., Shaw, P. W. & Carvalho, G. R. 1994. Schooling preferences for familiar fish in the guppy, *Poecilia reticulata*. *Journal of Fish Biology,* **45**, 401-406.

Maksymovitch, E. & Verrell, P. A. 1993. Divergence of mate recognition systems among conspecific populations of the plethodontid salamander *Desmognathus santeetlah* (Amphibia, Plethodontidae). *Biological Journal of the Linnean Society*, **49**, 19-29.

Markert, J. A., Arnegard, M. E., Danley, P. D. & Kocher, T. D. 1999. Biogeography and population genetics of the Lake Malawi cichlid *Melanochromis auratus*: habitat transience, philopatry and speciation. *Molecular Ecology*, **8**, 1013-1026.

Mather, K. & Jinks, J. L. 1982. *Biometrical Genetics*. 3rd Edn. Chapman and Hall, London.

Mathis, A., Chivers, D. P. & Smith, R. J. F. 1993. Population differences in response of fathead minnows (*Pimephales promelas*) to visual and chemical stimuli from predators. *Ethology*, **93**, 31-40.

McClure, M. 1999. Development and evolution of melanophore patterns of fishes of the genus *Danio* (Teleostei: Cyprinidae). *Journal of Morphology*, **241**, 83-105.

McDonald, C. G., Reimchen, T. E. & Hawryshyn, C. W. 1995. Nuptial color loss and signal masking in *Gasterosteus* - an analysis using video imaging. *Behaviour,* **132**, 963-977.

McGowan, R. A. & Martin, C. C. 1997. DNA methylation and genome imprinting in the zebrafish, *Danio rerio*: some evolutionary ramifications. *Biochemistry and Cell Biology*, **75**, 499-506.

McMillan, W. O., Weigt, L. A. & Palumbi, S. R. 1999. Color pattern evolution, assortative mating and genetic differentiation in brightly colored butterflyfishes (Chaetodontidae). *Evolution*, **53**, 247-260.

Menon, A. G. K. 1954. Further observations on the fish fauna of the Manipur state. *Records of the Indian Museum*, **52**, 21-26.

Metcalfe, N. B. & Thomson, B. C. 1995. Fish recognize and prefer to shoal with poor competitors. *Proceedings of the Royal Society of London, Series B,* **259**, 207-210.

Meyer, A., Biermann, C. H. & Orti, G. 1993. The phylogenetic position of the zebrafish (*Danio rerio*), a model system in developmental biology: an invitation to the comparative method. *Proceedings of the Royal Society of London, Series B*, **252**, 231-236.

Meyer, A., Morrissey, J. M. & Schartl, M. 1994. Recurrent origin of a sexually selected trait in *Xiphoporus* fishes inferred from a molecular phylogeny. *Nature,* **368**, 539-542.

Meyer, A., Ritchie, P. A. & Witte, K. -E. 1995. Predicting developmental processes from evolutionary patterns: a molecular phylogeny of the zebrafish (*Danio rerio*) and its relatives. *Philosophical Transactions of the Royal Society of London, Series B*, **349**, 103-111.

Milinski, M. & Bakker, T. C. M. 1990. Female sticklebacks use male coloration in mate choice and hence avoid parasitized males. *Nature*, **344**, 330-332.

Milinski, M., Külling, D. & Kettler, R. 1990. Tit for tat: sticklebacks 'trusting' a cooperating partner. *Behavioral Ecology* **1**, 7-12.

Morris, M. R. 1998. Female preference for trait symmetry in addition to trait size in swordtail fish. *Proceedings of the Royal Society of London, Series B,* 265, 907-911.

Morris, M. R., Mussel, M., and Ryan, M. J. 1995. Vertical bars on male *Xiphoporus multilineatus* - a signal that deters rival males and attracts females. *Behavioral Ecology,* **6,** 274-279.

Myers. 1924. American Museum Novitates, **150**, 1-7.

Nilsson, S. O. & Nilsson, G. E. 2000. Free choice by female sticklebacks: lack of preference for male dominance traits. *Canadian Journal of Zoology*, **78**, 1251-1258.

Nordeng, H. 1971. Is the local orientation of anadromous fish determined by pheromones? *Nature*, **233**, 411-413.

O'Connor, K. I., Metcalfe, N. B. & Taylor, A. C. 2000. Familiarity influences body darkening in territorial disputes between juvenile salmon. *Animal Behaviour*, **59**, 1095-1101.

Olsén, K. H. 1989. Sibling recognition in juvenile Arctic charr (*Salvelinus alpinus* (L.)). *Journal of Fish Biology* **34**, 571-581.

Olsén, K. H., Grahn, M., Lohm, J. & Langefors, A. 1998. MHC and kin discrimination in juvenile arctic charr, *Salvelinus alpinus* (L.). *Animal Behaviour* **56**, 319-327.

Orger, M. B., Smear, M. C., Anstis, S. M. & Baier, H. 2000. Perception of Fourier and non-Fourier motion by larval zebrafish. *Nature Neuroscience*, **3**, 1128-1133.

Ortolani, A. 1999. Spots, stripes, tail tips and dark eyes: Predicting the function of carnivore colour patterns using the comparative method. *Biological Journal of the Linnean Society*, **67**, 433-476.

Orr, H. A. & Coyne, J. A. 1992. The genetics of adaptation: a reassessment. *American Naturalist*, **140**, 725-742.

Otte, D. & Endler, J. A. (eds.). 1989. *Speciation and its Consequences*. Sinauer Associates Inc., Sunderland, Massachusetts.

Packard, G. C. & Boardman, T. J. 1987. The misuse of ratios to scale physiological data that vary allometrically with body size. In: M. E. Feder, A. F. Bennett, W. W. Burggren & R. B. Huey (eds.), *New Directions in Ecological Physiology*. pp. 216-239. Cambridge University Press, Cambridge.

Panhuis, T. M., Butlin, R., Zuk, M. & Tregenza, T. 2001. Sexual selection and speciation. *Trends in Ecology and Evolution.* In press.

Parichy, D. M., Rawls, J. F., Pratt, S. J., Whitfield, T. T. & Johnson, S. L. 1999. Zebrafish *sparse* corresponds to an orthologue of c-*kit* and is required for the morphogenesis of a subpopulation of melanocytes, but is not essential for hematopoiesis or primordial germ cell development. *Development*, **126**, 3425-3436.

Parichy, D. M., Ransom, D. G., Paw, B., Zon, L. I. & Johnson, S. L. 2000. An orthologue of the *kit*- related gene *fms* is required for development of neural crest-derived xanthophores and a subpopulation of adult melanocytes in the zebrafish, *Danio rerio*. *Development*, **127**, 3031-3044.

Petersson, E., Järvi, T., Olsén, H., Mayer, I. & Hedenskog, M. 1999. Male-male competition and female choice in brown trout. *Animal Behaviour*, **57**, 777-783.

Pettersson, L. B. & Bronmark, C. 1999. Energetic consequences of an inducible morphological defence in crucian carp. *Oecologia*, **121**, 12-18.

Peuhkuri, N. & Seppä, P. 1998. Do three-spined sticklebacks group with kin? *Annales Zoologici Fennici*, **35**, 21-27.

Pijnacker, L. P. & Ferwerda, M. A. 1995. Zebrafish chromosome-banding. *Genome*, **38**, 1052-1055.

Pilsatro, A., Giacomello, E. & Bisazza, A. 1997. Sexual selection for small size in male mosquitofish (*Gambusia holbrooki*). *Proceedings of the Royal Society of London, Series B,* **264**, 1125-1129.

Pitcher, T. J. & Parrish, J. K. 1993. Functions of shoaling behaviour in teleosts. In: T. J. Pitcher (ed.), *Behaviour of Teleost Fishes*. pp. 364-439. Chapman & Hall, London.

Pomiankowski, A. & Möller, A. P. 1995. A resolution of the lek paradox. *Proceedings of the Royal Society of London, Series B,* **260**, 21-29.

Postlethwait, J. H., Yan, Y. -L., Gates, M. A., Horne, S., Amores, A., Brownlie, A., Donovan, A., Egan, E. S., Force, A., Gong, Z., Goutel, C., Fritz, A., Kelsh, R., Knapik, E., Liao, E., Paw, B., Ransom, D., Singer, A., Thomson, M., Abduljabbar, T. S., Yelick, P., Beier, D., Joly, J. -S., Larhammar, D., Rosa, F., Westerfield, M., Zon, L. I., Johnson, S. L. & Talbot, W. S. 1998. Vertebrate genome evolution and the zebrafish gene map. *Nature Genetics*, **18**, 345-349.

Prashad, B. & Mukerji, D. D. 1929. The fish of the Indawgyi Lake and the streams of the Myitkyina district (Upper Burma). *Records of the Indian Museum*, **31**, 161-223.

Ptacek, M. B. 2000. The role of mating preferences in shaping interspecific divergence in mating signals in vertebrates. *Behavioural Processes*, **51**, 111-134.

Ptacek, M. B. and Travis, J. 1997. Mate choice in the sailfin molly, *Poecilia latipinna*. *Evolution,* **51**, 1217-1231.

Pyron, M. 1995. Mating patterns and a test for female mate choice in *Etheostoma spectabile* (Pisces, Percidae). *Behavioural Ecology and Sociobiology,* **36**, 407-412.

Quinn, T. P. & Busack, C. A. 1985. Chemosensory recognition of siblings in juvenile salmon (*Oncorhynchus kisutch*). *Animal Behaviour*, **33**, 51-56.

Quinn, T. P. & Hara, T. J. (1986). Sibling recognition and olfactory sensitivity in juvenile coho salmon (*Oncorhynchus kisutch*). *Canadian Journal of Zoology*, **64**, 921-925.

Quinn, T. P. & Tolson, G. M. 1986. Evidence of chemically mediated population recognition in coho salmon (*Oncorhynchus kisutch*). *Canadian Journal of Zoology*, **64**, 84-87.

Qvarnström, A. 1999. Genotype-by-environment interactions in the determination of the size of a secondary sexual character in the collared flycatcher (*Ficedula albicollis*). *Evolution*, **53**, 1564-1572.

Rawls, J. F. & Johnson, S. L. 2000. Zebrafish *kit* mutation reveals primary and secondary regulation of melanocyte development during fin stripe regeneration. *Development*, **127**, 3715-3724.

Ridgway, M. S. & McPhail, J. D. 1984. Ecology and evolution of sympatric sticklebacks (*Gasterosteus*) – mate choice and reproductive isolation in the Enos Lake species pair. *Canadian Journal of Zoology*, **62**, 1813-1818.

Riehl, R. & Baensch, H. A. 1986. *Aquarium Atlas* (2nd Edn.). Hans A. Baensch, Melle, Germany.

Ritchie, M. G. & Phillips, S. D. F. 1998. The genetics of sexual isolation. In: D. J. Howard & S. H. Berlocher (eds.), *Endless Forms: Species and Speciation*. Oxford University Press, Oxford.

Robinson, B. W. & Wilson, D. S. 1995. Experimentally-induced morphological diversity in Trinidadian guppies (*Poecilia reticulata*). *Copeia*, **1995**, 2, 294-305.

Robinson, B. W. & Wilson, D. S. 1996. Genetic variation and phenotypic plasticity in a trophically polymorphic population of pumpkinseed sunfish (*Lepomis gibbosus*). *Evolutionary Ecology*, **10**, 631-652.

Roff, D. A. 1997. *Evolutionary Quantitative Genetics*. Chapman and Hall, New York.

Rosenthal, G. G. & Evans, C. S. 1998. Female preference for large swords in *Xiphophorus helleri* reflects a bias for large apparent size. *Proceedings of the National Academy of Sciences, USA,* **95**, 4431-4436.

Rundle, H. D., Nagel, L., Boughman, J. W. & Schluter, D. 2000. Natural selection and parallel speciation in sympatric sticklebacks. *Science*, **287**, 306-308.

Ryan, M. J., Rand, A. S. & Weigt, L. A. 1996. Allozyme and advertisement call variation in the tungara frog, *Physalaemus pustulosus*. *Evolution*, **50**, 2435-2453.

Ryer, C. H. & Olla, B. L. 1992. Social mechanisms facilitating exploitation of spatially-variable ephemeral food patches in a pelagic marine fish. *Animal Behaviour*, **44**, 69-74.

Schluter, A., Parzefall, J. & Schlupp, I. 1998. Female preference for symmetrical vertical bars in male sailfin mollies. *Animal Behaviour*, **56**, 147-153.

Schluter, D. 1998. Ecological causes of speciation. In: D. J. Howard & S. H. Berlocher (eds.), *Endless Forms: Species and Speciation*. Oxford University Press, Oxford.

Seehausen, O. & van Alphen, J. J. M. 1998. The effect of male coloration on female mate choice in closely related Lake Victoria cichlids (*Haplochromis nyereri* complex). *Behavioural Ecology and Sociobiology,* **42**, 1-8.

Seehausen, O., Mayhew, P.J. & van Alphen, J. J. M. 1999. Evolution of colour patterns in East African cichlid fish. *Journal of Evolutionary Biology*, **12**, 514-534.

Seehausen, O., van Alphen, J. J. M. & Witte, F. 1997. Cichlid fish diversity threatened by eutrophication that curbs sexual selection. *Science*, **277**, 1808-1811.

Sen, N. & Dey, S. C. 1985. Two new fish species of the genus *Danio* Hamilton (Pisces: Cyprinidae) from Meghalaya, India. *Journal of the Assam Scientific Society*, **27**, 60-68.

Sharma, K. K., Sharma, O. P. & Tripathi, N. K. 1998. Female heterogamety in *Danio rerio* (Cypriniformes: Cyprinidae). *Proceedings of the National Academy of Science of India*, **68** (B), 123-126.

Shaw, R. G. 1987. Maximum-likelihood approaches to quantitative genetics of natural populations. *Evolution*, **41**, 812-826.

Sheppard, P. M., Turner, J. R. G., Brown, K. S., Benson, W. W. & Singer, M. C. 1985. Genetics and the evolution of muellerian mimicry in *Heliconius* butterflies. *Philosophical Transactions of the Royal Society of London, Series B*, **308**, 433-613.

Sheridan, L. & Pomiankowski, A. 1997. Female choice for spot asymmetry in the Trinidadian guppy. *Animal Behaviour*, **54**, 1523-1530.

Shimoda, N., Knapik. E. W., Ziniti, J., Sim, C., Yamada, E., Kaplan, S., Jackson, D., de Sauvage, F., Jacob, H. & Fishman, M. C. 1999. Zebrafish genetic map with 2000 microsatellite markers. *Genomics*, **58**, 219-132.

Sloman, K. A., Gilmour, K. M., Taylor, A. C. & Metcalfe, N. B. 2000. Physiological effects of dominance hierarchies within groups of brown trout, *Salmo trutta*, held under simulated natural conditions. *Fish Physiology and Biochemistry*, **22**, 11-20.

Spurway, H. 1953. Genetics of specific and subspecific differences in European newts. *Symposia of the Society for Experimental Biology*, **7**, 200-237.

Steck, N., Wedekind, C. & Milinski, M. 1999. No sibling odor preference in juvenile three-spined sticklebacks. *Behavioral Ecology* **10**, 493-497.

Stratton, G. E. & Uetz, G. W. 1986. The inheritance of courtship behavior and its role as a reproductive isolating mechanism in two species of *Schizocosa* wolf spiders (Araneae: Lycosidae). *Evolution*, **40**, 129-141.

Streisinger, G., Walker, C., Dower, N., Knauber, D. & Singer, F. 1981. Production of clones of homozygous diploid zebra fish (*Brachydanio rerio*). *Nature*, **291**, 293-296.

Swaddle, J. P. 1999. Visual signalling by asymmetry: a review of perceptual processes. *Philosophical Transactions of the Royal Society of London, Series B,* **354**, 1383-1393.

Swaddle, J.P. & Cuthill, I. C. 1994. Female zebra finches prefer males with symmetrical chest plumage. *Proceedings of the Royal Society of London, Series B,* **258**, 267-271.

Talwar, P. K. & Jhingran, A. G. 1992. *Inland Fishes of India and Adjacent Countries, Volume 1*. Oxford and IBH Publishing, New Delhi

Thresher, R. E. & Moyer, J. T. 1983. Male success, courtship complexity and patterns of sexual selection in three congeneric species of sexually monochromatic and dichromatic damselfishes (Pisces: Pomacentridae). *Animal Behaviour*, **31**, 113-127.

Turner, G. F. & Burrows, M. T. 1995. A model of sympatric speciation by sexual selection. *Proceedings of the Royal Society of London, Series B,* **260**, 287-292.

Uetz, G. W. & Smith, E. I. 1999. Asymmetry in a visual signalling character and sexual selection in a wolf spider. *Behavioural Ecology and Sociobiology,* **45**, 87-93.

Uy, J. A. C. & Borgia, G. 2000. Sexual selection drives rapid divergence in bowerbird display traits. *Evolution*, **54**, 273-278.

Van den Hurk, R. & Lambert, J. G. D. 1983. Ovarian steroid glucoronides function as sex pheromones for male zebrafish, *Brachydanio rerio. Canadian Journal of Zoology,* **61**, 2381-2387.

Van den Hurk, R. & Resink, J. W. 1992. Male reproductive-system as sex-pheromone producer in teleost fish. *Journal Of Experimental Zoology,* **261**, 204-213.

van Havre, N. & FitzGerald, G. J. 1988. Shoaling and kin recognition in the three-spine stickleback (*Gasterosteus aculeatus* L.). *Biology of Behaviour*, **13**, 190-201.

Vascotto, S. G., Beckham, Y. & Kelly, G. M. 1997. The zebrafish's swim to fame as an experimental model in biology. *Biochemistry and Cell Biology*, **75**, 479-485.

Vishwanath, W., Wahengbam, M., Laishram, K. & Keishing, S. S. 1998. Biodiversity of freshwater fishes of Manipur, India. *Italian Journal of Zoology*, **65**, *Supplement*, 321-324.

Waldman, B. 1988. The ecology of kin recognition. *Annual Review of Ecology and Systematics*, **19**, 543-571.

Warburton, K. & Lees, N. 1996. Species discrimination in guppies: learned response to visual cues. *Animal Behaviour*, **52**, 371–378.

Watson, P.J. & Thornhill, R. 1994. Fluctuating asymmetry and sexual selection. *Trends in Ecology and Evolution*, **9**, 21-25.

Weber, M. & de Beaufort, L. F. 1916. *The Fishes of the Indo-Australian Archipelago. Vol. III. Ostariophysi: Cyprinoidea, Apodes, Synbranchiii.* E. J. Brill, Leiden.

Westerfield, M. 1994. *The Zebrafish Book: a guide for the laboratory use of zebrafish* (*Brachydanio rerio*). Edn. 2.1. University of Oregon Press, Oregon.

Winberg, S. & Olsén, K. H. 1992. The influence of rearing conditions on the sibling odor preference of juvenile arctic charr, *Salvelinus alpinus* L. *Animal Behaviour* **44**, 157-164.

Wright, S. 1921. Systems of mating. I. The biometric relations between parents and offspring. *Genetics*, **6**, 144-161.

Wu, C. -I. & Palopoli, M. F. 1994. Genetics of postmating reproductive isolation in animals. *Annual Review of Genetics*, **28**, 283-308.

Wu, C. -I., Johnson, N. A. & Palopoli, M. F. 1996. Haldane's rule and its legacy: why are there so many sterile males? *Trends in Ecology and Evolution*, **11**, 281-284.

Zar, J. H. 1996. *Biostatistical Analysis*. 3rd Edn. Prentice Hall, London.